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Involvement of Lipopolysaccharide Binding Protein, CD14, and Toll-Like Receptors in the Initiation of Innate Immune Responses by Treponema Glycolipids

Nicolas W. J. Schröder,* Bastian Opitz,* Norbert Lamping,‡ Kathrin S. Michelsen,* Ulrich Zähringer,† Ulf B. Göbel,* and Ralf R. Schumann3*

Cultural supernatants from Treponema maltophilum associated with periodontitis in humans and Treponema brennaborense found in a bovine cattle disease accompanied with cachexia caused a dose-dependent TNF-α synthesis in human monocytes increasing with culture time. This activity could be reduced significantly by blocking the CD14-part of the LPS receptor using the My 4 mAb and by polymyxin B. In the murine macrophage cell line RAW 264.7, Treponema culture supernatants induced TNF-α secretion in a LPS binding protein (LBP)-dependent fashion. To enrich for active compounds, supernatants were extracted with butanol, while whole cells were extracted using a phenol/water method resulting in recovery of material exhibiting a similar activity profile.

An LPS-LBP binding competition assay revealed an interaction of the treponeme phenol/water extracts with LBP, while precipitation studies implied an affinity to polymyxin B and endotoxin neutralizing protein. Macrophages obtained from C3H/HeJ mice carrying a Toll-like receptor (TLR)-4 mutation were stimulated with treponeme extracts for NO release to assess the role of TLRs in cell activation. Furthermore, NF-κB translocation in TLR-2-negative Chinese hamster ovary (CHO) cells was studied. We found that phenol/water-extracts of the two strains use TLRs differently with T. brennaborense-stimulating cells in a TLR-4-dependent fashion, while T. maltophilum-mediated activation apparently involved TLR-2. These results indicate the presence of a novel class of glycolipids in Treponema initiating inflammatory responses involving LBP, CD14, and TLRs. The Journal of Immunology, 2000, 165: 2683–2693.

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4 Abbreviations used in this paper: LTA, lipoteichoic acid; CHO, Chinese hamster ovary; ENP, endotoxin neutralizing protein; Kdo, 3-deoxy-D-manno-octulosonic acid; LAL, Limulus amoeboocyte lysate; LBP, LPS binding protein; OMIZ-Pat, Treponema culture medium; PCP, phenol/chloroform/petroleum ether; PEM, peritoneal elicited macrophages; PG, peptidoglycan; TLR, Toll-like receptor; h, human; m, murine; GLC, gas-liquid chromatography; MS, mass spectrometry.
polypeptide known to bind lipid A, the active moiety of LPS (27). LPS effects can also be blocked by the mAb My 4 directed against CD14 (21). In the last years, it has been shown convincingly that members of the Toll-like receptor (TLR) family are involved in the recognition of pathogens by a wide host of organisms (28). In Drosophila, Toll has been shown to be involved in antifungal responses (29), while a homologous protein, 18-wheeler, induces antibacterial responses (30). In vertebrates, strong evidence has been presented that TLR-4 recognizes LPS of Gram-negative bacteria (31–33), while TLR-2 recognizes PG of Gram-positive bacteria, as well as lipoproteins of mycobacteria or Borrelia (34–39). Regarding LTA, results for an involvement of TLR-2 or -4 have been controversial (34, 35, 40).

Here we analyze the ability of two Treponema species isolated from a patient suffering from periodontitis and from a digital dermatitis lesion, respectively, to activate human monocytes, a murine macrophage cell line, macrophages obtained from C3H/HeJ mice, and Chinese hamster ovary (CHO) cells. First, we analyzed the involvement of the host LPS binding and receptor molecules LBP, CD14 (21). In the last years, it has been shown convincingly that members of the Toll-like receptor (TLR) family are involved in the recognition of pathogens by a wide host of organisms (28). In Drosophila, Toll has been shown to be involved in antifungal responses (29), while a homologous protein, 18-wheeler, induces antibacterial responses (30). In vertebrates, strong evidence has been presented that TLR-4 recognizes LPS of Gram-negative bacteria (31–33), while TLR-2 recognizes PG of Gram-positive bacteria, as well as lipoproteins of mycobacteria or Borrelia (34–39). Regarding LTA, results for an involvement of TLR-2 or -4 have been controversial (34, 35, 40).

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Materials and Methods

Treponeme culture and processing of culture supernatants

Frozen stocks of T. brennaborense and T. maltophilum cells (300 μl, each stored at –80°C) were inoculated in 3 ml of culture medium (OMIZ-Pat) as described previously (16). Bacteria were cultured under anaerobic conditions (Anaerogen, Oxoid, Germany) at 37°C for 3–4 days. The cultures were then transferred to a larger volume of OMIZ-Pat (20–100 ml) and further incubated for 1–2 days. Viability of treponemes and possible presence of contaminating bacteria were assessed by dark field microscopy. 400-fold magnification, BH2-RFCA microscope, Olympus, Hamburg, Germany. Sterility controls of the medium were performed by incubating OMIZ-Pat medium under aerobic and anaerobic conditions at 37°C for 1 wk. The pH value of the culture medium was measured repeatedly. Cultures were stopped at pH 6.0 and centrifuged at 12,000 × g at 4°C for 20 min. The supernatant was passed through 0.2-μm sterile filters (Schleicher & Schuell, Dassel, Germany). For some studies, culture supernatants were heat-inactivated at 100°C for 20 min and passed again through 0.2-μm sterile filters. OMIZ-Pat medium (16), treated similarly, was used as control.

Extraction of culture supernatants and whole Treponema cells

We used a modification of a published protocol for the extraction of LPS from Gram-negative cell walls using n-butanol (41). Briefly, filtered and heat-inactivated culture supernatants were mixed with an equal volume of n-butanol and incubated at 4°C for 1 h on a 180° shaker. Subsequently, the mixture was centrifuged at 26,000 × g at 4°C for 1 h, and the upper butanol phase was recovered. These steps were repeated once. Combined butanol phases were centrifuged and lyophilized. For stimulation experiments, lyophilized extracts were dissolved in RPMI 1640 medium (Life Technologies, Eggenstein, Germany) and added directly to the cultures. For some experiments, monoclonal anti-CD14 Ab My 4 (Coulter, Hamburg, Germany) was incubated with the cells at a concentration of 5 μg/ml at 37°C for 20 min before addition of stimuli to block CD14. After 4 h, supernatants were harvested and viability of cells was assessed via trypan blue staining. Additionally, 5 × 10⁴ RAW 264.7 cells per well (kindly provided by Dr. remodeling, Max-Delbrück-Centrum, Berlin, Germany) were cultured overnight in 96-well tissue culture plates using RPMI 1640 supplemented with 10% FCS. After repeated washing with RPMI 1640, stimulation was performed in the presence or absence of 1 μg/ml recombinant murine LBP (rmLBP) in a total volume of 100 μl. RAW 264.7 supernatants were harvested after 4 h of incubation, and cells were stained with trypan blue, ensuring integrity of cells.

Preparation and stimulation of peritoneal elicited macrophages (PEM)

Peritoneal macrophages were isolated from C3H/HeJ or C3H/HeN mice (Charles River, Sulzbach, Germany), by thioglycollate elicitation. Female 7- to 8-wk-old mice were injected i.p. with 1.5 ml of 3% thioglycollate broth (Sfin, Berlin, Germany). After 3 days, mice were sacrificed and peritoneal macrophages were harvested by injection of 10 ml of ice-cold HBSS (Life Technologies) i.p. followed by aspiration. Cells were washed twice with RPMI 1640, and 2 × 10⁵ cells were plated in 96-well tissue culture plates in RPMI 1640 containing 5% FCS. After 2 h, plates were washed twice with RPMI 1640 to remove nonadherent cells, and remaining cells were stimulated with treponeme phenol-water extracts or LPS for 24 h in RPMI 1640 containing 5% non-heat-inactivated FCS followed by NO detection as described below.

Quantitative detection of human and murine TNF-α and NO

Nunc MaxiSorp ELISA plates (Nune, Roskilde, Denmark) were coated with 0.5 μg/ml of anti-human TNF (anti-hTNF) Ab (PharMingen, Heidelberg, Germany) in 100 mM NaHCO₃, pH 8.3, and blocked with PBS containing 0.05% Tween 20 and 10% FCS. Cell supernatants and rTNF standard (R&D Systems, Wiesbaden, Germany) in PBS containing 10% FCS were incubated at 4°C overnight. Bound hTNF was detected using a biotinylated mouse anti-hTNF Ab (PharMingen) at a concentration of 0.5 μg/ml. Subsequently, 1 μg/ml streptavidin peroxidase conjugate (Sigma) was added with ortho-phenylene-diphosphate (Sigma) as substrate. The detection limit of this assay was ~10 pg/ml. For quantitation of murine TNF-α, MaxiSorp ELISA plates were coated with 3 μg/ml anti-murine TNF (anti-mTNF) Ab (PharMingen) in 100 mM NaHCO₃, pH 6.0. Samples and rmTNF standard (R&D Systems) were incubated at room temperature for 1 h, followed by detection with a biotin-conjugated anti-mTNF-Ab (PharMingen) and streptavidin-peroxidase with ortho-phenylene-diphosphate as substrate. The detection limit was ~15 pg/ml. All in vitro TNF-α results were assessed statistically by the Student’s t test, and the inhibitory effects of polymyxin B and My 4 as well as the enhancing effects of LBP were highly significant (p < 0.001). NO₂⁻ accumulation in culture medium was assessed according to a published protocol (44). In brief, 100 μl of Griess reagent (Sigma) was added to 100 μl of culture medium in 96-well plates and measured in a microplate reader at 540 nm with a standard of NaNO₂ diluted in RPMI 1640.
Estimation of NF-κB translocation

CHO cells transduced with human CD14 (CHO/CD14, generously provided by L. Hamann, Forschungszentrum Borstel, Germany) (45) were cultured overnight in six-well tissue culture plates at 4 × 10^5 cells per well with Ham’s nutrient medium F12 (PAA Laboratories, Linz, Austria) supplemented with 10% FCS and 400 μg/ml hygromycin B (Calbiochem, San Diego, CA). Before stimulation, cells were starved in FCS-free Ham’s medium for 3 h and incubated with LPS or treponeme extracts in the presence of 2% non-heat-inactivated FCS. After 1 h, cells were washed with ice-cold PBS containing 1 mM Na$_3$VO$_4$ and incubated in 150 μl of buffer A (1 mM Na$_3$VO$_4$, 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1 mM NaF). After 20 min, cells were harvested mechanically, transferred to 1.5-ml tubes, mixed with 25 μl Monutet P-40, and centrifuged at 13,000 × g at 4°C for 1 min. Pellets were resuspended in 50 μl of buffer B (400 mM NaCl, 1 mM Na$_3$VO$_4$, 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1 mM NaF), incubated for 30 min at 4°C, and spun at 13,000 × g at 4°C for 5 min. Supernatants containing nuclear proteins were collected, and nuclear extracts were analyzed by EMSA as described previously (46) using two synthetic oligonucleotides (Eurowicent, Seraing, Belgium) containing the NF-κB binding sequence of the murine Ig κ light chain enhancer.

Limulus assay

Treponema culture supernatants, both native and butanol extracted, as well as extracts of whole cells were assayed for endotoxin contamination by using a chromogenic Limulus amoebocyte lysate (LAL) assay (LPS, Sinntal-Oberzell, Germany). The endotoxin content of the OMIZ-Pat culture supernatants, both native and butanol extracted, and 30 μg/ml of butanol extracts, and 30 μg/ml of LPS solutions derived from E. coli 0111:B4 or Salmonella minnesota Re 595 LPS (Sigma) were boiled in sample buffer (2 ml 1 M Tris, 4 ml 1 M DTT, 800 mg SDS, 4 mg bromophenol blue, and 4 g glycerol ad 10 ml H$_2$O) for 5 min, loaded onto the gels, and submitted to electrophoresis. Gels were stained with the silver stain plus kit (Bio-Rad, Munich, Germany) according to the manufacturer’s instructions. In addition to the original protocol, gels were oxidized with 0.7% periodic acid after fixation (47). For some experiments, glycolipids were hydrolyzed and/or dephosphorylated as explained below.

Chemical analysis of the phenol/water-extracted cell wall fractions of T. maltophilia and T. brennaborense

Phosphate was determined according to Lowry (73), and 3-deoxy-Dmanno-2-octulosonic acid (Kdo) was estimated by the thiobarbituric acid assay as extracts of whole cells were assayed for endotoxin contamination by using a chromogenic Limulus amoebocyte lysate (LAL) assay (LPS, Sinntal-Oberzell, Germany). Before stimulation, cells were starved in FCS-free Ham’s medium for 3 h and 4.9 mg of T. brennaborense and 3.8 mg of T. maltophilia were added to 1 ml of buffer A containing 50 μM Na$_3$VO$_4$, 20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 1 mM NaF. After 20 min, cells were harvested mechanically, transferred to 1.5-ml tubes, mixed with 25 μl Monutet P-40, and centrifuged at 13,000 × g at 4°C for 1 min. Pellets were resuspended in 50 μl of buffer B (400 mM NaCl, 1 mM Na$_3$VO$_4$, 20 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, and 1 mM NaF), incubated for 30 min at 4°C, and spun at 13,000 × g at 4°C for 5 min. Supernatants containing nuclear proteins were collected, and nuclear extracts were analyzed by EMSA as described previously (46) using two synthetic oligonucleotides (Eurowicent, Seraing, Belgium) containing the NF-κB binding sequence of the murine Ig κ light chain enhancer.

Electrophoresis and silver staining of treponeme extracts

Stacking gels (5%) and separating gels (15, 16, and 20%, respectively) were prepared without SDS. Prestained and unstained low molecular mass markers ranging from 3 to 43 kDa (Life Technologies), 30 μl of bovine serum albumin (BSA), 30 μl of treponeme extracts was loaded onto the gel. Electrophoresis was carried out at 4°C with a 180° shaker. After centrifugation at 3000 × g for 10 min, supernatants were collected and loaded onto 15% SDS-PAGE gels, followed by silver staining as described above. Control samples were treated accordingly, however, without addition of any beads. Murine LBP was expressed in a baculovirus system and purified as described (26).

Results

Induction of TNF-α in myeloid cells by treponeme culture supernatants in the presence of serum, polymyxin B, mAb My 4, or rmLBP

Treponema culture supernatants induced TNF-α in freshly isolated human monocytes. This activity increased with culture time reaching a maximum at day 3 (Fig. 1). OMIZ-Pat culture medium alone, incubated with monocytes for the same period of time, failed to induce any detectable amounts of TNF-α (data not shown). For the

![FIGURE 1. Induction of TNF-α in human monocytes by Treponema culture supernatants. Freshly isolated human monocytes were incubated in a total volume of 80 μl. Then 20 μl of Treponema culture supernatants taken at the incubation time indicated from T. maltophilia and T. brennaborense cultures were added (20%). All experiments were performed in the presence of 5% human AB serum. TNF-α concentrations were measured by ELISA as described in Materials and Methods. Shown are mean values and SD of duplicate measurements. Experiments were conducted in duplicates with similar results.](http://www.jimmunol.org/DownloadedFrom)
following experiments, bacteria were cultured for 3 days. Cultures were monitored by pH measurement (6.0) to guarantee similar growth conditions. Both viability and motility of treponemes were assessed by dark field microscopy. To compare the activity of the treponeme supernatants with LPS, we performed experiments with monocytes in the presence and absence of serum. Cytokine induction caused by treponeme culture supernatants increased significantly in the presence of 5% human serum (Fig. 2A). As compared with *T. brennaborense*, serum-independent stimulation was significantly stronger for *T. maltophilum* culture supernatants (Fig. 2A). Both polymyxin B and the inhibitory monoclonal anti-CD14 Ab My 4 were able to significantly reduce cytokine levels induced by both treponeme cultures. However, the effect was more pronounced for LPS (Fig. 2B). The cytokine-inducing activity of *T. brennaborense* and *T. maltophilum* culture supernatants was inhibited in the presence of polymyxin B or My 4 mAb at least by 50%. In contrast, polymyxin B and My 4 did not influence cytokine induction caused by PMA, a phorbol ester causing cytokine induction by activating protein kinase C directly without receptor interaction (53) (data not shown). To investigate LBP effects on cytokine induction caused by treponemes, purified rmLBP and the murine macrophage cell line RAW 264.7 were used. In RAW 264.7 cells, TNF-α induction caused by both treponeme culture supernatants was significantly increased by addition of mLBP (Fig. 2C).

**Induction of cytokines by butanol extracts of culture supernatants and phenol/water extracts of whole cells in the presence of serum, polymyxin B, My 4, and rmLBP**

Based upon the notion that the stimulatory activity found within the supernatants shared characteristics with LPS, we purified the compounds from culture supernatants as well as from whole cells using extraction methods commonly used for LPS. Supernatants were treated with butanol, while whole cells were subjected to phenol/water or the PCP extraction. Yields obtained from whole cells with the phenol/water method were clearly higher; therefore, this method was used in the following experiments. To identify possible LPS contamination during the preparation, a mock extraction including all media and chemicals used during the procedure, was performed. TNF-α induction in human monocytes caused by butanol extracted *Treponema* supernatants was clearly reduced by both polymyxin B and My 4 (Fig. 3A). In RAW 264.7, an LBP-dependent cytokine induction was observed (Fig. 3B). In contrast to the culture supernatants, no TNF-α was induced in the absence of LBP. Similarly, phenol/water extracts of whole treponeme cells revealed a serum-dependent cell-stimulating capacity in human monocytes (data not shown). However, to achieve a TNF-α release equivalent to that caused by LPS, the concentrations of the extracts had to be increased by 1000-fold. Addition of polymyxin B and mAb My 4 led to a significant decrease of cytokine levels induced by LPS (Fig. 2B). In case of *T. maltophilum*, it was LBP dependent. In all experiments, the mock extracts did not cause cytokine induction.

**Induction of NO synthesis by treponeme phenol/water extracts in PEM derived from C3H/HeJ and C3H/HeN mice**

The role of TLR-4 in treponeme-mediated cell stimulation was analyzed using PEM derived from LPS hyporesponsive C3H/HeJ mice, a strain bearing a dominant negative mutation in the gene encoding TLR-4 (31). We isolated PEM from C3H/HeJ mice, as well as from the control C3H/HeN strain normally responsive to LPS. Cells were stimulated with increasing amounts of LPS and treponeme phenol/water extracts, followed by measurement of NO. LPS exhibited a significantly stronger stimulatory activity to-

![FIGURE 2. Effect of serum, LBP, polymyxin B, and anti-CD14 mAb My 4 on TNF-α induction in human monocytes and RAW 264.7 by *Treponema* supernatants. Freshly isolated human monocytes were stimulated with 10% of the *Treponema* cultures, and 1 ng/ml of *E. coli* 0111:B4 LPS both in the absence and presence of 5% human serum (A), 5 µg/ml polymyxin B, or 5 µg/ml of the anti-CD14 mAb My 4 (B). The murine macrophage cell line RAW 264.7 was stimulated with 10% of culture supernatants or 1 ng/ml of *E. coli* 0111:B4 LPS in the presence or absence of 1 µg/ml mLBP (C). Human and murine TNF-α concentrations were assessed by ELISA as described in Materials and Methods. Shown are mean values and SD of quadruplicate measurements. Experiments were repeated in quadruplicate (A) and twice (B) with similar results.](http://www.jimmunol.org/)

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ward C3H/HeN as compared with C3H/HeJ PEM (Fig. 4). While the mock extract failed to stimulate cells, phenol/water extracts derived from *T. brennaborense* revealed a stimulation pattern comparable to LPS, leading to a significantly weaker NO release by C3H/HeJ macrophages as compared with C3H/HeN cells. In contrast, extracts derived from *T. maltophilum* led to a comparable NO production in PEM of both strains, suggesting a less important role of TLR-4.

Translocation of NF-κB in CHO cells by treponeme phenol/water extracts

To elucidate the role of TLR-2 in treponeme-mediated signaling, we investigated CHO cells. These cells carry a mutation for TLR-2 leading to a defective receptor expression (54). CHO cells transfected with human CD14 inducing responsiveness to LPS (CHO/CD14) were stimulated with treponeme phenol/water extracts as well as with LPS. LPS induced a strong translocation of NF-κB in CHO/CD14 cells as shown in an EMSA (Fig. 5). *T. brennaborense* phenol/water extracts induced a translocation of NF-κB at concentrations of 1 μg/ml comparable to the LPS effect. In contrast, *T. maltophilum*-derived extracts, at 1 μg/ml, failed to induce NF-κB translocation, indicating an involvement of TLR-2.

SDS-PAGE silver stain analysis of native and butanol-extracted culture supernatants and whole cell phenol/water extracts

To identify and further characterize the active components in pure or butanol-extracted treponeme culture supernatants, the material was analyzed on 15% polyacrylamide gels that were silver stained subsequently. Visible bands appeared in the range of about 4 and 6 kDa in butanol-extracted supernatants from *T. maltophilum* and *T. brennaborense*.
The low molecular material reflected rough LPS derived from S. minnesota consisting only of lipid A and the inner core region with a size of 2.5 kDa (lane 1). The relative concentration present in the butanol extract of T. brennaborense revealed that this material contained about twice as much of the low molecular compound as the T. maltophilum extract. Digestion of both butanol extracts with pronase failed to change the profile of bands observed in SDS-PAGE, suggesting a nonproteinaceous nature of the immunostimulatory compound (data not shown). Phenol/water extracts of both strains were analyzed by silver-stained SDS-PAGE, revealing striking differences (Fig. 6B). The material obtained from T. brennaborense displayed a ladder-like pattern similar to that of smooth LPS presumably containing numerous repeating carbohydrate units. In contrast, T. maltophilum extracts exhibited few repeating units of larger molecular size. For both butanol-extracted culture supernatants and phenol/water extracts of whole cells, the size of their smallest units was similar.

Compositional and structural analysis of the phenol/water-extracted cell wall fractions of T. maltophilum and T. brennaborense

The results of a chemical analysis of phenol/water extracts from T. maltophilum and T. brennaborense are shown in Table I. Most of the components in both fractions could be analyzed and quantified (56% (w/w) for T. maltophilum and 71% for T. brennaborense). As expected from SDS-PAGE analysis, the amount of total fatty acids in the smaller glycolipid of T. maltophilum was significantly higher (8.3%, w/w) as compared with the high molecular T. brennaborense glycolipid (2.8%). LPS-characteristic β-hydroxylated fatty acids, as well as Kdo and heptose, were completely lacking in both strains. In T. maltophilum, galactosamine was identified to be the main sugar component (13%), whereas in T. brennaborense, glucose was identified as the dominating sugar (50.8%). In both preparations, a characteristically high amount of phosphate could be identified (5–10%). Besides traces of contaminating residual amino acids, alanine was the only amino acid identified in T. maltophilum, whereas T. brennaborense completely lacked amino acids.

After alkaline hydrolysis of both glycolipids by treatment with KOH, the material could not be visualized by silver staining, indicating the loss of ester-bound fatty acids (Fig. 7, A and B). To cleave phosphate-interlinked sugar chains, glycolipids were dephosphorylated by HF, resulting in material of ≤1 kDa size, which also could not be stained after KOH treatment (Fig. 7, A and B). This result suggests the presence of diacylglycerol as lipid anchor in both glycolipids and excludes sphingosine and steroids. For
ated glycosylglycerides were present in...

Identification of the lipid anchor in the glycolipid of *T. maltophilum*, extracts were peracylated after dephosphorylation with HF. GLC-MS analysis (CI mode) of the peracylated glycolipids from *T. maltophilum* revealed one major set of peaks ($t_R$, ~17 min) expressing pseudomolecular ions ([M + NH$_4$]$^+$) of m/z = 572 and 586, respectively. Their molecular masses ($M_0$ = 554 and 568 kDa, respectively) are consistent with monoacylated diacyl-glycerol carrying two tetradecanoic acid residues (14:0) and one pentadecanoic acid (15:0), respectively (Fig. 7C). However, attempts to identify a similar lipid anchor in *T. brevaborense* by using the same approach were unsuccessful, most likely due to the small proportion of the lipid in the whole molecule (see Table I).

For identification of the glycosyl part within the glycolipid, the peracylated glycolipids were further purified by silica gel chromatography, and fatty acids were released by alkaline hydrolysis, followed by permethylation of the remaining material. GLC-MS analysis of *T. maltophilum* revealed two glycosyl derivatives with retention times of 24.9 and 28.7 min expressing in the CI-mode pseudomolecular ions [M + NH$_4$]$^+$ with m/z = 764 ($M_0$ = 746 kDa) and [M + H$^+$]$^+$ m/z = 788 ($M_0$ = 787 kDa). These could be assigned to a permethylated trisaccharide glyceride with three hexoses (Hex$_3$-Gro), and a trisaccharide consisting of two hexoses and one hexosamine (Hex$_2$-HexNAc-Gro) (Fig. 7D). Both permethylated glycosylglycerides were present in ~1:9 proportion. The electron impact-mass spectrometry of the major permethylated glycosylglyceride showed diagnostic fragments derived from the reducing part of the molecule (m/z = 307, 557) as well as those from the nonreducing part (m/z = 464, 668), thus allowing the sequence of the trisaccharide to be assigned to Hex-HexNAc-Hex-Gro (Fig. 7D). Despite several attempts, the structure of the glycosyl part of the putative glycolipid isolated from *T. brevaborense* could not be revealed.

### Table I. Chemical analysis of *T. maltophilum* and *T. brevaborense* glycolipids

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<td><strong>Total</strong></td>
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*Chemical analysis was performed employing GLC and HPLC as described in Materials and Methods.

a The amino-dideoxy-hexose could not be quantified due to the lack of reference compound.
FIGURE 7. Silver stain analysis of hydrolyzed and dephosphorylated treponeme glycolipids, GLC-MS analysis of T. maltophilum-derived glycolipid, and proposed schematic structure. Treponeme glycolipids were either hydrolyzed by treatment with KOH, dephosphorylated by HF, or both, as explained in Materials and Methods. Resulting material, in comparison to untreated glycolipid, was further analyzed by silver staining (A, T. brennaborense, 16% gel; B, T. maltophilum, 20% gel). Before GLC-MS analysis, glycolipids of T. maltophilum were dephosphorylated (HF), dialyzed, and peracetylated. C, EI-MS of the major lipid mono-acetylated (deglycosylated) glycerol carrying two tetradecanoic acid residues (14:0). Dephosphorylated glycolipid was defrayed from fatty acids by alkaline hydrolysis and permethylated. D, The major glycosyl part of the T. maltophilum glycolipid carrying the Hex-HexNAc-Hex-Gro unit. E, A schematic structure proposal of the treponeme glycolipids investigated.
centrations ranging from 250 to 0.03 mLBP bound to the LPS-coated plates. Samples were incubated at con-

bound to immobilized LPS was detected by an Ab followed by colorimetric

LBP. After addition of increasing concentrations of the competitors, LBP

S. minnesota s-LPS) and

and polymyxin B.

A, 0111:B4 LPS or phenol/water extracts were incubated with ENP- or poly-

experiments.

46 kDa in size were loaded as control. Shown is one representative of two

followed by silver staining. Molecular mass standards ranging from 2.35 to

was mixed with loading buffer and loaded onto a 15% SDS-PAGE gel

FIGURE 8.

phenol/water extracts of Treponema bind to mLBP, ENP, and polymyxin B. A, LPS derived from E. coli 0111:B4 ("smooth"-LPS, s-LPS) and S. minnesota Re 595 ("rough"-LPS, r-LPS) and treponeme phenol/water extracts competed with immobilized LPS for binding to rm-

LBP. After addition of increasing concentrations of the competitors, LBP

bound to immobilized LPS was detected by an Ab followed by colorimetric
detection. The results shown as OD on the y-axis reflect the amount of

mLBP bound to the LPS-coated plates. Samples were incubated at concen-

trations ranging from 250 to 0.03 µg/ml, and mLBP was used at a

concentration of 100 ng/ml. Shown is one representative of three exper-

iments. B, Depletion of LPS and phenol/water extracts by ENP- or poly-

myxin B-conjugated beads. Solutions containing 100 µg/ml of E. coli

0111:B4 LPS or phenol/water extracts were incubated with ENP- or poly-

myxin B (PB)-conjugated beads. After centrifugation, 30 µl of supernatant

was mixed with loading buffer and loaded onto a 15% SDS-PAGE gel

followed by silver staining. Molecular mass standards ranging from 2.35 to

46 kDa in size were loaded as control. Shown is one representative of two

experiments.

and macrophages have been proposed to play a major role during this

process (55). Spirochetes may also directly stimulate resident defense cells for mediator release, although clear molecular mecha-

nisms for this process have to be defined.

Immunostimulatory elements of bacteria interact with soluble and cell-bound receptor molecules of the host organism, a key

element of the host’s repertoire to modulate an inflammatory reaction. As we have shown recently, the acute-phase response to

a systemic infection leading to elevated levels of the hepatic acute-phase protein LBP can greatly modulate the hosts re-

sponse to a systemic challenge with LPS (26). It is likely that LBP serves for toxic Treponema cell wall products as well as a

modulator in vivo and may be able to reduce or enhance the inflammatory reaction. Recent results by others and us provide
evidence that LBP interacts not only with LPS, but also with other bacterial products, i.e., LTA (7). Furthermore, our results
showing an involvement of the CD14 part of the LPS receptor are in agreement with CD14 acting as a pattern recognition
receptor (56, 57).

The immunostimulatory treponeme cell wall compounds de-

scribed here are apparently released by live bacteria or after cell
death. We present evidence that the compounds retained from the

supernatants correspond to the glycolipids extracted from whole
cells regarding size and biological characteristics. After cell death,
bacteria release immunostimulating particles such as LPS in

Gram-negative bacteria, causing a strong inflammatory response

potentially leading to septic shock and subsequent death of the host

(58, 59). In Gram-positive bacteria, elements like PG and LTA of

the outer cell wall, released after cell disintegration often following

antibiotic treatment, also stimulate cytokine release in host cells (3,

60). However, for spirochetes, the predominant inflammatory ac-
tive element of their cell wall has not yet been clearly identified.

For some spirochetes like T. hyodysenteriae and T. innocens, the

presence of LPS-like molecules has been described, while for oth-
eres it has been clearly ruled out (10). Borrelia burgdorferi fails to

contain LPS in the cell wall (8, 9); however, it contains PG (61)

and a set of outer membrane proteins eliciting inflammatory re-

sponses in immune cells (62–64). It is likely that these proteins are

identical with lipoproteins described to induce TNF-α-synthesis in

human monocytes (12, 65). T. pallidum possesses a number of

TNF-α-inducing membrane proteins, and for this spirochete the

presence of LPS was definitively ruled out after the completion of

the whole genome sequence (8, 11, 66). Our observations provide
evidence that treponemes contain a glycolipid-like material within

their membranes that is chemically different from LPS while ex-

hibiting comparable biological characteristics including, for one of

our isolates, involvement of TLR-4.

Analysis of the role of TLRs is important in light of the recent

paradigm of Gram-negative bacteria using TLR-4 via LPS and other

bacteria stimulating cells via TLR-2. Recently, two immu-

nostimulatory fractions isolated from the cell walls of T. denticola

have been compared, lipoproteins on one hand and lipooligosac-

charides, comparable to the glycolipids isolated in our study, on

the other hand (67). Both fractions were able to induce cytokines

and NO in host cells of normal and C3H/HeJ mice, suggesting a

TLR-4-independent activity, which is in line with our results for

T. maltophilum. Lipoproteins isolated from Borrelia recently also

have been found to stimulate host cells via TLR-2 similar to li-

poproteins isolated from mycobacteria (36, 37, 39). According to

our results, active cell wall compounds from genetically closely

related spirochetes stimulate cells via different TLRs. However,

chemical analysis revealed differences in the composition of the
glycolipids isolated. Thus structural differences may explain the

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differential use of pattern recognition receptors. While the *T. brennaborense* glycolipid contained significantly more carbohydrates and revealed the presence of a high number of small “repeating units” in silver gel analysis, *T. maltophilia* glycolipids displayed a small number of larger “repeats.” Recently, the TLR-2/TLR-4 paradigm was questioned by two other studies showing involvement of TLR-4 in non-LPS-mediated cell stimulation. Viable mycobacteria, in contrast to isolated lipoparabiaconnan, stimulated CHO cells overexpressing both TLR-2 or TLR-4 (68). A recent study comparing the TLR-2 and the TLR-4 knockout mouse provided evidence that LTA from Gram-positive bacteria also stimulate macrophages via TLR-4 (40). Furthermore, this group compared different types of LPS leading to a different degree of use of members of the TLR family (69).

We describe here the molecules involved in the reaction pattern of myelo-monocytic host cells to contact with cell wall components of recently identified spirochetes. Certain features of this interaction, i.e., involvement of LBP, CD14, and the use of TLR-4 by *T. brennaborense*, as well as the inhibitory effect of polymyxin B, resemble the cell stimulation pattern induced by LPS of Gram-negative bacteria. However, for polymyxin B, it recently has been shown that it interacts with numerous structures including phospholipids (70). Furthermore, the *Treponema* glycolipids described here bound to ENP, a protein usually considered to bind specifically to LPS. However, it is known that agents other than LPS cross-react in the Limulus assay, potentially due to similar physical properties (71, 72). Because the glycolipids described in this study interact with a range of other LPS-binding structures, it is likely that the discrete LAL activity observed is caused by the extracted compounds themselves and not by contaminating LPS. Furthermore, precipitation studies using ENP revealed a specific affinity of the treponeme extracts to this protein.

Our chemical analysis suggests a glycolipid structure in *T. maltophilia* and *T. brennaborense* differing significantly from that of LPS. This is based on the absence of structural components characteristic for LPS, such as heptose, Kdo, and β-hydroxy fatty acids. In contrast, *Treponema* glycolipids displayed LTA-like elements such as sugar, high phosphate, and alamine similar to that previously identified in *T. denticola* (19). This similarity was further supported by isolation and analysis of the dephosphorylated glycosyl part of the repeating units, being a hexasaccharide in *T. maltophilia* and a glucan in *T. brennaborense* (data not shown). Moreover, in *T. maltophilia* we identified two glycolipids composed of Hex3Gru and Hex-HexN-Hex-Gro (Fig. 7D). GLC-MS analysis of the lipid anchor revealed two monoacetylated diacylglycerols, the predominant one containing two tetradecanoic acids (14:0) (Fig. 7C). Our interpretation that *T. brennaborense* contains a glycolipid of similar structure is based on results obtained from SDS-PAGE (Fig. 7, A and B) and from TLC analysis (data not shown).

Taken together our chemical results indicate that *T. maltophilia* and *T. brennaborense* both exhibit a glycolipid consisting of a diacylglycerol-lipid anchor, a core region, in the case of *T. maltophilia* consisting of three sugars, and carbohydrate repeating units (Fig. 7E). As indicated by silver stain analysis, *T. maltophilia* exhibits a low number of large repeating units, each being composed of ~20–30 sugars, while *T. brennaborense* contains a high number of small repeating units, each being composed of ~5 sugars. Like in *T. denticola* (19), these glycolipids share structural characteristics with LTA and apparently represent the major membrane component. The differences in chemical composition between the two strains are significant considering the close genetic relatedness of both strains (15) and may be the cause for the different interactions with TLRs. A more detailed chemical analysis will be performed in our laboratories to further support this interpretation.

Our data complement the list of bacterial cell wall components recognized by TLRs and CD14, explaining results by others showing a CD14 involvement for spirochete-mediated host cell stimulation (13, 64). The differential use of TLRs by the treponeme glycolipids may help in understanding basic mechanisms of innate immunity caused by spirochetes as well as other microorganisms.

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References


