Lipoteichoic Acid Synergizes with Glycosphingolipids To Potently Stimulate Secretion of Interleukin-6 from Human Blood Cells

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In the present study, we found that lipoteichoic acid (LTA) synergizes with glycosphingolipids to stimulate human blood cells to secrete cytokines. We employed globoside, kerasin, and lactosylceramide as representative neutral glycosphingolipids and mixed gangliosides GM1 and GM3 as representative acidic glycosphingolipids. LTA and the glycosphingolipids enhanced cytokine secretion by human whole blood, peripheral blood mononuclear cells, and purified monocytes in a dose-dependent manner. The level of synergy ranged up to 10-fold greater than the additive stimulation caused by LTA and glycosphingolipid alone. The greatest synergy was observed with GM1. We also found that LTA synergizes with the synthetic bacterial lipopeptide mimic Pam3CysK4. In contrast, the glycosphingolipids suppressed the stimulation caused by Pam3CysK4. The stimulation of human cells requires the simultaneous presence of LTA and the glycosphingolipids and probably requires their physical interactions, as shown by dot blotting and nondenaturing polyacrylamide gel electrophoresis experiments. We hypothesize that the enhanced stimulation is due to heterooligomers that form between LTA and glycosphingolipids at the subcritical micelle concentrations used in these experiments. Previous studies showed that LTA also synergizes with hemoglobin. The data taken together suggest that LTA may be a pathogen-associated molecular pattern, although its full activity requires the presence of a synergistic partner(s).

It has been established that septic shock caused by gram-negative pathogens is primarily due to the ability of lipopolysaccharide (LPS) to trigger the excessive secretion of proinflammatory cytokines by macrophages (1, 10). In contrast, the bacterial component(s) responsible for septic shock caused by gram-positive bacteria has not been confirmed. As a result, a number of constituents of gram-positive bacteria have been studied for their abilities to cause or contribute to shock. These include proteinaceous pyrogenic toxins, which act in vitro as superantigens; peptidoglycan oligomers (28); and lipoteichoic acid (LTA), a bacterial cell membrane component that has structural similarities to LPS. Over the last several years, highly purified LTA free of LPS contamination has been shown to induce monocyte and macrophage secretion of proinflammatory cytokines (7, 14, 18–20, 30, 31), and it shares many other functional features with LPS (12, 32). However, a major difference between LPS and LTA is that the injection of purified LPS alone can result in shock in an animal model, whereas the injection of purified LTA alone has never been shown to induce shock. Even when one examines the levels at which LPS and LTA induce cytokine release from macrophages/monocytes in vitro, it is clear that LPS is a more potent immunostimulant than purified LTA.

Recently, our laboratories and others have begun to define molecules of both human and bacterial origin that synergize with LTA and greatly enhance its ability to stimulate cells of the immune system. Muramyl dipeptide, the minimal structural unit of peptidoglycan that retains immunostimulating activity, synergizes with LTA to enhance the induction of inflammatory cytokines in human monocytes (34). In addition, we have shown that hemoglobin synergizes with both weakly active phenol-extracted LTA and the more highly active butanol-extracted LTA to markedly enhance cytokine secretion by both human blood monocytes and mouse macrophages (7, 14). Synergism with hemoglobin in activating macrophages was noted for LTAs from a variety of different gram-positive bacteria (14) and also for LPS (4, 25, 26). The mechanisms for the synergy between LTA and these other molecules have not yet been defined at the molecular level. However, we have shown that LTA and hemoglobin physically interact, so one possible hypothesis is that LTA forms a complex with hemoglobin in a way that facilitates the presentation of LTA to the macrophage Toll-like receptors (TLRs).

One of the reasons for the notable difference in potency between LTA and LPS may be related to the structural differences between their lipid moieties and their interactions with specific TLRs (TLR2 and TLR4, respectively) (3, 11). Even when one examines only the interaction between different species of LPS and the LPS receptor, TLR4, it is clear that differences in the lipid moiety (e.g., the numbers of fatty acid chains) can result in markedly different levels of cytokine secretion. LPS containing less than six fatty acids (e.g., penta- or tetra-acylated LPS) exhibited less activity or was even nonactive, and in one case, signaling was even through a different
receptor (1, 6). Interestingly, it has been shown that the mixing of the poorly active penta- and tetra-acylated LPS molecules at concentrations below their critical micelle concentrations (CMCs) before addition of the mixture to cells results in increased cytokine secretion (5). This result raises the possibility that the heterooligomers that formed between these two LPS structures were better able to interact with their receptor, subsequently resulting in enhanced cytokine release.

We show here that when LTA is combined with various glycosphingolipids and incubated with either human whole blood or isolated cells, cytokine release is markedly enhanced. We also show that LTA and some of the glycolipids physically interact and that enhanced cytokine release requires that both components be incubated simultaneously with the blood cells. On the basis of these observations, we hypothesize that mixtures of LTA and various glycosphingolipids at concentrations below their CMCs may form heterooligomers that are better able to interact with their receptors. (The work performed by Shiri Meron-Sudai is in partial fulfillment of a Ph.D. degree from the Sackler Faculty of Medicine, Tel-Aviv University.)

MATERIALS AND METHODS

Reagents. Butanol-extracted Streptococcus pyogenes LTA was purified as described previously (14). The chain length of polyglycerophosphate and the percent α-analine content were analyzed by nuclear magnetic resonance (NMR) and were found to be 23 and 65%, respectively (14). The maximum level of LPS contamination of the LTA preparations was ≤0.004% (e.g., 400 pg/ml in 10 μg/ml LTA, as determined by the Limulus amoebocyte lysate assay) (14). Ten pg/ml polymyxin B (Sigma-Aldrich Co., St. Louis, MO) was added to all reaction mixtures to exclude stimulatory effects due to any residual LPS. The glycosphingolipids used in this study were purchased from Matreya LLC. Synthetic tri-malonylated bacterial lipopeptide Pam3CysK4 was purchased from InvivoGen (San Diego, CA).

Preparation of human PBMCs and monocytes. Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood with Ficoll-Hypaque (Amersham Biosciences), as described previously (17). Briefly, Ficoll-Hypaque was added to whole blood diluted 1:2 in Dulbecco's phosphate-buffered saline (PBS), and the mixture was centrifuged at 700 × g for 30 min at room temperature (RT). The PBMC layer was collected, and the cells were washed three times with cold Dulbecco's PBS. Monocytes were further isolated by allowing them to adhere to plastic. The PBMCs were resuspended in RPMI 1640 (supplemented with penicillin at 100 μg/ml and streptomycin at 100 U/ml) with 2% fetal calf serum (FCS) at a concentration of 5 × 10^6 cells/ml. The cells were incubated for 1 h at 37°C in tissue culture flasks to allow the monocytes to adhere to the flakes. Nonadherent cells were removed by washing the flakes three times with Earl's balanced salt solution. RPMI 1640 supplemented with 10% FCS was added to the adherent monocytes, and the cells were incubated overnight at 37°C, during which the monocytes detached (17). Monocytes purified in this way were collected, washed, and counted. PBMCs or monocytes, at 1.5 × 10^5 to 2 × 10^6 cells/200 μl in polypropylene tubes, were diluted in RPMI 1640 supplemented with 5% FCS or donor plasma and exposed to stimuli by overnight incubation.

Whole-blood assays. Heparinized blood obtained from volunteers was diluted 1:5 in saline, as described by Morath et al. (19, 20). Diluted blood was incubated with LTA, glycosphingolipids, or mixtures of both components that had been preincubated at 37°C for 1 h. After overnight incubation (i.e., 19 to 22 h), the supernatants were removed and assayed for the levels of interleukin-6 (IL-6) that had been secreted.

IL-6 measurements. Medium from the samples was collected and was assayed for the amount of IL-6 secreted by enzyme-linked immunosorbent assay, according to the manufacturer’s protocol (Pierce Endogen; Pierce Biotechnology, Inc.). This method is sensitive down to 300 pg/ml IL-6.

RESULTS

The ceramide-containing glycolipids (i.e., sphingolipids) are grouped according to the nature of the polar head group at position 1 of the ceramide backbone. One group, the glycosphingolipids, are further divided into two groups on the basis of the sugar composition of the head group. The neutral glycosphingolipids contain uncharged sugar residues, while the acidic glycosphingolipids, also known as gangliosides, contain charged sugar residues, such as sialic acid. In the present study, we employed galactosylceramide and lactosylceramide, which are the most predominant of the neutral glycosphingolipids, and globoside. As representatives of the acidic glycosphingolipids, we employed ganglioside GM1 or GM2 individually or as a mixture. The gangliosides vary by virtue of the overall size of their oligosaccharide chains, but they all contain only one sialic acid residue per molecule.

The addition of 15 μM of various neutral or acidic eukaryotic glycolipids markedly enhanced the level of IL-6 secretion by PBMCs in response to 0.062 μM LTA (Fig. 1). It should be noted that the magnitude of stimulation differed among the various sphingolipids, with the greatest level of synergy being found when LTA and GM2 were incubated with the PBMCs. This is of particular interest, since GM2 is the most prominent glycosphingolipid found in normal human serum (8, 22). By comparison, the level of IL-6 secretion in response to 0.062 μM LTA plus GM2 was equivalent to the level of IL-6 secretion in response to 50 ng LPS (114.53 ± 22 ng/ml and 149.23 ± 8.78 ng/ml, respectively). Results similar to those obtained with PBMCs were obtained in a separate set of experiments with LTA, GM2, and purified monocytes (Fig. 2). Human monocytes were also incubated with globoside mixed with LTA at concentrations ranging from 0 to 0.496 μM (which correspond to 0 to 2 μg/ml). This resulted in molar ratios of LTA to globoside that ranged from 1:480 to 1:60 (Fig. 3). The percent increases caused by the mixtures were the greatest at the lower ratios of LTA to globoside, and at a molar ratio of 1:60, there was no statistically significant increase in the level of stimulation.

This method is sensitive down to 300 pg/ml IL-6. Interestingly, it has been shown that the mixing of the poorly active penta- and tetra-acylated LPS molecules at concentrations below their critical micelle concentrations (CMCs) before addition of the mixture to cells results in increased cytokine secretion (5). This result raises the possibility that the heterooligomers that formed between these two LPS structures were better able to interact with their receptor, subsequently resulting in enhanced cytokine release.

Nonadherent cells were removed by washing the flakes three times with Earl’s balanced salt solution. RPMI 1640 supplemented with 10% FCS was added to whole blood diluted 1:2 in Dulbecco’s phosphate-buffered saline (PBS), and the mixture was centrifuged at 700 × g for 30 min at room temperature (RT). The PBMC layer was collected, and the cells were washed three times with cold Dulbecco’s PBS. Monocytes were further isolated by allowing them to adhere to plastic. The PBMCs were resuspended in RPMI 1640 (supplemented with penicillin at 100 μg/ml and streptomycin at 100 U/ml) with 2% fetal calf serum (FCS) at a concentration of 5 × 10^6 cells/ml. The cells were incubated for 1 h at 37°C in tissue culture flasks to allow the monocytes to adhere to the flakes. Nonadherent cells were removed by washing the flakes three times with Earl’s balanced salt solution. RPMI 1640 supplemented with 10% FCS was added to the adherent monocytes, and the cells were incubated overnight at 37°C, during which time the monocytes detached (17). Monocytes purified in this way were collected, washed, and counted. PBMCs or monocytes, at 1.5 × 10^5 to 2 × 10^6 cells/200 μl in polypropylene tubes, were diluted in RPMI 1640 supplemented with 5% FCS or donor plasma and exposed to stimuli by overnight incubation.
Interactions of LTA and sphingolipids. Having determined the ability of glycosphingolipids to potentiate the activity of LTA, we next sought to examine whether this potentiation was the result of the separate activity of each component on the cells or was a shared activity. For this purpose, either the monocytes were incubated for 1 h with 0.062 μM LTA, followed by washing and overnight incubation with 15 μM globoside, or they were incubated for 1 h with 15 μM globoside, followed by washing and overnight incubation with 0.062 μM LTA. As a positive control, the monocytes were also incubated overnight with a mixture of both components (Fig. 4). The synergistic effects were achieved only when both components were simultaneously incubated with the cells, suggesting the

FIG. 1. Potentiation of PBMC response to LTA by glycosphingolipids. PBMCs at 1.5 × 10^5 to 2 × 10^5 cells/200 μl were exposed to 0.062 μM LTA, 15 μM of the indicated glycosphingolipids, and mixtures of both components that had been preincubated at 37°C for 1 h. After overnight incubation (19 to 22 h), the supernatants were removed and assayed for IL-6 secretion. The data are given as the means ± standard deviations for triplicate or quadruplicate samples in a single representative experiment of five experiments performed. However, in all the experiments the magnitude of stimulation by the mixtures was significant compared to that by LTA or the glycosphingolipids alone (P ≤ 0.001). —LTA, without LTA; +LTA, with LTA.

FIG. 2. Stimulation of human monocytes by LTA is augmented by GM₃. Monocyte cultures were prepared as described in Materials and Methods. Monocytes at 2 × 10⁶ cells/200 μl were exposed to medium alone; 0.062 μM LTA alone; GM₃ alone at the indicated concentrations; and mixtures of 0.062 μM LTA with 0 μM (i.e., LTA control), 7.5 μM, or 15 μM GM₃ that had been preincubated at 37°C for 1 h before being added to macrophages. After overnight incubation of the macrophages with these various stimuli (i.e., 19 to 22 h), the supernatants were removed and assayed for IL-6 secretion. The data are given as the means ± standard deviations for triplicate samples in a single representative experiment of five experiments performed. Differences between responses with and without globoside were statistically significant (*, P ≤ 0.001) except when the LTA concentration was 0.496 μM, and at this concentration of LTA, no increase in the response over that achieved with LTA alone was observed.

FIG. 3. Stimulation of human monocytes by LTA is augmented by globoside. Monocyte cultures were prepared as described in Materials and Methods. Monocytes at 2 × 10⁶ cells/200 μl were exposed to medium alone, LTA at the indicated concentrations (0.031 to 0.496 μM), 15 μM globoside, and mixtures of both components that had been preincubated at 37°C for 1 h. After overnight incubation (i.e., 19 to 22 h), the supernatants were removed and assayed for IL-6 secretion. The data are given as the means ± standard deviations for triplicate samples in a single representative experiment of seven experiments performed. Differences between responses with and without globoside were statistically significant (*, P ≤ 0.003) except when the LTA concentration was 0.496 μM, and at this concentration of LTA, no increase in the response over that achieved with LTA alone was observed.

FIG. 4. Synergistic effect requires the simultaneous presence of stimuli. Monocytes were incubated either with 0.062 μM LTA alone for 1 h; with 15 μM globoside alone for 1 h; with LTA for 1 h, followed by washing and overnight incubation with 15 μM globoside (LTA→globoside); or with globoside for 1 h, followed by washing and overnight incubation with 0.062 μM LTA (globoside→LTA). The cells were also incubated overnight with a mixture of both components preincubated at 37°C for 1 h. The supernatants were removed and assayed for IL-6 secretion. The data are given as the means ± standard deviations for triplicate samples in a single representative experiment of three experiments performed (*, P ≤ 0.003).
when the ratio of the host glycolipid was increased, glycolipids had a dramatic effect on LTA migration. However, mixed gangliosides. At roughly equimolar ratios, neither of the ways, depending upon whether the glycolipid is globoside or acrylamide gel, the migration of LTA is affected in different solution (33). When LTA is incubated with two different ceramide-containing glycolipids before it is run on a nondenaturing gel (Fig. 5). This migration pattern may be explained by the ability of LTA to form micelles in aqueous solution (33). When LTA migrates on a sodium dodecyl sulfate-polyacrylamide gel at ~8 to 10 kDa, it migrates much more slowly and, because of its microheterogeneous nature, in a much more diffuse manner on a nondenaturing gel (Fig. 5). This migration pattern may be explained by the ability of LTA to form micelles in aqueous solution (33). When LTA is incubated with two different ceramide-containing glycolipids before it is run on a nondenaturing acrylamide gel, the migration of LTA is affected in different ways, depending upon whether the glycolipid is globoside or mixed gangliosides. At roughly equimolar ratios, neither of the glycolipids had a dramatic effect on LTA migration. However, when the ratio of the host glycolipid was increased ~10-fold, the use of globoside resulted in LTA migration that was slower and less diffuse, while the use of mixed gangliosides resulted in LTA migration that was more rapid (Fig. 5). The interaction with hemoglobin has also been shown to change the migration of LTA in a nondenaturing gel (14). LTA was also incubated with gelatin in the previous study to control for the nonspecific effect of the protein on LTA migration, and no alteration of migration was observed. In the dot blot assay, we spotted three glycosphingolipids on silica gel thin-layer chromatography plates, incubated them with LTA, and detected LTA binding by using an anti-LTA antiserum (Fig. 6). LTA bound strongly to lactosylceramide and kerasin, less so to GM2, and not at all to the negative control, polymyxin B. The negative control plate that was not incubated with LTA showed no binding of primary or secondary antibodies to kerasin, GM2, or polymyxin B. There appeared to be a weak cross-reaction with lactosylceramide, but this may have been due to the low titers of antibody against ceramides that can be found in normal mammalian serum (15, 21). Nevertheless, there was such a marked difference between the two blots that we are convinced that it successfully makes the point that LTA interacts with lactosylceramide.

Synergy of LTA preparations and glycosphingolipids is not due to lipoprotein contamination. Previous studies have suggested that the contamination of LTA preparations with small amounts of lipoproteins might be responsible for the ability of such LTA preparations to stimulate macrophages to secrete cytokines (13). Although NMR and amino acid analysis did not reveal any protein contamination in our LTA preparations (14), we examined the possibility that a trace amount of a lipoprotein or a lipopeptide other than LTA could be responsible for synergizing with glycolipids and enhancing monocyte-stimulating activity. We first examined whether the addition of very low concentrations of Pam3CysK4 to LTA would result in enhanced cytokine release (Fig. 7). The results showed that, indeed, low concentrations of Pam3CysK4 do synergize with LTA. However, when the same concentrations of Pam3CysK4 were added to the various glycosphingolipids, it was not able to synergize with any of the glycosphingolipids. Combinations of glycolipids with low concentrations of Pam3CysK4 give only an additive and not a synergistic effect, and this occurred only with globoside (Fig. 8). Furthermore, at higher concentrations of Pam3CysK4, glycolipids inhibited the secretion of IL-6 by the PBMCs. Taken together, these results suggest that the potentiation of stimulation by our LTA preparation and glycosphingolipid is not due to a contaminating Pam3CysK4-like lipopeptide.

Potentiation of LTA activity by glycosphingolipids in human whole blood. We next wished to compare the abilities of different glycolipids to synergize with LTA to stimulate monocytes in whole blood. In these experiments, we used LTA at 0.062 μM (0.5 μg/ml), because at this concentration LTA alone stimulated the cells only weakly. The concentrations of glycosphingolipids that we added to the cells ranged from the lowest to the highest of the known glycosphingolipid concentrations in normal human serum (8, 22), and the molar ratio between LTA and glycosphingolipids was kept at ~1:120.
Several neutral and acidic glycosphingolipids in a human whole-blood system were examined, and all of them showed an increase in IL-6 secretion when they were mixed with LTA (Fig. 9). There were no differences in the levels of stimulation when 7.5 /H9262 M glycosphingolipid (the low concentration of glycosphingolipids in normal human serum) or 15 /H9262 M glycosphin- 
golipid (the high concentration level of glycosphingolipids in normal human serum) was added. Similar results were ob-
tained when dealanylated LTA was used (data not shown).

In a separate set of experiments, we also assayed the culture 
supernatants for IL-1 /H9252 secretion. In these experiments, similar 
synergies were seen when LTA was combined with ceramide 
containing glycolipids, with GM3 again giving the highest level 
of enhancement, in which the level of IL-1 /H9252 secreted was 
roughly 10-fold higher than the level secreted in response to 50 
ng/ml LPS (Fig. 10). In future experiments, it will be of interest 
to more completely define the profiles of the cytokines se-
creted and to assess their effects in vivo.

**DISCUSSION**

LTA is one of the cell wall components of gram-positive 
bacteria long considered to be a possible contributor to septic 
shock caused by gram-positive organisms, but its activity in 
stimulating cells of the immune system to secrete proinflam-
bial or proinflammatory properties of LTA: (i) LTA is a gram-positive bacterial component that activates cells of the immune system (e.g., monocytes and macrophages) to secrete tumor necrosis factor alpha, IL-6, and other proinflammatory molecules; or (ii) a copurifying lipoprotein is the component of LTA preparations that stimulates cytokine secretion by monocytes and macrophages and LTA is essentially inactive (13). Since we had shown that our highly purified LTA preparations could synergize with glycolipids to enhance cytokine secretion from monocytes, we wanted to determine if the stimulation of monocytes with the lipopeptide Pam3CysK4 and glycolipids would also result in synergy. To the contrary, we found not only that there was no synergy but also that glycosphingolipids could actually inhibit the activity of Pam3CysK4. Furthermore, we showed that in a similar range of molar ratios, Pam3CysK4 did synergize with LTA and resulted in enhanced cytokine secretion. This result may also stem from the formation of heterooligomers between these two amphiphiles.

In summary, we currently favor the concept that LTA will exhibit potent stimulatory activity under certain conditions, such as when LTA is released from the pathogen (2) in the presence of hemoglobin (which is released from erythrocytes by a hemolytic toxin, such as streptolysin O), muramyl dipeptide (which is released from bacterial cell walls by lysozyme and amidases as part of the host response [29]), or glycosphingolipids (whose levels are known to be increased during sepsis [9]). It is also quite possible that there are other bacterial or host macromolecules that could synergize with LTA to increase proinflammatory cytokine secretion. Future experiments should focus on the synergistic effects of mixtures of LTA and other compounds.

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