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Bartonella quintana Lipopolysaccharide Is a Natural Antagonist of Toll-Like Receptor 4

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Bartonella quintana is a gram-negative microorganism that causes trench fever and chronic bacteremia. B. quintana lipopolysaccharide (LPS) was unable to induce the production of proinflammatory cytokines in human monocytes. Interestingly, B. quintana LPS is a potent antagonist of Toll-like receptor 4 (TLR4), as it inhibited both mRNA transcription and the release of tumor necrosis factor alpha, interleukin 1β (IL-1β), and IL-6 by Escherichia coli LPS in human monocytes, at ratios ranging from 1,000:1 to 10:1 (B. quintana LPS to E. coli LPS). Likewise, B. quintana LPS blocked the interaction of E. coli LPS with TLR4 in transfected cell lines. The extent of the inhibitory effect of B. quintana LPS was demonstrated in microarray studies, which showed downregulation of practically all genes induced by LPS in monocytes. Because of the role of TLR4 in inflammation, B. quintana LPS may prove useful as a potent anti-TLR4 agent with therapeutic potential in both infections and autoimmune inflammation.

Bartonella quintana is a gram-negative pathogen initially described during World War I as the agent of trench fever, a disease associated with recurrent fever and headaches. In the past few decades, B. quintana infection has been identified in homeless people (4). While most individuals with B. quintana infection recover, some 5 to 10% eventually develop chronic bacteremia (4) and subsequent complications, such as chronic endocarditis in the absence of preexisting heart valve lesions (20). New manifestations of B. quintana infections, such as bacillary angiomatosis, bacillary peliosis hepatitis, and chronic lymphadenopathy, have also been described (2). These manifestations have been attributed to proliferative and antiapoptotic effects of Bartonella spp. (6).

A characteristic of B. quintana bacteremia is the absence of symptoms of high fever and signs of septic shock, disseminated intravascular coagulation, or organ failure. Lipopolysaccharide (LPS, or endotoxin) is a main component of the outer membranes of gram-negative microorganisms, and the LPSs from gram-negative enteric bacteria (such as Escherichia coli and Salmonella enterica) are able to induce proinflammatory cytokines, chemokines, and adhesion molecules (23) and thereby to evoke the clinical signs of sepsis. The lipid A moiety of LPS interacts with a membrane receptor complex containing Toll-like receptor 4 (TLR4), MD-2, and CD14 (19, 27). Because of strong proinflammatory effects of most species of LPS, the apparent absence of sepsis syndrome in patients with B. quintana bacteremia is a puzzling aspect of the infection. As an explanation, overproduction of the anti-inflammatory cytokine interleukin-10 (IL-10) and an attenuated inflammatory cytokine profile during B. quintana bacteremia have been proposed (5), but the molecular mechanisms have remained elusive.

Recently, the LPS of the related organism Bartonella henselae was purified and characterized as a penta-acylated deep-rough LPS with low endotoxic activity (17, 27). In the present study, we investigated the biologic activities of B. quintana LPS in terms of induction of proinflammatory cytokines and interaction with TLRs and other species of LPS.

MATERIALS AND METHODS

Reagents and microorganisms. LPS (E. coli serotype O55:B5) was purchased from Sigma Chemical Co., and synthetic Pam3Cys was purchased from EMC Microcollections (Tübingen, Germany). The B. quintana Oklahoma strain was kindly provided by D. Raoult (Marseille, France) and grown on sheep blood agar at 37°C in a 5% CO2 atmosphere. For stimulation experiments, 5-day cultures of B. quintana were heat killed for 60 min at 56°C. B. quintana LPS was extracted either by a single-step phenol-water extraction, as previously described (13), or by a two-step extraction method (9) which eliminates contamination with proteins. Briefly, the gel was fixed in 40% methanol–10% acetic acid, sensitized by a two-step extraction method (9) which eliminates contamination with proteins. B. quintana LPS (100 µg of each) were run in a 10% polyacrylamide gel and subjected to silver staining to visualize contaminating proteins. B. quintana LPS was extracted either by a single-step phenol-water extraction, as previously described (13), or by a two-step extraction method (9) which eliminates contamination with proteins.

Signaling through human TLR2 and TLR4 in a transfected cell line. Chinese hamster ovary (CHO) fibroblasts stably transfected with human CD14 (3E10-CD14), a combination of CD14 and TLR4 (3E10-CD414), and TLR2 (3E10-TLR2) were a kind gift from Robin Ingalls. These cell lines express inducible membrane CD25 under the control of a region of the human E-selectin (ELAM-1) promoter containing NF-κB binding sites. Cells were maintained at 37°C in 5% CO2 in Ham's F-12 medium (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum, 0.01% L-glutamine, 50 µg/ml gentamicin, 400 U/ml hygromycin, and 0.5 mg/ml of G418 (for 3E10-TLR2) or 0.05 mg/ml of puromycin (for 3E10-TLR4) as an additional selection antibiotic.
TLR4 expression was confirmed by flow cytometry (Coultet Epiqs XL-MCL; Beckman Coulter, Mijdrecht, The Netherlands), using a phycoerythrin-labeled anti-TLR4 antibody (clone HTA125; Immunosource, Halle-Zoersel, Belgium).

For stimulation experiments, 500 µl of cells in culture medium at a density of 1 × 10^6/ml was plated in 24-well culture plates. After an overnight incubation, cells were incubated with control medium, Pam3Cys (10 µg/ml), E. coli LPS (1 µg/ml), B. quintana LPS (10 µg/ml), or a combination of E. coli LPS and B. quintana LPS for 20 h at 37°C. Thereafter, cells were harvested using trypsin-EDTA (Cambrex, East Rutherford, NY) and prepared for flow cytometry (Coulter FACScan). CD25 expression of the CHO cells was measured using fluorescein isothiocyanate-labeled anti-CD25 (DAKO, Glostrup, Denmark) and was expressed as the x-fold increase above the mean.

Isolation of PBMC and stimulation of cytokine production. After informed consent was obtained, venous blood was drawn from the cubital veins of six healthy volunteers into three 10-ml lithium-heparin tubes (Monocyte, ‘s Hertogenbosch, The Netherlands). The regional medical ethical committee approved the study protocol. The peripheral mononuclear cell (PBMC) fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Cells were washed twice in saline and suspended in culture medium (RPMI 1640 DM) supplemented with 10 µg/ml gentamicin, 10 mM t-glutamine, and 10 mM pyruvate. The cells were counted in a Coulter counter (Beckman Coulter, Mijdrecht, The Netherlands), and the number was adjusted to 5 × 10^6 cells/ml.

PBMC (5 × 10^5) in a 100-µl volume were added to round-bottomed 96-well plates (Greiner, Alphen/a/d Rijn, The Netherlands) and incubated with either 100 µl of culture medium (negative control) or one of the following stimuli: B. quintana bacteria (1 × 10^6 microorganisms/ml), B. quintana LPS (at concentrations of 100, 10, or 1 µg/ml), and E. coli LPS (10 ng/ml) 30 min before stimulating the cells with cytokine. RNAs were extracted by the TRIzol method (Sigma, St. Louis, MO). Subsequently, 200 µl chloroform and 500 µl 2-propanol (Merck, Darmstadt, Germany) were used to separate the RNAs from DNAs and proteins. Finally, after a wash step with 75% ethanol (Merck, Darmstadt, Germany), the dry RNAs were dissolved in 30 µl of water.

PCR amplification. To obtain cDNA, we reverse transcribed 1 µg DNA-treated total RNA with oligo(dT) primers (0.01 µg/µl, 10 min at 37°C, 72°C 10 min with 1000 units of reverse transcriptase). Subsequently, quantitative PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCRs to amplify glyceraldehyde-3-phosphate dehydrogenase, IL-1β, TNF-α, and IL-6 were performed using SYBR green PCR master mix (Applied Biosystems, Foster City, CA), 5 µl diluted (1:20) cDNA, and primers at a final concentration of 300 mmol/liter in a total volume of 25 µl. The following primers were developed using Primer Express (Applied Biosystems, Foster City, CA): CCTCCTGATGACCAACCCAGG (reverse primer) and TCTTCTCGAACCCCGAGTGAA (forward primer) for TNF-α; GGCAGCTTCT CCTATTGGAATTCC (reverse primer) and AGCCCTGGAAAGAGGACATGTA (forward primer) for IL-6; and TGGTGAATTGTCGGATCTACA CTT (reverse primer) and AAACCTGTACCTGCTCCGTTT (forward primer) for IL-1β. Quantification of the PCR signals for each sample was performed by comparing the cycle threshold values, in duplicate, for the gene of interest with the cycle threshold values for the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene. We validated all primers according to the manufacturer's instructions and were analyzed by using GCOS (Affymetrix, Santa Clara, CA). Normalization, model fitting, and filtering were performed by using dChip (Harvard) (12). Subsequently, pairwise and multiple comparisons were performed by dChip, applying different criteria (>1.2-fold change with P value of <0.05 and/or >2-fold change with P value of <0.05). High-level analyses such as cluster analysis, principal component analysis, and analysis of variance were performed by dChip on filtered genes and a gene list generated by multiple comparisons. Hierarchical cluster analysis was performed using algorithms that assemble all elements in a single tree based on computed similarity scores.

Microarray analysis. PBMC from two different healthy donors were preincubated with untreated for 8 h or treated with 1 ng/ml E. coli LPS. RNAs were extracted by the TRizol method (Sigma, St. Louis, MO). Protocols from Affymetrix (Santa Clara, CA) (1a) were followed as described previously, with some modifications, as briefly described below (21). Synthesis and labeling of double-stranded cDNA from 1 µg total RNA were performed through one-cycle amplification in vitro transcription/biotin labeling using a MessageAmp II rRNA amplification kit (Ambion, Austin, TX). After fragmentation, the hybridization mixture was spiked with Bio B, C, and D and cre and hybridized to human genome array U133 Plus2.0 (Affymetrix, Santa Clara, CA), containing over 47,000 probes.

After 16 h, hybridization arrays were washed and incubated with streptavidin-coupled phycoerythrin (Molecular Probes, Eugene, OR). The GeneChips were then scanned using a GeneChipScanner laser scanner (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions and were analyzed by using GCOS (Affymetrix, Santa Clara, CA). Normalization, model fitting, and filtering were performed by using dChip (Harvard) (12). Subsequently, pairwise and multiple comparisons were performed by dChip, applying different criteria (>1.2-fold change with P value of <0.05 and/or >2-fold change with P value of <0.05). High-level analyses such as cluster analysis, principal component analysis, and analysis of variance were performed by dChip on filtered genes and a gene list generated by multiple comparisons. Hierarchical cluster analysis was performed using algorithms that assemble all elements in a single tree based on computed similarity scores.

RESULTS

Role of TLR2 and TLR4 in recognition of B. quintana. The first sets of experiments were designed to explore the induction of proinflammatory cytokines by whole B. quintana microorganisms and the role of TLR2 and TLR4 in this induction. Interestingly, heat-killed B. quintana did not stimulate TLR4-transfected CHO cells, while TLR2-transfected CHO cells reacted to B. quintana, indicating that the microorganism is able to signal through this receptor (Fig. 1A to D). To further assess the biological significance of these findings, we investigated the production of proinflammatory cytokines by human PBMC upon stimulation with heat-killed B. quintana. As shown in Fig. 1E, a blocking TLR4 antibody did not influence the capacity of B. quintana to stimulate the production of TNF-α, IL-1β, and IL-6 from human PBMC. This finding implies that the stimulation of cytokines by B. quintana is not induced by its LPS component or that B. quintana LPS stimulates cytokines through a TLR4-independent mechanism.

B. quintana LPS is a TLR4 antagonist. To investigate the two above-mentioned possibilities, we isolated B. quintana LPS in a two-step purification process which warrants the absence of protein contaminants (14). The purity of both E. coli and B. quintana LPSs after the two-step purification was determined using silver staining for proteins. Silver staining revealed the absence of any protein contaminants in both LPS samples after the two-step purification was determined (Fig. 2A). As shown in Fig. 2B, purified B. quintana LPS did not stimulate the production of TNF-α, IL-1β, and IL-6 in human PBMC. This finding implies that the stimulation of cytokines by B. quintana is not induced by its LPS component or that B. quintana LPS stimulates cytokines through a TLR4-independent mechanism.

Microarray analysis. PBMC from two different healthy donors were preincubated with or not with 500 ng/ml B. quintana LPS for 1 h and, thereafter, were left untreated for 8 h or treated with 1 ng/ml E. coli LPS. RNAs were extracted by the TRIzol method (Sigma, St. Louis, MO). Protocols from Affymetrix (Santa Clara, CA) (1a) were followed as described previously, with some modifications, as briefly described below (21). Synthesis and labeling of double-stranded cDNA from 1 µg total RNA were performed through one-cycle amplification in vitro transcription/biotin labeling using a MessageAmp II rRNA amplification kit (Ambion, Austin, TX). After fragmentation, the hybridization mixture was spiked with Bio B, C, and D and cre and hybridized to human genome array U133 Plus2.0 (Affymetrix, Santa Clara, CA), containing over 47,000 probes.

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Statistical analysis. The differences between groups were analyzed by the unpaired Student t test and, where appropriate, the paired t test. The level of significance between groups was set to P values of <0.05. The data are given as means ± standard deviations (SD).
expression of proinflammatory cytokine TNF-α, IL-1β, and IL-6 mRNAs from *E. coli* LPS-challenged human PBMC (Fig. 3B). The inhibitory effect of *B. quintana* LPS was exerted by blocking TLR4, as further demonstrated by the blockade of *E. coli* LPS-induced stimulation of CHO cells transfected with TLR4 (Fig. 3C). In addition, the effects of *B. quintana* LPS are targeted specifically to TLR4, since the stimulation of TLR2 was not influenced by the presence or absence of *B. quintana* LPS in the cultures (not shown).

**Microarray analysis.** We performed oligonucleotide genome array (Affymetrix) analysis to evaluate the impact of *B. quintana* LPS on gene expression on its own and in combination with *E. coli* LPS. Cluster analysis revealed that samples treated with *E. coli* LPS clustered together, whereas samples treated with *B. quintana* LPS or *E. coli* LPS plus *B. quintana* LPS clustered together with the control (Fig. 4A). In spite of the strong influence of donor differences, the effect of *B. quintana* LPS was unambiguous, as the expression profile for *B. quintana* LPS alone or in combination with *E. coli* LPS was hardly different from that of the corresponding control. This indicated that *B. quintana* LPS exerted no effect on its own but totally inhibited the effect of *E. coli* LPS. The complete mode of inhibition indeed suggests that *B. quintana* LPS binds to TLR4 but does not transduce the signal, hereby acting as a competitive inhibitor of *E. coli* LPS. When the effects of different treatments on gene expression were compared, *E. coli* LPS
LPS regulated 679 genes (>2-fold change and $P < 0.05$; both up- and downregulation) compared to the control (Fig. 4B). In contrast, B. quintana LPS alone or in combination with E. coli LPS regulated no single gene compared to the control by these criteria.

**DISCUSSION**

In the present study, we show for the first time that the gram-negative bacterium B. quintana does not trigger host defense mechanisms via TLR4-dependent pathways and, instead, that its LPS component is a powerful TLR4 antagonist. Septic shock is often preceded by gram-negative bacteremia, and the LPS component of these pathogens is known to play a crucial role in this respect. Interestingly, B. quintana was previously described to induce chronic infections (4), whereas...
FIG. 4. (A) PBMC were isolated from two individual donors (1 and 2). They were preincubated or not with *B. quintana* LPS (Inh) for 15 min and thereafter stimulated or not with *E. coli* LPS (LPS). The numbers designate the donor origins. Gene expression profiling was performed and analyzed as described in Materials and Methods. Hierarchical cluster analysis was performed on genes (vertical dendrogram) and samples (horizontal dendrogram) according to the algorithm described in Materials and Methods to visualize the gene expression patterns and relatedness of samples. Red, upregulation; blue, downregulation; white, average regulation. The magnified view in the box shows sample information in color blocks (first row, individual sample color code; second row, treatment groups, with dark green for control, light green for LPS, dark brown for inhibitor alone, and light brown for LPS plus inhibitor; third row, treatment groups, with dark and light gray for with and without *E. coli* LPS, respectively) and clustering of samples according to the gene expression profiles. (B) Samples were compared to the control, and the numbers of differentially expressed genes were determined using the criteria of a $>2$-fold change and a $P$ value of $<0.05$. The number above each bar indicates the number of differentially expressed genes.
signs of an important acute response were absent in those infected with the pathogen (2, 20). In line with this, the results of our present study strongly suggest that TLR4 is not involved in the host defense against B. quintana, and this fact might also be responsible for the absence of hypotension and shock in B. quintana-infected persons. The limited role of LPS-TLR4 interaction in the induction of an immune response and shock in B. quintana spp. is not singular to B. quintana, since previous reports indicated that other systems, such as the proteinaceous VirB type IV secretion system (17), outer membrane proteins (8), and BadA (22), are more responsible than LPS for the stimulation of cytokines by the related microorganism B. henselae. In line with this, recent evidence suggests that evading TLR4-dependent responses may in fact contribute to the virulence of various gram-negative bacteria (16). On the other hand, other LPS species, such as those isolated from Legionella pneumophila, Leptospira interrogans, Helicobacter pylori, and Porphyromonas gingivalis (11, 17, 18, 26), interact with TLR2 rather than with TLR4. In a recent study, we found that cytokine stimulation by B. quintana is mediated almost entirely by TLR2 (G. Matera et al., submitted for publication), further substantiating the TLR4 independence of B. quintana-induced cytokine production.

Previous studies reported that certain LPS structures could act as TLR4 antagonists. We investigated whether this could also be the case for B. quintana LPS. Our results indicated that interaction of B. quintana LPS with TLR4 does not induce gene transcription, as demonstrated by the lack of gene regulation in an Affymetrix assay of cells incubated with culture medium containing B. quintana LPS. This contrasts with the potent stimulation of a variety of gene families, including the NF-κB proinflammatory cytokine genes, when human umbilical vein endothelial cells (7), but not HeLa229 cells (10), were stimulated with intact B. henselae microorganisms. This effect also differs from a recent report in which B. quintana LPS was indicated to induce the release of the chemokine IL-8 (15), while also being able to modulate the apoptosis of endothelial cells (13). One crucial difference with these initial reports is in the method of LPS purification. Indeed, when we stimulated PBMC with LPS only partially purified by the single-step method employed in the previous studies, we also observed TLR2-dependent stimulation of cytokines (not shown). However, single-step purification is prone to retain a high percentage of protein contaminants in the LPS preparation, which likely explains this discrepancy. B. quintana LPS is not the only LPS which is unable to stimulate cytokine production. Recently, another LPS-like molecule derived from Oscillatoria planktonthrix FP1, termed Cyp, was reported to have antagonistic effects on TLR4 (14). Similar properties have been described for the LPSs isolated from other microorganisms, such as Rhodobacter sphaeroides or Chromobacterium violaceum, which are similar to the lipid A precursor Ia (compound 406) (25).

The results of our study are the first to report the TLR4 antagonistic effects of an LPS from Bartonella spp. In line with a low endotoxic potency of an LPS from Bartonella spp., Zahringer and colleagues recently reported that LPS isolated from B. henselae is a penta-acylated deep-rough LPS with endotoxic activities at least 1,000-fold less potent than that of S. enterica LPS (27). B. quintana LPS has a similar deep-rough structure (24), which may explain part of the absent endotoxic activity. However, differences with B. henselae LPS must also be present, as B. quintana LPS is completely unable to stimulate cytokines at concentrations as high as 10 μg/ml. The nature of these differences remains to be established. It should be noted, however, that other LPS species with antagonistic properties, i.e., R. sphaeroides or C. violaceum LPS, have tetra-acylated lipid A structures (25). As we previously proposed, the three-dimensional structure of lipid A is responsible for the differential interaction with TLR4, with strictly cylindrical structures being antagonists, whereas more conical shapes function as strong TLR4 agonists (18). In addition, a hexa-acylated biphenyl LPS is likely to represent the structure that could best trigger a proinflammatory response upon binding to TLR4 (17). Other LPS structures with antagonistic properties for human TLR4, such as a mutant LPS from Neisseria meningitidis (T. Sprong, personal communication), have been shown to be agonists of murine TLR4. Initial studies suggest that this is not the case for B. quintana LPS (1).

The role of TLR4 in the pathogenesis of endotoxic shock has been demonstrated extensively, and recently the receptor was proposed to be a potential therapeutic target for several autoimmune diseases, including rheumatoid arthritis (3). In line with this, the results of our study underline the large potential of B. quintana LPS for therapeutic use in these diseases. Firstly, in experiments conducted in vitro, B. quintana LPS could block E. coli LPS-induced effects at ratios as small as 10:1. Secondly, B. quintana LPS specifically binds TLR4 and does not interfere with the stimulation of other receptor pathways. Finally, unlike studies using a selected number of genes, the genome array approach enables the evaluation of total transcript levels in an unbiased way. Therefore, these results clearly demonstrate that B. quintana LPS is a potent TLR4 antagonist and also suggest that its potential to interfere with other immunologic pathways, if any, is extremely limited. Eventually, this might be translated into a limitation in side effects in the case of therapeutic applications. This may give B. quintana LPS a significant advantage with respect to other biological drugs currently on the market.

In conclusion, we demonstrate in the present study that stimulation of cytokines by B. quintana is independent of both its LPS component and TLR4. Moreover, while being unable to stimulate cytokine production, B. quintana LPS is a potent TLR4 antagonist. Because TLR4 proinflammatory signals are involved in a variety of pathological inflammatory reactions, the use of the TLR4 antagonistic properties of B. quintana LPS may prove of potential therapeutic value.

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