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Lipopolysaccharides from *Bacteroides fragilis* Are Mitogenic for Spleen Cells from Endotoxin Responder and Nonresponder Mice

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The lipopolysaccharides (LPS) from *Bacteroides fragilis* are structurally atypical and give weak responses in most tests of endotoxic activity, but the mitogenic activity of LPS from *B. fragilis* has not been tested. We prepared LPS from *B. fragilis* 23745 by three methods and compared their mitogenic activity for murine spleen cells with that of LPS from *Escherichia coli* K235 prepared by similar techniques. LPS extracted from *B. fragilis* with hot phenol-water, with butanol-water, or by detergent separation from the outer membrane were mitogenic for spleen cells from C57BL/10ScN, C57BL/10ScCR, and C3H/HeJ mice. The outer membrane, the outer membrane protein-polysaccharide complex, and the capsular polysaccharide from *B. fragilis* were also mitogenic for spleen cells from the same murine strains. LPS extracted from *E. coli* K235 with hot phenol-water, butanol-water, or sodium deoxycholate were mitogenic for C57BL/10ScN spleen cells, but only the LPS extracted with butanol and deoxycholate were stimulatory for spleen cells from C57BL/10ScCR and C3H/HeJ mice. Two types of LPS varying in the 2-keto-3-deoxyoctonate-to-carbohydrate ratio were isolated from *E. coli* K235 with sodium deoxycholate; both endotoxins contained protein which was typical of lipid A or endotoxin protein. These results indicate that the LPS from *B. fragilis* is a potent mitogen for spleen cells from endotoxin responder and endotoxin nonresponder mice.

Bacterial lipopolysaccharides (LPS) are potent mitogens for murine B lymphocytes. LPS that have been tested for mitogenic activity for murine B lymphocytes have almost invariably been extracted from aerobic gram-negative bacteria, generally from bacteria of the family *Enterobacteriaceae* (e.g., *Escherichia coli, Salmonella* spp., *Serratia* spp.). The mitogenic activity of LPS from obligately anaerobic gram-negative bacteria has not been fully tested. *Bacteroides fragilis* is the obligate anaerobic bacterium most frequently isolated from clinical infections, and this organism is commonly isolated from the bloodstream of patients with life-threatening intraabdominal or pelvic infections. The structure of the LPS from *B. fragilis* is incompletely understood but appears to differ substantially from the typical structure of aerobic gram-negative bacterial LPS. The putative lipid A region of the *B. fragilis* LPS has an unusual fatty acid composition and contains no amide-linked β-hydroxymyristic acid (27); also, the linkage between the lipid and polysaccharide portions of the *B. fragilis* LPS is unlikely to be ketosidic because it lacks 2-keto-3-deoxyoctonate (KDO), an invariable constituent of other LPS (7, 26).

The LPS from *B. fragilis* are also unique in the weak responses they produce in usual tests of lipid A activity, such as *Limulus* lysate gelation, pyrogenicity, mouse lethality, chicken embryo toxicity, and dermal Schwartzman reactivity (7). Responses to the LPS from *B. fragilis* are 10- to 10,000-fold lower in these tests than are responses to endotoxin from *E. coli* or *Salmonella* spp. Disseminated intravascular coagulation, a complication of infection thought to be attributable to endotoxin, is rarely seen in patients with *Bacteroides* bacteremia.

To further understand the biology of LPS from *B. fragilis*, we studied the mitogenic activity of LPS and other outer membrane components of *B. fragilis* for murine B lymphocytes. LPS were prepared from *B. fragilis* by three methods and compared with similar preparations from *E. coli*. LPS were tested with spleen cells from mice which were either responsive or

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nonresponsive to the lipid A of *E. coli* LPS. Our results indicate that LPS from *B. fragilis* are potent mitogens for spleen cells from both endotoxin responder and endotoxin nonresponder mice.

MATERIALS AND METHODS

Bacteria. *B. fragilis* ATCC 23745 was obtained from the stock culture collection. *E. coli* K235 was the kind gift of Arthur Hirata and Jerry Petruska of Abbott Laboratories, North Chicago, Ill. *B. fragilis* 23745 was grown as previously described (8). *E. coli* K235 was grown for 18 h in tryptic soy broth supplemented with dextrose (14 g/liter). The pH was maintained at 6.9 to 7.0 during growth by the addition of 10 N NaOH with a pH titrator (Radiometer, Copenhagen, Denmark).

Heat-killed whole bacteria were prepared by heating end-log-phase cultures at 80°C for 10 min. Organisms were washed three times in phosphate-buffered saline and suspended to the desired concentration in supplemented RPMI 1640 medium (prepared as described below).

Bacterial antigens. LPS from *E. coli* K235 and *B. fragilis* 23745 were obtained or prepared by each of three extraction procedures as outlined below.

Phenol-water method. LPS extracted from *E. coli* K235 by the phenol-water method of McIntire et al. (13) were the generous gift of John Ryan (West Haven VA Hospital, New Haven, Conn.) and David Morrison (Scripps Clinic, La Jolla, Calif.). This method produces a nearly protein-free LPS preparation from *E. coli*. LPS were extracted from *B. fragilis* 23745 by a modification of this procedure. Washed whole organisms (70 g wet weight) were mixed with 70 ml of 88% phenol and 140 ml of distilled water at 4°C for 7 days in a separatory funnel. The aqueous layer and insoluble residue at the phenol-water interface were removed after centrifugation and then combined and brought to 350 ml by the addition of cold distilled water. This preparation was rapidly heated to 65°C, and preheated 88% phenol was added to bring the total phenol concentration to 44%. This preparation was incubated for 15 min at 65°C, rapidly cooled, and stored overnight at 4°C. The aqueous phase was separated by centrifugation, removed, dialyzed against distilled water, and repeatedly subjected to ultracentrifugation until the supernatant showed no absorbance at 260 or 280 nm. A portion of the final material was chromatographed on DEAE-Sephacel in 0.05 M Tris (pH 8.5) and eluted with 2 M NaCl in 0.05 M Tris.

Butanol-water method. LPS extracted from *E. coli* K235 by the butanol-water method of Morrison and Leive (18) also were kindly supplied by John Ryan and David Morrison. The procedure was applied to *B. fragilis* 23745 exactly as described. Briefly, whole bacteria were suspended in saline and extracted with an equal volume of water-saturated butanol for 30 min at 4°C. The aqueous phase was dialyzed and concentrated with an Amicon pressure cell, suspended in 0.05 M Tris (pH 7.4), and incubated overnight at 37°C with 0.5 mg of RNase and 0.1 mg of DNase per ml. The flocculent white precipitate and the bacterial pellet were separated by centrifugation, and the aqueous phase was chromatographed on Sepharose 4BCL. The single peak eluting at the void volume was pooled and lyophilized.

Deoxycholate method. LPS were extracted from the outer membrane of *B. fragilis* 23745 and *E. coli* K235 by the procedure of Kasper et al (7).

The outer membrane, protein-polysaccharide, and capsular polysaccharide preparations from *B. fragilis* 23745 were extracted as previously described (9). The protein-polysaccharide complex contains 45 to 52% protein and, when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), shows six major protein bands with molecular weights of 1.6×10^5, 1.3×10^5, 3.9×10^4, 2.5×10^4, 1.8×10^4, and 1.1×10^4, (9). The outer membrane fraction from *E. coli* K235 was extracted and purified exactly as described for *B. fragilis* (9). The outer membrane pellet was suspended in endotoxin-disaggregating buffer which contained 0.05 M glycine, 0.01 M EDTA, and 0.5% sodium deoxycholate (NaD) at pH 9.0. This sample was applied to a column (10 by 90 cm) containing Sephadex G100 in 0.5% NaD buffer. (See Fig. 1 for the elution profile of this chromatographic step.) The two peaks containing KDO (peaks II and III) were pooled separately, and NaD was removed by three sequential precipitations with cold 80% ethanol. The peak II and peak III materials were suspended in 0.05 M Tris buffer (pH 7.4) and chromatographed separately on a column (2.6 by 90 cm) containing Sepharose 4BCL (see below). LPS for pronase treatment were suspended at 1 mg/ml in 0.05 M Tris buffer (pH 7.4) and incubated overnight with 1 mg of pronase per 100 ml. The mixture was chromatographed on Sepharose 4BCL in 0.05 M Tris to separate pronase and the pronase digest from LPS.

Analytical methods. Total protein content was determined by the method of Lowry et al. (10), total carbohydrate was determined by the phenolsulfuric procedure with galactose standards (6), and KDO was determined by the method of Weissbach and Hurwitz (25) as modified by Osborn (19). SDS-PAGE of samples in 0.2% SDS–10% polyacrylamide gel was performed by the technique of Weber and Osborn (24). Gas-liquid chromatography of trimethylsilyl derivatives of the carbohydrates in LPS was done specifically to look for contaminating glucose. Prior studies have shown that *B. fragilis* contains a glucan which is found in antigen preparations (8).

Mice. C57BL/10ScN and C3H/HeJ mice were obtained from Jackson Laboratories, Bar Harbor, Maine. C57BL/10ScCR mice were obtained from Sprague-Dawley, Madison, Wis. All mice were females, aged 6 to 10 weeks.

Cell culture. Mouse spleen cells were obtained by gently homogenizing spleens in Hanks balanced salt solution. The spleen homogenate was filtered through 40-μm nylon mesh. Erythrocytes were lysed by suspending the cells in Tris-ammonium chloride buffer with 1% bovine serum albumin for 5 min at room temperature and washing them twice at 4°C in Hanks balanced salt solution. Cells were cultured in Microtest II flat-bottomed microtiter wells (Falcon Plastics, Oxnard, Calif.) at a density of 5×10^5 cells per culture well in 200 μl of RPMI 1640 medium supplemented with 2 mM glutamine, 5×10^{-5} M 2-mercaptoethanol, 50 U of penicillin per ml, 50 μg of streptomycin per ml, Eagle medium with minimal essential amino acids, 1 mM sodium pyruvate, and 1% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid). Fetal calf serum was not used to supplement any cultures.

TABLE 1. Chemical and physical characteristics of LPS from *B. fragilis* 23745 and *E. coli* K235

Organism and extraction procedure	Composition[a]				Major protein bands on SDS-PAGE (mol wt)
	% Total protein	% Total carbohydrate	% Nucleic acid	% KDO	
B. fragilis 23745					
Phenol-water	3.0	133.0[b]	<1	0	None detectable
Butanol-water	8.0	27.5	<1	0	33,000 47,000
NaD	14.5	ND[c]	<1	0	17,000 23,000 30,000
NaD + pronase	1.1	60–70[d]	<1	0	None detectable
E. coli K235					
Phenol-water	<0.5	93.0	<1	0.6	None detectable
Butanol-water	11.5	ND	ND	ND	16,000 20,000
NaD					
Peak II[e] (K_{av}, 0.31)[e]	9.1	56.0	<1	2.0	16,000 20,000 46,000
Peak III[e] (K_{av}, 0.43)[e]	6.9	34.5	<1	6.2	16,000 47,000

[a] Percentage of total by weight.
[b] Values exceeding 100% were attributable to the use of the galactose standard.
[c] ND, Not done.
[d] As reported in reference 9.
[e] On Sepharose 4BCL, with 0.05 M Tris (pH 7.4).

Mitogenesis was measured as described previously (12) after the addition of 1 μCi of [*methyl*-^{3}H]thymidine (5 Ci/mmol; New England Nuclear Corp., Boston, Mass.) during the final 16 h of culturing. Cultures were maintained from 24 to 96 h in preliminary experiments. Maximal responses were obtained at 48 h, and all results presented are for 48-h cultures. Cultures were harvested with a MASH II (Microbiological Associates, Bethesda, Md.) and counted in a scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). All mitogens were tested in triplicate (standard error of the mean within 10% of the mean) on at least two or three separate occasions. Results are expressed as Δcpm ($cpm_{sample} - cpm_{control}$) and as the stimulation index ($cpm_{sample}/cpm_{control}$), where cpm is counts per minute.

RESULTS

Chemical and physical characteristics of LPS. An analysis of some chemical and physical characteristics of the prepared LPS is given in Table 1. The NaD-extracted, pronase-treated *B. fragilis* LPS and the phenol-extracted *E. coli* LPS had minimal detectable protein contamination. Analysis by gas-liquid chromatography of NaD-extracted, pronase-treated *B. fragilis* LPS demonstrated a fatty acid composition similar to that reported by Wollenweber et al. (27) for phenol-extracted *B. fragilis* LPS. Analysis of the phenol-extracted *B. fragilis* LPS by gas-liquid chromatography showed significant contamination by a glucan. This has been previously demonstrated (8). All preparations of *B. fragilis* LPS lacked detectable KDO. Chromatography of *E. coli* K235 outer membrane components on Sephadex G100 equilibrated in NaD buffer resulted in the separation of two major KDO-containing peaks (peaks II and III in Fig. 1). The ratio of total carbohydrate to KDO was fivefold higher for peak II than for peak III, suggesting that peak II has a greater percentage of O-polysaccharide by weight than peak III does; this would be consistent with the larger molecular weight of peak II determined by chromatographic analysis in the disaggregating detergent buffer. Two forms of LPS from *E. coli* K235 with characteristics similar to our peak II and peak III preparations have been described by Goodman et al. (5). Our preparations contained more protein and KDO on a weight basis and chromatographed with a higher partition coefficient (K_{av}) than the preparations of Goodman et al. (5).

LPS were analyzed by SDS-PAGE. Gels were heavily loaded (250 to 300-mg sample) to permit the identification of protein bands (Table 1). The major bands identified in preparations from both *B. fragilis* and *E. coli* ranged between 1.6×10^4 and 4.7×10^4 daltons. No bands were visible in the NaD-extracted, pronase-treated *B. fragilis* NaD LPS or in the phenol-extracted *E. coli* LPS.

Mitogenic activity of outer membrane components from *B. fragilis* 23745. Initial studies demonstrated that outer membrane components (Fig. 2) and heat-killed organisms of *B. fragilis*

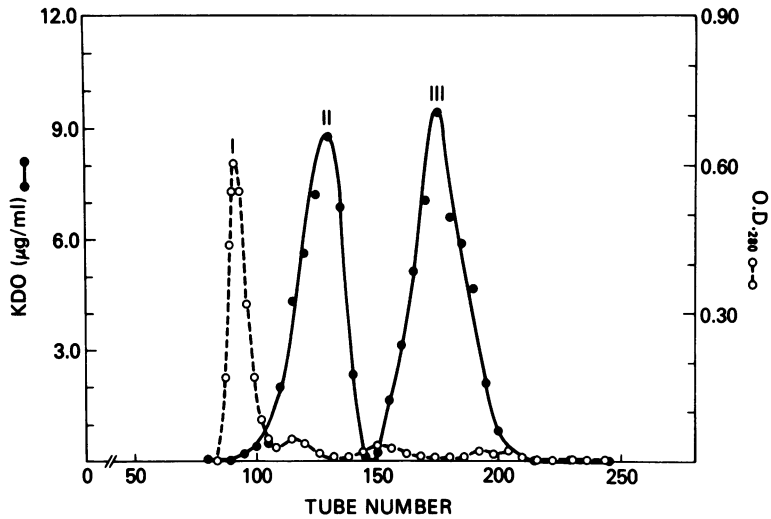

FIG. 1. Molecular sieve chromatography of the outer membrane components from *E. coli* K235 on Sephadex G100 in 0.5% NaD buffer. The outer membrane pellet from 120 g (wet weight) of *E. coli* K235 was suspended in 100 ml of 0.5% NaD buffer and applied to a column (10 by 90 cm) of Sephadex G100 in 0.5% NaD buffer. The sample was chromatographed at 25°C, 24-ml fractions were collected, and portions were assayed for absorbance at 280 nm (OD_{280}) and for KDO, as described in the text. One major peak-containing protein (I) and two major peaks containing KDO (II and III) were identified.

23745 were mitogenic for spleen cells from C57BL/10ScN mice. Maximal stimulation was observed at a concentration of 2.5 μg/ml for all mitogens except the polysaccharide capsule. Suspensions of heat-killed organisms were tested at concentrations ranging from $10^{5.3}$ to $10^{8.3}$ organisms per well. Maximal stimulation was observed at $10^{7.3}$ organisms per well (*B. fragilis*, 9.91×10^4 cpm; control, 2.7×10^3 cpm; stimulation index, 36.7). Stimulation indexes for $10^{5.3}$,

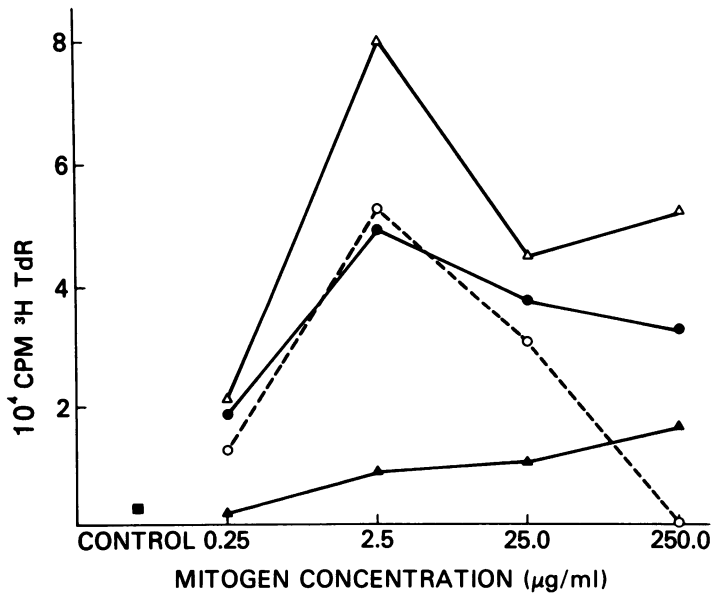

FIG. 2. Mitogenic activity of cell wall components from *B. fragilis* for spleen cells from C57BL/10ScN mice determined by incorporation of [*methyl*-^{3}H]thymidine (^{3}H TdR). Each point represents the mean for triplicate cultures of 5×10^5 spleen cells cultured for 48 h. Symbols: ■, control; ●, outer membrane; ○, LPS; △, protein-polysaccharide; ▲, capsular polysaccharide.

TABLE 2. Mitogenic activity of cell wall components of *B. fragilis* 23745 and *E. coli*. K235 for spleen cells from endotoxin nonresponder mice

Mitogen[a]	Murine spleen cell [*methyl*-^{3}H]thymidine incorporation (kcpm)[b]	
	C57BL/10ScCR	C3H/HeJ
B. fragilis outer membrane	160.9 (15.2)	121.1 (9.8)
B. fragilis LPS (extracted with NaD)	179.1 (16.9)	138.9 (11.2)
B. fragilis protein-polysaccharide	118.2 (11.2)	100.1 (8.1)
B. fragilis capsule	24.5 (2.3)	ND[c]
E. coli LPS (extracted with butanol)	58.2 (5.5)	51.0 (4.1)
E. coli LPS (extracted with phenol)	8.9 (0.8)	11.6 (0.9)
Control	10.6	12.4

[a] All mitogens were tested at 2.5 μg/ml.
[b] Numbers in parentheses represent the stimulation indexes.
[c] ND, Not done.

$10^{6.3}$, and $10^{8.3}$ organisms per well were 1.9, 5.7, and 10.8, respectively.

Cell wall components of *B. fragilis* were also mitogenic for spleen cells from two strains of mice (C57BL/10ScCR and C3H/HeJ) which are nonresponders to the lipid A moiety of enterobacterial LPS (Table 2). As anticipated (12), neither spleen cell preparation responded to *E. coli* LPS extracted with phenol, but mitogenic activity was demonstrated with *E. coli* LPS extracted with butanol.

Mitogenic activity of LPS prepared by three methods from *B. fragilis* and *E. coli*. The above results demonstrated that *B. fragilis* LPS extracted with NaD was mitogenic for spleen cells from endotoxin responder and nonresponder mice. It is possible that the mitogenic activity of this preparation was related to the method of preparation, particularly for the endotoxin nonresponder mice. This possibility was investigated by comparing the mitogenic activity of LPS prepared by three methods from *B. fragilis* and *E. coli*. The results for *B. fragilis* are shown in Table 3. All LPS preparations had significant mitogenic activity at 2.5 μg/ml for spleen cells from endotoxin responder and nonresponder mice. The marked stimulatory activity of NaD-extracted, pronase-treated *B. fragilis* LPS suggests that endotoxin protein is unlikely to account for all of the activity of this preparation. The significant protein content of the LPS extracted with phenol-water and butanol-water and of nonpronase-treated, NaD-extracted LPS prevents the drawing of any firm conclusion about the principal mitogenic moiety in these preparations.

The LPS extracted from *E. coli* with phenol-water, butanol-water, and NaD were mitogenic for spleen cells from the C57BL/10ScN mice (Table 4). Peak II and peak III materials from the NaD preparation were equivalent to one another in stimulatory activity at doses of 0.25, 2.5, and 25.0 μg/ml (all data not shown). The phenol-extracted *E. coli* LPS was not mitogenic for spleen cells from either strain of endotoxin nonresponder mice. The butanol-extracted and NaD-extracted LPS retained mitogenic activity for C57BL/10ScCr and C3H/HeJ spleen cells.

TABLE 3. Mitogenic activity of LPS from *B. fragilis* in endotoxin responder and nonresponder mice

Extraction procedure	*B. fragilis* LPS concn (μg/ml)	Murine spleen cell [*methyl*-^{3}H]thymidine incorporation (kcpm)[a]		
		C57BL/10ScN	C57BL/10ScCR	C3H/HeJ
NaD	2.5	102.0 (22.2)	114.6 (14.3)	81.3 (9.0)
	25.0	191.4 (41.6)	206.0 (25.8)	163.9 (18.2)
NaD + pronase	2.5	75.1 (16.3)	77.9 (9.7)	76.7 (8.5)
	25.0	166.8 (36.3)	179.7 (22.5)	153.2 (17.0)
Phenol-water	2.5	63.1 (13.7)	76.7 (9.6)	61.4 (6.8)
	25.0	43.9 (9.5)	77.7 (9.7)	61.5 (6.8)
Butanol-water	2.5	151.9 (33.0)	173.9 (21.7)	135.0 (15.0)
	25.0	5.0 (1.1)	8.4 (1.1)	6.4 (0.7)
Control		4.6	8.0	9.0

[a] Numbers in parentheses represent the stimulation indexes.

TABLE 4. Mitogenic activity of LPS from *E. coli* K235 in endotoxin responder and nonresponder mice

Extraction procedure	*E. coli* LPS concn (μg/ml)	Murine spleen cell [*methyl*-^{3}H]thymidine incorporation (kcpm)[a]		
		C57BL/10ScN	C57BL/10ScCR	C3H/HeJ
Phenol	2.5	105.4 (22.9)	9.6 (1.2)	9.7 (1.1)
	25.0	132.8 (28.9)	8.9 (1.1)	9.4 (1.1)
Butanol	2.5	88.0 (19.1)	58.2 (7.3)	51.0 (5.7)
	25.0	182.5 (39.7)	ND[b]	ND
NaD				
Peak II	2.5	138.7 (30.2)	100.2 (12.5)	70.9 (7.8)
	2.5[c]	125.6 (27.3)	47.0 (5.9)	33.6 (3.7)
	25.0	181.1 (39.4)	186.2 (23.3)	136.9 (15.2)
Peak III	2.5	151.6 (33.0)	102.1 (12.8)	77.6 (8.6)
	2.5[c]	154.6 (33.6)	40.5 (5.1)	27.0 (3.0)
	25.0	179.7 (39.1)	188.2 (23.5)	152.8 (17.0)
Control		4.6	8.0	9.0

[a] Numbers in parentheses represent the stimulation indexes.
[b] ND, Not done.
[c] Pronase treated.

The peak II and peak III NaD-extracted LPS from *E. coli* were treated with pronase under the same conditions as those used for pronase treatment of NaD-extracted LPS from *B. fragilis*. Pronase treatment of *E. coli* LPS did not alter the mitogenic activity for C57BL/10ScN spleen cells, but responses in the endotoxin nonresponder lines were decreased 50 to 70% (Table 4). Pronase treatment decreased the total protein content of the preparations by only 45 to 50%, and SDS-PAGE analysis demonstrated that low-molecular-weight proteolytic degradation fragments remained associated with the pronase-treated *E. coli* K235 LPS, as recently demonstrated by Goldman et al. (3) for *E. coli* $0111B_4$ LPS. Furthermore, a previous report by Goodman and Sultzer (4) demonstrated that the mitogenic activity of *E. coli* endotoxin protein for C3H/HeJ lymphocytes was unaffected by pronase treatment. It appears that pronase may have different effects on the endotoxin-associated proteins of *B. fragilis* and *E. coli*.

DISCUSSION

Previous studies have demonstrated that lipid A is the principle mitogenic moiety in LPS from *Enterobacteriaceae* (1, 20). However, lipid A from LPS in *Enterobacteriaceae* is not mitogenic for spleen cells from C3H/HeJ or C57BL/10ScCR mice, and these murine strains are designated endotoxin nonresponder lines. It has subsequently been shown that LPS extracted with hot trichloroacetic acid or aqueous butanol contain a lipid A-associated protein which is mitogenic for both endotoxin responder and endotoxin nonresponder spleen cells (17, 23). Since LPS extracted with hot aqueous phenol generally lack endotoxin protein, such preparations have no mitogenic activity for spleen cells from C3H/HeJ or C57BL/10ScCR mice.

We have shown that LPS extracted by three methods from *B. fragilis* are mitogenic for murine spleen cells. The pronase-treated, NaD-extracted LPS from *B. fragilis* are potent mitogens at a concentration of 2.5 μg/ml. The mitogenic activity of this preparation, which contains only 1% protein, is therefore unlikely to reside entirely in a lipid A-associated or endotoxin protein. The absence of a significant protein content in the NaD-extracted, pronase treated *B. fragilis* LPS also excludes the possibility that the mitogenic moiety is cell wall lipoprotein, an additional component of the gram-negative bacterial cell wall that has mitogenic activity for murine spleen cells (14).

The mitogenic activity of bacterial LPS is one measure of their endotoxicity. Elin et al. (2) compared mitogenicity, pyrogenicity, *Limulus* amoebocyte lysate reactivity, and complement-activating potential of 12 LPS or LPS derivatives. Only 1 of the 12 preparations, LPS from *Veillonella alcalescens*, was from a bacterial organism outside the family *Enterobacteriaceae*. A significant correlation was found between pyrogenicity, mitogenicity, and *Limulus* amebocyte lysate reactivity of these preparations. Our results with the *B. fragilis* LPS, which have weak pyrogenicity and *Limulus* amebocyte

lysate reactivity but potent mitogenic activity, suggest that correlations among these three parameters do not necessarily apply for nonenterobacterial LPS.

The discovery and understanding of the LPS nonresponsiveness of certain murine spleen cell preparations has evolved almost entirely with enterobacterial LPS. Recent reports have documented mitogenic activity of LPS from *Brucella abortus* (15) and protein-free LPS from *Pseudomonas aeruginosa* (21) for C3H/HeJ spleen cells. Taken in conjunction with the results from this study, it appears that the lack of a mitogenic response to LPS by spleen cells from C3H/HeJ and C57BL/10ScCr mice applies with certainty only to enterobacterial LPS. Furthermore, *B. abortus* (16), *P. aeruginosa* (21), and *B. fragilis* (2) LPS did not contain β-hydroxymyristic acid, which is the predominant amide-linked fatty acid in those bacterial LPS which have thus far been examined (reviewed in reference 22). For some LPS, amide-linked fatty acids appear to be necessary for mitogenic activity (11). Therefore, it is possible that endotoxin nonresponsiveness represents a selective defect in the ability to respond to the β-hydroxymyristic acid residue in lipid A.

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