INVESTIGATION OF LIPOTEICHOIC ACID STRUCTURE AND FUNCTION
TO ESTABLISH ITS ROLE IN GRAM-POSITIVE BACTERIAL INFECTIONS

by

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INVESTIGATION OF LIPOTEICHOIC ACID STRUCTURE AND FUNCTION TO ESTABLISH ITS ROLE IN GRAM-POSITIVE BACTERIAL INFECTIONS

HO SEONG SEO

MICROBIOLOGY

ABSTRACT

Lipoteichoic acid (LTA) is an essential bacterial membrane polysaccharide (cell wall component) that is linked to a glycerol backbone with two acyl chains. Since an antibody to LTA has been shown to protect from Gr+ bacterial infections or colonization, and the mutant Gr+ bacteria which produce less amount of LTA have less pathogenic than WT, LTA may be an important role in Gr+ bacterial infection and inflammation.

The currently accepted structure of pneumococcal LTA has the pentameric repeating unit bound to the lipid anchor \{Glc(\beta1\rightarrow3)AATGal(\beta1\rightarrow3)Glc(\alpha1\rightarrow3)-acyl2Gro\}. Unlike the lipid anchor of other Gr+ bacterial LTA, this pneumococcal LTA lipid anchor has not been detected in the bacterial membrane. In addition, the current structure does not explain the Forssman antigen properties of Pneumococcal LTA. Thus we propose here the new model of pneumococcal LTA structure and show the new model has correct structure to explain its functions with a mass spectrometry.

LTA can induce the production of various inflammatory molecules via Toll-like receptor 2 (TLR2) like LPS via TLR4. However, the role of LTA in Gr+ induced inflammation is still debated because there are many limitations to study LTA function. For instance, purified LTA is easily contaminated or damaged during purification step and it is impossible to make LTA-deficient Gr+ bacteria, because LTA is an essential
component of Gr+ bacteria. Interestingly, its biological function can be altered by removing acyl chains because monoacylated LTA is not active in mouse model. Thus, we examined several phospholipase A2 (PLA2) to develop LTA inactivation methods. We found that PAF-acetylhydrolase, a recombinant human PLA2, is the most efficient and specific enzyme to produce monoacylated LTA that is inactive in mouse model. In early bacterial infection, Gr+ bacteria release/shed TLR2 ligands which are essential PAMPs in early infection and may be important to initiate inflammatory diseases such as sepsis.

To define the molecules which are released in early stage, we identified pneumococcal molecules in the early bacteria culture supernatant with anti-pneumococcal antibody and we found LTA was present and may be dominant in early stage. Since LTA is potential TLR2 ligand, we hypothesis that LTA is an essential inflammatory component in early Gr+ bacterial infection. To investigate the role of LTA in early infection, three LTA inactivation methods were used to examine its role in the culture supernatants. Here we find that LTA is the primary TLR2 ligand in the early phase of Gr+ bacterial infection and remains a major ligand in the late phase when other TLR2 and TLR4 ligand(s) appear. Thus, our studies have established the importance of LTA in Gr+ bacteria induced inflammation. In addition, our findings suggest that LTA is important in the early sepsis process but other components become significant in late stages of bacterial infection.
DEDICATION

I dedicate this dissertation to my grandmother, Moon Gab-Saeng. She did not live to see the beginning of my study, but she have always been with me in my heart and never left me.
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I express my most sincere appreciation to my mentor, Dr. Moon H. Nahm, for the excellent training and helpful advice that I have received. I feel privileged to have had the great opportunity to work with and learn from him.

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<tbody>
<tr>
<td>AL</td>
<td>Alabama</td>
</tr>
<tr>
<td>2-thio PAF</td>
<td>2-thio Platelet-activating factor</td>
</tr>
<tr>
<td>AATGal</td>
<td>2,4-Diamino-2,4,6-tridesoxy-o-galactopyranose</td>
</tr>
<tr>
<td>acyl2Gro</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>AMU</td>
<td>Atomic mass unit</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BTK</td>
<td>Bruton tyrosine kinase</td>
</tr>
<tr>
<td>CaCl2</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CDM</td>
<td>Chemically defined media</td>
</tr>
<tr>
<td>CHO cells</td>
<td>Chines hamster ovary cells</td>
</tr>
<tr>
<td>C-PS</td>
<td>Cell wall polysaccharide (Teichoic acid)</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CYLD</td>
<td>Cylindromatosis</td>
</tr>
<tr>
<td>dFBS</td>
<td>Defined fetal bovine serum</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoyl phosphatidyl choline</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double strand ribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
</tr>
<tr>
<td>GB</td>
<td>Group B streptococcus (<em>Streptococcus agalactiae</em>)</td>
</tr>
<tr>
<td>Glc</td>
<td>Glucose</td>
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<table>
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<tbody>
<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
</tr>
<tr>
<td>Glx</td>
<td>Glutamine or Glutamic acid</td>
</tr>
<tr>
<td>GPCS</td>
<td>Gram-positive culture supernatant</td>
</tr>
<tr>
<td>Gr-</td>
<td>Gram-negative</td>
</tr>
<tr>
<td>Gr+</td>
<td>Gram-positive</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrofluoric acid</td>
</tr>
<tr>
<td>HSP60</td>
<td>Heat shock protein 60</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Interferon-alpha</td>
</tr>
<tr>
<td>IFN-β</td>
<td>Interferon-beta</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IgG1</td>
<td>Immunogloblin G1</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin-18</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor (IL-1R)–associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IRK</td>
<td>Insulin receptor (IR) tyrosine kinase</td>
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<tr>
<td>LPS</td>
<td>Lippolysaccharide</td>
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<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>Lyso-PtCho</td>
<td>Monoacyl phosphatidyl choline</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAL</td>
<td>MyD88-adapter-like</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption ionization-time of flight</td>
</tr>
<tr>
<td>MBP</td>
<td>Mannan binding protein</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyldipeptide</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MurNAc</td>
<td>N-acetylmuramic acid</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response protein</td>
</tr>
<tr>
<td>NaNO2</td>
<td>Sodium Nitrite</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain (NOD)-like receptors</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD receptor</td>
<td>Nucleotide-binding oligomerization domain receptor</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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</table>
PAF Platelet activating factor
PAF-AH Platelet activating factor (PAF)-acyethylhydrolase
PAFR Platelet activating factor receptor
PAMP Pathogen-associated molecular pattern
PC Phosphocholine
PGN Peptidoglycan
PLA2 Phospholipase A2
Ply Pneumolysin
PnLTA Pneumococcal LTA

LIST OF ABBREVIATIONS (Continued)

PRR Pattern recognition receptors
PS-A Polysaccharide A
PspA Pneumococcal surface protein A
PspC Pneumococcal surface protein C
PtCho Phosphatidyl choline
RAC Ras-Related C3 Botulinum Toxin Substrate
RIP Receptor-interacting protein
SA Staphylococcus aureus
Ser Serine
SP Streptococcus pneumoniae
ssRNA Single strand ribonucleotide
StLTA Staphylococcal LTA
TA  Teichoic acid, C-PS, Cell wall polysaccharide
TAB  TAK1-binding proteins
TAK  Transforming growth factor-β-activated kinase
TBK  Serine–threonine-protein kinase
TEPC-15  Anti-phosphocholine monoclonal antibody
THB  Todd-Hewitt broth
TLC  Thin layer chromatography
TLR  Toll-like receptor
TNF-α  Tumor necrosis factor alpha
TIRAP  TIR domain-containing adaptor protein
TRAM  TRIF-related adaptor molecule
TRAP6/7  Tumor necrosis factor receptor-associated factor
TRIF  TIR-domain-containing adaptor inducing interferon beta
TSB  Tryptic-Soybean broth
UDP  Uridine diphosphate
WT  Wild type
INTRODUCTION

Bacterial sepsis is a serious medical condition characterized by a whole-body inflammatory state caused by bacterial infection in the bloodstream (19, 170). Despite improvements in antibiotics and other supportive therapies, sepsis remains a leading cause of death within intensive care units (155). The incidence of sepsis increased about 8.7% annually, from about 164,000 cases to nearly 660,000 cases between 1979 and 2000 (134). Sepsis was traditionally associated with Gram-negative (Gr-) bacteria, but Gram-positive (Gr+) infections increased by an average of 26.3% per year with Gr+ bacteria becoming the predominant pathogens after 1987 (6, 7, 134). In 2000, Gr+ bacteria accounted for 52% of sepsis cases, whereas Gr- bacteria accounted for 37.6% (34, 112, 134). The increase in septicemia rates of Gr+ bacteria may be caused by multiple factors including an increase in antibiotic resistance among Gr+ bacteria (e.g., methicillin-resistant Staphylococcus aureus, penicillin-resistant Streptococcus pneumoniae, and vancomycin-resistant Enterococcus faecium) (2, 39, 40), an increased number of elderly individuals as a result of improvements in medical care, and an increased number of patients with metabolic, neoplastic, or immunodeficiency disorders (20-23, 170).

The clinical symptoms of sepsis are due to an uncontrolled inflammatory response to bacterial infections. Inflammatory responses begin when bacteria enter the body, trigger the innate immune system, and make host immune cells produce proinflammatory mediators, such as cytokines, chemokines, prostaglandins, and reactive oxygen species.
These molecules in turn rapidly induce vasodilatation and upregulation of adhesion molecules, resulting in extravasation of neutrophils and monocytes, and activation of leukocytes, lymphocytes and endothelial cells. Together these processes damage host cells, lead to organ failure and/or cause septic shock (155). Since the early response to bacterial infection is an essential step to progressive sepsis, we need to know the early innate immune responses induced by bacterial infection.

While both Gr- and Gr+ bacterial sepses display similar clinical symptoms, they elicit markedly different innate immune responses as shown by the pattern of proinflammatory mediator production (Table 2). The immunological response to Gr- bacteria mainly involves the production of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interferon-beta (IFN-β) and IL-6; whereas the response to Gr+ bacteria involves the release of relatively low levels of TNF-α, IL-1, and IL-6 and increased levels of IL-8, IFN-γ, and IL-18 (38, 50, 88, 170, 185). Also, the progression of Gr- sepsis is much faster than Gr+ sepsis. These differences might be due to the involvement of distinct receptors in the recognition of Gr- and Gr+ bacteria, in that different innate immune receptors (e.g., TLR4 vs. TLR2) might play critical roles in the different profiles of proinflammatory mediators (185).

The innate immune system is activated by receptors known as pattern recognition receptors (PRRs) in response to bacteria or their components. Examples of PRRs include the Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-reactive protein, serum amyloid protein, mannan binding protein, and scavenger receptor (57, 58, 91). In particular, TLRs are essential for detecting and
responding to early bacterial infections. Each TLR is triggered by a distinct set of microbial compounds (Figure 1) and results in the production of proinflammatory cytokines. For example, TLR4 is a receptor for lipopolysaccharide (LPS) and is essential for generating responses to Gr- bacteria (158), whereas TLR2 responds to Gr+ bacterial cell wall components, such as lipoteichoic acid (LTA), lipopeptide, and peptidoglycan (PGN) (75, 166, 193). In addition, TLR9 responds to DNA-containing unmethylated CpG motifs (85), and bacteria flagellin signals through TLR5 (82).

With the exception of TLR3, the activation of all TLRs triggers a common signaling pathway, the MyD88 pathway, that culminates in the activation of nuclear factor-κB (NF-κB) and the ultimate release of inflammatory cytokines and chemokines (179). TLR3 and TLR4 also couple to the adaptor TRIF (MyD88-independent pathway) (211), which triggers transcription of IFN-β (Figure 1). Other adaptors, such as TRAM, MAL, RAC, BTK, TRAP6/7 are differentially recruited by TLRs, but their functions are less clear (95, 139, 152, 212). TLR-mediated NF-κB activation is also negatively regulated. For instance, tumor suppressor cylindromatosis (CYLD) inhibits TLR2-mediated NF-κB activation via a deubiquitination-dependent mechanism (119, 213) and reduces pulmonary inflammation by S. pneumoniae and non-typeable Haemophilus influenzae (127, 128).

The NLRs are also important in responses to bacterial infections. In some cases, NLRs function in a similar manner to TLRs in that they also recognize microbial components, such as PGN and muramyldipeptide (MDP) (Figure 2). While the downstream signaling pathways that are involved in the TLR and NLR sytems are
markedly different, both eventually activate NF-κB (Figure 2). Among the NLRs, NOD1 and NOD2 respond to PGN fragments (42). Gr- PGN fragments activate NOD1, whereas Gr+ fragments activate NOD2 (65, 132, 161). NLRs also contribute to bacterial internalization and upregulate NF-κB mediated cytokine production (132, 151, 161).

The ligands for TLR2 and TLR4, which are thought to be critical to Gr+ and Gr- bacterial infections, appear to be present in the cell wall of these bacteria (Figure 3). A major component of Gr+ bacterial cell wall (comprising about 40–50%) is PGN, which consists of large networks of alternating glycan moieties of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) cross-linked by short peptides (Figure 4) (204, 205). Gr- bacteria also have a thin PGN layer (less than 10% of cell wall) with different peptide chains (Figure 3). It has been recognized that PGN activates immune cells via TLR2 (45, 167). However, the ability of PGN to activate TLR2 was recently debated (Table 1) (45, 60, 66, 100, 126, 154, 192, 203, 204, 218), following the discovery that PGN fragments stimulate NOD receptors (60, 65, 192).

Lipoproteins are another type of TLR2 ligand. Lipoproteins, which are found in both Gr+ and Gr- cell walls are a functionally diverse class of membrane bacterial proteins characterized by an N-terminal lipid moiety. The lipid moiety serves to anchor these proteins to the cell surface. Typically, between 1 % and 3 % of bacterial genomes encode lipoproteins (8). Lipoproteins may be divided into five general groups according to their function: adhesion and invasion, cell wall synthesis, nutrient uptake, degradative processes, and sensing and transmembrane signalling (178). While synthetic lipopeptides have proven to be potential TLR2 ligands, it is still unclear whether real bacterial
lipoproteins function the same as synthetic lipopeptides in Gr+ sepsis. In addition, early culture supernatant collected from staphylococci with a ΔlgtA mutation, which cannot produce lipoproteins, still have as much inflammatory activity as wild type culture supernatant (173).

In addition to PGN and lipoproteins, lipoteichoic acid (LTA) forms another group of TLR2 ligands. LTA is a polyphosphate polymer linked to a glycerol backbone with two acyl chains and accounts for 5% of the cell wall (Figure 4) (51, 53, 54, 148, 189). Like lipoproteins, LTA is anchored on the cell membrane via a lipid moiety. Unlike PGN and lipoprotein, however, LTA is found only on Gr+ bacteria, not on Gr- bacteria. Also LTA shares many of its biochemical and physiological properties with LPS (64). Thus, it might play an important role in the process of Gr+ bacterial sepsis as LPS does in Gr- sepsis (41, 75, 76, 89, 109, 114).

The structure of the “classical” LTA has a 1,3-linked polyglycerophosphate chain that is attached by a phosphodiester bond to a glycolipid, which occurs in the free state as a membrane component (Figure 4) (143, 145). This “classical” LTA is produced by many Gr+ bacterial species such as bacilli, enterococci, lactobacilli, lactococci, listeria, staphylococci and some streptococci (41, 52, 53, 194) and has widely served as the model LTA. However, several studies indicate that LTA is destroyed or altered during purification and growth in culture media (144). The structural variability of the “classical” LTA and difficulties in monitoring its structure have prevented researchers from obtaining “pure” and “intact” LTA for functional studies. Pneumococcal LTA structure is not a “classical” LTA structure, it has ribitol phosphate instead of
glycerolphosphate, and a tetrasaccharide is intercalated between the ribitol phosphate residues (Figure 4) (54, 55). Since pneumococcal LTA has less variability in its structure than the “classical” LTA, pneumococcal LTA could be a better model for studying structure and function relationships of LTA.

Recent studies from our laboratory found that purified pneumococcal LTA has a mass that does not reconcile the existing molecular model (114, 168). Mass spectrometry showed that pneumococcal LTA contains about 350 atomic mass unit (AMU) less than the predicted mass (114, 168). This raises the possibility that pneumococcal LTA becomes damaged during purification or, alternatively, the existing model of the LTA structure may not be correct. There are other difficulties in using the existing model of pneumococcal LTA. Its structure does not explain its biological and serological properties. For instance, the predicted lipid anchor could not be detected in pneumococcal membranes (54). The existing model does not contain terminal GalNAc(α1→3)GalNAc(β1→), which is necessary to explain the Forssman antigen properties of pneumococcal LTA (12, 84). Thus, we hypothesized the existing model to be incorrect, and we have investigated the structure of pneumococcal LTA. My work to test this hypothesis is present this dissertation.

Another problem in the study of LTA function is the presence of contaminants in the purified LTA preparation. Purified LTA generally contains small amounts of contaminants such as lipoprotein, PGN and/or membrane proteins. The presence of these contaminants greatly complicates biological studies of purified LTA. To eliminate the possibility that these contaminants are immunologically active, I investigated the effect of
LTA inactivation on the innate immune response to Gr+ bacteria. LTA was inactivated by monodeacylation or complete deacylation using enzymes or alkaline hydrolysis. In addition to inactivation, the LTA was removed from the preparation by using an LTA-specific antibody. These studies are also described in this dissertation. These studies demonstrate the importance of LTA in early Gr+ bacterial inflammation.
Figure 1. The TLR Signaling Pathway.

TLRs activate the signaling pathways through two main pathways – MyD88 and TRIF pathway. Stimulation of TLR2/1, TLR2/6, TLR4, TLR5, TLR7, TLR8, TLR9 and TLR11 with specific ligands trigger the MyD88 pathway, which activates NF-kB for early responses. TLR3 and TLR4 can stimulate the TRIF pathway, which activates IFR3 for late responses (IFN-β production).
Recognition of muramyl dipeptide (MDP) and γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP) through leucine-rich repeat (LRR) domains activates the NOD (nucleotide-binding oligomerization domain) proteins NOD2 and NOD1, respectively, which then recruit receptor-interacting serine/threonine kinase (RICK) through caspase-recruitment domain (CARD)–CARD interactions. Activation of RICK leads to the phosphorylation of IKKβ, as well as the phosphorylation of IκB and the release of nuclear factor-κB (NF-κB) for translocation to the nucleus. CARD12 negatively regulates RICK mediated NF-κB activation by both NOD1 and NOD2. (176).
Figure 3. Gram-positive and Gram-negative bacterial cell walls.

A. The cell wall of Gram-negative bacteria is composed of an outer membrane linked by lipoproteins to a thin, mainly single-layered, peptidoglycan. The peptidoglycan is located within the periplasmic space that exists between the outer and inner membranes. The outer membrane includes porins, which allow the passage of small hydrophilic molecules across the membrane, and lipopolysaccharide molecules that extend into the extracellular space. B. The cell wall of Gram-positive bacteria is composed of a thick, multilayered peptidoglycan sheath outside the cytoplasmic membrane. Teichoic acids are linked to and embedded in the peptidoglycan, and lipoteichoic acids extend into the cytoplasmic membrane.
Figure 4. Chemical structures of the surface polymers of the two major Gram-positive pathogens (*Staphylococcus aureus* and *Streptococcus pneumoniae*).

The lipoteichoic acid is a lipid-linked, membrane-bound form of teichoic acid (TA), a carbohydrate backbone formed by repeating glycerophosphate or ribitol phosphate. The cell wall is a network of peptidoglycan decorated by covalently linked teichoic acid. The peptidoglycan is a continuous backbone of repeating N-acetylglucosamine (G) and N-acetyl muramic acid (M) that is cross-linked to neighboring backbones by stem peptides. These stem peptides have a great variety of compositions as shown in the bottom half of the figure (% refers to relative abundance in overall wall composition). Adapted from (205).
**Table 1.** Threshold of bioactivity of cell wall components in vitro. Adapted from (205).

<table>
<thead>
<tr>
<th>Wall component</th>
<th>Minimal active concentration</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Staphylococi</td>
</tr>
<tr>
<td>Intact bacteria (cfu/ml)</td>
<td>$&lt;10^6$</td>
</tr>
<tr>
<td>LTA, LPS (ng/ml)</td>
<td>30</td>
</tr>
<tr>
<td>Insoluble PGN (ng/ml)</td>
<td>100</td>
</tr>
<tr>
<td>Soluble PGN (ng/ml)</td>
<td>100</td>
</tr>
<tr>
<td>LTA, LPS (ng/ml) with 10 ng/ml PGN</td>
<td>0.3</td>
</tr>
</tbody>
</table>
**Table 2.** The level of pro-inflammatory cytokines in human blood collected from sepsis patients (38, 50, 88, 170, 185).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Gram-negative sepsis</th>
<th>Gram-positive sepsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>High and Fast</td>
<td>Moderate and Slow</td>
</tr>
<tr>
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<td>IL-18</td>
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</tr>
<tr>
<td>IL-8</td>
<td>High</td>
<td>Low</td>
</tr>
</tbody>
</table>
GENERAL LIST OF REFERENCES


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CHAPTER 1

A NEW STRUCTURE OF PNEUMOCOCCAL LIPOTEichoIC ACID
EXPLAINS ITS BIOSYNTHeSIS AND SEROLOGICAL PROPErTY

by

Ho Seong Seo, Robert T. Cartee, David G. Pritchard, and Moon H. Nahm

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Format adapted and errata corrected for dissertation
**ABSTRACT**

Lipoteichoic acid (LTA) is an essential bacterial membrane polysaccharide (cell wall component) that is anchored to the membrane via a lipid anchor. According to the currently accepted structure of pneumococcal LTA, the polysaccharide is comprised of several repeating units, each of which starts with glucose and ends with ribitol, and the lipid anchor is predicted to be \( \text{Glc(} \beta_1 \rightarrow 3 \text{)AATGal(} \beta_1 \rightarrow 3 \text{)Glc(} \alpha_1 \rightarrow 3 \text{)-acyl}_2 \text{Gro.} \) However, this lipid anchor has not been detected in pneumococcal membranes. Furthermore the current structure does not explain the Forssman antigen properties of LTA, and predicts a molecular weight for LTA that is larger than its actual observed molecular weight. To resolve these problems, we analyzed the structure of LTA isolated from several \( S. \ pneumoniae \) strains using mass spectrometry. Our study found the R36A pneumococcal strain produces LTA that is more representative of pneumococci than that previously characterized from the R6 strain. Analysis of LTA fragments obtained after HF and HNO\(_2\) treatments showed the fragments that were consistent with the non-reducing terminus of LTA being \( \text{GalNAc(} \alpha_1 \rightarrow 3 \text{)GalNAc(} \beta_1 \rightarrow , \) which is the minimal structure for the Forssman antigen. Based on these data, we propose a revised structure of LTA: Its polysaccharide repeating unit begins with GalNAc and ends with AATGal, and its lipid anchor is \( \text{Glc(} \alpha_1 \rightarrow 3 \text{)-acyl}_2 \text{Gro,} \) a common lipid anchor found in pneumococcal membranes. This new model accurately predicts the observed molecular weights. The revised structure for LTA should facilitate investigation of the relationship between LTA’s structure and its function.
INTRODUCTION

As a major component of the membrane (cell wall) of all Gram-positive bacteria, lipoteichoic acid (LTA) is important for bacterial survival, growth and pathogenicity. LTA along with teichoic acid (TA) forms a polyanionic barrier, which provides protection against many cationic antimicrobial peptides such as bacteriocins (e.g., nisin) or host antimicrobial peptides (e.g., defensin) (1, 30, 118, 120). LTA is also important for bacterial growth since LTA can inhibit autolysin which is critical for cell wall remodeling and necessary for replication (93). In addition, LTA is critical in pathogenesis as it is involved in bacterial adhesion to host cells (11, 29, 32) and initiates inflammatory cascades by activating complement (98), TLR2 (47, 75, 167), and/or CD36, a C-type lectin (91, 131). In fact, a monoclonal antibody to LTA has been shown to protect animals from experimental infections (188, 202, 207).

As effectiveness of LTA in mediating these functions is highly dependent on small changes in LTA structure such as alanine or phosphocholine (PC) decorations (44, 114, 118, 216), it is important to know the exact structure of LTA. Fischer and his colleagues extensively studied LTA purified from the R6 strain of pneumococcus (54, 55) and showed that pneumococcal LTA is composed of a lipid anchor and a pentameric repeat unit that contains Glc, AATGal, two GalNAc, and ribitol-phosphate residues (Model A in Figure 5) (54, 55). Either one or both of the GalNAc residues is modified with PC. Based on this chemical structure, it is assumed that the polymer is formed by linking 2 to 8 repeating units, which begin with glucose and end with ribitol with phosphodiester bonds (Model A in Figure 5). The polymer would then be linked to a
cytoplasmic membrane lipid anchor: Glc(β1→3)AATGal(β1→3)Glc(α1→3)-acyl2Gro. The lipid anchor is critical for anchoring LTA to the cytoplasmic membrane (12, 53, 71, 115).

The currently accepted structure proposed for pneumococcal LTA (Model A) has several problems. For instance, the predicted lipid anchor could not be detected in pneumococcal membranes (54). The accepted structure does not contain terminal GalNAc(α1→3)GalNAc(β1→), which is necessary to explain Forssman antigen properties of pneumococcal LTA (12, 84). In addition, when we examined mass spectra of LTA from the R36A strain, we found its mass to be about 350 atomic mass unit (AMU) less than the predicted mass (114, 168). To investigate these problems, we hypothesized that the repeating unit biosynthesis begins with AATGal instead of ribitol and that the repeating unit is anchored to Glc(α1→3)-acyl2Gro (Model B in Figure 5), which is abundantly present in the pneumococcal membrane (27, 54, 108). Our evaluation of the two proposed LTA structures (Model A and Model B) follows.

**MATERIALS AND METHODS**

**Chemicals and bacterial isolates:** Forty eight percent hydrofluoric acid (HF) and sodium nitrite (NaNO₂) were obtained from Sigma-Aldrich (St. Louis, MO). *Streptococcus pneumoniae* R6 (ATCC BAA-255) and R36A (ATCC 12214) isolates were obtained from American Type Culture Collection (Manassas, VA). The parental strain of R6 is R36A, which is a nonencapsulated strain derived from a capsular type 2
clinical isolate (strain D39) (97, 99). A capsular type 3 isolate (WU2) was isolated as described (25). A capsular type 4 clinical isolate (TIGR4) (186) and a capsular type 6B clinical isolate (MX-73HIM), which was isolated from the pleural fluid of a pneumonia patient in Mexico, were provided by Dr. S. Hollingshead (University of Alabama at Birmingham, Birmingham, AL).

**Purification of lipoteichoic acid:** Pneumococcal LTA was purified by using Bligh-Dyer organic solvent extraction (18), Octyl-Sepharose chromatography, and an ion-exchange chromatography method, as described previously (12, 114). Briefly, pneumococci were cultured at 37°C for 10 h in Todd-Hewitt broth (Difco, Detroit, Mich.) with 0.5% yeast extract (Difco). Pelleted pneumococci were resuspended in 0.05 M sodium acetate (pH 4.0) and lysed by ultrasonication (Sonicator model W-220F from Heat Systems Ultrasonics, Inc., Plainview, N.Y.). After its extraction from the lysate with a chloroform and methanol mixture, the LTA was adsorbed onto an Octyl-Sepharose CL-4B column (Sigma-Aldrich) equilibrated in a mixture of 15% n-propanol and 0.05 M sodium acetate (pH 4.7). After eluting the LTA with 35% of n-propanol, the LTA was further purified by Q-Sepharose ion-exchange chromatography (Sigma-Aldrich) equilibrated in 10 mM 2-amino-2-methyl-1-propanol-HCl (pH 9.5) (Sigma-Aldrich) with 25% n-propanol. LTA was eluted using a 0 to 0.6 M sodium chloride gradient.

**Treatment with 48% hydrofluoric acid:** Five milligrams of purified R36A LTA in a 10-ml polypropylene tube was hydrolyzed with 1 ml of 48% HF at 4°C for 36 h to hydrolyze completely or for 3 h to hydrolyze partially. The samples were dried under a nitrogen stream for 1 h at room temperature to remove the HF. The samples were
adsorbed onto an Octyl-Sepharose CL-4B column (0.5×5 cm) and after washing the column with a mixture of 15% n-propanol and 50 mM sodium acetate (pH 4.7), the acylated LTA fragments were eluted from the column with 35% n-propanol.

**Deamination with HNO2:** Five milligrams of purified R36A LTA was dissolved in 0.5 ml of 0.2 M sodium acetate (pH 4.0) containing 0.5% sodium nitrite. After 40 min of incubation at room temperature, the sample was lyophilized and dissolved in 0.5 ml of PBS (pH 7.2).

**MALDI-TOF mass spectrometry:** Purified and treated LTA samples were analyzed using a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry from PerSeptive Biosystems (Framingham, MA) in the Mass Spectrometry Shared Facility at the University of Alabama at Birmingham. Briefly, a mixture of 1 µl of sample and 1 µl of matrix solution (0.5 M 2,5-dihydroxybenzoic acid and 0.1% trifluoroacetic acid in methanol) was applied to a sample plate. After drying, the sample was analyzed with a MALDI-TOF mass spectrometer.

**Tandem mass spectrometry:** The tandem mass spectral analyses of LTA fragments were performed in the Mass Spectrometry Shared Facility at the University of Alabama at Birmingham with a Micromass Q-TOF2 mass spectrometer (Micromass Ltd., Manchester, United Kingdom) equipped with an electro-spray ion source. After being dissolved in distilled water, the samples were injected into the mass spectrometer along with running buffer (acetonitrile-water [1:1] containing 0.1% formic acid) at a rate of 1 µl/min using a Harvard syringe pump. The injected sample was positively ionized with electrospray (needle voltage of 2.8 kV) and detected with a time-of-flight mass
spectrometer. For tandem mass spectrometry (MS/MS), the parent ion was fragmented into daughter ions by energizing it to 34-50 eV before collision with argon gas. The daughter ions were analyzed with a time-of-flight mass spectrometer. The MS/MS spectra were processed using the Max-Ent3 module of a MassLynx 3.5.

RESULTS

1) R36A LTA is representative of pneumococcal species.

In our previous studies, we observed that the mass of LTA isolated from the R36A strain is about 350 AMU less than the mass predicted by the currently accepted structure of LTA (114). Since the accepted model of LTA structure was established with LTA isolated from S. pneumoniae strain R6 (12), we wanted to exclude strain differences as the basis for the observed mass discrepancy. To do this we isolated LTA using a method based on Fischer’s classical method (12, 114) and directly compared the mass spectra of LTA preparations from both the R36A and R6 strains. Even though the classical method is known to remove alanine groups from ribitol (44), we used the method to facilitate comparison of Fischer’s results with ours. Consistent with our previous report (114), the mass spectrum of R36A LTA showed three major peaks at approximately m/z 7272, 8573, and 9872 (Figure 6B). The three peaks correspond to LTA with 5, 6, and 7 repeating units and a lipid anchor of about 756 mass units (i.e., 8573=6•1299+756+Na⁺). Each major peak was accompanied by several satellite peaks
Figure 5 Models of the molecular structure of *S. pneumoniae* LTA. Model A depicts the currently accepted structure (54) and Model B depicts a newly proposed structure. Models A and B have lipid anchors with 1105 and 756 AMUs, respectively, but both models have repeating units with 1299 AMU. For the LTA with 6 repeating units, Models A and B predict 8922 and 8573 AMUs respectively.
### Predicted Mass of LTA (Model A)

<table>
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<tr>
<th>Repeating unit (RU)</th>
<th>Missing PC groups</th>
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<tbody>
<tr>
<td></td>
<td>none</td>
</tr>
<tr>
<td>5 RU</td>
<td>7623</td>
</tr>
<tr>
<td>6 RU</td>
<td>8922</td>
</tr>
<tr>
<td>7 RU</td>
<td>10221</td>
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### Predicted Mass of LTA (Model B)

<table>
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<th>Repeating unit (RU)</th>
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<tr>
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</tr>
<tr>
<td>6 RU</td>
<td>8572</td>
</tr>
<tr>
<td>7 RU</td>
<td>9871</td>
</tr>
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</table>
Figure 6  Panels A to F show the mass spectra of LTA isolated from several strains. Strains are identified at the top of each panel. Each major peak is labeled with its own AMU. Each peak has at least three satellite peaks that differ from the major peak by 26-28 AMU. The satellite peaks are shown for the mass spectra of R36A LTA (insert in Panel B). R6 LTA (Panel A) has three groups of peaks (labeled X, Y, and Z), with the peaks within a group being separated by about 165 AMU. Panels G and H show the predicted masses of LTA with 5, 6, or 7 repeating units (RU) and with 0, 1, 2, or 3 PC groups missing using Model A (Panel G) or Model B (Panel H). The formula for the predicted masses was: \((1299 \times \text{number of repeating units}) + \text{l lipid anchor} - (\text{number of missing PC groups} \times 165) + \text{Na}^+ \) (23). The mass of the lipid anchor is 1105 AMU for Model A and 756 AMU for Model B.
with about 28- or 26-AMU differences (insert in Figure 6B). These satellite peaks reflect the microheterogeneity in acyl groups (12). The mass difference between the major peaks is 1299-1302 AMU, which corresponds to an oligosaccharide repeating unit that contains 2 PC groups. The currently accepted LTA structure shown as Model A in Figure 1 predicts 8922 AMU for LTA with 6 repeating units (Figure 6G). Since Model A also has a repeating unit mass of 1299, the mass discrepancy of 349 AMU (8922 vs. 8573) is likely not in the repeating unit but rather in the lipid anchor (Figure 6G and 6H).

In contrast, LTA from the R6 strain shows a different mass spectrum (Figure 6A). Instead of three isolated peaks, it shows three groups (X, Y, and Z) of peaks, with each group containing several distinct peaks that are separated by about 165 AMU, which corresponds to the mass of PC. For instance, group Y has seven peaks, three of which are heavier and three of which are lighter than the 8075 peak. The seven peaks can be explained if they represent LTA with 6 repeating units but missing 0-6 PC groups. The 8075 peak should represent LTA missing 3 PC groups since the three heavier peaks should represent LTA missing 0, 1, or 2 PC groups and the three lighter peaks should represent LTA missing 4, 5, or 6 PC groups. Furthermore, the masses of these peaks are correctly predicted by Model B (Figure 6H) but not by Model A (Figure 6G).

Groups X and Z have six and eight distinct peaks, respectively, and the mass difference among the fourth peaks (from the right) in each of the three groups (6774, 8075, and 9375) is roughly 1300 AMU. The mass spectra suggest that group X represents LTA with 5 repeating units and that its peaks represent LTA missing 0-5 PC groups. Similarly, group Z peaks represent LTA with 7 repeating units missing 0-7 PC groups. The masses of the peaks in groups X and Z could also be correctly predicted by
Model B (Figure 6H). Consistent with our findings, the repeating units of the R6 LTA were reported to have either one or two PC residues (12). Thus, the R6 and R36A LTAs differ in their mass spectra, but this difference is only due to the variable number of PC residues per repeating unit.

Since the R6 and R36A LTAs differ in structure, we determined the typical structure of pneumococcal LTA by studying LTAs purified from several different strains of \textit{S. pneumoniae}, including some clinical isolates (Figure 6C-F). All of the purified LTAs showed three major peaks and satellite peaks that are almost identical to the peaks of R36A LTA (Figure 6). Based on these data, we conclude that R36A LTA is more representative of pneumococcal LTA than is R6 LTA (Figure 6).

2) \textbf{Complete hydrolysis of LTA with 48\% HF yields molecular products predicted only by Model B.}

To determine if Model B correctly predicts the structure of LTA, we subjected LTA purified from R36A to hydrolysis with 48\% HF. HF rapidly cleaves phosphodiester and phosphomonoester bonds and then slowly cleaves the linkage between GalNAc and ribitol (12, 54, 102, 130). As a result of these hydrolysis reactions occurring at different rates, oligosaccharides without any PC (product B1 in Figure 7) would be produced due to the hydrolysis of the phosphodiester bond between the ribitol and Glc residues and due to the loss of the PC residues. Following these reactions, some of the resulting oligosaccharides would slowly lose the ribitol (product B2 in Figure 7). If Model B is the correct structure of LTA, then HF treatment would yield hydrolysis products A1 and A2.
Figure 7 Mass spectra of LTA fragments after complete 48% HF hydrolysis. Panel A shows the currently accepted pneumococcal LTA structure (Model A), and Panel B shows the newly proposed LTA structure (Model B). Dashed lines indicate the cleavage site by 48% HF hydrolysis. A1 and A2 indicate the fragments from the terminus that are uniquely found in Model B. B1 and B2 indicate the repeating unit. C1 and C2 indicate lipidated fragments. Panel C shows mass spectrum of the hydrolysates of LTA. The major peaks are identified with labels. Na\(^+\) indicates the sodium adducts; these peaks are 22 AMU heavier than the mass of the Na\(^+\) free ion or 22+1 AMU heavier than the mass of their structure alone.
(Figure 7B), whereas if Model A is correct, then these products would not be observed (Figure 7A).

Following HF treatment for 36 h at 4°C and analysis of the hydrolysate of R36A LTA by mass spectrometry, we observed peaks at \( m/z \) 581 and 599, which, respectively, correspond to the sodium adducts of product A1 that are anhydrous and hydrated (Figure 7C). The spectra also showed peaks at \( m/z \) 425 and 447, which correspond to product A2 and its sodium adduct (Figure 7C). The chemical nature of these four peaks was further confirmed by analyzing their daughter ions with a tandem mass spectrometer (data not shown). Since the peaks representing products A1 and A2 cannot be explained with Model A, these data support Model B as the correct structure of pneumococcal LTA. Products A1 and A2 were also observed with R6 LTA, indicating that Model B is also appropriate for R6 LTA.

The mass spectra of R36A and R6 LTA also showed peaks with masses that were consistent with the hydrolysis products labeled B1, B2, C1, and C2 in Figure 3B. When the chemical nature of these peaks was investigated with tandem mass spectrometry, the B1 and B2 peaks were found to correspond, respectively, to oligosaccharide with or without ribitol, and the C1 and C2 peaks were found to correspond, respectively, to lipid anchors with two or one acyl chains. The satellite peaks with +26 or -28 AMU differences associated with C1 and C2 also reflect the microheterogeneity of their acyl chains. Our findings are consistent with the previous report (12), which showed that the primary peak has C18:1 and C16:0 acyl chains, the +26 AMU peak has two C18:1 acyl chains, and the -28 AMU peak has C16:1 and C16:0 acyl chains.
Figure 8  Panel A shows the fragments predicted by Model A in response to a mild HF hydrolysis reaction and Panel B shows the fragments predicted by Model B in response to a mild HF hydrolysis reaction. Dotted lines indicate the preferential cleavage sites. The molecular weights of the fragments are shown. Panel C shows the mass spectrum of LTA before the hydrolysis reaction. Peaks at m/z 9874, 8574, and 7274 indicate LTA molecules with 5, 6, and 7 repeating units. Peaks at m/z 4941, 4292, and 3640 reflect ions with two positive charges and respectively correspond to ions with 9874, 8574, and 7274 AMUs. Panel D shows the mass spectrum of the acylated LTA fragments after a mild HF hydrolysis reaction. Acylated fragments were obtained by Octyl-Sepharose chromatography. The underlined numbers indicate the peaks representing the pre-hydrolysis LTA. The other numbers indicate the peaks that appeared after hydrolysis. Dotted arrows show the reduction in the mass (-952 AMU) between the pre-hydrolysis and post-hydrolysis peaks.
3) Partial hydrolysis of LTA with HF produces new peaks that have shifted down by 952 m/z units.

Hydrolysis with 48% HF for a very short time (3 h, 4°C) should only cleave the phosphodiester and phosphomonoester bonds of the LTA either removing one PC residue or making a single cut at the phosphodiester bond between the ribitol and Glc (indicated with dotted lines in Figure 8A and B). If Model A is correct, the partial digest would remove one or more complete repeating units (Figure 8A). Since each repeating unit has 1299 AMU, the mass of new peaks would be reduced by multiples of 1299 AMU and all new peaks would be “in phase” with original peaks. In contrast, if Model B is correct, this partial hydrolysis would remove the terminal GalNAc-GalNAc-Rib oligosaccharide and “out-of-phase” peaks that differ by 951 AMU would be observed (Figure 8B). For MALDI-TOF spectroscopy, masses of molecular fragments were calculated for their sodium adducts since phospholipids and LTA generally appear as ions with sodium adducts in MALDI-TOF studies (12, 59, 164).

Following a partial digestion of the R36A LTA and selection of acylated fragments by purification over Octyl-Sephrose, peaks with 952 AMU less than the original peaks (m/z 8923, 7624, and 6322) were observed by MALDI-TOF mass spectroscopy (Figure 9D). Additional “out-of-phase” peaks at m/z 5020 and 3718 were also observed. These peaks, which are separated by 1299-1302 AMUs, reflect the ions that have lost additional repeating units in addition to the 952 AMU loss. In addition to these peaks, new peaks were found at m/z 7109 and 8410. These peaks have values about 165 AMU less than the original peaks and should represent the loss of one PC residue,
A

B

C. Before reaction

D. After reaction (HNO₂)
Figure 9  Panel A shows the fragments predicted by Model B in response to a mild deamination reaction. Arrows with dotted lines indicate the preferential cleavage sites (129) and the molecular weight of the fragment that becomes dissociated from the acylated fragments. Panel B shows the mass spectrum of LTA before the deamination reaction. Peaks at \( m/z \) 4291 and 4941 represent ions with two charges. Panel C shows the mass spectrum of Panel B LTA after the deamination reaction. The underlined numbers indicate the peaks found in untreated LTA. Acylated fragments have satellite peaks and identified with * . The other numbers indicate the peaks that appeared after the deamination reaction.
which is also expected to occur during HF hydrolysis. Thus, these findings support Model B, not Model A.

4) Partial deamination of LTA with HNO does not create new peaks.

A deamination reaction with NaNO₂ in acid primarily cleaves the glycosidic bond on the reducing end of AATGal, although the reaction can secondarily cleave the glycosidic bond on the non-reducing end of AATGal (12, 55, 129). If deamination reaction conditions can be adjusted to permit only one cut at the primary cleavage site, the partial deamination could also be used to distinguish between the two models of LTA structure. Specifically, Model A predicts the production of “out-of-phase” peaks shifted by 348 AMU (Figure 9A), while Model B does not (Figure 9B). Pneumococcal LTA was subjected to a mild deamination and was examined by MALDI-TOF mass spectroscopy. The reaction product, which was not purified over the Octyl-Sepharose column, contained both acylated and non-acylated fragments. Reflecting this, MALDI-TOF spectra showed peaks with and without satellite peaks (Figure 9D). New peaks without satellite peaks were found at \( m/z \) 3914, 5215, 6516, 7816, and 9117. New peaks with satellite peaks were found at \( m/z \) 3367, 4668, and 5970.

Those with satellite peaks represent LTA that has lost 2 to 4 repeating units but that still has the lipid anchor. The other group without the satellite peaks corresponds to products containing 3 to 7 oligosaccharide repeating units that were released from the lipid anchor. The chemical nature of the repeating unit was further confirmed by analyzing daughter ions of the peak at \( m/z \) 1317, which was produced after a complete deamination reaction (Data not shown). None of the “out-of-phase” peaks predicted by
Model A were observed in the spectra, thus, the structure of LTA is consistent with Model B but not with Model A.

DISCUSSION

Model A in Figure 5 represents the currently accepted structure of pneumococcal LTA based on the study by Fischer’s group (12, 54). However, Model A predicts results that are inconsistent with our actual mass spectrometry results obtained with LTA from various pneumococcal isolates. Model B, which differs from Model A only in where the repeating unit biosynthesis begins, does predict results that are consistent with our new mass spectrometry results obtained following HF hydrolysis and deamination reactions. Model B is also consistent with the previous data reported by Fischer’s group. For instance, when they studied LTA after HF hydrolysis, they found molecular fragments labeled B1, B2, C1, and C2 in Figure 7B. Their study did not detect the molecular fragments labeled A1 and A2, which represent an incomplete repeating unit. This can also be readily explained because, following the hydrolysis step, Fischer’s group purified the intact repeating units before conducting their mass spectrometry studies (12, 54).

In addition to being consistent with all the biochemical study results, Model B resolves a serological dilemma that has been associated with Model A (12): Pneumococcal LTA has been known to express the Forssman antigen, which is found on oligosaccharides expressing GalNAc(α1→3)GalNAc(β1→) at their terminus (12, 84). Since Model A has GalNAc(α1→3)GalNAc(β1→) in the middle of the structure and not
at the terminus, Forssman antigenicity has been a significant problem for Model A (12, 54). On the other hand, Model B places GalNAc(α1→3)GalNAc(β1→) at the terminus of an intact LTA molecule as well as at its repeating units. In fact, Model B predicts repeating units with the minimal structure of Forssman antigens \{GalNAc(α1→3)GalNAc(β1→)\} prior to their decoration with PC.

Model B also explains a conundrum in LTA biosynthesis, which requires the linkage of the polymerized repeating units to a lipid anchor as the last step of LTA biosynthesis. According to Model A, the lipid anchor is Glc-AATGal-Glc-acyl2Gro. However, this lipid anchor has not been detected in pneumococcal membranes (54), and there are still no explanations as to how it becomes a part of LTA. In contrast, according to Model B, the required lipid anchor is Glc(α1→3)-acyl2Gro, which is known to be a major glycolipid of pneumococcal membranes (27, 54, 108).

Model B may resolve yet another problem associated with biosynthesis of LTA. The repeating unit of staphylococcal LTA is a simple glycerol phosphate and is directly polymerized into LTA by a single enzyme, LtaS (71, 72). However, the pneumococcal LTA repeating unit is much more complex (Figure 5), and the repeating unit assembly would, like the majority of the pneumococcal capsules (214), require an initial transferase to transfer the initial sugar onto a polypr enyl phosphate acceptor in the membrane. According to Model A, the initial transferase should transfer ribitol phosphate to the polypr enyl phosphate lipid acceptor; however, to date, no bacterial polysaccharide has been shown to initiate the formation of repeating units by the addition of ribitol phosphate. Even staphylococci with polyribitol phosphate TA do not begin the
polymerization of TA with the transfer of ribitol. Instead, their polymerization begins with the initial transfer of GlcNAc-1-phosphate to a polyprenyl phosphate lipid acceptor and is followed by repeated addition of the ribitol (149, 159). In contrast, Model B predicts that the initial sugar transferred to the polyprenyl phosphate lipid acceptor would be AATGal-1-phosphate. PS-A polysaccharide of *B. fragilis* also has a repeating unit that is initiated by the addition of AATGal, with the enzyme catalyzing this addition being encoded by *wcfS* (36). TIGR4 *S. pneumoniae* genome has SP1838, which is 44% identical to and 66% similar to *wcfS* (36). SP1838, which is presumed to be essential for pneumococcal survival (187), is also found in the pneumococcal genomes of D39 (SPD1619), R6 (Spr1654), and others (97, 122, 186). While additional studies are required, based on the revised structure of LTA (Model B), SP1838 could be the initial transferase for LTA synthesis.

Furthermore, since both TA and LTA of *S. pneumoniae* have repeating units with identical structures and are both likely synthesized by the same set of enzymes, SP1838 would also initiate TA repeating unit synthesis. Although LTA is attached to pneumococci by its lipid anchor, TA is attached to the cell wall through the muramic acid of the peptidoglycan (169). As with LTA, it has been thought that the repeating units of TA are linked together with phosphodiester bonds and that TA is attached to the cell wall by linking its ribitol to an unknown oligosaccharide linker via a phosphodiester bond (169). This view of TA structure is based on chemical analyses of TA and does not reflect the biological steps involved in the synthesis and polymerization of TA repeating units. As with LTA, the biological synthesis of TA repeating units should start with
AATGal and end with GalNAc, and AATGal may be involved in the attachment of TA to the peptidoglycan.

We found LTAs from pneumococcal strains R6 and R36A to be different in structure. Strain R6 was derived from strain R36A, which was, in turn, derived from strain D39. The mass spectrometry pattern of R36A LTA is similar to that of LTA from several strains, including two recent clinical isolates (TIGR4 and MX-73HIM). Also, experimental strains expressing fewer PCs are less virulent than are a wild-type strain (216). Thus, R6 LTA should be considered as a variant and R36A LTA should be considered representative of pneumococcal LTA. Mass spectral data indicated that about half of typical R6 LTA molecules have repeating units with 1 PC group and the other half have repeating units with 2 PC groups. Our findings are consistent with previous findings, which found 2 PC groups in 78% of R6 LTA repeating units and 1 PC group in 22% (12). licD1 and licD2 gene products are involved in transferring PC onto LTA/TA repeating units (111, 216), and an enzyme, Pce, is involved in removing PC from LTA/TA (200). Pneumococcal strains with a mutation in licD2 have only 1 PC group per repeating unit (216). However, inspection of R6 and D39 genome sequences shows identical DNA sequences for the three genes. Additional studies are needed to explain the structural differences between R6 and D39 LTAs.

Many species of Gram-positive bacteria can produce LTA with small structural alterations. In addition to the heterogeneity in PC, pneumococcal LTA may have galactose instead of glucose in the repeating units (199) or may have ribitol decorated with alanine (44, 118). Similarly, LTA from other bacterial species can be variably
decorated with alanyl or glucosyl groups (160). It is also becoming clear that these small structural changes have a significant impact on LTA functions such as resistance to a bacteriocin (118), adhesion to host cells (216), or pathogenic potential (1, 206). Yet it is difficult to investigate the impact of the small structural alterations on LTA function using LTA from many bacterial species (e.g., staphylococci) because a structural feature of their LTA (highly variable but large numbers of small glycerophosphate repeating units) prevents us to delineate their exact structure. With knowledge of and the ability to determine the precise pneumococcal LTA structure, pneumococci can be a model for investigating the impact of small alterations in LTA structure on bacterial survival, growth, and pathogenicity.
REFERENCES


CHAPTER 2

PLATELET-ACTIVATING FACTOR-ACETYLYHYDROLASE CAN MONODEACYLATE AND INACTIVATE LIPOTEICHOIC ACID

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ABSTRACT

Bacterial lipoteichoic acid (LTA) shares a structural motif with platelet activating factor (PAF). Both molecules are strong inflammatory agents and have a glycerol backbone with two lipid chains at the $sn$-1 and $sn$-2 positions. PAF is normally inactivated by PAF-acetylhydrolase (PAF-AH), a phospholipase A2 (PLA2), which removes a short acyl group at the $sn$-2 position. To investigate whether PAF-AH can similarly degrade LTA, we studied the effect of porcine PLA2, bee venom PLA2, and recombinant human PAF-AH on pneumococcal LTA (PnLTA) and staphylococcal LTA (StLTA). After incubation with a porcine or bee venom PLA2, a large fraction of PnLTA lost 264 Daltons, which correspond to the mass of oleic acid group at $sn$-2 position. After incubation with recombinant human PAF-AH, PnLTA lost 264 daltons and the reduction did not occur when PAF-AH was exposed to Pefabloc SC, an irreversible inhibitor of the PAF-AH active site. Following PAF-AH treatment, PnLTA and StLTA were not able to stimulate mouse RAW264.7 cells to produce TNF-$\alpha$ but could stimulate CHO cells expressing human TLR2. This stimulation pattern has been observed with monoacyl PnLTA prepared by mild alkali hydrolysis (114). Taken together, we conclude that PAF-AH can remove one acyl chain at the $sn$-2 position of LTA and produce a monoacyl-LTA that is inactive to mouse cells.
INTRODUCTION

A major component of the cell walls of Gram-positive bacteria is lipoteichoic acid (LTA), which is a polyphosphate polymer linked to a glycerol backbone with two acyl chains. The acyl chains anchor the LTA molecule to the bacterial plasma membrane like the acyl chains of LPS of Gram-negative bacteria. Like LPS, LTA is an amphipathic molecule and an important pathogen-associated molecular pattern (PAMP) capable of stimulating innate immunity and responsible for Gram-positive bacterial sepsis (75, 89, 203). For instance, staphylococcal LTA (StLTA) has been shown to induce septic shock like changes in rats when peptidoglycan is co-administered (41, 109, 110). Lastly, LTA stimulates toll-like receptor 2 (TLR2) and induces the production of various inflammatory molecules including TNF-α, IL-1 and nitric oxide (75, 181, 191) as LPS does via TLR4 (40).

In addition to resembling LPS, LTA has some similarities to PAF, which is a potent intermediate in the host response and can replicate many symptoms observed during bacterial sepsis (135). Both LTA and PAF molecules share structural similarity with a glycerol backbone with acyl groups at \( sn-1 \) and \( sn-2 \) positions (124). LTA may stimulate platelet activating factor receptor (PAFR) either directly (124) or indirectly by inducing production of endogenous PAF (219). In addition to PAFR stimulation, LTA may be able to modulate the degradation of PAF by competitive inhibition of PAF-AH.

PAF degradation is primarily dependent on plasma PAF-AH (PAF-AH) inasmuch as plasma PAF-AH deficient persons have a marked inability to clear PAF (106). PAF-
AH is a phospholipase A2 (PLA2) and inactivates PAF by removing the acetyl group at the sn-2 position (17, 81, 137, 171, 175). In addition to PAF, PAF-AH can remove acyl chains at the sn-2 position from various glycerolipids including diacylglycerols, triacylglycerols, and acetylated alkanols (141). However, PAF-AH has not been shown to deacylate a long acyl chain at sn-2 position and it is not expected to remove the long acyl chain found in LTA. If PAF-AH can degrade LTA however, the finding would have a significant impact in our understanding of the inflammatory properties of LTA. We have therefore investigated the ability of PAF-AH to deacylate LTA.

**MATERIALS AND METHODS**

**Reagents:** Recombinant human plasma PAF-AH was kindly provided by ICOS Corporation (Bothell, WA). This enzyme was prepared by expressing the full length cDNA of human plasma PAF-AH in Escherichia coli (190) and is as active as native plasma PAF-AH enzyme and has been used in clinical studies (86, 92). LPS of *E. coli* O55:B5 was purchased from Sigma-Aldrich (St. Louis, MO) and impurities were removed by further purification as described (90). Phosphatidylcholine, bee venom phospholipase A2, pancreas phospholipase A2 and Pefabloc SC were obtained from Sigma-Aldrich (St. Louis, MO). A synthetic lipoprotein (Pam3CSK4) was obtained from InvivoGen (San Diego, CA).
**Cell lines and bacteria:** The mouse macrophage cell line RAW264.7 (ATCC TIB-71) was obtained from American Type Culture Collection (Manassas, VA) and were cultured with Dulbecco’s modified Eagle’s medium (DMEM, Cellgro Mediatech, Herndon, VA) supplemented with 10% defined fetal bovine serum (HyClone, Logan, Utah), 2 mM L-glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. 3E10-TLR2 cell line, which constitutively expresses human CD14 and human TLR2, was from Dr. D. Golenbock (Boston Medical Center, Boston, MA). The cell line expresses CD25 on the cells surface in response to TLR2 ligands. The cells were grown in Ham’s F-12 medium (GIBCO-BRL, Rockville, MD) supplemented with 10% defined fetal bovine serum (HyClone, Logan, Utah), 1 mg/ml of G418 (Calbiochem, La Jolla, CA), and 400 U/ml of hygromycin B (Calbiochem, La Jolla, CA) at 37°C in a 5% CO₂ humidified incubator.

*S. pneumoniae* strain R36A (ATCC 12214) and *S. aureus* (ATCC 6538) were used for LTA purification. R36A was grown in Todd-Hewitt broth (Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.5% yeast extract (Becton Dickinson) until late log phase (OD₆₀₀=0.6–1.0). *S. aureus* was cultured in Tryptic-Soybean broth (Becton Dickinson) until late log phase.

**Purification of pneumococcal lipoteichoic acid:** Pneumococcal LTA was purified according to the method used by Behr et al. (12) with an additional purification step: ion-exchange chromatography. Briefly, *S. pneumoniae* (strain R36A) was cultured overnight at 37°C in Todd-Hewitt broth (Difco, Detroit, MI) with the supplement of 0.5% yeast extract (Difco) or chemically defined media (196). Cells were pelleted,
resuspended in 0.05 M sodium acetate buffer (pH 4.0), and disrupted by ultrasonication (Sonicator™ Model W-220F from Heat Systems Ultrasonics, Inc. (Plainview, N.Y.)). LTA was extracted from the lysate with a chloroform-methanol-water (1.0:1.0:0.9) mixture, and the aqueous phase containing LTA was collected following phase separation. LTA was then adsorbed onto an octyl-Sepharose CL-4B column (Sigma Chemical) equilibrated in 0.05 M sodium acetate buffer (pH 4.7) containing 15% n-propanol. LTA was eluted from the column with a stepwise n-propanol gradient (20%, 35%, and 45%), and column fractions containing the LTA were pooled and dialyzed against water. To further purify LTA by ion-exchange chromatography, LTA was adsorbed to a Q-Sepharose Fast Flow column (Sigma Chemical) equilibrated in a 10 mM 2-amino-2-methyl-1-propanol·HCl buffer (Sigma Chemical, pH 9.5 or pH 10.5) containing 20% n-propanol. LTA was eluted from the column with a continuous linear salt gradient (0.0 – 0.3 M of NaCl in the equilibration buffer), and the eluent was collected in 2-ml aliquots. Each fraction was individually dialyzed against pyrogen-free water, evaporated under vacuum, and stored at -20°C.

Highly purified lipoteichoic acid was isolated from *S. aureus* (ATCC 6538) by *n*-butanol extraction, as previously described (143). Briefly, the cells were cultured aerobically in Tryptic Soy broth (Difco, Detroit, MI) for 16 h at 37°C in a shaking incubator. The cells were harvested, suspended in 0.1 M sodium citrate buffer (pH 4.7), and disintegrated by ultrasonication (Sonicator™ Model W-220F). The cells were then mixed with an equal volume of *n*-butanol by stirring them for 30 min at room temperature. After centrifugation at 13,000 × g for 20 min, the aqueous phase was evaporated, dialyzed against pyrogen-free water, and equilibrated with 0.1 M sodium
acetate buffer containing 15% \textit{n}-propanol (pH 4.7). The LTA was first purified by hydrophobic interaction chromatography on an octyl-Sepharose CL-4B (Sigma) column (2.5 \texttimes 20 \text{ cm}). The column was eluted with a stepwise \textit{n}-propanol gradient (100 ml of 20\% \textit{n}-propanol, 200 ml of 35\% \textit{n}-propanol, and 100 ml of 45\% \textit{n}-propanol). Then, the column fractions containing LTA were pooled after inorganic phosphate assay and the pool was dialyzed against water. The LTA-containing fractions were further subjected to DEAE-Sepharose ion-exchange chromatography (Fast Flow, Sigma, 2.5 \texttimes 9.5 \text{ cm}) equilibrated in the 0.1 M sodium acetate buffer (pH 4.7) containing 20\% \textit{n}-propanol. The column was eluted with 300 ml of a linear salt gradient (0 – 1 M NaCl in the equilibration buffer) and the eluate was collected in 10 ml aliquots. The quantity of the purified LTA was determined by inorganic phosphate assay.

\textbf{Structure confirmation and purity determination of LTAs:} LTA molecules were characterized by MALDI-TOF mass spectrometry in the Mass Spectrometry Shared Facility in the University of Alabama at Birmingham. Briefly, 1 \mu l of a sample and 1 \mu l of matrix solution (0.5 M 2,5-dihydroxybenzoic acid, 0.1\% trifluoroacetic acid in methanol) were applied to a sample plate. After drying, the sample was analyzed with a mass spectrometer (Voyager Biospectrometry DE Pro workstation) from PerSeptive Biosystems (Framingham, MA).

The purities of the LTAs were determined by measuring their endotoxin contents with the Limulus Amebocyte Lysate assay (BioWhittaker, Walkersville, MD) and their DNA, RNA, or protein contamination with UV-absorbance at 260/280 nm. To assess protein contaminants, the samples were separated by polyacrylamide-gel electrophoresis.
Then the electrophoresis gel was stained with silver nitrate to visualize all protein bands and with the Western blot technique using TEPC-15 (33) to visualize the LTA bands.

**Quantification of LTAs:** The content of the pneumococcal LTAs was determined by measuring both inorganic phosphorus (49) and the amount of LTA equivalent to C-PS. For phosphorus determinations, samples were digested with a nitric acid-sulfuric acid mixture, and treated with molybdate and stannous chloride. OD at 600 nm of the samples was converted to phosphorus concentrations. The C-PS equivalence assay was an inhibition ELISA conducted as follows: Immulon 2 HB flat-bottom 96-well plates (Dynex Technologies Inc., Chantilly, VA) were coated by incubating them overnight with C-PS (1 μg/ml) in PBS and for 1 h with blocking buffer (1% BSA/PBS/0.05% sodium azide/0.05% Tween-20). Pneumococcal LTAs or C-PS that was serially diluted in the blocking buffer was added to the ELISA plates along with TEPC-15 antibody. After 1 h incubation at 37°C, the plates were washed 3 times with the wash buffer (PBS/0.05% Tween-20). Then alkaline phosphatase-conjugated goat-anti-mouse immunoglobulins were added to each well. After another 1 h incubation at 37°C, the plates were washed 3 times with the wash buffer, and para-nitrophenyl phosphate (Sigma Chemical) solution in diethanolamine buffer (pH 9.8) was added. When the colors developed, the absorbance was measured at 405 nm, and the absorbance was converted to LTA concentrations using the standard curve obtained with C-PS.

**Thin layer chromatography (TLC):** Five μl of phospholipid samples were applied onto a TLC plate (5 × 20 cm Silica gel 60C) (EMDscience, Hawthorne, NY). After drying, the plate was placed in a TLC chamber containing a 65:30:5 (v/v/v) mixture
of chloroform/methanol/ammonia (25%) at the bottom and the chromatography was performed for 90 min (153, 184). For visualization of phospholipids, TLC plates were then sprayed with 1.3 % of molybdenum blue spray reagent (Sigma) (121, 147).

**Determination of TNF-α production**: Peritoneal cells from C57BL/6 mice were obtained with PBS. The cells (10⁶ cells/ml) were suspended in RPMI 1640 supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell suspension was placed in 96-well plates (200 μl/well) overnight and was stimulated with LTA (or its variants) for 20 h.

RAW264.7 cells were placed in 96-well plates (Costar, Corning, NY) (2×10⁵ cells/well) and the cells were stimulated with 50 μg/ml of PnLTA, 1 μg/ml of StLTA, 0.1 μg/ml of LPS or 0.2 μg/ml of Pam3CSK4 for 24 hours. In some cases, the stimulants were treated with PAF-AH for 2 hours at 37 ºC at the indicated doses and PAF-AH was inactivated by incubating at 65 ºC for 2 hours or by adding 100 μM of Pefabloc SC prior to stimulation. The amount of TNF-α in the culture supernatant was determined with the sandwich type ELISA Ready-SET-Go kit (eBioscience, San Diego, CA), and the manufacturer’s protocol was followed.

**CD25 expressions by 3E10-TLR2 cells**: 3E10-TLR2 cells were placed in 6-well plates (Costar, Corning, NY) (5×10⁵ cells/well) and after 24 hours when the cells were 70% confluent, the cells were stimulated with various molecular preparations. After 16 hours, the cells were washed once with PBS (pH 7.3) and detached with 2 mM EDTA in
PBS. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD25 (Becton Dickinson, San Diego, CA), and their CD25 expression was determined on a FACSCalibur flow cytometer with CellQuest acquisition analysis software (Becton Dickinson, San Diego, CA).

RESULTS

1) Highly purified LTA stimulated TLR2, but not TLR4

To examine the ability of LTA to stimulate TLR, highly pure and structurally intact LTA was isolated from *S. pneumoniae* and *S. aureus*. First is that LTA preparation was added to the two NF-κB reporter cell lines (CHO/CD14/TLR2 and CHO/CD14/TLR4), and the percentage of CD25<sup>+</sup> cells was determined by flow cytometry after 16 h of culture. Prior to the exposure to any stimulants, about 10-15% of CHO/CD14/TLR2 or CHO/CD14/TLR4 cells expressed detectable amount of CD25. As expected, *E. coli* LPS (1 μg/ml) induced the expression of CD25 on CHO/CD14/TLR4 cells and synthetic lipoptotein (Pam3CSK4) (1 μg/ml) on CHO/CD14/TLR2 cells (Figure 10A). Upon exposure to 30 μg/ml of pneumococcal LTA and 3 μg/ml of staphylococcal LTA, about 58% and 78% of CHO/CD14/TLR2 cells expressed CD25 (Figure 10A). In contrast, the fraction of CD25<sup>+</sup> cells among CHO/CD14/TLR4 cells remained low (about 10%).
**Figure 10** LTA induces the inflammation via TLR2. (A), 3E10-TLR2 or 4 cells were treated with 30 μg/ml of pneumococcal LTA (PnLTA), 3 μg/ml of staphylococcal LTA (StLTA) or 1 μg/ml of LPS. Cellular activation of TLR-dependent NF-κB was determined by flow cytometry with measurement of CD25 reporter gene expression. (B), Mouse peritoneal macrophages harvested from C57BL/6 (Black bar) or C57BL/6 TLR2−/− (White bar) mice were stimulated with indicated concentrations of PnLTA, StLTA, or LPS for 24 hours. The concentrations of TNF-α released into the culture supernatants were determined by ELISA. Results are the mean ± SD of a representative experiment in triplicate. N.D. indicates “less than 50 pg/ml”.
Second approach is that preparation was added to the mouse peritoneal macrophages collected from WT and TLR2-/- mouse and the TNF-α production was determined. WT peritoneal macrophages produced TNF-α in response to StLTA, PnLTA, Pam3CSK4 and LPS, whereas TLR2-/- peritoneal macrophages produced TNF-α only in response to LPS. Thus our highly pure PnLTA and StLTA are TLR2 ligands, but not TLR4 (Figure 10B).

2) An acyl chain is essential in TLR2 stimulation by the pneumococcal LTA.

We next examined the molecular moieties necessary for the stimulation. For these studies, two LTA variants were produced: LTA-1 with only one acyl chain and LTA-0 with no acyl chains. To assess the impact of acyl chains on LTA function, we examined the abilities of LTA-2 (diacyl LTA), LTA-1 (monoacylated LTA) and LTA-0 (Deacylated LTA) to stimulate mouse peritoneal macrophage cells and mouse macrophage cell line (RAW264.7) to produce TNF-α. Each LTA preparation was purified using our pneumococcal purification method (114). When the mouse cells were stimulated with each LTA preparations at 50 μg/ml, LTA-2 induced TNF-α production about 10 fold more than did LTA-1 (Figure 11). This residual activity of LTA-1 may be due to a small amount of residual diacyl LTA (i.e., LTA-2). Completely deacylated LTA did not stimulate mouse cells (Figure 11). Taken together, these data suggest that LTA-1 do not stimulate mouse cells.
**Figure 11** Mono- and de-acylated pneumococcal LTA do not stimulate mouse cells. (A), RAW264.7 or (B) C57BL/6 mice peritoneal macrophages were stimulated with 50 μg/ml of deacylated LTA (LTA-0), monoacylated LTA (LTA-1) or diacylated LTA (LTA-2) for 24 hours. The concentrations of TNF-α released into the culture supernatants were determined by ELISA. Results are the mean ± SD of a representative experiment in triplicate.
3) PAF-AH can monodeacylate PnLTA.

To investigate the effect of PAF-AH on PnLTA, we incubated PnLTA (250 μg/ml in PBS) with various concentrations of PAF-AH (0.5, 5, 10 μg/ml) for 2 hours at 37°C and examined the reaction mixture by mass spectrometry. A previous study showed that changes in the mass spectra can be used to monitor the structural alterations of PnLTA (114). Prior to incubation with PAF-AH, PnLTA has distinct mass spectra with three major peaks (7297, 8598, and 9900 m/z), each corresponding to LTA with 5, 6, or 7 repeating units (Figure 12A). As previously observed, the major peaks had satellite peaks with 28 m/z difference (e.g., 8571 vs. 8598) (Figure 12B) consistent with the heterogeneity in the acyl chains (114). When PnLTA was incubated with high PAF-AH concentrations (5~10 μg/ml), a new peak at 8334 became dominant and the original peak at 8598 decreased significantly (Figure 12C) or became undetectable (Figure 12D). The new peak was very small but detectable when PnLTA was incubated with a low (0.5 μg/ml) concentration of PAF-AH (Figure 12B). This peak was barely detectable even before any PAF-AH treatment (Figure 12A, indicated with an arrow head), probably as a result of chemical instability of the second acyl chain (22). The appearance of the new peak was time-dependent; about 50% of LTA shifted in 1 hr and almost 100% in 6 hrs in the presence of 1 μg/ml of PAF-AH (Figure 12E). The peaks at 7297 or 9900 also lost 264 m/z in the same patterns.

Since PAF-AH is a PLA2, it is likely that the sn-2 acyl chain is removed. Indeed, the mass difference between the old and new peaks (264 m/z) is consistent with the loss of an oleic acid (Figure 12C). To further confirm that the sn-2 acyl chain was removed,
**Figure 12.** Mass-spectrum of PnLTA (250 μg/ml) before reacting with PAF-AH (A) and after reacting for 2 hours with different concentrations of PAF-AH (0.5, 5, and 10 μg/ml) (B, C, and D respectively). The peak heights were shown as “% intensity”, which indicates relative percentage of each peak height to the tallest peak height in each spectrum. Positions of various prominent peaks were shown in the Figure. (E) shows the height of the peaks at 8598 (square) and 8334 (solid circle) m/z units at various times. The x-axis indicates the number of hours LTA was incubated with PAF-AH at 37 °C.
Figure 13. Mass-spectrum of PnLTA (250 μg/ml) before any reaction (A) and after reacting with 10 μg/ml of PAF-AH for 3 hours at 37 °C (B), porcine pancreas PLA2 for 48 hours at 37 °C (C) and bee venom PLA2 for 48 hours at RT (D). The molecular weight of porcine pancreas PLA2 is about 14,000 (14) and the peak at 7006 observed in (C) corresponds to the porcine PLA2 with two charge units.
we treated PnLTA with two additional PLA2 enzymes- porcine pancreas and bee venom PLA2 and examined the reaction products with MALDI-TOF (Figure 13) (210). Untreated PnLTA showed the three major peaks as before although the peak position has shifted down by about 10 m/z unit compared to Figure 12. PnLTA (250 μg/ml) was incubated with PLA2 (50 μg/ml) in a buffer (160 mM HEPES (pH 7.4) and 10 mM CaCl2) for 24 or 48 hours (101). Although the enzyme reaction with these PLA2 enzymes was inefficient compared to PAF-AH, the two PLA2 enzyme treatments produced new peaks with 264- 267 mass units less than the original peaks (8591 vs 8324 in Figure 13C and 8589 vs 8325 in Figure 13D) as PAF-AH treatment did (Figure 12B). A peak with 7006 m/z was found only in porcine pancreas treated LTA (Figure 13C). This peak corresponds to porcine pancreas PLA2 with 2 charges (14). These findings lend further support that PAF-AH removed the acyl chain at the sn-2 position of PnLTA.

4) PAF-AH does not deacylate 1, 2-dipalmitoyl-phosphatidylcholine (DPPC) or lipoprotein

PAF-AH is only known to remove short acyl chains yet we observed deacylation of a long acyl chain from PnLTA. Therefore, we re-examined our preparation of PAF-AH for its ability to deacylate DPPC. As was done for PnLTA, DPPC (250 μg/ml) was incubated with 10 μg /ml of PAF-AH at RT for 6 hours, and the reaction mixture was analyzed by TLC. In TLC, monoacyl phosphatidyl choline (labeled as Lyso-PtCho in Figure 14A) can be easily distinguished from diacyl phosphatidyl choline (labeled as L-Pt-Chol in Figure 14A) by their differences in mobility (Figure 14A). The reaction
Figure 14. (A) The thin layer chromatogram of dipalmitoyl phosphatidyl choline (DPPC) (lane 1), lyso-DPPC (lane 2), and DPPC incubated with PBS (lane 3), and DPPC incubated with PBS containing PAF-AH (10 μg/ml) (lane 4). DPPC and lyso-DPPC were labeled L-PtCho and lyso-PtCho respectively. The concentration of DPPC or lyso-DPPC was 250 μg/ml and the incubation time was 6 hours. (B) the mass spectrum of a synthetic bacterial lipoprotein (Pam3CSK4, 250 μg/ml) before (top panel) and after (bottom panel) a reaction with 10 μg/ml of PAF-AH for 6 hours at 37 °C. The two major peaks correspond to Pam3CSK4 (at 1518) and its sodium salt (at 1540) (105).
mixture did not show any monoacyl phosphatidyl choline. Thus, our preparation of PAF-AH does not deacylate long acyl chain associated with DPPC.

Bacterial lipoproteins are important PAMPs and they have two acyl chains on a cysteine backbone (28). To investigate whether PAF-AH can deacylate these acyl chains, we incubated a synthetic lipoprotein, Pam3CSK4 with PAF-AH as we did for LTA. We examined the alterations in the molecular structure with mass spectrometry. Two major peaks at 1518 and 1540 correspond to Pam3CSK4 and its sodium salt respectively (105). Unlike LTA, we did not observe any changes in the molecular structure following the PAF-AH treatment (Figure 14B). Thus, PAF-AH does not deacylate Pam3CSK4 and is unlikely able to deacylate bacterial lipoproteins.

5) PAF-AH can be inactivated by heat or by adding serine protease inhibitors

The enzymatically active site of PAF-AH responsible for PAF deacylation can be neutralized by heating at 65 °C (48) or by treatment with Pefabloc SC (100 μM) (48, 68), a serine protease inhibitor. To investigate whether the active site of PAF-AH is responsible for deacylating LTA as well as PAF, we investigated whether heating or Pefabloc SC can inactivate PAF-AH’s activity on deacylating PnLTA. When PnLTA was incubated with PAF-AH at 37 °C for 2 hours, the major peak shifted from 8598 (Figure 15A) to 8336 (Figure 15B) as expected. Similar changes were observed for other peaks. However, such a change was not observed when PAF-AH was preincubated at 65 °C for 2 hours (Figure 15C) or was incubated with 100 μM of Pefabloc SC for 2 hours at
Figure 15. Mass spectra of PnLTA before (A) or after reacting with 5 μg/ml of PAF-AH for 2 hours (B, C and D) were shown. PAF-AH was inactivated by incubating it at 65 °C for 2 hours (C) or by incubating with 100 μM of Pefabloc SC for 2 hours at 37 °C (D).
37 °C (Figure 15D). Thus, the active site of PAF-AH used for PnLTA is likely identical to the active site used for PAF.

6) PAF-AH treatment alters functionality of LTA from PnLTA and StLTA.

We have previously noted that a controlled mild alkali hydrolysis produces monodeacylated PnLTA that can stimulate human cells but not mouse cells (114). To determine if the PAF-AH treated PnLTA behaves similarly, PnLTA was incubated with different concentrations of PAF-AH for 2 hours at 37 °C and treated with Pefabloc SC to stop PAF-AH activity. Pefabloc SC was not removed from the reaction mixture because it does not affect TNF-α or CD25 production by target cells (Figure 16 I and J). We stimulated mouse RAW264.7 cells and human 3E10-TLR2 cells with reaction mixtures for 24 hours. PAF-AH-treated PnLTA did not induce RAW264.7 cells to produce TNF-α (Figure 16A), but induced CD25 expression by 3E10-TLR2 cells (Figure 16B). Completely deacylated PnLTA did not stimulate either human or mouse cells (Figure 11).

Unlike PnLTA, the StLTA structure cannot be monitored by mass spectrometry. We considered that PAF-AH may monodeacylate StLTA and the resulting monoacyl StLTA stimulates human cells but not mouse cells as PnLTA. To investigate whether PAF-AH also monodeacylate StLTA, we incubated highly purified StLTA with PAF-AH and examined the biological properties of the resulting StLTA. As hypothesized, StLTA treated with PAF-AH was able to stimulate human cells (Figure 16D) but not mouse cells (Figure 16C), although untreated StLTA stimulated both mouse and human cells.
Figure 16. TNF-α production by mouse cells (left panels) and CD25 expression by human cells (right panels) in response to 50 μg/ml of PnLTA (A and B), 3 μg/ml of StLTA (C and D), 0.2 μg/ml of Pam3CSK4 (E and F), and 0.1 μg/ml of LPS (G and H) were shown. To examine the effect of Pefabloc SC itself, cells were stimulated with various stimulants (Panels I and J) - 50 μg/ml of PnLTA, 3 μg/ml of StLTA, 0.2 μg/ml of Pam3CSK4 and 0.1 μg/ml of LPS in the presence (black bar) or absence (white bar) of 100 μM of Pefabloc SC. Mouse cells (RAW264.7) were stimulated for 48 hours and the TNF-α level was determined by ELISA. 3E10-TLR2 cells were stimulated for 16 hours and CD25 expression was determined by flow cytometry. The stimulants were treated with PAF-AH for 2 hours at 37 °C at the indicated doses and incubated at 65 °C for 2 hours or with 100 μM of Pefabloc SC before stimulation.
(Figure 16C and D). Completely deacylated StLTA produced by alkali hydrolysis did not stimulate both human and mouse cells (Data not shown). PAF-AH treatment did not alter the capacity of another TLR2 ligand Pam3CSK4 (200 ng/ml) or a TLR4 agonist, lipopolysaccharide (100 ng/ml) to stimulate human or mouse cells (Figure 16E, F, G and H) and low concentration of Pam3CSK4 (20 ng/ml) and LPS (10 ng/ml) were also not altered by PAF-AH treatment (Data not shown). Taken together, deacylation is specific for LTA and PAF-AH can monodeacylate not only PnLTA but also StLTA.

**DISCUSSION**

Here we demonstrate that PAF-AH can remove an acyl chain from PnLTA in a time- and concentration-dependent manner, apparently using the enzymatic site used for PAF. PAF-AH removed the acyl chain at the sn-2 position as shown by the size of the mass loss and also because two other PLA2 enzymes produced the same mass loss. Furthermore, PAF-AH appears to be able to deacylate StLTA as well as PnLTA since PAF-AH treatment endows both LTAs with the characteristic functional pattern of monoacyl LTA (114); active on human cells but inactive on mouse cells. The deacylation appears to be specific for LTA since PAF-AH did not remove acyl chains from DPPC or Pam3CSK4, a model bacterial lipoprotein.

PAF-AH has only been shown to remove short (4-6 carbons) acyl chains at the sn-2 position (172) although it can remove a slightly longer acyl chain if carboxylic or aldehydic groups are present at the ω site (174). Since LTA has a very long acyl chain
(18 carbons) at $sn$-2 position and lacks carboxylic or aldehydic groups at the $\omega$ end, our finding was therefore totally unexpected. PAF-AH, unlike other PLA2, reacts on substrates in solution but not those in micelles (141). Perhaps, PAF-AH can deacylate LTA since a large portion of LTA exists as free molecules in solution due to a large hydrophilic repeating unit at the $sn$-3.

Inactivation of PAF has been considered to be the physiological role of PAF-AH. But its physiological role may now include degradation of LTA from various Gram-positive bacterial species. In support of this idea, we have observed that culture supernatants of several Gram-positive bacterial species such as *Streptococcus mutans* and group B streptococcus become unable to stimulate RAW264.7 cells to produce TNF-$\alpha$ following PAF-AH treatment (Data not shown). Since we used 1-10 $\mu$g/ml for PAF-AH treatments and normal blood levels of PAF-AH are about 1 $\mu$g/ml in blood (150), blood should have enough PAF-AH to inactivate biologically relevant amounts of LTA. Indeed, plasma lipoproteins, which harbor PAF-AH in plasma (141), have been shown to inactivate LTA in the presence of LBP (73).

The importance of PAF in experimental sepsis is clearly established. Injection of PAF can cause many symptoms associated with sepsis (32). PAF levels are elevated and PAF-AH levels decrease during clinical sepsis (150). PAF-AH was therefore considered as a pharmacologic agent for reducing PAF and treating bacterial sepsis. Recent clinical trials of PAF-AH however, did not show clear clinical benefits (150). We have shown here that PAF-AH monodeacylates LTA, which is shown to be important in an experimental model of Gram-positive sepsis in animals (109). Since about half of
bacterial sepsis cases are due to Gram-positive bacteria (34, 94), one should consider that deacylation of LTA as another pharmacological action of PAF-AH.

PAF-AH appears to use the same enzymatic site to deacylate PAF and LTA. Thus LTA, if it is present in a high concentration, may interfere with the ability of PAF-AH to deactivate PAF. LTA may be present in body fluids at high concentrations for several reasons. LTA is shed from bacteria easily and early bacterial cultures in vitro can achieve 1 μg/ml (1 μM) of LTA (198). Antibiotics such as penicillin can kill a large number of bacteria and release their LTA as a burst (136, 142). Further, bacteria and their LTA are confined to a small anatomic area during meningeal or middle ear infections. It would be interesting to study whether LTA can function as a competitive inhibitor of PAF-AH inactivating PAF in these clinical situations.
REFERENCES


CHAPTER 3
LIPOTEichoIC ACID IS IMPORTANT IN INNATE IMMUNE RESPONSE TO
GRAM POSITIVE BACTERIA

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ABSTRACT

To define the role of lipoteichoic acid (LTA) in innate immunity to Gram-positive (Gr+) bacteria, we investigated the production of TNF-α by macrophages stimulated with Gr+ bacterial culture supernatants (GPCSs) after their LTA was removed or inactivated. GPCSs were obtained from three Gr+ species (pneumococci, staphylococci, and group B streptococci) during the exponential growth phase (labeled early GPCS) or at the senescent stage (labeled late GPCS). LTA was removed using an anti-LTA antibody or was inactivated by alkaline hydrolysis or platelet activating factor-acetylhydrolase (PAF-AH) treatment. Both early and late GPCS from the three Gr+ bacteria stimulated macrophages to produce TNF-α primarily via TLR2 although late pneumococcal supernatant could stimulate via TLR4 as well. Following both LTA inactivation methods, early GPCS lost about 85-100% of its activity and late GPCS lost about 50-70%. Both early and late culture supernatants from Escherichia coli could be inactivated by alkali hydrolysis, but not by PAF-AH. In addition, removal of LTA from an early staphylococcal culture supernatant with a monoclonal antibody reduced about 70-85% of its potency. Reconstitution of inactivated early GPCS with a highly purified LTA restored its inflammatory activity but the restored GPCS had higher activity than the pure LTA alone. These findings indicate that LTA is the primary TLR2 ligand in the early phase of Gr+ bacterial infection and remains a major ligand in the late phase when other TLR2 and TLR4 ligand(s) appear. In addition, our findings suggest that other Gr+ bacterial factor(s) synergize with LTA in inducing inflammatory responses.
INTRODUCTION

Innate immunity to bacteria provides the first line of defense against bacterial invasion by triggering the host’s initial inflammatory responses (16, 43, 156). Central to innate immunity are Toll-like receptors (TLRs), which recognize the conserved pathogen-associated molecular patterns (PAMPs) and trigger the innate immune response (3, 4, 195). In the case of Gram-negative (Gr-) bacteria, TLR4 is the primary receptor and mainly senses LPS, which is a major component of the outer membrane (90, 96, 183). For Gram-positive (Gr+) bacteria, TLR2 is the primary host receptor involved in inflammatory responses to the bacteria (46, 116, 180), but the nature of TLR2 ligands is unclear.

Several components of Gr+ bacterial cell wall have been proposed to be TLR2 ligands. Although there are controversies (60, 91, 100), peptidoglycan (PGN) may be a TLR2 ligand (45). Many studies suggested that lipoteichoic acid (LTA) is the key TLR2 ligand (44, 166, 167, 193). Indeed, highly purified LTA as well as chemically synthesized LTA analogs can stimulate TLR2 (145). Despite this body of evidence, a recent study reported evidence that a contaminating lipoprotein, not LTA, may be the TLR2 ligand even when LTA was purified using an updated method (77, 79, 201). This controversy arises primarily because of difficulties in obtaining LTA without structural damage and/or biologically active contaminants although methods of purifying LTA have been greatly improved (47, 56, 114).

Therefore, to evaluate the role of LTA in innate immune responses to Gr+ bacteria, we investigated the effects of inactivating LTA on the inflammatory properties
of bacterial culture supernatants, which contain molecules that are shed or released from bacteria (83, 87, 104, 177, 197, 208). Selective inactivation was possible since we have shown that platelet activating factor-acetylhydrolase (PAF-AH), a phospholipase A₂ (PLA₂), selectively inactivates staphylococcal and pneumococcal LTAs by removing their acyl-2 chain (168), but does not affect other acylated bacterial molecules such as LPS and phosphatidylcholine (168). We now report the effects of LTA inactivation on the inflammatory properties of Gr+ bacterial culture supernatants (GPCS).

**MATERIALS AND METHODS**

**Reagents:** Recombinant human plasma PAF-AH was kindly provided by ICOS Corporation (Bothell, WA). This enzyme was prepared by expressing the full-length cDNA of human plasma PAF-AH in *Escherichia coli* (39), is as active as native plasma PAF-AH enzyme, and has been used in clinical studies (12, 15). Pefabloc SC and anti-phosphorylcholine (PC) antibody (TEPC-15) were obtained from Sigma-Aldrich (St. Louis, MO). Mouse anti-LTA monoclonal IgG1 antibody (BD1701) and rabbit anti-staphylococcal LTA polyclonal antibody were obtained from BioDesign (Saco, ME). A monoclonal antibody (mAb) to pneumolysin (NCL-SPNm) was purchased from NovoCastra (Newcastle, UK). mAb to pneumococcal surface protein A (PspA) (Xir126) (37) and rabbit polyclonal antibody to pneumococcal surface protein C (PspC) (26) were obtained, respectively, from Dr. D. Briles (University of Alabama at Birmingham, Birmingham, AL) and Dr. L. McDaniel (University of Mississippi Medical Center, Jackson, MS). A synthetic lipoprotein (Pam3CSK4) and synthetic muramyl dipeptide
(MDP) were obtained from Calbiochem (San Diego, CA). The TLR ligands Poly (I:C), FSL-1, ODN1826, and imiquimod (R837) were purchased from Invivogen (San Diego, CA). Protein-G conjugated agarose was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit anti-pneumococci antiserum was prepared by immunizing a rabbit with three monthly intramuscular injections of $10^8$ heat-killed R36A strain of pneumococcus in incomplete Freund’s adjuvant and by obtaining serum 1 month after the last immunization.

**Cells and culture conditions:** The mouse macrophage cell line RAW264.7 (ATCC TIB-71) was obtained from American Type Culture Collection (Manassas, VA) and was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Cellgro Mediatech, Herndon, VA) supplemented with 10% defined FBS (HyClone, Logan, UT), 2 mM L-glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. Two Chinese hamster ovary (CHO) cell lines 3E10-TLR2 or 3E10-TLR4, which constitutively express human CD14 and human TLR2 or TLR4 respectively, were obtained from Dr. D. Golenbock (Boston Medical Center, Boston, MA) (138). All cell lines express inducible membrane CD25 under control of a region from the human E-selectin (ELAM-1) promoter containing NF-κB binding sites. The cells were grown in Ham’s F-12 medium (GIBCO-BRL, Rockville, MD) supplemented with 10% defined FBS (HyClone, Logan, Utah), 1 mg/ml of G418 (Calbiochem, La Jolla, CA), and 400 U/ml of hygromycin B (Calbiochem, La Jolla, CA) at 37°C in a 5% CO₂ humidified incubator. To determine the responses of these CHO cell lines to bacterial products, CHO cells ($1 \times 10^5$/ml) were incubated with various bacterial culture supernatants for 16 h, harvested using 10mM EDTA, stained for CD25 expression with
PE-labeled anti-CD25 (Becton Dickinson, San Diego, CA), and analyzed by flow cytometry.

**Bacterial strains and the generation of bacterial supernatants:** *S. pneumoniae* strain R36A (ATCC 12214), *S. aureus* (ATCC 6538), group B streptococcus (GBS) strain COH1, and *E. coli* ER2357 were cultured in DMEM with 5% FBS, and supernatants were harvested at mid-log phase (OD$_{600}$=0.4–0.47) and late-stationary phase culture (at 16 h). *S. pneumoniae* strain D39 and its isogenic mutants PLN-A ($\Delta$Pneumolysin) (15), JY53 ($\Delta$PspA) (215), and Tre108 ($\Delta$PspC) (9) were cultured in a mixture of 8 volumes of DMEM with 5% FBS and 2 volumes of Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco).

**Mouse peritoneal macrophages:** C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and TLR2$^{-/-}$ and TLR4$^{-/-}$ mice on a C57BL/6 background were bred in our animal facility by Dr. S. Michalek using breeding pairs obtained under a material transfer agreement from Dr. S. Akira (Osaka University, Osaka, Japan) via Dr. D. Golenbock. Peritoneal macrophages were collected 3 days after i.p. injection of 3 ml of 3% thioglycollate. Cells were resuspended in DMEM (10% FBS), and $10^6$ cells were seeded onto wells of a 96-well plate. After 4 h of incubation at 37°C, unbound cells were removed by washing the wells three times with medium. Cells were stimulated with various bacteria culture supernatants for 24 h at 37 °C in a 5% CO$_2$ humidified incubator.

**Neutralizing LTA activity in bacterial culture supernatants:** Three different approaches were used to inactivate or remove LTA in bacterial culture supernatants. In
the first approach, 9 volumes of bacterial supernatants were mixed with 1 volume of 2 N NaOH, incubated for 2 h at 37°C, and neutralized with 6 N HCl to a pH of 6.5-7.5. In the second approach, the supernatants were incubated at 37°C for 2 h with varying concentrations of PAF-AH and then mixed with Pefabloc SC (100 μM final concentration) as we have described previously (168). In the third approach, LTA was removed from staphylococcal GPCS with an anti-LTA mAb (BD1701). Briefly, the mAb was first immobilized to protein G-coupled agarose beads by incubating the two reagents together (final concentrations: 40 % beads and 10 μg/ml of mAb) with gentle shaking for 6 h at 4°C. After washing the agarose beads twice with PBS, the agarose bead pellets were mixed with the same volume of staphylococcal GPCS and the mixture was gently shaken for 2 h at 4°C. LTA-depleted GPCS was obtained by removing agarose beads with centrifugation.

Purification of lipoteichoic acid: Pneumococcal and staphylococcal LTA were prepared using organic solvent extraction, octyl-Sepharose, and an ion-exchange chromatography method, as previously described (47, 56, 114). The level of endotoxin contamination was determined using the QCL-1000 quantitative chromogenic Limulus amoebocyte lysate assay, according to the manufacturer’s directions (Bio-Whittaker). The resulting LTA preparations contained less than 10 pg of endotoxin per mg of LTA.

TNF-α measurement: RAW264.7 cells (2×10^5 cells/well) and mouse peritoneal macrophages were cultured in 96-well plates (Costar, Corning, NY), and stimulated with bacterial culture supernatants for 24 h. The amount of mouse TNF-α in the culture
supernatant was determined with a commercially available sandwich-type ELISA (eBioscience, San Diego, CA) using the manufacturer’s protocol.

**Western blotting analysis:** Bacterial culture supernatants and purified staphylococcal LTAs were loaded onto a 15% native PAGE gel. After 100 min of electrophoresis at 60-100 v/cm, the gel was blotted onto a nitrocellulose membrane. The membrane was blocked with 1.5% BSA in PBS (0.05% Tween20) and incubated with an anti-LTA mAb BD1701 (1:5000), TEPC-15 (1:5,000), or anti-pneumococci rabbit serum (1:10,000). The membrane was then washed and incubated with a 1:5,000 dilution of peroxidase conjugates of a rabbit anti-mouse immunoglobulin (Southern Biotech, Birmingham, AL) or a goat anti-rabbit immunoglobulin (Southern Biotech) reagent. After washing, the membrane-bound peroxidase was detected with Lumiglo Reagent (Cell Signaling, Danvers, MA).

**Data analysis:** All of the experiments in this study were conducted at least three times. The data shown are representative results. Experimental values are given as means ± standard deviations (SD). The statistical significance of differences between two means was evaluated with Student’s unpaired t test.
RESULTS

1) GPCSs induce inflammatory responses by mouse macrophages and human epithelial cells.

To define the inflammatory molecules of Gr+ bacteria at different stages of infections, we obtained GPCSs during mid-logarithmic growth (early-GPCS [early GPCS]) and after 16 h of culture (late GPCS) from three different Gr+ bacterial species [S. pneumoniae (SP), S. aureus (SA), and GBS (GB)] and evaluated their ability to stimulate RAW264.7 macrophages to secrete TNF-α following incubation with different amounts of GPCSs (Figure 17A). TNF-α production increased with increasing amounts of early GPCS, with 20% of early GPCS being as potent as $10^8$ heat killed bacteria induced TNF-α production (Figure 17B). The late GPCSs also induced the production of TNF-α, which was several times higher than that seen with early GPCSs (Figure 17A). Interestingly, pneumococcal late GPCS was the most potent among the three late GPCSs, whereas staphylococcal early GPCS was the most potent among the early GPCSs. These results clearly showed presence of inflammatory molecules in early and late GPCS which induce TNF-α production as much as potential amount of heat killed bacteria. Based on these results, 10% GPCS was used in all subsequent experiments. Additionally, 10% of early and late GPCS efficiently stimulated human lung epithelial cells (A549) to produce IL-8 (Figure 17C) suggesting that GPCS contains potential PAMP which stimulate both mouse and human cells.
2) TLR2 but not TLR4 is primarily responsible for GPCS-induced inflammation.

To establish whether the GPCS stimulates cells via TLR2, which is known to be the primary receptor for innate immunity to Gr+ bacteria (117, 182), we used two different approaches to study early GPCS and late GPCS. Our first approach was to determine if early GPCS can activate the CHO cell lines 3E10-TLR2 and 3E10-TLR4. All early GPCSs induced an up-regulation in CD25 expression on 3E10-TLR2, but not on 3E10-TLR4 (Figure 18A). In contrast, the 1% culture supernatant of *E. coli* activated both 3E10-TLR2 and 3E10-TLR4. The response with the 3E10-TLR2 cell line was not unexpected since it is known that the CHO cells express hamster TLR4, but nonfunctional hamster TLR2 (67, 166).

In our second approach, we directly stimulated mouse peritoneal macrophages from C57BL/6 wild type (WT), TLR2−/−, or TLR4−/− mice with GPCS and measured TNF-α production (Figure 18B and 18C). WT and TLR4−/−, but not TLR2−/−, peritoneal macrophages secreted TNF-α in response to all early GPCS, although a higher percentage of GPCS was required for the peritoneal macrophages than for RAW264.7 cells. In contrast, *E. coli* culture supernatant stimulated WT and TLR2−/−, but not TLR4−/−, macrophages (Figure 18D). Therefore, the early GPCSs primarily contain TLR2 ligands, whereas the *E. coli* culture supernatant contains TLR4 ligands which would be LPS.
Figure 17 TNF-α production by RAW264.7 cells in response to early (Panel A) or late (Panel B) culture supernatants of *S. pneumoniae* (marked SP), *S. aureus* (marked SA), Group B streptococci (marked GB), or *E. coli* (marked EC). *E. coli* culture supernatant was used at 1%; the percentages of the GPCS culture supernatants are indicated in the Figure. Bars indicate the mean of results of triplicate wells of a representative experiment. Error bars indicate SD.
When we examined late GPCS for its ability to stimulate the CHO cell lines expressing human TLRs, we found that SA and GB late GPCS stimulated only 3E10-TLR2 and did not stimulate 3E10-TLR4 (Figure 19A). In contrast, pneumococcal late GPCS stimulated both 3E10-TLR2 and 3E10-TLR4 (Figure 19A). Since pneumococci produce pneumolysin, which is a TLR4 ligand (133) that is released from dead pneumococci (13), we also examined the inflammatory properties of early and late GPCS harvested from the D39 strain or a pneumolysin-deficient D39 isogenic mutant strain (PLN-A). Early GPCS from both pneumococcal strains did not stimulate the 3E10-TLR4 cells (Figure 19B). However, the late GPCS of D39 (but not of PLN-A) stimulated 3E10-TLR4 cells (Figure 19B). These results suggest that TLR2 ligands are the main PAMPs in early GPCS and that the pneumolysin present in the pneumococcal late GPCSs activated cells via TLR4.

Next we examined that TLR2 ligand was major component even in late GPCS by directly stimulating mouse peritoneal macrophages from C57BL/6 wild type (WT), TLR2−/−, or TLR4−/− mice and measured TNF-α production (Figure 19C). WT and TLR4−/− peritoneal macrophages secreted TNF-α in response to all late GPCS, TLR2−/− peritoneal macrophages did not produce TNF-α or slightly produced TNF-α only in high concentration of Staphylococcal late GPCS (Figure 19C). E. coli culture supernatant stimulated WT and TLR2−/−, but not TLR4−/−, macrophages (Figure 19D). Therefore, unlike early GPCS, the late GPCS contain primarily TLR2 ligand, but Staphylococcal late GPCS has other PAMP which induce TNF-α independent with TLR2 and TLR4.
Figure 18 Panel A shows percentage of CD25+ 3E10-TLR2 (open bar) or 3E10-TLR4 (black bar) cells in response to the bacterial culture medium alone (labeled Med), pneumococcal early GPCS (labeled SP), staphylococcal early GPCS (labeled SA), Group B streptococcal early GPCS (labeled GB), and early E. coli culture supernatant (labeled EC). All GPCSs were used at 10%, and E. coli supernatant was at 1%. The amount of TNF-α produced by mouse peritoneal macrophages collected from WT (open square), TLR2+/− (solid circle), and TLR4+/− (open triangle) mice in response to the indicated concentrations of early culture supernatants collected from S. pneumoniae (Panel B), S. aureus (Panel C) and E. coli (Panel D). Bars or data points indicate the mean of results of triplicate wells of a representative experiment. Error bars indicate SD.
Figure 19. Panel A shows percentage of CD25+ 3E10-0.1RL2 (open bar) and 3E10-TLR4 (solid bar) cells in response to the bacterial culture medium alone (labeled Med), pneumococcal late GPCS (labeled SP), staphylococcal late GPCS (labeled SA), Group B streptococcal late GPCS (labeled GB), and late E. coli culture supernatant (labeled EC). All GPCSs were used at 10%, and E. coli supernatant was at 1%. Panel B shows percentage of CD25+ 3E10-TLR4 cells in response to indicated concentration of early and late culture supernatants collected from S. pneumoniae strain D39 (labeled WT) and its pneumolysin-deficient isogenic mutant (labeled PLN-A). Bars indicate the mean of results of triplicate wells of a representative experiment. Error bars indicate SD.
3) LTA is a major molecular component found in early GPCS and late GPCS.

To begin identifying the TLR2 ligands in GPCS, we examined pneumococcal culture supernatants by PAGE and Western blotting using a rabbit antiserum against pneumococci. At least four groups of molecular bands were detected in the culture supernatants after a 3-h culture. These bands were labeled as a (70 kDa), b (60 kDa), c (50 kDa), and d (10-30 kDa) in Figure 20A. Bands a, b, and c do not correspond to PspA, PspC, or pneumolysin—three well-known pneumococcal proteins with inflammatory properties (69, 133)—because the PspA, PspC, and pneumolysin were absent in early GPCS and were found at different molecular weight positions: 90 KDa for PspA, 95 kDa for Since LTA is considered a TLR2 ligand (44, 75), we also investigated its presence and amount in GPCS from SA and GB using mAb to LTA (BD1701). In both culture supernatants, the mAb visualized diffuse bands at 10 kDa (Figure 20C), similar to that reported for staphylococcal or GBS LTA (70, 217). Western blot analysis suggested the presence of about 0.2-1 μg/ml of LTA in early GPCS and about 1-5 μg/ml of LTA in late GPCS (Figure 20C). The amount of LTA in staphylococcal GPCS was confirmed with a sandwich-type ELISA using mouse and rabbit anti-LTA antibodies (Figure 20D). No LTA was detected in the E. coli culture supernatants with either TEPC-15 or BD1701 (data not shown). Since 0.1 μg/ml of staphylococcal LTA is known to be inflammatory (145, 166), our results indicate that both the early and late GPCS contain biologically relevant amounts of LTA.
Figure 20. Panel A shows molecules in GPCS of *S. pneumoniae* (R36A strain) harvested at indicated times (in hours), separated by PAGE, and detected with a rabbit anti-pneumococcal antiserum. O/N indicates that the R36A culture supernatant was harvested after an overnight culture (16 h). Optical densities at 600 nm at the time of harvest were indicated at the top of each lane. The left two lanes were loaded with 20 μl of purified LTA at the indicated concentrations. Panel B shows pneumolysin (labeled Ply), PspA (labeled PspA), or PspC (labeled PspC) in R36A culture supernatants that were harvested at the indicated times and detected with specific antibodies. Twenty microliter of lysates of $10^8$ and $10^7$ R36A pneumococci were loaded in the left two lanes as positive controls. In Panel C, 20 μl of purified LTA were loaded in the left three lanes, of early GPCS in the middle three lanes, and of late GPCS in the right three lanes. The specific late GPCSs were *S. pneumoniae* (labeled SP), *S. aureus* (labeled SA), and Group B streptococcus (labeled GB). Concentrations of purified LTA and GPCS are indicated at the top of each lane. The molecules in the samples were separated by PAGE and visualized with anti-phosphorylcholine antibody (TEPC-15) for pneumococcal LTA or with an anti-LTA mouse mAb (BD1701) for the others. Panel D shows the amount of LTA in the bacteria culture medium alone (labeled Med) and in staphylococcal early GPCS (labeled E), staphylococcal late GPCS (labeled L), early culture supernatants of pneumococcal (labeled E-SP) and *E. coli* (labeled E-EC). The amount of LTA was determined with a sandwich-type ELISA using a mouse mAb and a rabbit antiserum specific to staphylococcal LTA.
4) The inflammatory capacity of GPCS is significantly reduced (almost lost) by LTA inactivation.

We have previously reported (168) that PAF-AH can monodeacylate pneumococcal and staphylococcal LTAs, which makes them inactive in stimulating RAW264.7 cells. Although it is unlikely that PAF-AH, a phospholipase A2, will affect other PAMP, we next investigated the effect of PAF-AH on other TLR ligands including Poly (I:C) (a TLR3 ligand), LPS (a TLR4 ligand), R837 (a TLR7 or TLR8 ligand), ODN1826 (a TLR9 ligand), and MDP (a NOD2 ligand) (Fig. 21). We found that the treatment did inactivate LTA, as we previously reported (168), but all other ligands were unaffected by treatment with 10 μg/ml of PAF-AH (Fig. 21). Included in the ligands tested, were peptidoglycan (PGN) and two synthetic lipoproteins, Pam3CSK4 (a TLR2/1 ligand) and FSC-1 (a TLR2/6 ligand) (Fig. 21), which represent other TLR2 ligands of Gr+ bacteria (45, 79). Therefore, PAF-AH is a highly specific LTA inhibitor.

To determine the role of LTA in early GPCS, we next investigated the inflammatory properties of early (Fig. 22A) and late (Fig. 22B) GPCSs after treatment with PAF-AH or alkaline hydrolysis (0.2 N NaOH). Based on preliminary studies, we used 10% of early- and 5% of late GPCSs. Untreated early and late culture supernatants dramatically increased TNF-α production (Fig. 22A and Fig. 22B). When culture supernatants were treated with alkaline hydrolysis and neutralized with HCl, early culture supernatants from the two Gr+ bacteria species as well as the E. coli retained less than 10% of the original activity (Fig. 22A). The hydrolysis also reduced activity of late culture supernatants by 50 – 60% (Fig. 22B). Alkaline hydrolysis can inactivate LTA,
Figure 21. TNF-α production by RAW264.7 cells in response to stimulation by pneumococcal LTA (PnLTA), staphylococcal LTA (StLTA), staphylococcal PGN (PGN), Pam3CSK4 (P3Cys), FSL-1, Poly (I:C) [P(I:C)], LPS, imiquimod (R837), ODN1826 (ODN), or MDP before (open bar) or after (black bar) 10 μg/ml PAF-AH treatment or 0.2N NaOH for 2 hrs at 37°C. The ligand concentrations (in μg/ml) and receptor specificities are identified at the bottom of the figure. Bars indicate the mean of results of triplicate wells of a representative experiment. Error bars indicate SD. The p values are indicated in the figure.
synthetic lipopeptides, and LPS, but does not inactivate PGN or MDP (Fig. 21). Thus, LTA, lipoprotein or LPS may be important inflammatory factors in these culture supernatants.

To selectively inactivate LTA, bacterial culture supernatants were treated with PAF-AH. When early supernatants of pneumococci and staphylococci were treated with PAF-AH, 1 μg/ml PAH-AH reduced TNF-α production by 70-90% and 10 μg/ml of PAF-AH reduced TNF-α production almost completely (>90%) (Fig. 22A). When the late culture supernatants were investigated, the PAF-AH treatment (10 μg/ml) reduced their ability to induce TNF-α production by 50% and 70%, respectively (Fig. 22B). The inactivation procedure itself did not affect GPCS activity since inactivation with 0 μg/ml of PAF-AH did not alter GPCS activity (Fig. 22A and 22B), and the addition of 10 μg/ml of PAF-AH alone did not affect the activities of RAW264.7 cells (Data not shown) (168). PAF-AH treatments of early and late GBS culture supernatants also reduced their TNF-α inducing activity by >90% (Data not shown). In contrast to GPCS, treatment of early and late *E. coli* culture supernatants with 10 μg/ml of PAF-AH had no effect on its inflammatory activity (Fig. 22A and 22B). These results strongly suggest that LTA is the dominant inflammatory factor in early GPCSs and is still a major factor in late GPCSs.

To further confirm the inflammatory role of LTA in GPCS, we depleted LTA in the staphylococcal GPCS with anti-LTA antibody and examined the antibody-treated GPCS for its ability to induce RAW264.7 cells to produce TNF-α (Fig. 23). Treatment with mAb BD1701 reduced the inflammatory properties of early staphylococcal supernatants by 80%, but reduced late supernatants by only 65%. The control mouse
A

S. pneumoniae early GPCS (10%)

B

S. pneumoniae late GPCS (5%)

S. aureus early GPCS (10%)

S. aureus late GPCS (5%)

E. coli early Supernatant (0.1%)

E. coli late Supernatant (0.01%)
Figure 22. TNF-α production by RAW264.7 cells in response to bacterial culture supernatants of *S. pneumonia, S. aureus*, or *E. coli*. The two bars in the left indicate controls: un-stimulated (first bars) or stimulated with untreated bacterial culture supernatants (second bars). The third bars indicate the cells stimulated with culture supernatant treated with alkali hydrolysis and the remaining bars (4th to 7th) indicate the cells stimulated with supernatants treated with PAF-AH at indicated concentrations (in μg/ml). PAF-AH was inactivated with 100 μM Pefabloc SC after the reaction. Early-GPCSs were used at 10%, late-GPCSs were at 5%, early *E. coli* supernatant was at 0.1%, and late *E. coli* supernatant was at 0.01%. Bars indicate the mean of results of triplicate wells of a representative experiment. Error bars indicate SD. The *p* values are indicated in the figure.
IgG1 antibody had no effect on the inflammatory properties of either GPCS (Fig. 23). Also, mAb BD1701 did not reduce the inflammatory capacity of *E. coli* culture supernatant and LPS. Thus, all three independent approaches to removing LTA support the contention that LTA is essential to the ability of early GPCS to stimulate RAW264.7 cells and is important to the parallel ability of late GPCS.

5) Addition of purified LTA restores the inflammatory capacity of LTA-inactivated early GPCS.

To investigate whether the treatments used above to inactivate LTA also removed other critical molecules other than LTA, we examined the effect of restoring deactivated early GPCS with purified LTA. Ten percent of alkali-inactivated early staphylococcal supernatants, which originally contained about 0.02-0.1 μg/ml LTA prior to inactivation (Fig. 1D), regained its original activity when 0.1 μg/ml of staphylococcal LTA was added (Fig. 5A). In contrast, LTA, by itself, induced several times less TNF-α than the same amount of LTA mixed with inactivated early GPCS (Fig. 24A). PAF-AH-inactivated staphylococcal supernatants showed almost identical results (Fig. 24B). These findings demonstrate the inflammatory activity of LTA and suggest that early GPCS contains a factor(s) synergizing with LTA for inflammatory activity.

When the pneumococcal culture supernatants were similarly investigated, we found that pneumococcal culture supernatants could also be reconstituted with purified LTA. However, the restoration required about 3-10 μg/ml of LTA (Fig. 24C and D), whereas 10 % of early pneumococcal supernatant has only about 0.02-0.1 μg/ml. Thus, the restoration of inactivated culture supernatants required about 10 times more purified LTA.
**Figure 23.** TNF-α production by RAW264.7 cells in response to bacterial culture medium (labeled Med), staphylococcal early GPCS (labeled Early), staphylococcal late GPCS (labeled Late), purified staphylococcal LTA (labeled LTA), *E. coli* early culture supernatant (labeled Early), or *E. coli* LPS (labeled LPS). Each stimulant was used unabsorbed (black bars), after absorption with an irrelevant isotype-matched control mAb (open bars), or after absorption with an anti-LTA mAb (hatched bars). Bars indicate the mean of results of triplicate wells of a representative experiment. Error bars indicate SD. The *p* values are indicated in the figure.
**Figure 24.** TNF-α production by RAW264.7 cells in response to early GPCS from staphylococci (Panels A and B, Early SA) or from pneumococci (Panels C and D, Early SP). Early GPCS was inactivated by alkali hydrolysis (labeled as 0.2 N NaOH) or with 10 μg/ml PAF-AH (labeled as PAF-AH). Then an indicated concentration (mg/L) of purified pneumococcal LTA (PnLTA) or staphylococcal LTA (StLTA) was added to respective inactivated culture supernatants (10%). Bars indicate the mean of results of triplicate wells of a representative experiment. Error bars indicate SD.
LTA. In addition, the activity of purified pneumococcal LTA was enhanced several folds with the inactivated pneumococcal culture supernatants (Fig. 24C and D). This suggests that pneumococcal culture supernatants also contain factors that synergize with LTA for inflammatory activity.

**DISCUSSION**

The significance of LTA as a TLR2 ligand is controversial (78, 79), mainly because “purified” LTA is often contaminated (45, 62, 79) or structurally damaged (144). To avoid these limitations, we examined bacterial culture supernatants after selectively removing or inactivating LTA. Our LTA inactivation/removal methods are based on PAF-AH and mAb (BD1701) that do not inactivate the synthetic lipoprotein Pam3CSK4 and various model ligands to other TLRs (Figure 21) (168). We found that the inactivation of LTA almost completely reduces the TLR2-mediated inflammatory properties of early GPCS (Figure 23 and 24). Also, the inflammatory property of the LTA-depleted early GPCS can be quantitatively restored with LTA (Figure 25). These findings clearly show that LTA is the dominant TLR2 ligand in early GPCS.

The LTA inactivation also made late GPCSs of several Gr+ bacteria less inflammatory (Figure 23). However, unlike early-phase supernatants, the LTA inactivation left late GPCS with significant residual amounts of inflammatory activity. Thus, late GPCS must have a TLR2 ligand(s) other than LTA, which may be a lipoprotein. Lipoproteins should be present in late GPCS as many bacteria begin to die in
the late phase of culture and lipoprotein(s) account for the majority of inflammatory properties of dead Gr+ bacteria (10, 125, 157, 173). Thus, our data suggest that the dominant TLR2 ligands may vary during the stages of infection: LTA may be critically important in the early phase, but other molecules such as lipoproteins may become significant as TLR2 ligands in the late phase of infection.

There are debates whether lipoprotein or LTA is the dominant inflammatory molecules of Gr+ bacteria or their lysates. Staphylococci with a ΔlgtA mutation cannot produce lipoproteins and their lysates are less stimulatory than the wild-type staphylococcal lysates (173). Yet, a chemically synthesized staphylococcal LTA analog is strongly stimulatory (145). In addition, there are disagreements in the ability of LTA extracted from ΔlgtA mutant bacteria to stimulate human blood cells (77, 201). Since we did not investigate Gr+ bacteria themselves, our study cannot directly address the debates. However, our data does show that LTA is the dominant TLR2 ligand in early GPCS and is still a significant ligand in late GPCS. Thus, LTA should be considered as an important TLR2 ligand. This may occur because LTA is readily released into and is abundant in culture supernatants (104, 208) whereas lipoproteins are primarily associated with bacteria and not released into supernatants (173). In fact, consistent with our conclusions, the culture supernatant of the lipoprotein-deficient bacteria (ΔlgtA) is as inflammatory as wild-type bacterial culture supernatant (173).

In the case of pneumococci but not the other two Gr+ bacteria evaluated in this study, late GPCS could stimulate macrophages via TLR4 (Figure 20A). This is consistent with the observations that pneumolysin can stimulate via TLR4 (133) and that
dead pneumococci induce inflammation primarily via TLR4, whereas live pneumococci induce inflammation via TLR2 (165). In fact, we found that an late GPCS from a pneumolysin-deficient pneumococcal strain completely lost the ability to stimulate via TLR4 (Figure 20B). TLR4 stimulation can activate additional intracellular signaling pathways involved in inflammatory responses and would enhance the inflammatory properties of pneumococci (74). The presence of pneumolysin may explain why pneumococci are more inflammatory than other Gr+ bacteria (204). This further illustrates that bacteria provide additional inflammatory molecules at different stages of infections.

Studies of purified LTA, on their own, cannot demonstrate changes in the functional potency of LTA during its purification process. This situation has been a serious limitation to the study of the inflammatory properties of LTA. However, the selective inactivation approach used here permits one to investigate the functional potency of LTA. When we reconstituted LTA in the LTA-inactivated early GPCS to the natural early GPCS level (about 1 μg/ml) (Figure 21C and 21D), the inflammatory potency of the staphylococcal early GPCS was quantitatively restored (Figure 25A and 25B). This finding strongly suggests that staphylococcal LTA is not damaged by the new LTA isolation procedure. In contrast to staphylococcal LTA, we needed about 10 fold more pneumococcal LTA to regain the inflammatory capacity of pneumococcal early GPCS (Figure 25C and 25D). This strongly suggests that the widely used procedure for purifying pneumococcal LTA yields pneumococcal LTA with reduced activity. Consistent with our conclusion, Draing et al. reported, during the preparation of this
manuscript, that the classical purification procedure removes the alanyl group from pneumococcal LTA and thus reduces its inflammatory potency (44).

Although LTA is well known as a TLR2 ligand, LTA can bind other receptors (CD36) or activate PAFR (76, 124), which can amplify inflammatory signals (31). Also, LTA can be synergistic with another PAMP like peptidoglycan or MDP (109, 113, 140, 203), as well as unidentified factor(s) we have detected in GPCS in the present study. The spectrum of available PAMPs could vary during different stages of infection, and other receptors expressed on host cells could vary depending on their cell types and/or activation status. Thus, the role of LTA in Gr+ infections should be assessed in the context of a specific pathophysiological process. For instance, pneumolysin may be more prominent than LTA in pneumonia where a large number of dead pneumococci are present. Indeed, deletion or neutralization of pneumolysin makes pneumococci less effective in causing pneumonia than in sepsis (5, 24). It would be interesting to investigate whether LTA is important when the pneumococcal density is low, such as in nasopharyngeal carriage or at the beginning of a pathologic process.
REFERENCES


63. **von Aulock, S., T. Hartung, and C. Hermann.** 2007. Comment on "Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in *Staphylococcus aureus*". J Immunol **178:**2610.


FINAL SUMMARY

Minor structural alteration of LTA can affect its function. For instance, the loss of D-alanine on LTA alters the anionic charges of the cell membrane and wall (1, 30, 118, 120, 209), and significantly alter LTA properties such as resistance to a bacteriocin (118), adhesion to host cells (216), pathogenic potential (1, 206), and neutrophil extracellular trap (NET)-mediated killing. It may also reduce endogenous DNA binding to enhance transformability. Thus, understanding the role of LTA in Gr+ bacterial infections requires exact knowledge of its molecular structure.

With our ability to precisely monitor pneumococcal LTA structure, we discovered an unexpected variation. Although both the R6 and R36A strains of S. pneumoniae were derived from the clinical isolate D39 strain, we found the LTAs from R6 and R36A to be different in structure (Figure 6A and B in Page 43). The mass spectrometry pattern of R36A LTA includes two PC per repeating unit, whereas R6 expresses one or two PC per repeating unit of LTA (Figure 6A and B in Page 43). These differences could be due to genetic differences in their LTA synthesis genes, because these two strains have many genetic differences despite their common origin (122). While previous studies have used R6 as the model for LTA, LTA appears to be a variant, while the R36A LTA structure appears to be more representative of the structure found in many other pneumococcal strains including clinical isolates (Figure 6 in Page 43). This emphasizes the need to use a standardized bacterial strain, which produces LTA that closely resemble a wild type LTA, such as that is seen in R36A, rather than a R6 strain.
Our investigations of pneumococcal LTA structure showed inconsistencies in the accepted model of LTA (Figure 5, Model A in Page 42). The model, which was proposed by Fischer’s group (12), could not explain several biological and serological properties of LTA (Figure 5, Model A in Page 42). For instance, the predicted lipid anchor could not be detected in pneumococcal membranes (54). The previously proposed structure does not contain terminal GalNAc(\(\alpha_1\rightarrow3\))GalNAc(\(\beta_1\rightarrow\)), which is necessary to explain the Forssman antigen properties of pneumococcal LTA (12, 84). In addition, the mass spectrum of pneumococcal LTA showed about 350 atomic mass unit (AMU) less than predicted for the accepted model (Figure 6 G and H in Page 43) (114, 168). To resolve these inconsistencies, we investigated pneumococcal LTA using mass spectrometry and various hydrolysis conditions. Based on these studies we revised the model of the pneumococcal LTA structure (Part I).

In addition, the revised model explains a conundrum in LTA biosynthesis. The final step of LTA synthesis requires linkage of the polymerized repeating units to a lipid anchor which is present on the cell membrane (Figure 25, Step 4 in Page 141). However, Fischer’s LTA model (Figure 5, Model A in Page 42) requires Glc-AATGal-Glc-acyl_2Gro as a lipid anchor, which has not been detected in pneumococcal membranes (54). In contrast, according to our revised LTA model (Figure 5, Model B in Page 42), the required lipid anchor is Glc(\(\alpha_1\rightarrow3\))-acyl_2Gro, which is abundantly present as a pneumococcal membrane component (27, 54, 108).

The revised model also suggests a putative mechanism of pneumococcal LTA biosynthesis (Figure 25, Step 1 in Page 141). The new structure suggests that repeating
unit synthesis should begin with the transfer of AAT-Gal to the polyprenyl phosphate lipid acceptor by an initial transferase. Studies of the pneumococcal genome suggest that SP1838 (TacA) of TIGR4 *S. pneumoniae* may encode the initial transferase (36, 97, 122, 186). Each subsequent sugar addition to the repeating unit may be added by a set of glycosyltransferases (TacB, TacC, TacD, TacE) that utilize a nucleotide sugar as a substrate (Figure 25, Step 2 in Page 141). Phosphocholine is then transferred to one or both GalNAc residues by *licABC*, and *licD1* and *licD2*. Next, the completed repeat units are flipped out to the outer leaflet of the membrane by TacF, and repeating units are then polymerized by an unidentified polymerase (Figure 25, Step 3 in Page 141). In the final step, polymerization occurs by transferring the undecaprenyl polyphosphate-linked repeating unit chain to the lipid anchor [Glc(α1→3)-acyl2Gro] (Figure 26, Step 4 in Page 141).

The revised model can also resolve the Forssman antigen properties of purified LTA. Previous studies showed that purified immature LTA with fewer repeating units possess higher Forssman antigen properties than a mature full-length LTA (54). Thus, mature LTA might lose some of the Forssman antigen properties due to PC incorporation, and having too many repeating units to the polyprenyl phosphate lipid anchor (Figure 25 in Page 141). According to this hypothesis, pneumococcal Forssman antigen should be expressed on the inside of the cell membrane and can be released when bacteria are lysed.

The immunological functions of LTA have been extensively debated. For instance, some studies reported that LTA is the priming TLR2 ligand that elicits production of various inflammatory molecules including TNF-α, IL-1 and nitric oxide.
(Figure 10 in Page 77) (75, 181, 191). However, some recent studies have reported that the TLR2 ligand is not LTA, but a contaminating lipoprotein found in LTA preparations (77, 79, 201). These controversies exist because of difficulties in obtaining pure LTA without structural damage or biologically active contaminants.

To resolve whether LTA is a key component in Gr+ bacteria-induced inflammation, we developed a method to directly inactivate the LTA of Gr+ bacteria without destroying other components. This approach avoids the need to purify LTA (Part 2). Monoacyl LTA produced by alkaline hydrolysis of intact LTA does not stimulate murine macrophages. This observation is analogous to previous finding showing that the number of acyl chains is critical to the biological potency of bacterial PAMPs (145, 146). However, hydrolysis was very difficult to control and could inactivate many other PAMPs (Figure 21 in Page 116) (114). We discovered that PAF-AH, a phospholipase A2, could remove one acyl chain and inactivate LTA completely (Figures 12 in Page 81 and Figure 16 in Page 88). In addition, PAF-AH does not inactivate other PAMPs such as LPS, PGN, MDP, Pam3CSK4, and FSC-2 (Figure 21 in Page 116). Thus, PAF-AH was found to be useful in inactivating LTA without destroying other bacterial components.

PAF-AH is known to be an enzyme that inactivates endogenous PAF. PAF induces a strong inflammatory response by stimulating PAF receptors (123, 135, 137) and is up-regulated following TLR stimulation. LTA shares a structural motif with PAF and can stimulate the PAF receptor either directly (124) or indirectly (219). Our preliminary data obtained in collaboration with Dr. Diana Stafforini (University of Utah) show that LTA is a competitive inhibitor of PAF-AH competing with PAF for the same
enzymatic site(s) of PAF-AH. Since LTA would be available at a high concentration at the infection site, it may reduce the ability of PAF-AH to deactivate PAF and might enhance endogenous PAF-mediated inflammation (Figure 26 in Page 142).

We used the new LTA inactivation method to study the role of LTA in inflammation without purifying it. Specifically, we investigated the effects of inactivating LTA in early and late GPCS. Early and late GPCS were used to mimic early and late stages of Gr+ bacterial infections. To establish whether the GPCS stimulates cells via TLR2, which is known to be the primary receptor for innate immunity to Gr+ bacteria (117, 182), we directly stimulated mouse peritoneal macrophages from C57BL/6 wild type (WT), TLR2−/−, or TLR4−/− mice with GPCS and measured TNF-α production (Figure 18 in Page 110 and Figure 19 in Page 111). The studies showed that both the early and late GPCSs contain primarily TLR2 ligands, whereas the E. coli culture supernatant contains TLR4 ligands, illustrating, a key difference in the pathogenesis of Gr+ and Gr- infections (Figure 18 in Page 110 and Figure 19 in Page 111).

Following LTA inactivation, early GPCS almost completely lost its inflammatory properties (Figure 22A in Page 118). These findings clearly demonstrate that LTA is the dominant inflammatory agent in early GPCS. LTA inactivation also made late GPCSs of several Gr+ bacteria significantly less inflammatory (Figure 22B in Page 118). However, unlike early culture supernatants, late GPCS retained significant amounts of residual inflammatory activity. Late GPCS might therefore have a TLR2 ligand(s) other than LTA that is perhaps released from lysed bacteria. Thus, our data suggest that the dominant TLR2 ligands may vary during the stages of infection: LTA may be critically
important in the early phase, but other molecules such as lipoproteins may become significant TLR2 ligands in the late phase of infection.

When we added highly purified LTA to the LTA-inactivated early GPCS, the inflammatory potency of the early GPCS was quantitatively restored (Figure 24 in Page 122). This confirms that our LTA-inactivation method does not degrade factors other than LTA. Furthermore, the reconstituted GPCS induced several times more TNF-α than would be expected with purified LTA alone (Figure 24 in Page 122). This enhanced response suggests that bacterial culture supernatants also contain at least one substance that synergizes with LTA for inflammatory activity, and that these synergy factors may be critical in LTA-induced inflammation.

These are several potential synergy. For instance, LTA can synergize with NOD ligands (MDP, PGN), TLR4 ligands (LPS), or TLR3 ligands (dsRNA) (109, 113, 140, 203) (Data not shown), but not TLR2/1, TLR2/6, TLR7 or TLR9 ligands (63, 163). Hemoglobin also was reported to be synergistic with LTA (35, 80), and there could be novel molecules. In view of these possibilities, additional work is necessary to identify the synergy partner(s).

Late GPCS from pneumococci can stimulate macrophages via TLR4 (Figure 19 in Page 111). Pneumolysin from dead pneumococci induces inflammation primarily via TLR4 (133), and deletion or neutralization of pneumolysin makes pneumococci less effective in causing pneumonia (5, 24). We found that late GPCS from a pneumolysin-deficient pneumococcal strain does not stimulate via TLR4 (Figure 19B in Page 111). TLR4 stimulation would enhance the inflammatory properties of pneumococci, because it
can activate additional intracellular signaling pathways involved in inflammatory responses not produced by TLR2 stimulation (74). Interestingly, late GPCS from two other Gr+ bacteria (S. aureus and Streptococcus agalactae) do not stimulate TLR4. The presence of pneumolysin may suggest why pneumococci are so inflammatory (204).

Our findings suggest that the spectrum of available PAMPs may vary during different stages of infection and at different sites of infections. LTA is important when the pneumococcal density is low, such as in nasopharyngeal carriage or in early sepsis, whereas both pneumolysin and LTA may act together when the pneumococcal density is high, such as in pneumonia and late stage pneumococcal infection. Additionally, other molecules such as lipoproteins and PGN might be partially required for the progress in Gr+ bacteria-induced inflammation (Figure 28 in Page143).

Our studies have established the importance of LTA in Gr+ bacterial-induced inflammation. We found that LTA is important in the early sepsis process, but that other components become significant in late stages of bacterial infection. However, we provide evidence of a synergy factor or factors that enhance the potency of LTA. Thus, it is unlikely that LTA alone is responsible for early Gr+ bacterial-induced inflammation. Future studies should investigate possible synergy factors.

Studies investigating possible synergy factors will require highly purified intact LTA. Our model of the structure of pneumococcal LTA should greatly facilitate its purification. Once synergy factors are identified, it will be possible to replicate Gr+ bacterial sepsis with purified molecular components and gain a deeper understanding of the pathophysiology of Gr+ sepsis.
Figure 25. Model for the synthesis of TA and LTA. Pneumococcal TA and LTA are likely synthesized by the Wzy polymerase-dependent mechanism of bacterial polysaccharide synthesis. This mechanism involves four steps (1) repeat unit formation; (2) transport; (3) polymerization; and (4) transfer.
Figure 26. Model of LTA mediated inflammation and regulation of inflammation by competitive inhibition with PAF-AH.
Figure 27. The model of early and late Gr+ bacteria induced inflammation
GENERAL LIST OF REFERENCES


APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM
NOTICE OF APPROVAL WITH STIPULATIONS

DATE: April 19, 2007
TO: Moon Nahm, M.D.
EBRR-614 2170
FAX: 975-2149

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Pneumococcal Conjugate Vaccines and Old Adults
Sponsor: NIH
Animal Project Number: 070407797

On April 3, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>C</td>
<td>350</td>
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Approval is granted with the following stipulation(s):

Animal procurement and initiation of studies may not commence until veterinary observation of the procedures is scheduled with one of the ARP veterinarians. Please Joe Goodwin at 934-7856 to schedule the observation. Once the IACUC is notified by ARP that the observation has been scheduled, this stipulation will be lifted.

Animal use is scheduled for review one year from April 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 070407797 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7992.
APPENDIX B

REPRINT PERMISSION LETTER