

Bacteroides fragilis-Derived Lipopolysaccharide Produces Cell Activation and Lethal Toxicity via Toll-Like Receptor 4

Giuseppe Mancuso,¹ Angelina Midiri,¹ Carmelo Biondo,¹ Concetta Beninati,¹
Maria Gambuzza,¹ Daniele Macrì,² Antonella Bellantoni,¹
Andrej Weintraub,³ Terje Espevik,⁴
and Giuseppe Teti^{1*}

Department of Pathology and Experimental Microbiology, University of Messina, Messina, Italy I-98125¹; ASL No. 4, Enna, Italy²; Department of Laboratory Medicine, Karolinska Institute, S-141 86 Stockholm, Sweden³; and Norwegian University of Science and Technology, N-7489 Trondheim, Norway⁴

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Bacteroides fragilis, which is part of the normal intestinal flora, is a frequent cause of serious disease, especially in diabetic and surgical patients. In these conditions, *B. fragilis* lipopolysaccharide (LPS) is likely to play a major pathophysiologic role. *B. fragilis* LPS is structurally different from classical enterobacterial LPS, whose biological activities are mediated by Toll-like receptor 4 (TLR4) activation. The ability of *B. fragilis* LPS to activate TLR4 and TLR2 was investigated here, since evidence on this issue is scarce and controversial. Each of four different protein-free *B. fragilis* LPS preparations could induce interleukin-8 responses in cells cotransfected with TLR4/CD14/MD2 but not TLR4/CD14 alone. Two of the preparations also induced cytokine production in cells cotransfected with TLR2/CD14 or in peritoneal macrophages from TLR4 mutant C3H/HeJ mice. Both of these activities, however, were lost after repurification with a modified phenol reextraction procedure. Importantly, all preparations could induce endotoxic shock in TLR2-deficient mice, but not in TLR4 mutant C3H/HeJ mice. Consistent with these findings, anti-TLR4 and anti-CD14, but not anti-TLR2, antibodies could inhibit *B. fragilis* LPS-induced cytokine production in human monocytes. Collectively, these results indicate that *B. fragilis* LPS signals via a TLR4/CD14/MD2-dependent pathway, and it is unable to activate TLR2. Moreover, our data document the occurrence of TLR2-activating contaminants even in highly purified *B. fragilis* LPS preparations. This may explain earlier contradictory findings on the ability of *B. fragilis* LPS to activate cells in the absence of functional TLR4. These data may be useful to devise strategies to prevent the pathophysiologic changes observed during *B. fragilis* sepsis and to better understand structure-activity relationships of LPS.

Bacteroides fragilis is the most important human anaerobic pathogen. This gram-negative bacterium, which is part of the normal intestinal flora, causes infections arising from exposure of normally sterile body sites to even minute amounts of fecal material. *B. fragilis* has a marked tendency to provoke abscess formation and is frequently isolated from the blood during life-threatening sepsis in association with intra-abdominal or pelvic abscesses (2, 6, 9). Lipopolysaccharide (LPS), a major constituent of the cell wall of gram-negative bacteria, plays an important role in the pathophysiology of sepsis (7). Exposure of host cells to small quantities of LPS or of its bioactive center, lipid A, results in the rapid activation of a number of responses, including the production of proinflammatory cytokines by monocytes, macrophages, and neutrophils (5, 7). Recent studies have indicated that Toll-like receptors (TLRs) are responsible for the recognition of LPS and other microbial products and play a central role in the initiation of innate immune responses, including cytokine release (1, 26, 34). The TLR family consists of at least 13 different paralogues, each recognizing a specific group of pathogen-derived molecules (1). Engagement of TLRs by microbial products results in

stimulation of signal transduction pathways through adaptor molecules, the best characterized of which is myeloid differentiation factor 88 (MyD88), an adaptor used by most TLRs and by the interleukin 1 (IL-1) receptor (1, 26, 34). The best-characterized TLRs, to date, are TLR2 and TLR4. TLR2 is involved in the recognition of gram-positive bacteria and mycobacteria (10, 24, 31, 39), whereas TLR4 recognizes LPS produced by enterobacteria (3, 15, 29) in conjunction with the accessory protein MD2 (32, 35). It was reported that TLR2 is involved in signaling in response to some nonenterobacterial LPS preparations, including LPS from *Leptospira interrogans* (21) and *Porphyromonas gingivalis* (19, 37).

B. fragilis lipid A is structurally similar, but not identical, to *P. gingivalis* lipid A (7, 44). Both have a monophosphorylated disaccharide backbone and relatively long (15 to 17 carbon atoms) fatty acids that are not present in enterobacterial LPS, which carries a biphosphorylated disaccharide and 12 to 14 carbon fatty acids. These structural differences probably account for the considerably (100- to 1,000-fold) lower endotoxicity of *B. fragilis* and *P. gingivalis* LPS relative to enterobacterial LPS (7, 44). Very little is known of the molecular mechanisms involved in *B. fragilis* LPS-induced cell activation. The scant information available is contradictory in regard to the ability of *B. fragilis* LPS to trigger responses in LPS non-responder mice, which harbor mutations in the TLR4-encoding gene. *B. fragilis* LPS was found to be an equally potent

* Corresponding author. Mailing address: Policlinico Universitario, Via C. Valeria 1, I-98125 Messina, Italy. Phone: 39-090-221-3310. Fax: 39-090-221-3312. E-mail: teti@eniware.it.

mitogen for spleen cells from LPS responder and hyporesponder mice (16). Subsequent studies demonstrated that the mitogenic effects on LPS hyporesponder splenocytes were detectable only under special culture conditions (high cell densities) and that LPS hyporesponsive mice could not produce a number of in vivo and in vitro responses to *B. fragilis* LPS that were readily detectable in LPS responder mice (43). Recent evidence, however, has indicated that *B. fragilis* LPS could stimulate macrophage inflammatory protein 2 expression in the absence of functional TLR4 in a macrophage cell line derived from the LPS hyporesponder mouse strain C57BL/10ScCr (22). In a different report, *B. fragilis* LPS was able to induce IL-8 production in cells transfected with TLR2 and CD14 (8). These data raise the possibility that, similar to other nonenterobacterial preparations, *B. fragilis* LPS stimulates cells through TLR2. However, since this hypothesis has not been tested in TLR2-deficient animals, it is currently unclear whether the biological activities of *B. fragilis* LPS are mediated by activation of TLR4, TLR2, or other TLRs.

In the present study we examined the involvement of TLR2 and TLR4 in *B. fragilis* LPS-induced activation phenomena by using highly purified and well-characterized preparations (44–46), as well as different experimental systems, including genetically defective mice, TLR-transfected cells, and anti-receptor antibodies. Our results suggest that, as for enterobacterial LPS, the proinflammatory effects of *B. fragilis* LPS are mediated by the TLR4/CD14/MD2 pathway and suggest the possibility that unknown, nonprotein contaminants may induce cell activation via TLR2-dependent signaling.

MATERIALS AND METHODS

Reagents. Chemicals were obtained from Sigma Chimica (Milan, Italy), unless indicated otherwise. Bacterial lipoprotein [BLP; palmitoyl-Cys(RS)-2,3-di-(palmitoyloxy)-propyl]-Ala-Gly-OH] was obtained from Bachem (Bubendorf, Switzerland). Monoclonal antibody (MAb) 3C10 (anti-human CD14) and TL2.1 (anti-human TLR2) were generated as previously described (10). MAb HTA125 (anti-human TLR4) was kindly provided by Kensuke Miyake (Saga Medical School, Saga, Japan) (32).

LPS preparations. *Salmonella enterica* serovar Minnesota R595 ultrapure LPS was obtained from List Biologicals (Campbell, CA [distributed by Vinci-Biochem, Vinci, Italy]). This preparation contained 225 EU/ng, as determined by a chromogenic *Limulus* amoebocyte lysate (LAL) assay (Pyrochrome Associates of Cape Cod, Falmouth, MA). Four different *B. fragilis* LPS preparations were used in the present study. All were obtained by phenol-water extraction, followed by phenol-chloroform-petroleum ether extraction, as described previously (44–46). Three *B. fragilis* LPS preparations (9343-XVI, 9343-XVIIe, and 9343-IV) were from the *B. fragilis* strain NCTC 9343 (obtained from the National Collection of Type Cultures, London, United Kingdom). 9343-XVI and 9343-IV were different batches obtained in different occasions using identical procedures, whereas 9343-XVIIe was obtained from 9343-XVI by electro dialysis and conversion to a triethylamine (TEA) salt form, as described previously (17). The fourth preparation (5631e) was extracted from VPI 5631 strain (obtained from the Virginia Polytechnic Institute and State University, Blacksburg, VA) and converted to the TEA salt form by electro dialysis, as described previously (17). The four *B. fragilis* LPS preparations showed similar specific activities in the LAL assay, ranging from 18 to 60 EU/ng.

SDS-PAGE and protein staining procedure. To detect protein contamination, a sensitive gold staining technique was used. *B. fragilis* LPS was overloaded (5 μ g per lane) on 15% polyacrylamide gel slabs and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Unstained slabs were blotted onto nitrocellulose transfer membranes that were stained in a colloidal gold solution (Enhanced Colloidal Gold Total Protein Detection kit; Bio-Rad Laboratories s.r.l., Milan, Italy), as described previously (19). Bovine serum albumin (BSA; 0.01 to 100 ng per lane) was used as a positive control. In initial experiments it was found that this colloidal gold staining could detect as little as 1 ng of BSA.

Repurification of *B. fragilis* LPS by modified phenol-water reextraction. LPS preparations were repurified according to the modified extraction method described by Hirschfeld et al. (13), as follows. A total of 5 mg of LPS was resuspended in 1 ml of endotoxin-free water containing 0.2% TEA and deoxycholate was added to a final concentration of 0.5%, followed by the addition of 1 ml of water-saturated phenol. After intermittent vortexing for 5 min, the phases were allowed to separate at room temperature for 5 min. Samples were placed on ice for 5 min, followed by centrifugation at 4°C for 2 min at 10,000 \times g. The top aqueous layer was transferred to a new tube, and the phenol phase was subjected to reextraction with 1 ml of 0.2% TEA–0.5% deoxycholate. The aqueous phases were pooled and reextracted with 2 ml of water-saturated phenol. The pooled aqueous phases were adjusted to 75% ethanol and 30 mM sodium acetate and were allowed to precipitate at –20°C for 1 h. The precipitates were centrifuged at 4°C for 10 min at 10,000 \times g, washed in 1 ml of cold 100% ethanol, and air dried. The precipitates were resuspended in 1 ml of endotoxin-free water containing 0.2% TEA. The reextraction procedure resulted in no loss of LPS, as shown by similar activities, in the LAL assay, of the *B. fragilis* LPS preparations before and after repurification. To further exclude loss of LPS as a result of migration to the phenol phase during repurification, phenol-extracted material was tested for LAL activity. To this end, combined phenol phases were extensively dialyzed against methanol, and the contents of the dialysis bag were allowed to evaporate in a glass tube. After the addition of 1 ml of 0.2% TEA in water, the tube was vortexed, and the solution was tested by LAL. LAL activity was consistently 4 orders of magnitude lower in the phenol-phase material compared to repurified *B. fragilis* LPS. These data indicate that there was no significant loss of *B. fragilis* LPS as of result of the reextraction procedure.

Cell cultures. Human embryonic kidney (HEK) 293 cells stably transfected with TLR2 (HEK 293-TLR2) or TLR4 (HEK 293-TLR4) or cotransfected with TLR2/CD14 (HEK 293-TLR2/CD14), TLR4/CD14 (HEK 293-TLR4/CD14), or TLR4/CD14/MD2 (HEK 293-TLR4/CD14/MD2) were grown in low-glucose Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum (FCS), HEPES (10 mM), L-glutamine (2 mM), penicillin (50 IU/ml), streptomycin (50 mg/ml; all obtained from Invitrogen Life Technologies, San Giuliano Milanese, Italy), and puromycin (1 μ g/ml). For stimulation experiments, HEK 293 cells were plated in 24-well tissue culture plates (5×10^5 /ml) and induced for 5 h with the indicated stimuli. Culture supernatants were collected and stored at –70°C until assayed for IL-8 production.

Mice. TLR2-deficient mice, engineered as described previously (36), were obtained from S. Akira (Osaka University, Osaka, Japan) through D. Golenbock (University of Massachusetts, Boston, MA). C57BL/6 mice (Charles River Italia, Calco, Italy) served as controls for the TLR2-deficient mice. C3H/HeJ (lps defective) and C3H/HeN control wild-type mice were also purchased from Charles River. Mice used in the present study were housed under specific-pathogen-free conditions in enclosed filter top cages of the Department of Pathology and Experimental Microbiology of the University of Messina (Messina, Italy). The mice were fed clean food and water ad libitum. All of the procedures described in the present study were in agreement with the European Union guidelines of animal care and were approved by the relevant local committees.

Peritoneal macrophages. Peritoneal cells were isolated from the peritoneal cavity by washing with ice-cold phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl [pH 7.2]), pelleted by centrifugation, and resuspended in RPMI 1640 supplemented with 2% FCS, 50 IU of penicillin/ml, and 50 μ g of streptomycin/ml. Peritoneal cells were then seeded in 96-well dishes at a density of 2×10^5 /well and incubated at 37°C in a 5% humidified CO₂ environment. After 24 h, nonadherent cells were removed by washing with medium, and the adherent cells were stimulated for 22 h with various concentrations of the different LPS preparations. Unstimulated cells were included as baseline control. Culture supernatants were collected and stored at –70°C until assayed for cytokine measurement.

Lethal toxicity test. The lethality test was performed as previously described (11) with 8-week-old male mice. To increase LPS susceptibility, mice were pretreated intraperitoneally with 30 mg of D-galactosamine 3 h before intravenous administration of the indicated doses of LPS dissolved in 0.1 ml of phosphate-buffered saline.

Human monocyte cultures. Mononuclear cells were obtained from the peripheral blood of healthy adult donors by centrifugation on Ficoll-Hypaque (Amersham Biosciences, Milan, Italy) (25, 42). Cells at the interface were extensively washed, resuspended to a concentration of 1.5×10^6 /ml in RPMI 1640 supplemented with 10% FCS, streptomycin (50 μ g/ml), and benzylpenicillin (50 IU/ml), and cultured in 24-well culture plates for 2 h at 37°C in 5% CO₂. Thereafter, nonadherent cells were aspirate, and adherent monocytes were washed twice in RPMI 1640. Monolayers were incubated with control immunoglobulin Gs or



FIG. 1. Purity of *B. fragilis* LPS preparations. Five micrograms of *B. fragilis* LPS 9343-XVI (lane 2), 5631e (lane 3), 9343-IV (lane 4), and 9343-XVIIe (lane 5) were resolved on a 15% gel and transferred to a nitrocellulose membrane. Proteins were visualized by a colloidal gold stain. A total of 10 ng of BSA (lane 1) was used as positive control.

blocking MAbs at the indicated concentrations for 30 min at 37°C before addition of the LPS preparations. After a 22-h incubation, culture supernatants were collected and stored at -70°C until assayed for tumor necrosis factor alpha (TNF- α) and IL-6.

Cytokine determination. Murine TNF- α and IL-6 concentrations in culture supernatants were determined as described previously (4) by using commercial mouse enzyme-linked immunosorbent assay (ELISA) kits (a mouse TNF- α module set [Bender MedSystems, Vienna, Austria] and a murine IL-6 reagent set [Euroclone, Wetherby, United Kingdom], respectively). The lower detection limit of both assays was 16 pg/ml. Human TNF- α , IL-6, and IL-8 measurements were performed, respectively, by human TNF- α , IL-6, and IL-8 ELISA reagent set (Euroclone; sensitivity of <15 pg/ml for all assays).

RESULTS

Lack of protein contamination in *B. fragilis* LPS preparations. None of the four *B. fragilis* LPS preparations used here contained detectable amounts (≥ 5 μ g/mg) of proteins, as assayed by the method of Lowry (23). However, since previous studies have indicated that even trace amounts of protein contaminants can induce TLR2 activation (13), we sought to assess protein contamination by using more stringent methods. To this end, all of the four *B. fragilis* LPS preparations used here were subjected to SDS-PAGE in overloaded gels (5 μ g per lane), blotted on nitrocellulose, and stained by a sensitive colloidal gold stain, which could detect as little as 1 ng of protein. No bands appeared in any of the *B. fragilis* LPS preparations, indicating that the protein contamination was <0.02% (Fig. 1).

Activation of HEK 293 transfected cells. The ability of *B. fragilis* LPS to activate TLR2 or TLR4 was tested in HEK 293

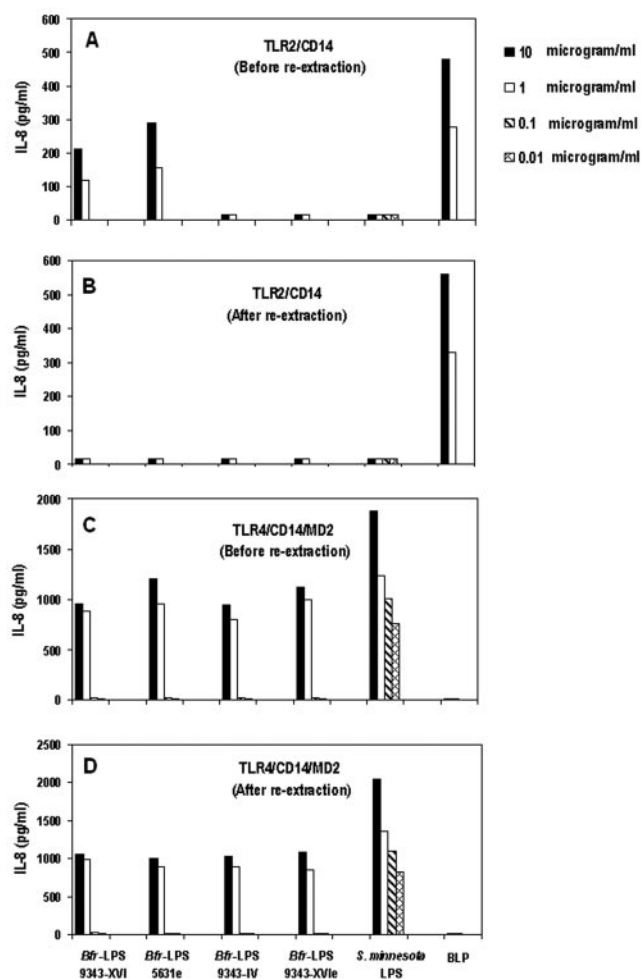


FIG. 2. *B. fragilis* LPS-induced activation in TLR-transfected cells. HEK 293 cells stably cotransfected with TLR2/CD14 (A and B) or TLR4/CD14/MD2 (C and D) were stimulated for 5 h with the indicated *B. fragilis* LPS doses, and culture supernatants were assayed for IL-8 production. *S. enterica* serovar Minnesota R595 ultrapure LPS and BLP were used as positive controls. Four different *B. fragilis* LPS preparations were used both before (A and C) and after (B and D) re-purification by phenol reextraction procedure. The results from one experiment, representative of three, are shown.

cells stably transfected with human TLR2, TLR2/CD14, or TLR4/CD14/MD2. After cell stimulation, IL-8 was measured in culture supernatants. BLP and *S. enterica* serovar Minnesota LPS were included in these experiments as control agonists for, respectively, TLR2 and TLR4. None of the four *B. fragilis* LPS preparations examined was able to stimulate IL-8 production in cells stably transfected with TLR2 alone (data not shown). However, two of the four preparations (9343-XVI and 5631-e) induced IL-8 release in cells cotransfected with TLR2/CD14, while the other two (9343-IV and 9343-XVIIe) did not (Fig. 2A).

There was no known feature of the *B. fragilis* LPS preparations, including source strain or electro dialysis treatment, that could be related to the ability to stimulate TLR2/CD14 transfected cells. For example, 9343-XVI (stimulatory) and 9343-IV (nonstimulatory) were obtained in two different occasions us-

ing the same strain and the same extraction-purification procedure.

It was previously shown that the TLR2 stimulating activity of enterobacterial LPS could be abolished after removal of protein contaminants by a modified phenol reextraction (13, 38). Therefore, our preparations were repurified by this procedure and tested again with TLR2/CD14-cotransfected cells. Figure 2B shows that the repurified preparations completely lost their ability to stimulate these cells, indicating that unidentified contaminants were responsible for the TLR2-stimulating activity of these preparations.

In further experiments, cells cotransfected with TLR4/CD14/MD2 were stimulated with unextracted and phenol reextracted *B. fragilis* LPS preparations. Figure 2C and D shows that all unextracted or phenol extracted preparations could induce significant IL-8 production. Therefore, phenol reextraction did not influence these effects. In contrast, no IL-8 production was detected in cells transfected with TLR4 alone or cotransfected with TLR4/CD14 (not shown). These data indicated that *B. fragilis* LPS can simulate TLR4 to produce cell activation and that the presence of CD14 and MD2 is also required for this activity.

Cytokine production in macrophages from LPS hyporesponder mice. Further experiments were conducted to assess whether TLR4 or TLR2 are involved in *B. fragilis* LPS responses under more physiological conditions than those provided by receptor overexpression in transfected cells. Therefore, the TNF- α -inducing ability of *B. fragilis* LPS preparations was tested in peritoneal macrophages from LPS hyporesponder (C3H/HeJ) mice, which have a loss-of-function mutation in the gene encoding TLR4. The results were compared to those observed in LPS normoresponder (C3H/HeN) mice. Figure 3 (upper panels) shows that, even before repurification, all four *B. fragilis* LPS preparations tested induced considerably higher TNF- α responses in C3H/HeN, relative to C3H/HeJ mice. However, the same two *B. fragilis* LPS preparations (*B. fragilis* LPS 9343-XVI and 5631e) that were previously found to activate TLR2/CD14 cotransfected cells (Fig. 2) could produce modest TNF- α responses in C3H/HeJ mice (Fig. 3, upper panels). This activity, however, was completely lost after phenol repurification (Fig. 3, lower panels).

Next, to further exclude the involvement of TLR2 in *B. fragilis* LPS-induced stimulation, we used peritoneal macrophages from TLR2-deficient mice (Fig. 4). In these experiments, two representative *B. fragilis* LPS preparations were tested before and after phenol repurification. Both repurified and nonrepurified preparations induced comparable TNF- α responses in peritoneal macrophages from control C57BL/6 and TLR2 gene-disrupted mice (Fig. 4), indicating that TLR2-dependent stimulation was negligible or absent under physiological conditions of cell activation. When IL-6 levels, instead of TNF- α , were measured in the culture supernatants from the experiments reported in Fig. 3 and 4, we obtained results very similar to those observed with TNF- α (data not shown).

Lethal toxicity of *B. fragilis* LPS on galactosamine-sensitized mice. The lethal toxicity of the 9343-XVIe *B. fragilis* LPS was tested in galactosamine-sensitized mice using C3H/HeJ LPS-hyporesponsive mice or in TLR2^{-/-} mice (Table 1). Serovar Minnesota LPS was used as a control. Treatment of LPS-responsive, control C3H/HeN mice with 1 or 10 ng of serovar

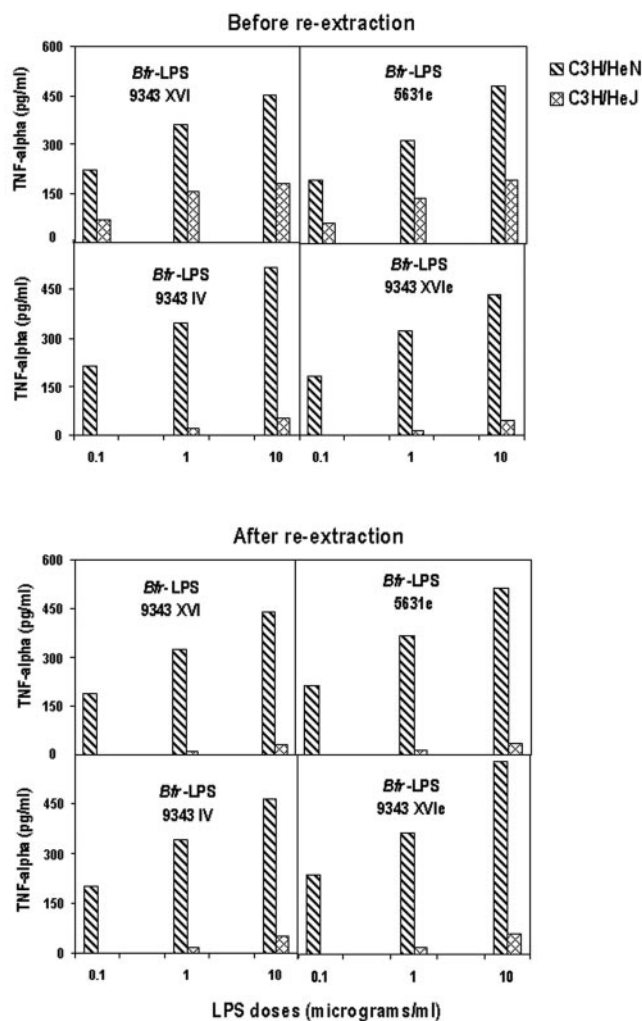


FIG. 3. Repurified *B. fragilis* LPS fails to induce TNF- α production in macrophages from LPS hyporesponder mice. Peritoneal macrophages from LPS responder (C3H/HeN) or LPS hyporesponder (C3H/HeJ) mice were stimulated for 22 h with various concentrations of *B. fragilis* LPS preparations obtained before and after repurification with a phenol reextraction procedure. Culture supernatants were assayed for TNF- α release. The results from one experiment, representative of three, are shown.

Minnesota LPS resulted in 33 or 100% lethality, respectively. At least 3-log-higher doses were needed to induce mortality with *B. fragilis* LPS, which killed, respectively, 25 and 70% of the C3H/HeN mice with doses of 10 and 100 μ g, respectively (Table 1). In contrast, C3H/HeJ mice were completely resistant to challenge with up to 100 μ g of either serovar Minnesota or *B. fragilis* LPS.

Table 1 also shows that both TLR2^{-/-} mice and their wild-type controls were highly sensitive to *B. fragilis* LPS-induced toxicity. These results indicate the crucial role of TLR4 in *B. fragilis* LPS-mediated lethal toxicity and the lack of such a role for TLR2. In further experiments, we tested the lethal toxicity of a nonrepurified *B. fragilis* LPS preparation (9343-XVI) that in previous experiments was found to stimulate TLR2/CD14-transfected cells (see Fig. 2, upper panel). We were unable to induce lethal toxicity with up to 200 μ g per animal in C3H/HeJ

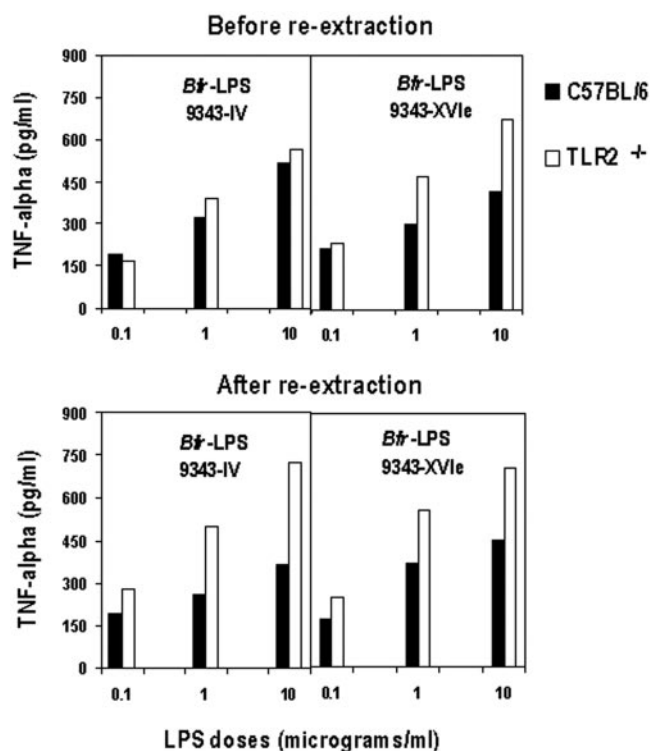


FIG. 4. TNF- α release by peritoneal macrophages from TLR2-deficient mice stimulated with *B. fragilis* LPS. Peritoneal macrophages from wild-type C57BL/6 or TLR2^{-/-} mice were stimulated for 22 h with various concentrations of two different *B. fragilis* LPS preparations and supernatants were tested for TNF- α production. Both unextracted and reextracted *B. fragilis* LPS preparations were used. The results of one experiment, representative of three, are shown.

hypo-responder mice, indicating that, even in the presence of TLR2-stimulating contaminants, *B. fragilis* LPS preparations were devoid of TLR4-independent endotoxin-like activity (data not shown).

Effect of TLR-blocking antibodies on cytokine release by human mononuclear phagocytes. To study the involvement of human TLRs in *B. fragilis* LPS-induced cell activation, human monocytes were pretreated with specific blocking anti-human TLR2 or anti-TLR4 monoclonal antibodies, prior to the addition of LPS, and supernatants were tested for TNF- α or IL-6 production. Serovar Minnesota LPS was used as a control. As shown in Fig. 5, serovar Minnesota LPS (upper panels) was considerably more potent than *B. fragilis* LPS (lower panels) in inducing the production of either TNF- α (left panels) or IL-6 (right panels). This was evidenced by the fact that ca. 2-log-higher *B. fragilis* LPS doses were needed to induce cytokine elevations over baseline values, relative to serovar Minnesota LPS. Moreover, the maximal levels of TNF- α or IL-6 induced by serovar Minnesota LPS were at least threefold higher than those induced by *B. fragilis* LPS. Pretreatment of monocytes with MAb HTA125 (anti-TLR4) decreased by 40 to 50% TNF- α or IL-6 production induced by both serovar Minnesota and *B. fragilis* LPS. Anti-TLR2 MAb failed to inhibit cytokine production induced by either serovar Minnesota LPS or *B. fragilis* LPS, whereas the addition of the CD14 MAb resulted in the almost-complete reduction of immunologically detectable

TABLE 1. *B. fragilis* LPS fails to induce lethality in LPS hypo-responder mice^a

Mouse strain	LPS type	No. dead/total no. at dose (μ g/mouse) of:				
		0.001	0.01	1	10	100
C3H/HeN	<i>S. enterica</i> serovar Minnesota	2/6	3/3			
	<i>B. fragilis</i>			0/3	1/4	4/6
C3H/HeJ	<i>S. enterica</i> serovar Minnesota		0/3	0/3	0/3	0/3
	<i>B. fragilis</i>				0/4	0/6
C57BL/6	<i>S. enterica</i> serovar Minnesota	1/3	3/3			
	<i>B. fragilis</i>			0/4	1/6	3/6
TLR2 ^{-/-}	<i>S. enterica</i> serovar Minnesota	2/4	4/4			
	<i>B. fragilis</i>			0/4	3/6	6/6

^a Mice were pretreated intraperitoneally with 30 mg of D-galactosamine 3 h before intravenous administration of different doses of Serovar Minnesota or *B. fragilis* LPS.

TNF- α and IL-6 (Fig. 5). These data confirm that the TLR4, but not the TLR2, pathway is involved in *B. fragilis* LPS stimulation of human monocytes.

DISCUSSION

The discovery that the great majority of innate immune responses originate from TLRs has opened new exciting possibilities to treat infectious disease and chronic inflammatory conditions, including inflammatory bowel disease (1). A prerequisite for applying these new approaches, however, is the correct identification of the TLRs that recognize major microbial components. Since it far outnumbers other colonic species, including *Enterobacteriaceae*, the *B. fragilis* group is likely to represent the major source of endogenous LPS in the human body (40).

Moreover, bacteria of the *B. fragilis* group, which includes among others *B. thetaiotaomicron*, are key regulators of the human mucosal immune system and of bowel development (18, 47). These bacteria, together with other members of the gut microbiota, act to induce tolerance to commonly encountered environmental antigens and to fortify the mucosal barrier (18, 47). In view of the potentially important role of *B. fragilis* LPS in human biology and disease, it was of interest to study its ability to stimulate TLRs.

B. fragilis LPS was found here to induce cell activation and lethal toxicity via TLR4 and not via TLR2. Several lines of evidence supported this conclusion. First, repurified *B. fragilis* LPS was totally unable to induce TNF- α responses in the absence of a functional TLR4, as shown in TLR4 mutant LPS hypo-responsive mice. Second, none of the *B. fragilis* LPS preparations tested (both repurified and nonrepurified) was able to induce lethal shock in these mice. In contrast, TNF- α responses and lethality could be readily induced in TLR2-defective mice. Similarly, in human cells, TLR4, but not TLR2, appeared to mediate *B. fragilis*-induced activation. In fact, anti-TLR4 and anti-CD14, but not anti-TLR2, antibodies could

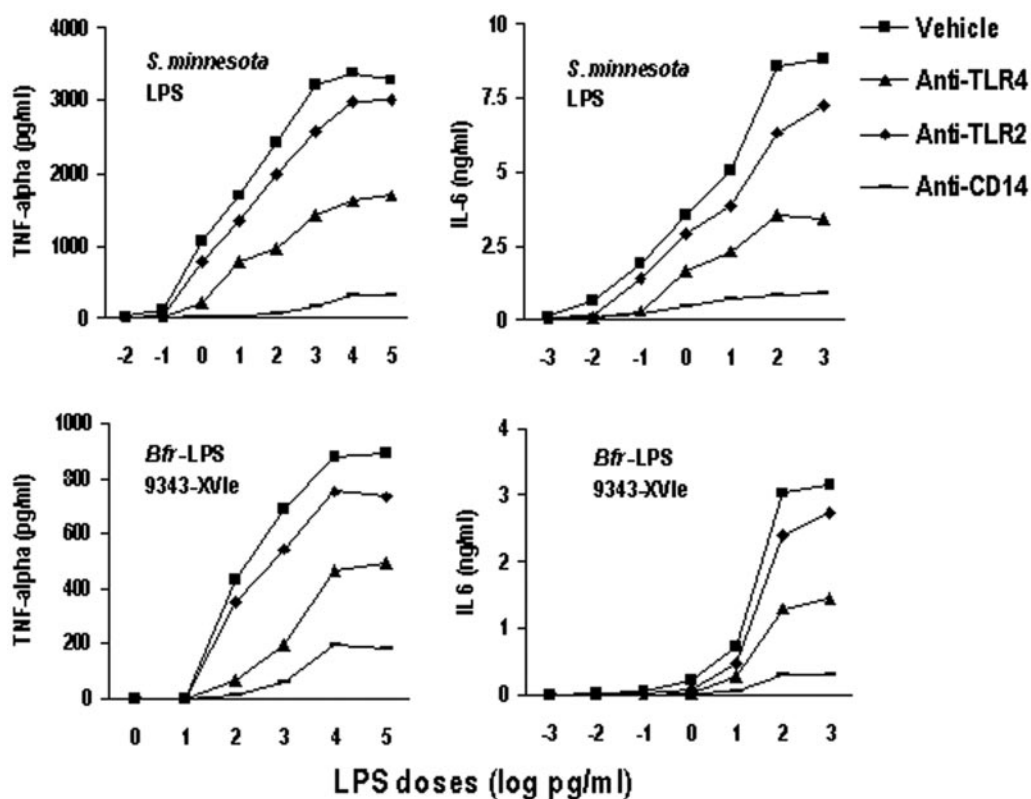


FIG. 5. Anti-TLR4 or anti-CD14 antibodies block *B. fragilis* LPS-induced cytokine release by human monocytes. Human monocytes were pretreated with specific blocking anti-human TLR2, anti-TLR4 or anti-CD14 monoclonal antibodies (10 μ g/ml) prior to the addition of 9343-XVIe *B. fragilis* LPS. *S. enterica* serovar Minnesota LPS was used as a control. The results of one experiment, representative of three, are shown.

inhibit *B. fragilis* LPS-induced cytokine production in human monocytes. Moreover, repurified *B. fragilis* LPS could induce IL-8 production in cells cotransfected with human TLR4/CD14/MD2 but not in cells cotransfected with human TLR2/CD14. These data suggested that, in addition to TLR4, the presence of the MD2 protein and CD14 is necessary for *B. fragilis* LPS-induced cell activation. Therefore, *B. fragilis* LPS uses the same receptors and coreceptors used by enterobacterial LPS, although its potency is \sim 1,000-fold lower (7) (Fig. 5 and Table 1 of the present study).

The ability of *B. fragilis*-LPS to stimulate TLR4, rather than TLR2, may have important functional consequences in its interaction with the human host. TLR4 and TLR2 agonists induce the differential expression of a large number of genes in various cell types, including macrophages (14), dendritic cells (30), and mast cells (33). Although the great majority of the genes activated by TLR2 stimulation are also activated by TLR4, there are a number of key genes, including IL-12, as well as both beta and gamma interferons, whose expression is increased by TLR4, but not TLR2, agonists (41). Further studies involving in vivo colonization and infection models are clearly needed to better assess the role of the TLR4 pathway in host-*B. fragilis* interactions.

In 2000 the important observation was made that removal of contaminants from enteric LPS by phenol-water reextraction abrogates TLR2, but not TLR4 agonistic activity (13, 38). Our results extend these findings to *B. fragilis* LPS and resolve the

contradictory findings of the earlier reports. In 1982, *B. fragilis* LPS was found to be mitogenic for splenocytes from C3H/HeJ mice (16), but after 2 years it was shown that *B. fragilis* LPS could not produce, in these mice, many effects observed in wild-type animals (43). More recently, Lorenz et al. showed that *B. fragilis* LPS could stimulate macrophage inflammatory protein 2 secretion in the absence of functional TLR4 in a cell line from the mouse strain C57BL/10ScNcr (22). However, as pointed out by these authors, the presence of contaminants could not be excluded (22). Erridge et al. recently reported that *B. fragilis* LPS could stimulate TLR2-transfected cells even after repurification by phenol extraction (8). These authors did not examine the effects of their preparation in TLR-defective mice. We interpret these data to indicate that TLR2-activating contaminants cannot be always removed by phenol reextraction. Alternatively, differences between our data and those of Erridge et al. may be due to differences in the TLR-transfected cell lines used. Irrespective of the reasons for these discrepancies, data obtained using only protein hyperexpression systems, such as TLR-transfected cell lines, should be taken with caution since they do not necessarily reflect the physiological situation.

It is generally assumed that the contaminants frequently found in enterobacterial preparations are of protein nature and recently, two lipoproteins, Lip19 and Lip12, with TLR2-stimulating activity have been identified in phenol extracts from *Escherichia coli* LPS (20). Although we have not at-

tempted to characterize them, it is unlikely that the TLR2-stimulating contaminants of our *B. fragilis* LPS are proteins, since these were not detectable in our preparations. Similar data were obtained by Muroi et al., who found, in phenol extracts of enterobacterial LPS, nonprotein components with LPS-like activities (27). In the present study, the presence of TLR2-stimulating material in *B. fragilis* LPS preparations could not be related to the strain or the purification procedure used. For example, two preparations (9343-IV and 9343-XVI) obtained using the same strain and the same purification protocols produced different results when tested for their ability to activate TLR2. Therefore, subtle, as-yet-undefined, variations occurring during preparation of *B. fragilis* LPS may result in the presence of TLR2-stimulating contaminants. Studies are under way to analyze the chemico-physical factors in extraction-purification procedures influencing the presence of TLR2-stimulating activity in *B. fragilis* LPS preparations.

Protein-free phenol reextracted *P. gingivalis* LPS preparations were previously reported to activate TLR2 (14). *B. fragilis* lipid A is similar to that of *P. gingivalis*, since both lack phosphorus at position 4 of the disaccharide backbone and carry five fatty acids with lengths of 15 to 17 carbon residues. The position of the various fatty acids, however, is different (7). It is possible that this subtle structural difference accounts for the different receptor specificity of *P. gingivalis* and *B. fragilis* LPS. Alternatively, *P. gingivalis* preparations may contain TLR2-stimulating contaminants that cannot be removed by phenol extraction. In favor of the latter hypothesis, a synthetic triacylated *P. gingivalis* lipid A was shown to stimulate TLR4, but not TLR2 (28). Moreover, Hashimoto et al. identified, in *P. gingivalis* LPS preparations, a lipoprotein with potent TLR2-stimulating activity (12).

In conclusion, our data indicate that, as with enterobacterial LPS, the proinflammatory effects of *B. fragilis* LPS are mediated by the TLR4/CD14/MD2 pathway. These data may be useful to devise alternative strategies to prevent the pathophysiologic changes observed during *B. fragilis* sepsis and to further elucidate the mechanisms underlying immune homeostasis in the intestinal tract.

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