INFECTION AND IMMUNITY, Jan. 2002, p. 211–217 Vol. 70, No. 1
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.1.211–217.2002

Biological Characterization of Lipopolysaccharide from *Treponema pectinovorum*

Lakshmyya Kesavalu,[1]* Clinton W. Falk,[1] Kenneth J. Davis,[2] Michelle J. Steffen,[1] Xiaoping Xu,[2] Stanley C. Holt,[3] and Jeffrey L. Ebersole[1]

Center for Oral Health Research, College of Dentistry, University of Kentucky, Lexington, Kentucky 40536[1]; Department of Microbiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284[2]; and The Forsyth Institute, Boston, Massachusetts 02115[3]

Received 6 August 2001/Returned for modification 28 August 2001/Accepted 11 October 2001

This study investigated the endotoxic and biological properties of purified lipopolysaccharide (LPS) isolated from an oral spirochete, *Treponema pectinovorum*. Endotoxicity, measured by *Limulus* amoebocyte lysate kinetic assay, showed that the LPS contained 1.28 endotoxin units per μg of purified LPS, which was approximately 4,000 times less than *Escherichia coli* O55:B5 LPS. To determine in vivo endotoxicity, LPS responder mice were administered LPS following galactosamine (GalN) sensitization. The LPS induced neither endotoxic symptoms nor lethality for 96 h, suggesting negligible or very low endotoxicity. In contrast, infection with live *T. pectinovorum* induced 100% lethality within 12 h in GalN-sensitized LPS responder mice, indicating an endotoxin-like property of this treponeme. Heat-killed microorganisms exhibited no lethality in GalN-sensitized mice, suggesting that the endotoxicity was associated with heat-labile components. To determine cytokine and chemokine induction by LPS, human gingival fibroblasts were stimulated and secretion of interleukin 1β (IL-1β), granulocyte-macrophage colony-stimulating factor, gamma interferon, IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1) was assessed. The purified LPS induced significant amounts of only IL-6, IL-8, and MCP-1, although they were substantially lower than levels after challenge with live *T. pectinovorum*. After injection of LPS or live or heat-killed *T. pectinovorum*, serum was collected from mice and analyzed for proinflammatory cytokines IL-1β, tumor necrosis factor alpha (TNF-α), and IL-6. LPS induced only IL-6 consistently. Both live and heat-killed *T. pectinovorum* induced serum IL-6, which was higher than the level detected following LPS administration. Importantly, live bacteria elicited systemic TNF-α and IL-1β levels similar to those induced by a lethal dose of live *E. coli* O111. The results indicated that *T. pectinovorum* LPS has very low or no endotoxicity, although it can elicit low levels of cytokines from host cells. In contrast to the LPS, live *T. pectinovorum* demonstrated in vivo toxicity, which was associated with serum IL-1β, TNF-α, and IL-6, suggesting an endotoxin-like property of a heat-labile molecule(s) of the spirochete.

The oral cavity provides multiple ecological niches for a wide array of bacterial species. Many of these microorganisms can be considered commensal inhabitants that exist in symbiosis with the host. However, bacteriologic studies have also documented a succession of genera and species which comprise the supragingival and subgingival microbiota of the soft and hard tissues within the oral cavity (32). Associated with the progression from periodontal health through gingivitis to periodontitis, there is a shift towards a more gram-negative anaerobic microbiota at sites of periodontal destruction. Within this group of proposed virulent species are the spirochetes, which appear to colonize the subgingival plaque and are linked with periodontal disease and tissue destruction (31, 32). *Treponema pectinovorum* is an oral anaerobic spirochete that has recently been found to be associated with periodontitis in human immunodeficiency virus-infected patients (22, 31, 32, 36).

Lipopolysaccharide (LPS), or endotoxin, elicits a wide assortment of biological effects in the host, which reflect a specific interaction with cellular targets. Mononuclear phagocytes/macrophages mediate the lethal function of endotoxin, and tumor necrosis factor alpha (TNF-α) has been identified as the major cytokine mediator in the expression of endotoxicity during sepsis due to gram-negative bacteria (23). LPS activation of host cells is mediated by the interaction of LPS with LPS binding protein and subsequently to CD14 receptors on the cell surface (14). Recently, toll-like receptor 2 (TLR2) (12, 19, 38) and TLR4 (3) have been identified as the molecules conferring or mediating LPS-induced signal transduction. Moreover, lipoproteins (non-LPS components) activate cells through CD14 and TLR2 proteins (11, 38). Recognition of LPS by this innate immune system results in an inflammatory response characterized by the production of cytokines such as TNF, interleukin 1 (IL-1), IL-6, and IL-8 as well as gene activation of cellular receptors like intercellular adhesion molecule-1 and E-selectin, among others. Importantly, LPS molecules from oral bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Fusobacterium nucleatum* have been suggested to play an important role in the inflammatory responses in the pathogenesis of chronic periodontitis (13, 39, 40). The detection of an LPS-like structure in the outer membrane of oral treponemes has been suggested in only two cultivable species, but inconsistencies in the characteristics of the molecule have not allowed definitive identification (4, 20, 40). Recently, we have described *T. pectinovorum* associated with periodontal disease and have detailed the isolation and chem-

* Corresponding author. Mailing address: Center for Oral Health Research, College of Dentistry, 159 Health Science Research Building, University of Kentucky, Lexington, KY 40536-0305. Phone: (859) 323-0045. Fax: (859) 257-6566. E-mail: knlaks0@pop.uky.edu.

ical characterization of a molecule isolated from the outer membrane which possesses many of the characteristics of an LPS (36, 37). The purpose of this study was to examine the biological functions of the purified *T. pectinovorum* LPS using both in vitro and in vivo assays. The results indicated that this LPS molecule has very low endotoxicity as determined by using classic biomarkers of endotoxin.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *T. pectinovorum* ATCC 33768 and a clinical isolate, S1, were grown in GM-1 medium with 3% pectin (Sigma, St. Louis, Mo.) in a Coy anaerobic chamber in an atmosphere of 85% N_2, 5% CO_2, and 10% H_2 for 24 to 72 h (16, 17). *Escherichia coli* O111 was grown in Luria-Bertani broth overnight at 37°C aerobically (*E. coli* Reference Center, University Park, Pa.). *T. pectinovorum* was heat killed at 100°C for 60 min in a water bath (29). For the analyses, the bacteria were centrifuged at 11,000 rpm for 20 min (Sorvall Centrifuges, Wilmington, Del.) and washed, and the cells were resuspended in phosphate-buffered saline (0.05 M phosphate, pH 7.2). An aliquot of the culture was removed from the chamber, and 10-fold dilutions were made for estimating total counts using a Petroff-Hausser bacterial counting chamber. Purity of the treponeme culture was determined by phase-contrast and dark-field microscopic observations, Gram staining, and plating on blood agar plates.

LPS isolation. The total cell membrane, outer membrane, and LPS were isolated from *T. pectinovorum* ATCC 33768 and S1 as described previously (36, 37). Briefly, bacteria were freeze-thawed for 40 cycles and then centrifuged at 6,000 rpm for 10 min (Sorvall Centrifuges, Wilmington, Del.) to separate the cloudy supernatant from the broken cellular debris. The supernatant was centrifuged at 17,000 rpm for 30 min at 4°C to obtain a membrane fraction. The membranes were extracted with 1% Zwittergent 3.14 (Calbiochem Corporation, La Jolla, Calif.), and the detergent soluble outer membrane fraction, containing the major outer membrane proteins (36) and the LPS, was processed by the method of Darveau and Hancock (6) and Walker et al. (37) to obtain purified LPS. The LPS was purified at 200,000 × *g* for 2 h at 4°C and suspended in a small volume of distilled water. 3-Deoxy-D-*manno*-octulosonic acid was estimated using the method of Karkhanis et al. (15), and protein content was estimated using the method of Markwell et al. (21). The characteristics of *T. pectinovorum* LPS used in this investigation were as reported previously by Walker et al. (37).

Endotoxin assay. Endotoxin activity of *T. pectinovorum* LPS was determined by a kinetic *Limulus* amoebocyte lysate (LAL) assay using an endochrome-K assay kit (Charles River Endosafe, Charleston, S.C.). Briefly, a standard curve ranging from 50 to 0.0005 endotoxin units (EU)/ml was prepared in duplicate from *E. coli* O55:B5 control standard endotoxin supplied by the manufacturer. *T. pectinovorum* LPS was serially diluted from 50 μg to 5 ng of LPS/ml into microtiter plates using LAL reagent water. Reconstituted LAL reagent was added to the endotoxin standards, LPS sample wells, and controls in duplicate. A timed observation (60 min, at 2 min intervals) of the absorbance of microtiter plate at 405 nm using Dynatech Biolinx software (Dynatech, Chantilly, Va.) was initiated immediately to detect endotoxin activity. After the completion of the monitoring interval, endotoxin activity of LPS was calculated from a control standard endotoxin and expressed in EU per milliliter and EU per nanogram of LPS.

HGFs. A normal human gingival fibroblast (HGF) cell culture (Gin-7) was grown to confluence in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), antibiotics (penicillin [100/ml] and streptomycin [100/ml]) (Gibco, Grand Island, N.Y.), and L-glutamine (2 mM) (Gibco) at 37°C in 5% CO_2 and moist air (34). The cells routinely reached confluence by approximately 2 days and were passaged by a 1:3 split. All experiments were carried out with HGF at fewer than 12 passages.

In vitro LPS–gingival-fibroblast interactions. The HGFs were plated in 24-well microtiter plates at 10^5 cells/well. The HGFs were propagated to confluence (~5 × 10^5/well) for 2 days prior to experimentation. *T. pectinovorum* LPS was suspended in Dulbecco modified Eagle medium with 1% fetal bovine serum at concentrations of 100, 10, 1, and 0.1 μg/ml. One milliter of the diluted stimulant was added to the test wells, and all assessments were made in triplicate. Supernatants were collected at 1, 3, 6, 12, 24, and 48 h following challenge, centrifuged (13,000 × *g* for 5 min), and frozen in multiple aliquots at −70°C until analyzed.

ELISA for cytokines. A sequential enzyme-linked immunosorbent assay (ELISA) for the analysis of multiple cytokines was performed to detect IL-1β, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein 1 (MCP-1), and gamma interferon (IFN-γ) from fibroblast culture supernatants and mouse serum (33). The samples were assayed in the sequence of the expected least to most prominent cytokine in the fluid or serum to be analyzed as well as the minimal detectable level of the assays. Briefly, individual microtiter plates were incubated with 0.2 ml of 5-μg/ml mouse monoclonal antibody to each of the cytokines in carbonate-bicarbonate buffer as a capture antibody. After 3 to 4 h incubation at 37°C, the solution was removed and 1% bovine serum albumin (Sigma) in phosphate-buffered saline was added to block unbound sites in the wells. The plates were stored with the bovine serum albumin at 4°C at least overnight. A pooled recombinant standard of cytokines was used on all plates. Each of the cytokines in the pooled standard was adjusted to 1,000 pg/0.2 ml and diluted serially twofold to 1.95 pg/0.2 ml.

Samples and standards were added to the first plate (i.e., anti-IL-1β) in the sequence, and the plate was incubated for 1 h at 37°C. The second plate in the sequence (i.e., anti-IL-8) was washed, and the samples from the first plate were transferred in replicate onto the second. The second plate was incubated as the first, the first plate was thoroughly washed, and rabbit antisera to the cytokine were added to the plate as a developing system. The plate was incubated overnight. When the incubation from the second plate was completed, the samples were transferred in replicate onto a third plate (i.e., anti-IL-6) and incubated, and the second plate was developed as the first. This was repeated until the samples had been incubated on all of the plates coated with the monoclonal antibodies to the individual cytokines of interest.

The following day, all plates were washed, and an alkaline phosphatase-conjugated goat anti-rabbit antiserum (Boehringer Mannheim) was added to the plates. The plates were incubated for an additional 4 h, washed, and developed with *p*-nitrophenylphosphate (Sigma) as the substrate. The levels of cytokines in the samples were determined by using Dynatech Biolinx software with a sigmoidal fit. Intraplate and interplate variability was accepted with a sample duplicate variation of ≤15%, and the standard curve between plates required (i) a maximum optical density of at least 1.0 (no more than 20% variation), (ii) no significant difference ($P > 0.05$) in the slopes, and (iii) a background of <0.15.

The commercial sources of the various reagents include the following, in the order recombinant standard, monoclonal capture, and polyclonal developing reagent: for IL-1β, R & D Systems (Minneapolis, Minn.), Biosource (Camarillo, Calif.), and Sigma; for IL-6, R & D, Biosource, and Sigma; for IL-8, Biosource, Biosource, and Endogen (Cambridge, Mass.); for IFN-γ, R & D, Biosource, and Genzyme; for GM-CSF, R & D, Genzyme, and Genzyme; and for MCP-1, Sigma, Sigma and Chemicon (Temecula, Calif.).

In vivo lethality induced by *T. pectinovorum* LPS. C3H/HeN Tac-MTV (LPS responder, Taconic, Germantown, N.Y.), C3H/HeJ (LPS nonresponder; The Jackson Laboratory, Bar Harbor, Maine), and ICR (Tac:Icr:Ha [ICR]; Taconic) outbred mice (female, 6 to 8 weeks old) were used for testing the lethal toxicity of *T. pectinovorum* ATCC 33768 LPS. The animals were housed in isolator cages in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility at the University of Texas Health Science Center at San Antonio and were provided autoclaved TEKLAD chow (Harlan Sprague Dawley, Inc., Madison, Wis.) and water ad libitum. *T. pectinovorum* LPS and *E. coli* O111:B4 LPS (Sigma) lethal toxicity assays were carried out in LPS responder and LPS nonresponder mice (five to six mice/group). *T. pectinovorum* LPS and *E. coli* LPS were injected intraperitoneally (i.p.) as a mixture with D-galactosamine (GalN; 20 mg/mouse; Aldrich Chemicals) in 0.2 ml of LAL reagent water (9). Six experimental groups were used in these studies: LPS responder mice received 5 μg, 500 ng, or 50 ng of *T. pectinovorum* LPS with GalN; LPS nonresponder mice received 5 μg of *T. pectinovorum* LPS with GalN; LPS responder mice received 50 ng of *E. coli* LPS with GalN (positive control); and LPS responder mice received GalN only (negative control). Deaths were recorded for 4 days postinjection.

Toxicity of live and heat-killed *T. pectinovorum* in GalN-sensitized mice. To determine lethal toxicity, groups of LPS responder, LPS nonresponder, and ICR mice were injected i.p. with live or heat-killed (29) *T. pectinovorum* ATCC 33768 or S1 (10^7, 10^8, or 10^9 organisms) or live *E. coli* O111 (10^5, 10^7, or 10^9 organisms) with and without GalN. Death of the mice due to toxicity was observed over a 24- to 48-h period. Subsets of these animals were euthanized at 3, 8, and 12 h postinjection. Blood was collected for analysis of the proinflammatory cytokines IL-1β, TNF-α, and IL-6.

Serum cytokines IL-1β, TNF-α, and IL-6 induced by LPS. The second analysis of in vivo endotoxicity utilized evaluation of the level of the proinflammatory cytokines IL-1β, TNF-α, and IL-6, which have been associated with lethality resulting from sepsis due to gram-negative bacteria (23) and endotoxemia (1). LPS responder mice were injected i.p. with *T. pectinovorum* ATCC 33768 LPS (5 μg, 500 ng, and 50 ng) with GalN (20 mg/mouse). After 3, 8, and 12 h, three animals each from groups I, II, and III were euthanized by CO_2 inhalation, and blood was collected. Serum was separated after centrifugation and stored at −70°C.

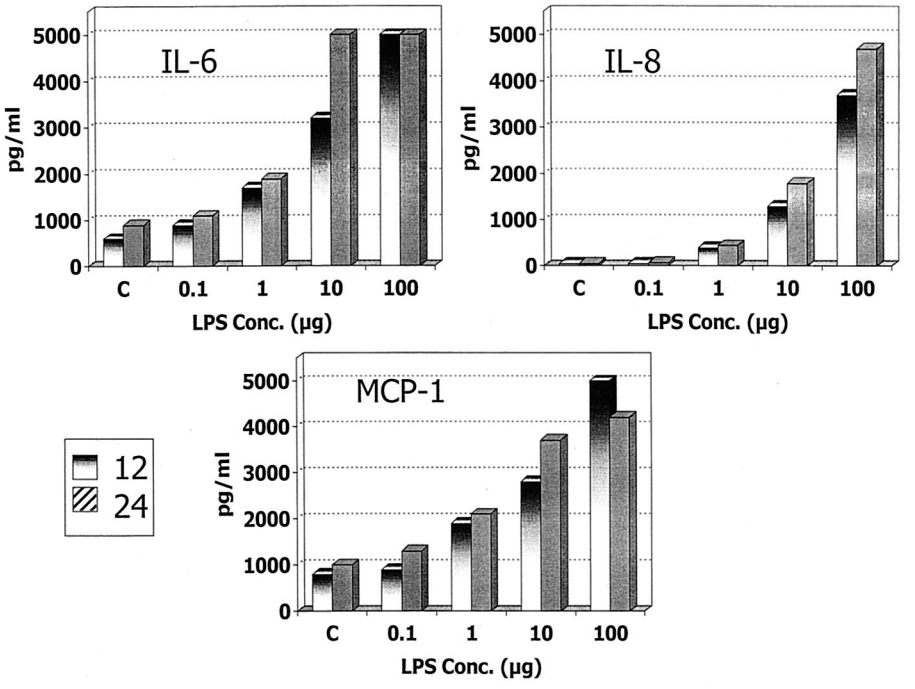

FIG. 1. IL-6, IL-8, and MCP-1 secretion by HGFs (Gin-7) challenged with purified *T. pectinovorum* ATCC 33768 LPS at 0.1, 1, 10, and 100 μg per ml. Supernatants were collected at 1, 3, 6, 12, 24, and 48 h after LPS challenge. Each cytokine concentration was determined by sequencial ELISA. The cells were stimulated with 5×10^9 bacteria. The bars denote the mean level of cytokine detected during 12-and 24-h culture periods from triplicate determinations.

The serum was analyzed for the individual cytokines using a sequential ELISA as described previously (33). The reagents included rat monoclonal antibodies (IL-1β and IL-6; R & D Systems) or goat polyclonal antibodies (TNF-α; R & D Systems) specific for mouse cytokines as capture reagents coating the wells at 1 μg/well. Following incubation of the serum, the system was developed with biotinylated goat polyclonal antibody specific for each cytokine (IL-1β, TNF-α, and IL-6; R & D Systems), streptavidin-alkaline phosphatase (Zymed, San Francisco, Calif.), and *p*-nitrophenylphosphate (Sigma). The levels were determined by comparison with standard curves generated from purified murine recombinant cytokines (IL-1β, TNF-α, and IL-6; R & D Systems).

Statistical analyses. Statistical differences in cytokine production by gingival fibroblasts and serum cytokine levels were determined using a Wilcoxon–Mann-Whitney U test (Minitab, State College, Pa).

RESULTS

In vitro endotoxicity of LPS. The endotoxicity of purified LPS from *T. pectinovorum* strains was determined by LAL assay, and its endotoxic activity was compared to that of *E. coli* LPS. The results showed that *T. pectinovorum* ATCC 33768 LPS did exhibit very minimal endotoxicity: it was 4,170 times less endotoxic than the control *E. coli* O55:B5 LPS standard. In contrast, live *T. pectinovorum* strains ATCC 33768 and S1 at 10^7 organisms demonstrated substantial endotoxicity comparable with a dose of 10^4 *E. coli* organisms. Heat-killed *T. pectinovorum* ATCC 33768 at 10^7 cells also demonstrated some minimal endotoxin activity at a level similar to that of 10^2 *E. coli* cells.

In vitro cytokine induction by *T. pectinovorum* LPS. HGFs were challenged with purified *T. pectinovorum* ATCC 33768 LPS, and the supernatants were evaluated for levels of cytokine and chemokine secretion. The *T. pectinovorum* LPS was capable of stimulating the secretion of IL-6, IL-8, and MCP-1 from the HGFs (Fig. 1). However, to observe this activity, high levels of *T. pectinovorum* LPS were required. After an initial 6-h lag period, a dose-response increase in IL-6 secretion by 12 h after LPS stimulation was noted compared to the medium control. IL-1β, GM-CSF, and IFN-γ were not detected in HGF supernatants following challenge with *T. pectinovorum* LPS.

In vivo endotoxic activity of LPS and *T. pectinovorum*. Groups of LPS responder and nonresponder mice were challenged with *T. pectinovorum* ATCC 33768 LPS in the presence of GalN. Mortality was compared with that obtained with a positive control, *E. coli* O111 LPS. The results showed that *T. pectinovorum* LPS at 5, 0.5, and 0.05 μg was uniformly nontoxic in GalN-sensitized mice (Table 1). None of the *T. pectinovorum* LPS-challenged animals exhibited endotoxic symptoms or death, whereas control mice given *E. coli* O111 LPS at 0.05 μg exhibited classical endotoxic symptoms by 3 h and 100% lethality within 9 to 36 h. The results indicated that *T. pectinovorum* LPS lacked this endotoxic property.

LPS responder mice challenged i.p. with live *T. pectinovorum* ATCC 33768 at 10^7 to 10^9 cells and GalN became moribund by 3 to 8 h, and 100% of animals died by 12 h (Table 2). None of the animals died when GalN was omitted, but animals were sick for 24 to 36 h. In contrast, heat-killed *T. pectinovorum* cells at 10^8 per mouse in the presence or absence of GalN were not lethal in LPS responder mice, indicating that heat treatment abolished the in vivo toxic activity of the spirochetes. LPS responder mice inoculated with live *E. coli* O111 at 10^5, 10^7, and 10^9 cells and GalN became moribund by 8 h, and all died. Similarly, *T. pectinovorum* strains ATCC 33768 and S1 were lethal in ICR animals following challenge with GalN and 10^7 to

TABLE 1. Endotoxicity of *T. pectinovorum* LPS in LPS responder and nonresponder mice[a]

Mice	LPS (dose [μg])	Endotoxicity	Lethality[b]
LPS responders	*T. pectinovorum* (5)	No toxic symptoms	0/6
LPS responders	*T. pectinovorum* (0.5)	No toxic symptoms	0/6
LPS responders	*T. pectinovorum* (0.05)	No toxic symptoms	0/6
LPS nonresponders	*T. pectinovorum* (5)	No toxic symptoms	0/5
LPS responders	*E. coli* (0.05) (LPS control)	9- to 36-h lethality	5/5
LPS responders (GalN control)		No symptoms of GalN toxicity	0/5

[a] All mice received 20 mg of GalN.
[b] Number of animals that died/number tested.

10^{10} cells. The animals became moribund by 3 to 8 h, and 100% of animals died within 12 h. Thus, the GalN sensitized the mice to a lethal outcome following challenge with live *T. pectinovorum*, although this toxicity was approximately 100-fold lower than that of *E. coli*.

Induction of systemic proinflammatory cytokines. To investigate the role of systemic cytokines IL-1β, TNF-α, and IL-6 in mortality, *T. pectinovorum* ATCC 33768 LPS and live and heat-killed *T. pectinovorum* cells were administered with and without GalN to groups of LPS responder mice. Sera from the various groups of mice were analyzed for proinflammatory-cytokine levels. The results demonstrated that *T. pectinovorum* LPS elicited high levels of IL-6 in serum. These levels tended to occur as early as 3 h and generally peaked by 12 h. The levels of IL-6 in serum were independent of lethality. Neither of the other cytokines was induced by *T. pectinovorum* LPS challenge (Fig. 2). Both live *T. pectinovorum* and *E. coli* induced significant levels of IL-1β, TNF-α, and IL-6 in serum, which correlated very well with the early cascade of septic shock events by 3 h and lethality by 8 h. The heat-killed *T. pectinovorum* cells stimulated neither IL-1β nor TNF-α, with no evidence of septic shock syndrome or lethality. However, heat-killed cells with and without GalN stimulated significant serum IL-6, indicating that IL-6 may not contribute to mortality in this model (Fig. 3).

TABLE 2. Lethality of live and heat-killed *T. pectinovorum* in GalN-sensitized mice

Bacterium	Mice	GalN[a]	Infection dose (no. of cells)	Lethality[b]
T. pectinovorum ATCC 33768, live	ICR	+	10^9	5/5
		+	10^{10}	5/5
		−	10^9	0/5
		−	10^{10}	0/5
T. pectinovorum ATCC 33768, live	C3H/HeN	+	10^7	4/4
		+	10^8	9/9
		+	10^9	4/4
		−	10^8	0/5
T. pectinovorum ATCC 33768, H-K[c]	C3H/HeN	+	10^8	0/5
		−	10^8	0/5
T. pectinovorum ATCC 33768, live	C3H/HeJ	+	10^9	5/5
T. pectinovorum S1, live	ICR	+	10^7	0/5
		+	10^8	0/5
		+	10^9	8/10
		+	10^{10}	10/10
		−	10^7	0/5
		−	10^8	0/5
		−	10^9	0/5
		−	10^{10}	0/5
E. coli O111, live	C3H/HeN	+	10^5	4/4
		+	10^7	4/4
		+	10^9	3/3
None	ICR	+		0/5

[a] GalN was mixed with an appropriate concentration of treponemes and administered i.p. to groups of mice. Control animals received either GalN or an appropriate concentration of treponemes only i.p.
[b] Number of animals that died/total injected.
[c] *T. pectinovorum* was heat killed (H-K) at 100°C for 60 min in a water bath.

DISCUSSION

We have previously reported on the identification and purification of a molecule from the outer membrane of *T. pectinovorum*, which demonstrated chemical characteristics similar to those of LPSs from other gram-negative prokaryotes (36). However, there were differences from other classical enteric bacterial LPS molecules. The *T. pectinovorum* LPS lacks a classical ladder-like O-antigenic sugar structure and appeared

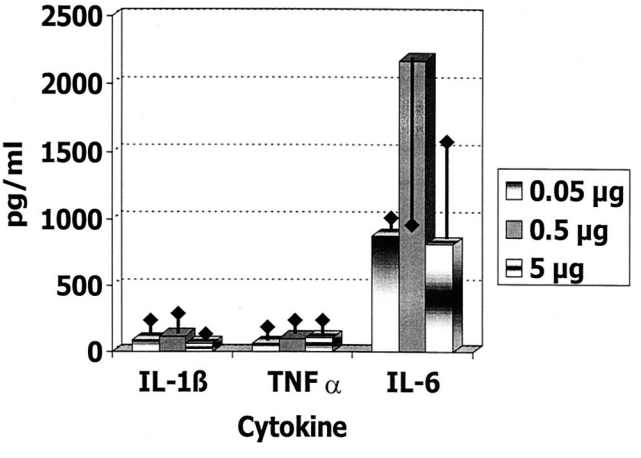

FIG. 2. Cytokine (IL-1β, TNF-α, and IL-6) pattern in C3H/HeN Tac-MTV (LPS responder) mice after LPS treatment. Mice ($n = 3$) were injected i.p. with 5, 0.5, and 0.05 μg of *T. pectinovorum* ATCC 33768 LPS with GalN as indicated in Materials and Methods. Serum samples were taken at 3, 8, and 12 h after LPS administration. Each cytokine concentration was determined by ELISA. Results are the means and standard deviations for three mice at 12 h.

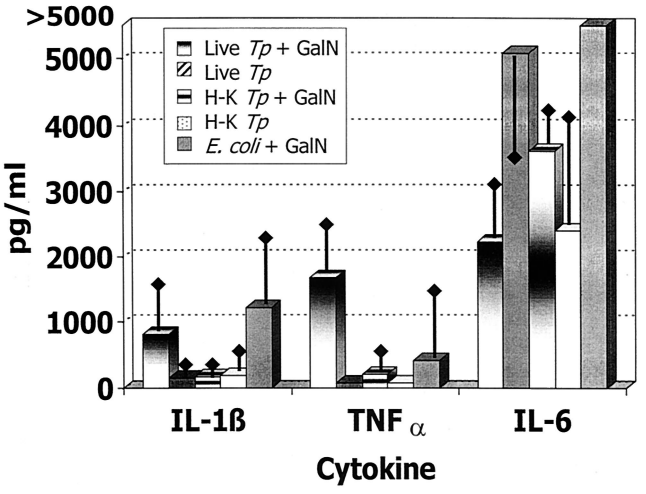

FIG. 3. Proinflammatory-cytokine (IL-1β, TNF-α, and IL-6) patterns in C3H/HeN Tac-MTV (LPS responder) mice were analyzed after i.p. challenge infection with live (10^8 cells) and heat-killed (H-K) (10^8 cells) *T. pectinovorum* ATCC 33768 and live *E. coli* O111 (10^5 cells) with and without GalN as described in Materials and Methods. Serum samples were taken at 3, 8, and 12 h postinfection. Each cytokine concentration was determined by sequential ELISA. Results are the means and standard deviations for three mice at 12 h.

to consist of single low- and high-molecular-weight species without a ladder-like series of bands (36). The presence of the classical 3-deoxy-D-*manno*-octulosonic acid molecule in the *T. pectinovorum* LPS provides strong evidence that this oral treponeme in fact contains a bona fide LPS. Kurimoto et al. (20) and Yotis et al. (40) have previously determined that an LPS-like extract from *Treponema denticola* and *Treponema vincentii* had biological activities consistent with LPS from enteric bacteria. Neither *Treponema pallidum* nor *Borrelia burgdorferi* possesses LPS, whereas *Leptospira interrogans* possesses an atypical LPS that differs from classical LPS from gram-negative organisms in several biochemical, physical, and biological properties (7).

In the present study, we characterized the biological functions of *T. pectinovorum* LPS using in vitro and in vivo models. In vitro, the *T. pectinovorum* LPS was evaluated for endotoxicity with an LAL assay. This is a classical method for determination of endotoxin activity, although other molecules and gram-positive bacteria can also activate this primitive host response. We did note that the *T. pectinovorum* LPS activated this system, consistent with LPS-like preparations from other oral treponemes (20, 40, 41). However, the endotoxicity was very low compared with the endotoxic activity of more classical *E. coli* LPS. Consequently, we predicted that the *T. pectinovorum* LPS would exhibit rather low endotoxicity and immunobiological activities. This concept was validated by demonstrating undetectable or very low serum TNF-α and IL-1β and a total lack of endotoxicity in vivo in a murine model system.

While the *T. pectinovorum* LPS did not induce lethal endotoxemia in mice, it did induce a systemic response manifested by elevations in serum IL-6 levels. Similarly, LPS and lipid A from periodontal pathogens, such as *Prevotella intermedia* and *Porphyromonas gingivalis*, exhibited very low or no endotoxicity in GalN-loaded mice and LAL assays (14, 26). In contrast, the endotoxin activities of *Veillonella* and *F. nucleatum* LPSs were comparable to those of *Salmonella enterica* serovar Enteritidis and *E. coli* (28, 35). Thus, low endotoxic activity of LPS is not a uniform characteristic of oral gram-negative bacteria. Lipid A from the enteric pathogen *Helicobacter pylori* also exhibited negligible lethal toxicity in GalN-loaded mice, pyrogenicity for rabbits, and activity in the LAL test (27). Moreover, virulent *Brachyspira* (formerly *Treponema) hyodysenteriae* and avirulent *Treponema innocens* LPS and endotoxin preparations were poor adjuvants, failed to induce a dermal Shwartzman reaction, and were not pyrogenic (10). Consequently, accumulating evidence supports the idea that numerous disparate pathogens produce structurally distinct LPSs that do not fit the classic paradigm of bioactive endotoxins. The potential that these molecules may still represent a virulence strategy of these microorganisms has been coupled to concepts of pattern recognition receptors and innate immune mechanisms (5).

We also evaluated the biologic functions of the *T. pectinovorum* LPS by assessing its ability to elicit cytokine responses from gingival fibroblasts, which are the resident cells comprising the connective tissue of the periodontium. As in all cells, the fibroblasts express surface receptors that can be triggered to initiate a series of events inside the cell, some of which result in the production of cytokines contributing to cell communication and amplification of the immune system to control infections (2, 8, 42; E. Shupp, M. J. Steffen, and J. L. Ebersole, Am. Assoc. Dent. Res. Meet., abstr. 2736, 2000). We utilized these biological phenomena to assess the capacity of *T. pectinovorum* LPS to interact with the gingival fibroblasts. Previous studies examining the challenge of gingival fibroblasts with various bacteria have indicated that these cells preferentially respond to produce IL-6 and IL-8 (8, 24). Recently, it was reported that *T. pectinovorum* can also induce high levels of IL-6, IL-8, and MCP-1 from gingival fibroblasts (24; Shupp et al., Am. Assoc. Dent. Res. Meet.). A previous study showed that neither *T. pectinovorum* nor *T. denticola* stimulated IL-1α, IL-1β, IL-10, platelet-derived growth factor, GM-CSF, IFN-γ, or TNF-α production by the gingival fibroblasts (24). As with the bacteria, the *T. pectinovorum* LPS did not elicit production of IL-1β, GM-CSF, or IFN-γ by the gingival fibroblasts. However, we detected a time course and dose-response induction of IL-6, IL-8, and MCP-1 following gingival fibroblast challenge with LPS. While this was clearly discernible above basal level production by the cells, rather high levels of the LPS were required to elicit these responses. Thus, we concluded that in contrast to LPS from other oral microorganisms, the *T. pectinovorum* LPS is not particularly biologically active, as determined using standard assays of endotoxicity and as a host cell ligand for inflammation. Therefore, it may play a more important role as a structural macromolecule than as a virulence component. These observations are consistent with reports for other oral treponemes, which appear to provide minimal stimulatory signals for the host inflammatory and immune system. This may, in fact, be a strategy used by this group of microorganisms to enhance their capacity to colonize and compete in the host subgingival microenvironment.

In contrast to the relative minimal biologic function of the LPS, intact *T. pectinovorum* was quite active in a number of the assays employed. Live *T. pectinovorum* ATCC 33768 demonstrated in vitro endotoxic activity in the LAL assay, although

the extent of this activity was significantly lower than that noted with *E. coli* O111. Importantly, and consistent with isolated LPS, this endotoxicity was abolished following heat killing of the spirochete. In general, heating gram-negative bacteria has been classically used to differentiate the functions of LPS (e.g., heat resistance) from other bacterial components, which are most often heat labile (23). This in vitro endotoxicity was further supported by the observation that live *T. pectinovorum* was lethal in GalN-sensitized mice. This method has classically been used to document in vivo endotoxicity of bacteria or biomolecules. Furthermore, challenge of mice with live *T. pectinovorum* elicited increases in serum IL-1β and TNF-α, which correlated with lethality, similar to lethal challenge with *E. coli*. Heat killing of *T. pectinovorum* eliminated the in vivo toxicity, suggesting that both endotoxin assays may have been activated by non-LPS, heat-labile molecule(s) expressed by this oral treponeme. Alternatively, live *T. pectinovorum* may secrete soluble factors extracellularly that mediated in vivo toxicity in the mice. Additional studies will be required to distinguish between these interpretations. It has been demonstrated that *T. pallidum* and *B. burgdorferi* possess no LPS but their abundant membrane lipoproteins are potent immunopotentiators of proinflammatory activities of monocytes via a CD14-dependent pathway distinct from that used by LPS (30). In this manner, both in vitro and in vivo studies implicate lipoproteins and lipopeptides (non-LPS components) as inflammatory mediators that contribute to the immunopathogenesis of syphilis and Lyme disease (25). Thus, our major findings are consistent with heat-labile non-LPS components serving as inflammatory mediators contributing to in vivo toxicity. Live *T. pectinovorum* also stimulated gingival fibroblasts to secrete elevated levels of IL-6, IL-8, and MCP-1, which were significantly higher than those stimulated by any dose of *T. pectinovorum* LPS. Previous observations also showed that killed *T. pectinovorum* induced a higher level of IL-6 and IL-8 in vitro than the live bacteria (24). As with the LPS, three cytokines, IL-6, IL-8, and MCP-1, were produced by gingival fibroblasts in response to *T. pectinovorum* challenge. The levels of these cytokines were significantly higher following stimulation with the microorganism than with the isolated *T. pectinovorum* LPS, supporting a contribution by non-LPS components mediating cytokine induction (24).

These experiments demonstrate that the *T. pectinovorum* LPS does not exhibit high levels of biologic activity as determined by using measures of endotoxin function. However, *T. pectinovorum* appears to produce other macromolecules, which can activate systems classically triggered by endotoxins. This includes the induction of serum IL-1β and TNF-α. These host cytokines have been clearly defined as the systemically acting molecules resulting in endotoxic shock (23). Consequently, it appears that *T. pectinovorum* can induce a systemic endotoxemia-like reaction via classic mechanisms but not through the activity of LPS. The physicochemical characteristics of these unique molecules will need to be delineated as potential unique virulence determinants of this oral treponeme.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grant DE-11368 from the National Institute of Dental and Craniofacial Research.

We thank S. G. Walker for assistance with LPS preparation.

REFERENCES

1. **Beutler, B. I., W. Milsark, and A. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor (TNF) protects mice from the lethal effect of endotoxin. Science **229:**869–871.
2. **Birkedal-Hansen, H.** 1993. Role of cytokines and inflammatory mediators in tissue destruction. J. Periodontal Res. **28:**500–510.
3. **Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky.** 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J. Biol. Chem. **274:**10689–10692.
4. **Dahle, U. R., L. Tronstad, and I. Olsen.** 1996. 3-Hydroxy fatty acids in a lipopolysaccharide-like material from *Treponema denticola* strain FM. Endod. Dent. Traumatol. **12:**202–205.
5. **Darveau, R. P.** 2000. Oral innate host defense responses: interactions with microbial communities and their role in the development of disease, p. 169–218. *In* H. K. Kuramitsu and R. P. Ellen (ed.), Oral bacterial ecology: the molecular basis. Horizon Scientific Press, Wymondham, United Kingdom.
6. **Darveau, R. P., and R. E. W. Hancock.** 1983. Procedure for isolation of bacterial lipopolysaccharides from both smooth and rough *Pseudomonas aeruginosa* and *Salmonella typhimurium* strains. J. Bacteriol. **155:**831–838.
7. **de Souza, L., and M. C. Koury.** 1992. Isolation and biological activities of endotoxin from *Leptospira interrogans*. Can. J. Microbiol. **38:**284–289.
8. **Dongari-Bagtzoglou, A., and J. L. Ebersole.** 1996. Production of inflammatory mediators and cytokines by human gingival fibroblasts following bacterial challenge. J. Periodontal Res. **31:**90–98.
9. **Galanos, C., M. A. Freudenberg, and W. Feutter.** 1979. Galactosamine-induced sensitization to the lethal effects of endotoxin. Proc. Natl. Acad. Sci. USA **76:**5939–5943.
10. **Greer, J. M., and M. J. Wannemuhler.** 1989. Comparison of the biological responses induced by lipopolysaccharide and endotoxin of *Treponema hyodysenteriae* and *Treponema innocens*. Infect. Immun. **57:**717–723.
11. **Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Wanis.** 1999. Cutting edge: inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by toll-like receptor 2. J. Immunol. **163:**2382–2386.
12. **Hirschfeld, M., J. J. Weis, V. Toshchakov, C. A. Salkowski, M. J. Cody, D. C. Ward, N. Qureshi, S. M. Michalek, and S. N. Vogel.** 2001. Signaling by toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. Infect. Immun. **69:**1477–1482.
13. **Holt, S. C., and T. E. Bramanti.** 1991. Factors in virulence expression and their role in periodontal disease pathogenesis. Crit. Rev. Oral Biol. Med. **2:**177–281.
14. **Johne, B., and K. Bryn.** 1986. Chemical composition and biological properties of a lipopolysaccharide from *Bacteroides intermedius*. Acta. Pathol. Microbiol. Immunol. Scand. **94:**265–271.
15. **Karkhanis, Y. D., J. Zeltner, J. J. Jackson, and D. J. Carlo.** 1978. A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of Gram-negative bacteria. Anal. Biochem. **85:**595–601.
16. **Kesavalu, L., S. G. Walker, S. C. Holt, R. R. Crawley, and J. L. Ebersole.** 1997. Virulence characteristics of oral treponemes in a murine model. Infect. Immun. **65:**5096–5102.
17. **Kesavalu, L., S. C. Holt, and J. L. Ebersole.** 1999. Environmental modulation of oral treponeme virulence in a murine model. Infect. Immun. **67:**2783–2789.
18. **Kielian, T. L., and F. Blecha.** 1995. CD14 and other recognition molecules for lipopolysaccharide: a review. Immunopharmacology **29:**187–205.
19. **Kirschning, C. J., H. Wesche, T. M. Ayres, and M. Rothe.** 1998. Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. J. Exp. Med. **188:**2091–2097.
20. **Kurimoto, T., M. Suzuki, and T. Watanabe.** 1990. Chemical composition and biological activities of lipopolysaccharides extracted from *Treponema denticola* and *Treponema vincentii*. Shigaku **78:**208–232.
21. **Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
22. **Moore, W. E. C., and L. V. H. Moore.** 1994. The bacteria of periodontal diseases. Periodontol. 2000 **5:**66–77.
23. **Morrison, D. C., and J. L. Ryan.** 1992. Bacterial endotoxic lipopolysaccharides. CRC Press, London, United Kingdom.
24. **Nixon, C. S., M. J. Steffen, and J. L. Ebersole.** 2000. Cytokine responses *to Treponema pectinovorum* and *Treponema denticola* in human gingival fibroblasts. Infect. Immun. **68:**5284–5292.
25. **Norgard, M. V., B. S. Riley, J. A. Richardson, and J. D. Radolf.** 1996. Dermal inflammation elicited by synthetic analogs of *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins. Infect. Immun. **63:**1507–1515.
26. **Ogawa, T.** 1994. Immunobiological properties of chemically defined lipid A from lipopolysaccharide of *Porphyromonas (Bacteroides) gingivalis*. Eur. J. Biochem. **219:**737–742.
27. **Ogawa, T., Y. Suda, W. Kashihara, T. Hayashi, T. Shimoyama, S. Kusumoto, and T. Tamura.** 1997. Immunobiological activities of chemically defined lipid A from *Helicobacter pylori* LPS in comparison with *Porphyromonas gingivalis*

lipid A and *Escherichia coli*-type synthetic lipid A (compound 506). Vaccine **15:**1598–605.
28. **Onoue, S., M. Niwa, Y. Isshiki, and K. Kawahara.** 1996. Extraction and characterization of the smooth-type lipopolysaccharide from *Fusobacterium nucleatum* JCM 8532 and its biological activities. Microbiol. Immunol. **40:** 323–331.
29. **Schutze, E., J. Hildebrandt, E. Liehl, and C. Lam.** 1994. Protection of mice from mortality caused by living and heat-killed bacteria by SDZ MRL 953. Circ. Shock **42:**121–127.
30. **Sellati, T. J., D. A. Bouis, R. L. Kitchens, R. P. Darveau, J. Pugin, R. J. Ulevitch, S. C. Gangloff, S. M. Goyert, M. V. Norgard, and J. D. Radolf.** 1998. *Treponema pallidum* and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytic cells via a CD14-dependent pathway distinct from that used by lipopolysaccharide. J. Immunol. **160:**5455–5464.
31. **Simonson, L. G., C. H. Goodman, J. J. Bial, and H. E. Morton.** 1988. Quantitative relationship of *Treponema denticola* to severity of periodontal disease. Infect. Immun. **56:**726–728.
32. **Socransky, S. S., A. D. Haffajee, L. A. Ximenez-Fyvie, M. Feres, and D. Mager.** 1999. Ecological considerations in the treatment of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* periodontal infections. Periodontol. 2000 **20:**341–362.
33. **Steffen, M. J., and J. L. Ebersole.** 1996. Sequential ELISA for cytokine levels in limited volumes of biological fluids. BioTechniques **21:**504–509.
34. **Steffen, M., S. C. Holt, and J. L. Ebersole.** 2000. *Porphyromonas gingivalis* induction of mediator and cytokine secretion by human gingival fibroblasts. Oral Microbiol. Immunol. **15:**172–180.
35. **Sveen, K., T. Hofstad, and K. C. Milner.** 1977. Lethality for mice and chick embryos, pyrogenicity in rabbits and ability to gelate lysate from amoebocytes of Limulus polyphemus by lipopolysaccharides from *Bacteroides, Fusobacterium* and *Veillonella*. Acta Pathol. Microbiol. Scand. **85B:**388–396.
36. **Walker, S. G., J. L. Ebersole, and S. C. Holt.** 1997. Identification, isolation, and characterization of the 42-kilodalton major outer membrane protein (MompA) from *Treponema pectinovorum* ATCC 33768. J. Bacteriol. **179:** 6441–6447.
37. **Walker, S. G., X. Xu, E. Altman, K. J. Davies, J. L. Ebersole, and S. C. Holt.** 1999. Isolation, and chemical analysis of a lipopolysaccharide from the outer membrane of the oral anaerobic spirochete *Treponema pectinovorum* ATCC 33768. Oral Microbiol. Immunol. **14:**304–308.
38. **Werts, C., R. I. Tapping, J. C. Mathison, T. Chuang, V. Kravchenko, I. S. Girons, D. A. Haake, P. J. Godowski, F. Hayashi, A. Ozinsky, D. M. Underhill, C. J. Kirschning, H. Wagner, A. Aderem, P. S. Tobias, and R. J. Ulevitch.** 2001. Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. Nat. Immunol. **2:**346–352.
39. **Wilson, M.** 1995. Biological activities of lipopolysaccharides from oral bacteria and their relevance to the pathogenesis of chronic periodontitis. Sci. Prog. **78:**19–34.
40. **Yotis, W. W., F. Macaluso, and C. Gopalsami.** 1995. Immunochemical features of a macromolecule of *Treponema denticola*. J. Basic Microbiol. **35:** 255–268.
41. **Yotis, W. W., V. K. Sharma, C. Gopalsami, S. Chegini, J. McNulty, K. Hoerman, J. Keene, Jr., and L. G. Simonson.** 1991. Biochemical properties of the outer membrane of *Treponema denticola*. J. Clin. Microbiol. **29:**1397–1406.
42. **Yu, X., and D. Graves.** 1995. Fibroblasts, mononuclear phagocytes, and endothelial cells express monocyte chemoattractant protein-1 (MCP-1) in inflamed human gingiva. J. Periodontol. **66:**80–88.

Editor: J. D. Clements