L-Ficolin Specifically Binds to Lipoteichoic Acid, a Cell Wall Constituent of Gram-Positive Bacteria, and Activates the Lectin Pathway of Complement

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L-Ficolin Specifically Binds to Lipoteichoic Acid, a Cell Wall Constituent of Gram-Positive Bacteria, and Activates the Lectin Pathway of Complement

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The lectin pathway of complement is activated when a carbohydrate recognition complex and associated serine proteases binds to the surface of a pathogen. Three recognition subcomponents have been shown to form active initiation complexes: mannan-binding lectin (MBL), L-ficolin, and H-ficolin. The importance of MBL in antimicrobial host defense is well recognized, but the role of the ficolins remains largely undefined. This report shows that L-ficolin specifically binds to lipoteichoic acid (LTA), a cell wall component found in all Gram-positive bacteria. Immobilized LTA from Staphylococcus aureus binds L-ficolin complexes from sera, and these complexes initiate lectin pathway-dependent C4 turnover. C4 activation correlates with serum L-ficolin concentrations, but not with serum MBL levels. L-ficolin binding and corresponding levels of C4 turnover were observed on LTA purified from other clinically important bacteria, including Streptococcus pyogenes and Streptococcus agalactiae. None of the LTA preparations bound MBL, H-ficolin, or the classical pathway recognition molecule, C1q. The Journal of Immunology, 2004, 172: 1198–1202.

The lectin pathway of complement activation provides an essential route of innate antimicrobial host defense. Activation of the lectin pathway occurs in response to carbohydrate structures present on microbial surfaces and is initiated through multimolecular fluid-phase complexes composed of a carbohydrate recognition subcomponent and the lectin pathway serine protease, mannan-binding lectin-associated serine protease-2 (MASP-2). Three different carbohydrate recognition subcomponents that form complexes and activate complement via MASP-2 have been described: mannan-binding lectin (MBL), L-ficolin, and H-ficolin (previously described as Hakata Ag) (1–4). All recognition subcomponents consist of homotrimers of a single polypeptide chain with an N-terminal collagen-like domain, a neck region, and a C-terminal carbohydrate-binding domain (5). In MBL, this carbohydrate recognition domain is a classical C-type lectin domain, while the carbohydrate recognition domains of ficolins show a fibrinogen-like domain structure. In plasma, the recognition subcomponents are present as higher-order oligomers of the homotrimeric subunits that form complexes with MASP-2 and two other serine proteases, MASP-1 and MASP-3, to compose a lectin pathway activation complex (6–9). Of these, only MASP-2 was shown to translate the binding of lectin pathway complexes to microbial carbohydrates into activation of complement by cleavage of C4 and C4b bound C2 (8, 10–12). It has been shown that MBL binds to a range of clinically important microorganisms including fungi, viruses, and both Gram-negative and Gram-positive bacteria (13, 14). In contrast, little is known about the binding specificities of the ficolins. H-ficolin was shown to bind to Staphylococcus typhimurium, Salmonella minnesota, and Escherichia coli (15), while L-ficolin was shown to activate the lectin pathway after binding to S. typhimurium (16). This is the first report that identifies a defined cell wall constituent of Gram-positive bacteria, namely lipoteichoic acid (LTA), to be specifically recognized by a lectin pathway recognition subcomponent. LTA is increasingly regarded as the Gram-positive counterpart of LPS. It is a potent immunostimulant that induces cytokine release from mononuclear phagocytes and whole blood (17, 18). We demonstrate that the L-ficolin-LTA interaction initiates an innate anti-microbial immune response by triggering the lectin pathway of complement activation.

Materials and Methods

Materials

 Unless otherwise stated, all reagents were obtained from Sigma-Aldrich (St. Louis, MO). Sera were collected from healthy volunteers, with the approval of the institutional ethical review board, and were assayed for MBL as described by Haurum et al. (19). C1q-depleted serum was prepared from pooled normal human serum (NHS) using protein A-coupled Dynabeads (Dynal Biotech, Oslo, Norway) coated with rabbit anti-human C1q IgG (DAKO, Glostrup, Denmark), according to the supplier’s instructions. L-ficolin and L-ficolin/MASP complexes were purified from human serum as previously described (16), and their concentrations were determined using a proprietary Lowry assay kit (Sigma-Aldrich). PSA, a polysaccharide produced by Aerococcus viridans, was prepared as previously described (20). Formalin-fixed Staphylococcus aureus DSM20233 were prepared as follows: bacteria were grown overnight at 37°C in tryptic soy blood medium, washed three times with PBS, then fixed for 1 h at room temperature in PBS/0.5% formalin, and washed a further three times with PBS, before being resuspended in 15 mM Na2CO3, 35 mM NaHCO3, pH 9.6 (coating buffer).
Materials and Methods
was measured as described in (21). The purity of the LTA was greater than 99%, according to nuclear magnetic resonance and mass spectrometry.

Extraction and purification of LTA
Pure LTA, free from endotoxin and other contaminants, was purified from cell extracts of S. aureus (DSM20233), Bacillus subtilis (DSM1087), Bio-

solid-phase binding assays
Nunc Maxisorb microtiter plates were coated with LTA, mannan, mAb 4H5, or formalin-fixed S. aureus as described above, PSA from A. viridans (2 µg/well in coating buffer), 1 µg/well of the L-ficolin-specific mAb GN4 (3), or immune complexes generated in situ by coating with BSA (1 µg/well in coating buffer), then adding rabbit anti-BSA (2 µg/ml in wash buffer). Wells were blocked with 300 µl of 0.1% HSA in TBS for 1.5 h at room temperature, then washed with wash buffer. Serum samples or purified L-ficolin/MASP complexes were diluted in 100 µl of 10 mM Tris-Cl, 140 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, pH 7.4, and added to the plates and incubated overnight at 4°C. "C. After washing, bound proteins were detected using rabbit anti-human L-ficolin IgG (24), rabbit anti-human H-ficolin antisemur (18), or goat anti-human C1q (Atlantic Abs, Stillwater, MN). Secondary Abs were alkaline phosphatase-conju-
gated goat anti-rabbit IgG or rabbit anti-goat IgG, as appropriate, and bound Ab was detected using the colorimetric substrate p-nitrophenyl phosphate. For negative controls, the primary Ab was omitted or replaced with preimmune rabbit IgG, rabbit serum, or goat serum. A standard serum was included on each plate to allow cross-plate normalization of the results.

L-ficolin ELISA
Nunc Maxisorb microtiter plates were coated with 1 µg/well mAb GN4 in coating buffer. Wells were blocked, diluted serum samples added, and L-ficolin detected using rabbit anti-human L-ficolin IgG (24), as described in Solid-phase binding assays.

Flow cytometry
One hundred microliters of S. aureus DSM20233 (freshly isolated; OD560 = 1.4) were suspended in Veronal-buffered saline supplemented with 0.1% gelatin, 2 mM CaCl2, and 0.5 mM MgCl2 (GBV) and spun down. The pellets were incubated at 37°C for 30 min with 20 µl of FITC-conjugated anti-mouse IgG (100 µg/ml; DAKO). The cells were washed twice with GBV between each reaction. Reactivities were evaluated by FACSCalibur 4A flow cytometry (BD Biosciences, Mountain View, CA). F(ab')2 of the murine anti-human L-ficolin Ab mAb 2F5 (IgG1) were generated by pepsin cleavage using a proprietary kit (Pierce Biotech-

Results
A C4 cleavage assay that monitors complement activation via the lectin pathway was used to determine serum responses to very pure LTA preparations derived from the cell wall of S. aureus strain DSM 20233. As shown in Figs. 1, A and B, lectin pathway-medi-
ated C4 cleavage occurred in both MBL-sufficient and MBL-deficient (MBL ≤ 50 ng/ml) sera, suggesting that MBL was not the recognition molecule involved in LTA-dependent complement activation. Similar results were obtained using recalci
dated plasma in place of serum (data not shown). Moreover, depletion of C1q had no effect on the ability of serum to activate C4 in response to S. aureus (Fig. 1A). A sensitive MBL-binding assay detected as little as 50 ng/ml MBL when ELISA wells were coated with mannan, but no MBL binding was detected when wells were coated with LTA from DSM20233 (data not shown). L-ficolin binding to LTA could be demonstrated with all of the sera tested and the level of C4 activation correlated closely with the concentration of L-ficolin in the sera (Fig. 1C). There was no corresponding correlation with the MBL concentrations in these sera. C4 activation on LTA-coated wells could be completely inhibited by pre-incubating the serum with excess fluid-phase LTA, while fluid-phase mannan (which inhibits MBL-driven C4 activation) had no effect (Fig. 1D). Initially, the plates were coated with LTA dissolved in methanol, to protect the alkali-labile ω-alanine esters on the phosphate backbone, which are essential for LTA-mediated cytokine release (17, 18). However, it was found that L-ficolin binding and C4 activation were similar on LTA that had been dissolved in carbonate buffer at pH 9.2, suggesting that ω-alanine substitution is not essential for L-ficolin binding.

Two experiments demonstrate that H-ficolin does not contribute to the C4 activation seen on LTA from S. aureus strain DSM20233: First, an H-ficolin-specific ELISA showed that, although H-ficolin binds to the anti-H-ficolin mAb GN4 and to PSA from A. viridans (a known ligand for H-ficolin), it binds neither to whole formalin-fixed S. aureus nor to LTA (Fig. 2A). Second, coating plates with mAb 4H5 leads to H-ficolin-dependent activation of the lectin pathway that can be specifically inhibited by adding excess fluid-phase PSA, but not by adding LTA (data not shown). Fig. 2B illustrates the absence of a direct interaction between C1q and LTA at physiological salt concentrations.

Next, the sera were replaced with purified L-ficolin-MASP complexes (16). Concentration-dependent binding of L-ficolin-MASP complexes was observed on wells coated with the L-ficolin-specific mAb GN4, LTA from DSM20233, and formalin-fixed
DSM20233, but not on wells coated with PSA or mannan (Fig. 3A). Likewise, concentration-dependent C4 activation was seen on LTA coated wells, but not on those coated with mannan (Fig. 3B).

Preparations of pure LTA from other Gram-positive bacteria were tested for C4 activation, ficolin binding, and MBL binding. L-ficolin binding and C4 activation on LTA from B. subtilis (DSM1087), S. pyogenes, and S. agalactiae (two isolates) were remarkably similar to that seen for LTA from S. aureus DSM20233 (Fig. 4). LTA from B. animalis bound significantly less L-ficolin, and the C4 activation was correspondingly low. Neither MBL nor H-ficolin bound to any of the LTA preparations tested (data not shown).

Flow cytometry was used to demonstrate binding of purified L-ficolin-p35 to whole S. aureus DSM20233, and this binding could also be inhibited by excess fluid-phase LTA (Fig. 5, A and B). C4 activation on whole formalin-fixed DSM20233 could be inhibited to roughly equal extents by both mannan and LTA (Fig. 5C), and the effect of the two inhibitors was additive, implying that approximately half of the C4 activation observed on the whole bacteria is a consequence of MBL binding to cell wall components other than LTA, probably to the mannose-rich peptidoglycan.

Discussion

Previous studies have shown that on LTA preparations isolated from various bacterial strains complement activation occurs in an Ab-independent fashion (25–27). The mode of activation, however, remained unclear. Based on measurements demonstrating a significant consumption of complement components C4 and C2 (and a moderate consumption of hemolytically active C1), the most recent of these reports suggested that LTA might activate complement through activation of the classical pathway involving a direct interaction of LTA with C1q (27). No direct binding between LTA and C1q, however, was described.

This study demonstrates that complement activation occurs via the lectin pathway through specific binding of L-ficolin to LTA preparations from different Gram-positive bacterial strains, including S. aureus strain DSM 20233. The binding of L-ficolin to LTA

FIGURE 4. C4 cleavage and L-ficolin binding by LTA from different Gram-positive bacteria. Plates were coated with 1 μg/well of purified LTA from S. aureus, B. animalis, S. pyogenes, B. subtilis, and two isolates of S. agalactiae (DSM6313 and COH1). Diluted standard serum was added, and C4 deposition or L-ficolin binding was assayed. Results are relative C4 cleavage and relative L-ficolin binding, normalized to LTA from S. aureus (n = 4, error bars represent the SD).

FIGURE 5. S. aureus binds L-ficolin and activates the lectin pathway of complement. S. aureus DSM20233 was incubated with purified L-ficolin in the presence of various concentrations of purified LTA. L-ficolin binding was detected by flow cytometry using the F(ab)2 of mAb 2F5 and FITC-conjugated anti-mouse IgG F(ab)2. A, Black peak, negative control (no L-ficolin); solid line, L-ficolin (without LTA); dashed line, L-ficolin pre-incubated with 8 μg/ml fluid-phase LTA. B, Inhibition of L-ficolin binding to S. aureus by LTA. Results are the means of three independent experiments, error bars represent the SD, and the solid line shows binding as a percentage of that seen for L-ficolin alone. C, Inhibition of C4 activation on microtiter plates coated with formalin-fixed S. aureus. Normal serum was pre-incubated with various amounts of LTA, mannan, or LTA and mannan (abscissa), then added to the coated plates and C4 activation assayed as described in Materials and Methods. Results are means of two independent experiments and are relative to the serum with no added inhibitors.
was highly specific, none of the LTA preparations bound MBL or H-ficolin. These findings are consistent with those from Polotsky and coworkers (28), who reported that recombinant human MBL binds to LTA from Enterococcus spp. (in which the polyglycerolphosphate chain is substituted with glycosyl groups), but not to LTA from nine other species, including S. aureus, S. pyogenes, and Bifidobacterium.

Inhibition assays indicated that L-ficolin is responsible for ~50% of the total lectin pathway-dependent C4 activation seen on whole formalin-fixed S. aureus; the remaining C4 activation could be inhibited with mannan and is therefore attributable to MBL binding to cell wall components other than LTA. This finding may explain the observation that the depletion of C4 and iC3b on S. aureus, and the opsonophagocytosis of S. aureus, in MBL-deficient serum is approximately half of that seen in MBL-deficient serum reconstituted with MBL-MASP complexes (29).

The levels of L-ficolin binding and lectin pathway-dependent C4 activation detected on LTA purified from B. subtilis, S. pyogenes, and S. agalactiae were similar to those seen on LTA from S. aureus, while LTA from B. animalis had a drastically reduced capacity to bind serum L-ficolin (~30% of the amount bound by the same concentration of the other LTAs tested) and showed correspondingly little C4 activation. The low level of binding to Bifidobacterium LTA is probably a consequence of its backbone structure; Bifidobacterium spp LTA differs from the others in that its backbone consists of lipofuranan instead of polyglycerophosphate and it is substituted with monoglycosylphosphate groups instead of N-acetylated carbohydrate groups (30).

Our results indicate that the repertoire of microbial organisms recognized by L-ficolin could both overlap and extend that recognized by MBL. The ability of several fluid-phase carbohydrate recognition molecules to initiate the lectin pathway of complement activation in response to different pathogen-associated molecular patterns broadens the spectrum for the innate response toward invading microbial organisms.

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