Distribution and Kinetics of Lipoprotein-Bound Lipoteichoic Acid

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Lipoteichoic acid (LTA), a major cell wall component of gram-positive bacteria, is an amphipathic anionic glycolipid with structural similarities to lipopolysaccharide (LPS) from gram-negative bacteria. LTA has been implicated as one of the primary immunostimulatory components that may trigger the systemic inflammatory response syndrome. Plasma lipoproteins have been shown to sequester LPS, which results in attenuation of the host response to infection, but little is known about the LTA binding characteristics of plasma lipid particles. In this study, we have examined the LTA binding capacities and association kinetics of the major lipoprotein classes under simulated physiological conditions in human whole blood (ex vivo) by using biologically active, fluorescently labeled LTA and high-performance gel permeation chromatography. The average distribution of an LTA preparation from Staphylococcus aureus in whole blood from 10 human volunteers revealed that >95% of the LTA was associated with total plasma lipoproteins in the following proportions: high-density lipoprotein (HDL), 68% ± 10%; low-density lipoprotein (LDL), 28% ± 8%; and very low density lipoprotein (VLDL), 4% ± 5%. The saturation capacity of lipoproteins for LTA was in excess of 150 μg/ml. The LTA distribution was temperature dependent, with an optimal binding between 22 and 37°C. The binding of LTA by lipoproteins was essentially complete within 10 min and was followed by a subsequent redistribution from HDL and VLDL to LDL. We conclude that HDL has the highest binding capacity for LTA and propose that the loading and redistribution of LTA among plasma lipoproteins is a specific process that closely resembles that previously described for LPS (J. H. M. Levels, P. R. Abraham, A. van den Ende, and S. J. H. van Deventer, Infect. Immun. 68:2821-2828, 2001).

Lipoteichoic acid (LTA), the major cell wall component of most gram-positive bacteria, is a member of a structurally related group of macroamphiphiles, the glycolipids, which consist of a hydrophobic diacylglycerol membrane anchor and a hydrophilic head group exposed on the outer bacterial surface (28). Experimental evidence has shown that LTA is a potent endotoxin capable during severe infection of inducing hemodynamic, hematological, and metabolic changes of a magnitude similar to those induced by lipopolysaccharide (LPS) from gram-negative bacteria (4). LTA preparations stimulate cells associated with cellular immunity to produce high levels of endogenous mediators of inflammation, such as tumor necrosis factor alpha (TNF-α) and the interleukins (IL-6, IL-1β, and IL-8), which are capable of sustaining an inflammatory state that may lead to septic shock and multiorgan failure (2, 4). Recognition of LPS by monocytes and macrophages is effected by the membrane-bound G-protein-linked receptor CD14 in a process which is accelerated by LPS binding protein (LBP) (14, 31). LBP acts together with soluble CD14 to monomerize LPS micelles and facilitate transport of the endotoxin to lipoproteins and macrophage receptors (29, 30). LBP and soluble CD14 have been found to bind LTA (22, 23) but with lower affinity than they bind LPS. In addition, Toll-like receptor proteins TLR-2 and TLR-4 of the macrophages have recently been implicated in endotoxin-induced intracellular signaling by LTA (9) and LPS (24, 25), respectively. In contrast to that of LPS, little is known about the mechanism of processing and clearing of LTA in the host.

Lipid metabolism appears to be extensively regulated during the host response to infection by increased levels of proinflammatory cytokines such as TNF-α, IL-1, and IL-6 or after cytokine administration in experimental animals and in humans (10). Disturbances in lipoprotein homeostasis appear to be characteristic of bacterial infection (1, 3, 11). The reduction in total cholesterol and in the apolipoprotein A-I and B contents of high-density lipoprotein (HDL) and low-density lipoprotein (LDL), respectively, coupled with an increase in very low density lipoprotein (VLDL) triglycerides, has been previously described (10). The magnitude of these alterations in lipoprotein composition appears to be related to the severity of the infection. Remarkably, all of these changes in the plasma lipid profiles were independent of the underlying diseases or the infectious agent responsible for initiating systemic inflammatory response syndrome. It has recently been proposed that disturbances in lipid metabolism may contribute to host defense, because the immune response is intimately linked to the metabolic response (12).

The expression of the scavenger receptor BI, an important mediator of cellular metabolism of HDL in the adrenal gland, also gives strong indications that the scavenger receptor BI may play a role in the processing of bacterial endotoxin during sepsis caused by gram-negative organisms (15). These changes in lipid metabolism also appear to form an integral part of the acute-phase response.

It has previously been shown that all lipoprotein classes are...
capable of binding LPS (26, 5, 21), which results in the attenuation of the host response to infection (6, 8, 19), and that lipoproteins are capable of inhibiting macrophage activation by isolated LTA preparations (13). The binding characteristics and kinetics of fluorescently labeled biologically active LPS with native plasma lipoproteins analyzed by high-performance gel chromatography (HPGC) have been recently described (16). To address the question of whether the interaction of LTA with lipoproteins is comparable to that of LPS, we examined the binding characteristics of plasma lipoproteins from healthy human subjects by using fluorescently labeled LTA and HPGC lipoprotein analysis. Here we report the LTA binding capacities of lipoproteins and the distribution and kinetics of lipoprotein-bound LTA under simulated physiological conditions in whole blood (ex vivo).

MATERIALS AND METHODS

Reagents. LTA preparations from Staphylococcus aureus were from Sigma Chemical Co. (St Louis, Mo.) and were labeled as described by Fischer et al. (7). Pyrogen-free distilled water used throughout the experiments was from Ecolab (Braun Medical AG, Melsungen, Germany). The fluorescent label 4,4-difluoro-1,3-dimethyl-5-(4-methoxyphenyl)-4-bora-3a,4a-diaza-s-indacene-2-propanoyl-tetramethyl-rhodamine (BODIPY-TMR) was from Molecular Probes Inc. (Eugene, Oreg.). 4-Amino-antipiperidoxy (PAF) reagent for postcolumn cholesterol detection was from Biorad (Marcy l’Etoile, France). Pyrogen-free heparin was from LEO Pharmaceuticals B.V. (Weesp, The Netherlands). The Limulus amebocyte lysate (LAL) Coastest endotoxin was obtained from Chromogenix AB (Möndal, Sweden). TNF-α was analyzed with the Pellekin human TNF-α enzyme-linked immunosorbent assay kit (CLB, Amsterdam, The Netherlands).

Fluorescent labeling of LTA. LTA was labeled with the water-soluble BODIPY-TMR 4-sulfotetrafluorophenyl ester (Molecular Probes) by using modifications of the manufacturer’s protocol for oligosaccharide labeling. LTA was prepared for labeling by sonication of a suspension at a concentration of 2 mg/ml in pyrogen-free water with a Branson sonifier at maximum output for a total of 10 min on ice. A molecular mass of 8 kDa was used as the average size of the LTA monomer (M, 7,000 to 10,000) (7). LTA at a final concentration of 1 mg/ml in 0.1 M sodium bicarbonate buffer, pH 8.3, was derivatized in polypropylene tubes by the addition of a fivefold molar excess of BODIPY-TMR disolved in pyrogen-free water, and the reaction was allowed to proceed for 2 h in the dark at room temperature. Nonconjugated BODIPY label remaining after the derivatization was allowed to react with a 20-fold molar excess of glycine for a further 30 min. The BODIPY-LTA conjugate was separated from BODIPY-glycine by gel filtration on a 10-ml Sephadex G-15 column (Pharmacia Biotech, Uppsala, Sweden) by using pyrogen-free water. The BODIPY-LTA micelles elute in the void volume, while BODIPY-glycine is retained by the matrix. The concentration of the peak fraction of labeled LTA was approximately 0.80 mg/ml. The efficiency of label incorporation was determined by measurement of the optical density at 542 nm by using the quoted extinction coefficient of 60,000 cm⁻¹ M⁻¹, and the stoichiometry of labeling was found to be approximately two BODIPY molecules to one LTA molecule. The LTA preparation was tested for purity and LTA labeling specificity by high-performance liquid chromatography analysis. Comparison with highly purified LTA (18) (kindly provided by Thomas Hartung from the University of Constance) by reversed-phase high-performance liquid chromatography (20) revealed that the labeled LTA preparation had a comparable fingerprint. The labeling specificity was 90% ± 4% (standard deviation [SD]).

Blood sampling and handling. Whole blood was drawn by venipuncture from healthy volunteers after informed consent and collected in pyrogen-free polypropylene tubes containing heparin (2 U/ml) or in some instances sodium citrate (0.32% [wt/vol]. Becton Dickinson, Lincoln Park, N.J.). Blood samples or cell-free plasma samples obtained by centrifugation (2,000 × g for 20 min at 12°C) were always used for experimentation within 1 h after collection.

Biological activity of LTA. Labeled or unlabeled LTA was added to heparinized blood (2 U/ml) from five healthy volunteers to final concentrations ranging from 10 to 10,000 ng/ml of blood. Prior to LTA addition, polymyxin B (10 μg/ml) was added to neutralize potential LPS contamination (17). After incubation for 4 h at 37°C, TNF-α production was determined in cell-free plasma obtained by centrifugation for 20 min at 2,000 × g at 4°C by using a specific and sensitive cytokine TNF-α enzyme-linked immunosorbent assay kit (Sanquin, Amsterdam, The Netherlands).
These results demonstrate the inverse relationship of LTA binding and the lipoprotein cholesterol content. The saturation capacity of the lipoproteins for LTA was investigated by adding LTA to plasma to achieve concentrations from 5 to 200 μg/ml and by analysis of the fluorescence distribution (Fig. 4). HDL shows the highest binding capacity for LTA, with saturation approaching 170 μg/ml, whereas LDL and VLDL approached saturation at LTA concentrations of 150 μg/ml.

Temperature dependence of LTA distribution. To investigate the influence of temperature on the LTA distribution among the lipoprotein classes in plasma, experiments were done at temperatures ranging from 4 to 60°C (Fig. 5). The highest overall LTA fluorescence signal was observed at 37°C. In addition, the association of LTA with LDL and to a lesser extent with HDL appeared to be temperature dependent, with...
optimum association occurring between 22 and 37°C. A contin-
uous increase in LTA signal with increasing temperature
was evident only for VLDL, which contains a high proportion
of triglycerides relative to the other lipoprotein particles.

Kinetics of LTA binding. To study the binding kinetics of
lipoproteins, the distribution of fluorescent LTA among the
lipoprotein classes in plasma was analyzed after incubations
from 10 to 120 min with LTA at 10, 20, and 40 μg/ml (Fig. 6).
We observed that the loading of lipoproteins with LTA was
essentially complete within 10 min and that a redistribution of
LTA occurred during the subsequent 110 min. The median
decreases of HDL- and VLDL-bound LTA of 23% ± 4% and
13% ± 20%, respectively, were accompanied by an increase in
LDL-bound LTA of 28% ± 13% relative to the amount of
LTA bound by the respective lipoprotein classes at 10 min.

DISCUSSION

For this study, we present the overall distribution, temper-
ature dependence, saturation characteristics, and association
kinetics of a purified *S. aureus* LTA preparation among the
major plasma lipoprotein classes by using HPGC for quantita-
tion of fluorescent LTA binding to the lipid particles.

A recent report has drawn our attention to potential LPS
contamination in commercial LTA preparations (28). The
preparations used in our studies were, therefore, examined for
the presence of LPS by using the sensitive LAL endotoxin
assay and were found to contain trace amounts of LPS in the
order of 19 ng of LPS/mg of LTA, which represents a contam-
ination of 0.0019% (wt/wt). Thus, more than 99.9% of the
measured BODIPY fluorescence was of LTA origin, and com-
petition for lipoprotein binding by fluorescent LPS in these
amounts was found to be negligible (data not shown).

In our experimental design, we observed an average recov-
ery of LTA fluorescence of 68, 28, and 4% in the HDL, LDL,
and VLDL fractions, respectively, after incubation for 1 h at
37°C. This demonstrates that HDL has the highest affinity for LTA and that the overall distribution of LTA among the major lipoprotein classes (Fig. 1) is directly comparable with the recently described LPS distribution among plasma lipoproteins (16). Furthermore, since LTA binding appears to be independent of the lipoprotein cholesterol content, the specificity of LTA binding appears to be determined by factors other than lipid composition. A reasonable assumption would be that the LTA distribution is not only dependent on nonlipid factors, especially with regard to HDL, but is also directly proportional to the total number of these small lipid particles. Current evidence indicates that HDL-associated lipid and LPS transfer to the total number of these small lipid particles. Current especially with regard to HDL, but is also directly proportional to the total number of these small lipid particles. 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