BACTERIAL PATHOGENICITY

A re-appraisal of the biological activity of bacteroides LPS

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Summary. Lipopolysaccharides (LPS) were extracted from seven *Bacteroides* strains by three different techniques: the phenol-water (PW), phenol-chloroform-petroleum (PCP) and Triton-Mg²⁺ methods. The strains selected included two different *B. fragilis* strains, one of which was grown in two different media. Yields varied between the strains, growth media and extraction technique, but generally the highest yield by weight was from the PCP method and the lowest from the PW method. The PW method was selected for the greatest amounts of carbohydrate and KDO, and the PCP method for the least. Phosphorus levels were more uniform among all extraction methods. Protein contamination was found in all *Bacteroides* LPS extracts, with extremely low levels in PW-LPS and the highest levels in material extracted by the PCP and Triton-Mg²⁺ techniques. No protein contamination could be detected after proteinase K treatment. After silver staining LPS PAGE profiles showed ladder patterns characteristic of smooth LPS for *B. vulgatus*, *B. thetaiotaomicron* and the control *Escherichia coli* O18:K⁻ strains, whereas the other *Bacteroides* strains showed mainly rough and low M_r material only. The PCP method did not select for high M_r material in the *B. fragilis* strains; otherwise the LPS profiles for all extraction methods were identical. The biological activities of native and sodium salt form LPS were investigated on a weight for weight basis and compared to that of *E. coli* O18:K⁻ PW-LPS. Amongst the LPS from *Bacteroides* strains, those prepared by the PW method were found to have a significantly higher activity in a galactosamine mouse lethality model, in induction of TNF and the *Limulus* amoebocyte lysate (LAL) assay, than LPS extracted by the PCP or Triton-Mg²⁺ methods. LPS from *Bacteroides* strains extracted by the PCP method had consistently low activity in all assays. Comparing PW-LPS from *Bacteroides* strains with that from *E. coli* O18:K⁻ in the galactosamine mouse model, the *E. coli* O18:K⁻ LPS was c. 5000-fold more active than the most active bacteroides LPS. However, in the LAL assay native PW-LPS from both the *B. fragilis* strains, and *B. caccae* had higher activities (up to 30-fold) than *E. coli* O18:K⁻ LPS, with the PW-LPS from the other *Bacteroides* spp. being up to 15-fold less active than the *E. coli* O18:K⁻ PW-LPS. In the TNF induction assay, *E. coli* O18:K⁻ PW-LPS was 4–50-fold more active than bacteroides PW-LPS. In the LAL assay and galactosamine mouse model, native LPS had more activity (c. two-fold) than sodium salt form LPS. There was no clear difference in activity between native and sodium salt form LPS in the TNF induction assay. The results for the LAL and TNF induction assay were re-evaluated relative to KDO concentration. In the TNF induction assay, previously low activities seen on a weight for weight basis were due in part to less KDO being present. However, LAL activity for PCP-LPS was still low after re-evaluation relative to KDO concentration. The molecular basis for the differences in biological activity of bacteroides LPS in relation to extraction methods and chemical composition is not yet understood.

Introduction

The gram-negative bacillus *Bacteroides fragilis* is the anaerobe most frequently isolated from human infec-

tions including intra-abdominal, vaginal, brain and lung abscesses and peritonitis. The source is usually the faecal flora. Faeces contain 10¹¹–10¹² bacteria/g and anaerobes constitute c. 99.9% of the faecal bacterial mass. Of this, the species belonging to the genus *Bacteroides sensu stricto* (formerly members of the *B. fragilis* group of *Bacteroides*) account for 20–30% of

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the species isolated.¹ The genus *Bacteroides* constitutes *B. fragilis*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron*, *B. vulgatus*, with *B. eggerthii*, *B. uniformis*, *B. caccae*, *B. merdae* and *B. stercoris* being more recently added to the group due to changes in classification. Other species formerly classified as *Bacteroides* have been reclassified into the genera *Porphyromonas* and *Prevotella*.^{2,3}

In the past 25 years, the lipopolysaccharide (LPS) of *B. fragilis* has been studied by many groups and has yielded many controversial results regarding its rough or smooth structure, and presence of KDO or not, but it is generally agreed that it is weakly endotoxic.⁴⁻⁷ It was thought initially that *B. fragilis* LPS contained no repeating long chains of polysaccharide linked to lipid A core. This was due in part to the use of the phenol-chloroform-petroleum extraction method which tends to select for rough type LPS⁵ and it was not recognised that subpopulations existed that expressed different surface molecules.³ In 1991⁸ Maskell investigated by polyacrylamide gel electrophoresis LPS from 10 species of *Bacteroides* prepared by the non-selective proteinase K method. He showed that some *Bacteroides* spp. produced predominantly rough LPS whereas others such as *B. vulgatus* and *B. caccae* produced smooth LPS similar to that of facultative enterobacteria. Other species such as *B. fragilis* produced poorly resolved high molecular mass (M_r) material as well as rough LPS. It is now well established that *B. fragilis* LPS does contain KDO, but only in a phosphorylated form which renders it undetectable in the standard thiobarbituric acid assay. Prior dephosphorylation by hydrofluoric acid treatment is required for its detection.⁹ It is well documented that the lipid A of *B. fragilis* LPS contains different fatty acids from the lipid A of enterobacterial LPS and is monophosphorylated, and this is one reason why it is less endotoxic than enterobacterial LPS.⁷

Endotoxaemia can occur as a primary event, for example in patients with intra-abdominal or urinary tract sepsis. However, in critically ill patients without a confirmed bacteraemia, it is thought that the gut is the major source of "endotoxin"¹⁰ which subsequently leads to the systemic inflammatory response syndrome (SIRS),¹¹ a consequence of which can be multiple organ failure and death in up to 80% of patients. Although it has been shown that the LPS of *B. fragilis* has lower activity than enterobacterial endotoxin,⁷ *Bacteroides* spp. are numerically far more predominant (c. 1000-fold) in the gut than enterobacteria such as *E. coli*, thus representing a vast pool of potentially biologically active LPS.

In this paper, the biological activity of LPS from seven species of *Bacteroides* is re-examined by comparing the activity of LPS extracted by three different methods in mouse lethality and TNF induction models, and an attempt is made to relate activity to chemical composition. The *Limulus* amoebocyte lysate (LAL) assay was included in the study as the standard in-vitro method for detection of endotoxin.

Materials and methods

Bacterial strains

A list of strains used and their origin is shown in table I.

Culture methods

Bacteria were grown in 15–16-L batches in a 20-L capacity fermenter (LH Engineering, Stoke Poges). *Bacteroides* spp. were grown in defined broth¹² or proteose-peptone yeast extract (PPY) broth, anaerobically, in N₂ 100% at 37°C. *Escherichia coli* O18:K⁻ was grown in nutrient broth (NB) anaerobically, in N₂ 100% at 37°C, or aerobically (pO₂ 50%) at 37°C. Strict purity checks were made for each organism at the end of each fermentation run.

Extraction of LPS

LPS was extracted by the phenol water (PW) method as described by Westphal and Luderitz,¹³ by the phenol-chloroform-petroleum (PCP) method as described by Galanos *et al.*,¹⁴ and by the Triton-Mg²⁺ method described by Uchida and Mizushima.¹⁵ All native LPS (5 mg/ml) were freed from protein contamination by treating with proteinase K 20 µg/ml at 65°C for 2 h. Proteinase K was removed by two washes with pyrogen-free water (PFH₂O) at 100 000 *g*. Batches of de-ionised and sodium salt form LPS were prepared by the method of Galanos as described by Hancock and Poxton.¹⁶ Further native enterobacterial LPS used in the TNF induction assays were obtained from PW or PCP extracts prepared by F. McLoughlin, Department of Medical Microbiology, Edinburgh University Medical School.

Polyacrylamide gel electrophoresis

Polyacrylamide gels (14% without SDS) were prepared with the buffer system of Laemmli.¹⁷ Each extract was dissolved to a concentration of LPS 1 mg/ml in the SDS-2-mercaptoethanol solubilisation buffer of Laemmli¹⁷ and heated at 100°C for 3 min. Samples (20 µl) were applied to a non-SDS polyacrylamide 14% gel. To visualise LPS, gels were oxidised with periodate and stained with silver by a method modified from that of Tsai and Frasch.^{16,18} Gels were stained for protein by the Coomassie Blue method as described by Hancock and Poxton.¹⁶

Chemical analysis of LPS

Neutral sugars were quantified by the colorimetric method of Dubois *et al.*¹⁹ The phosphorus content was measured by the method of Chen *et al.*²⁰ *Bacteroides* LPS were dephosphorylated by hydrofluoric acid as described by Beckmann *et al.*⁹ and the KDO content of

Table I. Bacterial strains

Species	Strain no.	Origin or source
<i>B. fragilis</i>	MPRL 1669	Appendix, NCTC 9343
<i>B. fragilis</i>	MPRL 1504	Wound
<i>B. vulgatus</i>	MPRL 1985	Blood
<i>B. thetaiotaomicron</i>	MPRL 1720	Faeces
<i>B. caccae</i>	MPRL 1555	Wound
<i>B. uniformis</i>	MPRL 1721	?, ATCC 8492
<i>B. ovatus</i>	MPRL 1709	?, ATCC 8483
<i>E. coli</i> O18:K ⁻ (bort)	MPRL 1274	Dr A. S. Cross, Walter Reed Army Institute for Research, Washington DC, USA

NCTC, National Collection of Type Cultures, PHLS, Colindale Avenue, London; ATCC, American Type Culture Collection, Rockville, MD, USA; MPRL, departmental stock culture.

Table II. Details of LPS library

Strain	Growth medium	Culture volume (L)	Yield from different extraction methods*			
				PW	PCP	Triton
<i>B. fragilis</i> NCTC 9343	VT	16	1	6.87	6.87	6.87
			2	41.4	227.5	175.6
			3	0.60	3.31	2.56
<i>B. fragilis</i> NCTC 9343	PPY	15	1	2.34	2.39	2.36
			2	37.4	99.2	47.2
			3	1.60	4.42	2.00
<i>B. fragilis</i> MPRL 1504	PPY	15	1	2.85	2.57	2.50
			2	63.7	64.5	47.6
			3	2.24	2.51	1.91
<i>B. vulgatus</i> MPRL 1985	PPY	15	1	2.42	2.47	2.44
			2	90.7	96.9	53.2
			3	3.74	3.92	2.18
<i>B. thetaiotaomicron</i> MPRL 1720	PPY	15	1	3.23	3.67	3.77
			2	29.8	103.8	83.0
			3	0.92	2.83	2.20
<i>B. caccae</i> MPRL 1555	PPY	15	1	7.40	7.63	7.50
			2	20.3	98.6	20.7
			3	0.27	1.29	0.28
<i>B. uniformis</i> MPRL 1721	PPY	15	1	6.75	5.21	5.60
			2	25.8	213.4	57.7
			3	0.38	4.24	1.03
<i>B. ovatus</i> MPRL 1709	PPY	15	1	3.04	3.00	2.97
			2	54.9	124.0	73.3
			3	0.81	4.14	2.47
<i>E. coli</i> O18:K ⁻ MPRL 1275	NB Aerobic	15	1	7.63	—	—
			2	169.8	—	—
			3	2.23	—	—
<i>E. coli</i> O18:K ⁻ MPRL 1275	NB Anaerobic	16	1	1.18	—	—
			2	47.5	—	—
			3	4.04	—	—

VT, Van Tassell and Wilkins' medium; PPY, proteose-peptone yeast extract broth; NB, nutrient broth; PW, phenol-water; PCP, phenol-chloroform-petroleum.

* 1, dry weight of cells (g); 2, yield of LPS (mg); 3, % yield.

all LPS was determined by a modified thiobarbiturate assay.¹⁶ Protein was measured by the Folin assay.²¹

Mouse lethality: galactosamine model

D-Galactosamine (12 mg/mouse) was administered intraperitoneally to groups of three 6–8-week-old male C57 black mice just before intraperitoneal administration of LPS in the range 5 ng–5 µg. Survival was recorded up to 24 h. Animal experiments were performed in accordance with Home Office guidelines.

Limulus amoebocyte lysate (LAL) assay

LPS samples were diluted to ranges of 50–0.5 ng/ml in PFH₂O. Each sample (50 µl) was added in duplicate to wells of microtitration plates (Greiner). Chromogenic LAL reagent (Coatest Endotoxin, Chromogenix, Sweden) was added to each well through a transfer plate to ensure that each well received the LAL reagent at the same time. To minimise error through temperature gradients the outer wells of the plate were left unfilled. The plate was read kinetically every 19 s at

Fig. 1. Silver stain profile of LPS library. R, position of rough LPS; X, band present in PCP preparations. **a:** track 1, NCTC 9343 VT PW; 2, NCTC 9343 VT PCP; 3, NCTC 9343 VT TRITON; 4, NCTC 9343 PPY PW; 5, NCTC 9343 PPY PCP; 6, NCTC 9343 PPY TRITON; 7, MPRL 1504 PPY PW; 8, MPRL 1504 PPY PCP; 9, MPRL 1504 PPY TRITON; 10, MPRL 1985 PPY PW; 11, MPRL 1985 PPY PCP; 12, MPRL 1985 PPY TRITON. **b:** track 1, MPRL 1720 PPY PW; 2, MPRL 1720 PPY PCP; 3, MPRL 1720 PPY TRITON; 4, MPRL 1555 PPY PW; 5, MPRL 1555 PPY PCP; 6, MPRL 1555 PPY TRITON; 7, MPRL 1721 PPY PW; 8, MPRL 1721 PPY PCP; 9, MPRL 1721 PPY TRITON; 10, MPRL 1709 PPY PW; 11, MPRL 1709 PPY PCP; 12, MPRL 1709 PPY TRITON. **c:** track 1, *E. coli* O18:K⁻ LPS grown aerobically; 2, *E. coli* O18:K⁻ LPS grown anaerobically.

405 nm (reference background 650 nm) for 90 min in a Thermomax plate reader (Molecular Devices) at 37°C. The replicate mean onset time for test samples was standardised against an endotoxin of known potency (*E. coli* O111:B4, Coatest Kit endotoxin standard, Chromogenix).

LPS-induced TNF secretion

Mononuclear leucocytes (MNL: *c.* 30% monocytes) from freshly collected human buffy coats (obtained from the Blood Transfusion Service, Edinburgh) were prepared by sedimentation on lymphocyte separation medium (ICN Flow). Cells (8×10^6 /ml) were cultured in RPMI 1640 (Blood Transfusion Service, Edinburgh) supplemented with penicillin

100 U/ml, streptomycin 100 µg/ml, 1 mM L-glutamine and fetal calf serum (FCS) 10% in the presence or absence of varying (ng) concentrations of LPS. Culture supernates were collected after 4 h and stored at -20°C for TNF determination.

Determination of TNF content

The L929 mouse fibroblast cell line, which is sensitive to TNF, was cultured in growth medium—MEM (Sigma) containing FCS 5% supplemented with penicillin 100 U/ml, streptomycin 100 µg/ml and 1 mM L-glutamine, and maintained by splitting 1 in 10 twice weekly. Cells were dislodged by a solution of trypsin 0.05%, EDTA 0.02% to avoid cell clumping, washed and resuspended in a growth medium to

Table III. Estimation of carbohydrate, phosphorus and KDO content in native LPS samples

LPS sample/medium	Extraction method	CHO ($\mu\text{g}/\text{mg}$ dry wt)	P ($\mu\text{g}/\text{mg}$ dry wt)	KDO ($\mu\text{g}/\text{mg}$ dry wt)
<i>B. fragilis</i>	PW	415.8	16.4	7.8
NCTC 9393/VT	PCP	614.4	33.1	0.8
	Triton	229.2	29.3	3.5
<i>B. fragilis</i>	PW	172.2	39.2	3.8
NCTC 9343/PPY	PCP	90.8	33.6	1.4
	Triton	147.8	49.0	2.1
<i>B. fragilis</i>	PW	198.2	55.3	6.2
MPRL 1504/PPY	PCP	120.8	72.4	0.5
	Triton	130.0	28.5	3.7
<i>B. vulgatus</i>	PW	267.6	78.9	2.7
MPRL 1985/PPY	PCP	67.4	47.4	0.9
	Triton	197.2	37.8	2.6
<i>B. thetaiotaomicron</i>	PW	363.8	54.2	—
MPRL 1720/PPY	PCP	43.8	47.4	0.4
	Triton	262.4	79.6	4.0
<i>B. caccae</i>	PW	240.2	54.3	—
MPRL 1555/PPY	PCP	29.6	33.8	—
	Triton	148.6	24.9	—
<i>B. uniformis</i>	PW	221.6	102.0	—
MPRL 1721/PPY	PCP	53.0	38.2	—
	Triton	173.4	85.5	—
<i>B. ovatus</i>	PW	281.0	43.1	5.7
MPRL 1709/PPY	PCP	110.8	39.6	1.1
	Triton	178.0	41.6	2.4
<i>E. coli</i> O18:K ⁻ / NB aerobic	PW	254.8	36.5	2.2
<i>E. coli</i> O18:K ⁻ / NB anaerobic	PW	260.0	34.0	2.4

PW, phenol-water; PCP, phenol-chloroform-petroleum; VT, Van Tassell and Wilkins' medium; PPY, proteose-peptone yeast extract broth; NB, nutrient broth; CHO, carbohydrate; P, phosphorus; —, not done. All assays were repeated on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for each experiment.

3×10^5 cells/ml. Cells were dispensed in flat-bottomed microtitration plates (Greiner) at $100 \mu\text{l}/\text{well}$ and incubated in CO_2 5% at 37°C for 20 h. The growth medium was then aspirated, discarded and replaced with $100 \mu\text{l}/\text{well}$ of assay medium—MEM containing FCS 5% supplemented with 1 mM glutamine and actinomycin-D (to stop further cell growth without killing the cells) $2 \mu\text{g}/\text{ml}$. To experimental wells, $100 \mu\text{l}$ of test supernate diluted 1 in 5 in assay medium was added. A standard of recombinant TNF (National Institute for Biological Standards and Controls) diluted serially 1 in 5 from a starting concentration of 1000 IU/ml and wells without TNF were included. Plates were sealed and incubated in CO_2 5% at 37°C for 24 h. The medium was then discarded and replaced with filtered ($0.22 \mu\text{m}$) crystal violet solution (crystal violet 0.5% in methanol 20% v/v in distilled water) $100 \mu\text{l}/\text{well}$ which stains surviving cells. After 2 min, the plates were washed vigorously under tap water and dried. The crystals were dissolved by addition of $100 \mu\text{l}$ of acetic acid 20% v/v to all wells and the plate was read at 585 nm on a Vmax plate reader (Molecular Devices). The content of TNF was calculated relative to the standard curve.

Results

Comparison of different extraction methods

The yield of native LPS obtained with each extraction method is shown in table II. The PCP extraction method gave the highest yield of LPS for each organism, whereas the PW method gave the lowest yield for most organisms. Those organisms that gave a high yield of dry cells were very mucoid and the yield of LPS was generally poorer.

Polyacrylamide gels were run and stained for LPS and protein. Contaminating protein was found in bacteroides LPS extracted by all three methods, especially those extracted by the PCP and Triton methods (data not shown). LPS extracts were assayed for protein content:²¹ in PCP extracts protein content ranged from undetectable to $> 200 \mu\text{g}/\text{mg}$ dry weight; in all Triton preparations, with the exception of that from *B. vulgatus* MPRL 1985 in which none was detected, protein contamination was in the range $60\text{--}500 \mu\text{g}/\text{mg}$ dry weight; and none was detectable in the PW extracts.

All LPS preparations were then treated with proteinase K. On repeating the protein assays, none was detected and no Coomassie Blue-staining material was

Table IV. LPS-induced mouse lethality (galactosamine model)

LPS sample	Number of survivors after				
	5 ng	50 ng	5 µg	10 µg	20 µg
<i>B. fragilis</i> NCTC 9343 VT PW	—	—	3/3	1/3	0/3
<i>B. fragilis</i> NCTC 9343 PPY PW	—	—	3/3	2/3	1/3
<i>B. fragilis</i> MPRL 1504 PW	—	—	3/3	3/3	2/3
<i>B. vulgatus</i> MPRL 1985 PW	—	—	3/3	3/3	0/3
<i>B. thetaiotaomicron</i> MPRL 1720 PW	—	—	3/3	3/3	1/3
<i>B. caccae</i> MPRL 1555 PW	—	—	3/3	3/3	1/3
<i>B. uniformis</i> MPRL 1721 PW	—	—	3/3	3/3	3/3
<i>B. ovatus</i> MPRL 1709 PW	—	—	3/3	3/3	3/3
<i>B. fragilis</i> NCTC 9343 PPY PCP	—	—	3/3	3/3	3/3
<i>B. fragilis</i> NCTC 9343 PPY Triton	—	—	3/3	3/3	3/3
* <i>B. fragilis</i> NCTC 9343 PPY PW	—	—	3/3	2/3	0/3
* <i>B. fragilis</i> NCTC 9343 PPY PCP	—	—	3/3	3/3	3/3
* <i>B. fragilis</i> NCTC 9343 PPY Triton	—	—	3/3	3/3	3/3
<i>E. coli</i> O18:K ⁻ aerobic PW	3/3	0/3	0/3	—	—
<i>E. coli</i> O18:K ⁻ anaerobic PW	3/3	0/3	0/3	—	—

All LPS are native samples unless indicated.

* Sodium salt form LPS; see table III for other abbreviations.

Table V. Endotoxic activities of sodium salt and native LPS (5 ng/ml) samples

LPS sample	LAL IU/ml	
	Sodium salt form	Native form
(i) PW extracts		
<i>B. fragilis</i> NCTC 9343 VT	75.4	617.4
<i>B. fragilis</i> NCTC 9343 PPY	48.7	227.8
<i>B. fragilis</i> MPRL 1504	136.2	1863
<i>B. vulgatus</i> MPRL 1985	3.3	15.6
<i>B. thetaiotaomicron</i> MPRL 1720	1.0	4.1
<i>B. caccae</i> MPRL 1555	67.4	234.9
<i>B. uniformis</i> MPRL 1721	0.9	13.7
<i>B. ovatus</i> MPRL 1709	12.5	4.3
<i>E. coli</i> O18:K ⁻ aerobically grown PW	6.8	62.2
<i>E. coli</i> O18:K ⁻ anaerobically grown PW	2.2	31.7
(ii) PCP extracts		
<i>B. fragilis</i> NCTC 9343 VT	1.1	0.5
<i>B. fragilis</i> NCTC 9343 PPY	4.9	0.6
<i>B. fragilis</i> MPRL 1504	0.8	0.9
<i>B. vulgatus</i> MPRL 1985	4.2	0.4
<i>B. thetaiotaomicron</i> MPRL 1720	0.2	0.8
<i>B. caccae</i> MPRL 1555	0.2	0.1
<i>B. uniformis</i> MPRL 1721	7.9	20.7
<i>B. ovatus</i> MPRL 1709	0.1	0.6
<i>E. coli</i> O18:K ⁻ aerobically grown PW	10.4	45.4
<i>E. coli</i> O18:K ⁻ anaerobically grown PW	6.0	23.9
(iii) Triton extracts		
<i>B. fragilis</i> NCTC 9343 VT	226.2	480.4
<i>B. fragilis</i> NCTC 9343 PPY	455.7	71.7
<i>B. fragilis</i> MPRL 1504	1020	704
<i>B. vulgatus</i> MPRL 1985	34.6	11.1
<i>B. thetaiotaomicron</i> MPRL 1720	63.5	17.4
<i>B. caccae</i> MPRL 1555	6.0	50.6
<i>B. uniformis</i> MPRL 1721	22.2	42.3
<i>B. ovatus</i> MPRL 1709	124.4	46.3
<i>E. coli</i> O18:K ⁻ aerobically grown PW	9.2	83.6
<i>E. coli</i> O18:K ⁻ anaerobically grown PW	8.8	42.3

All assays were repeated on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for all assays. See table III for abbreviations.

Fig. 2. TNF concentration (IU/ml) from human mononuclear leucocytes (8×10^6 cells/ml) after stimulation for 4 h with *B. fragilis* NCTC 9343 PPY (■), *B. vulgatus* MPRL 1985 (●) and aerobically grown *E. coli* O18:K⁻ (◆) native LPS samples extracted by the PW method. Each point is the mean of two readings.

observed in PAGE. LPS profiles after silver staining were identical to those before proteinase K treatment (data not shown). The following results are from LPS preparations which had been treated with proteinase K to remove protein contamination.

Fig. 1 shows LPS profiles on PAGE for each strain and extraction method after separation and silver staining. A banding pattern typical of smooth LPS was seen for *E. coli* O18:K⁻ (gel c, tracks 1 and 2), *B. vulgatus* MPRL 1985 (gel a, tracks 10, 11 and 12) and *B. thetaiotaomicron* MPRL 1720 (gel b, tracks 1, 2 and 3). Material of high M_r was seen in *B. fragilis* LPS extracted by both the PW and Triton methods. Rough, low M_r material was seen in extracts prepared for all species by each method, and was "dumbbell shaped" for *B. caccae* and *B. ovatus* (gel b, tracks 4, 5 and 6, and 10, 11 and 12). The PCP extraction method produced a pronounced band (X) present behind the main front band (R) in all preparations. For *B. fragilis*, the PCP extraction method did not select for high M_r material. Silver stain profiles for sodium salt form LPS were identical to profiles of native forms (data not shown). LPS profiles for *E. coli* O18:K⁻ grown aerobically and anaerobically were identical.

Chemical analysis of LPS samples

Samples of dry extract were resuspended in PFH₂O to a concentration of 5 mg/ml and assayed for carbohydrate, phosphorus and KDO content. The relative amounts of carbohydrate, phosphorus and

KDO are shown in table III. *Bacteroides* spp. showed a negligible amount or no KDO content before treatment with hydrofluoric acid. The PW extraction method selected for the greatest amount of carbohydrate and KDO material and the PCP extraction method for the least. Phosphorus levels were more uniform among all extraction methods. No difference was observed between LPS extracts from *E. coli* O18:K⁻ grown in different atmospheres.

LPS induced mouse lethality (galactosamine model)

E. coli O18:K⁻ LPS was lethal to all mice at 50 ng/mouse (table IV) whereas *B. fragilis* NCTC 9343 VT PW-LPS and *B. vulgatus* MPRL 1985 PW-LPS were only lethal to mice at 20 µg/mouse. No difference in lethality was seen for native and sodium salt form *B. fragilis* NCTC 9343 VT PW-LPS. Mice were not killed by the maximum dose of *B. fragilis* LPS extracted by PCP or Triton, nor by *B. uniformis* and *B. ovatus* LPS extracted by PW.

Reactivity of LPS samples in LAL assay

Endotoxic activities in the LAL assay of native and sodium salt form LPS are illustrated in table V (i), (ii) and (iii) for each extraction method. On a weight for weight basis, the greatest activity was seen in the PW extracts and the least activity seen in the PCP extracts. Most native PW-LPS were more active than sodium

Table VI. Measured TNF concentration from human MNL (8×10^6 cells/ml) after stimulation for 4 h with native and sodium salt form LPS (50 ng/ml) from *E. coli* O18:K⁻ and *Bacteroides* spp. extracted by three different methods

LPS sample	Extraction method	TNF concentration (IU/ml)	
		Native LPS	Sodium salt LPS
<i>B. fragilis</i> NCTC 9343 VT	PW	23.16	11.92
	PCP	0.30	0.71
	Triton	2.99	1.07
<i>B. fragilis</i> NCTC 9343 PPY	PW	18.81	4.60
	PCP	2.53	1.78
	Triton	0.51	0.31
<i>B. fragilis</i> MPRL 1504	PW	4.93	2.51
	PCP	0.42	0.25
	Triton	0.99	0.47
<i>B. vulgatus</i> MPRL 1985	PW	2.58	0.72
	PCP	0.15	0.51
	Triton	0.39	0.31
<i>B. thetaiotaomicron</i> MPRL 1720	PW	6.40	6.47
	PCP	0.03	0.43
	Triton	0.17	0.49
<i>B. caccae</i> MPRL 1555	PW	13.89	15.25
	PCP	0.22	0.74
	Triton	2.48	2.44
<i>B. uniformis</i> MPRL 1721	PW	10.10	35.34
	PCP	4.81	4.81
	Triton	10.87	5.09
<i>B. ovatus</i> MPRL 1709	PW	8.66	13.89
	PCP	1.44	5.37
	Triton	1.25	2.32
<i>E. coli</i> O18:K ⁻ aerobically grown	PW	94.61	43.21
<i>E. coli</i> O18:K ⁻ anaerobically grown	PW	99.00	43.21
<i>E. coli</i> O6	PW	59.00	—
<i>E. coli</i> O12	PW	58.20	—
<i>E. coli</i> O15	PW	68.96	—
<i>K. pneumoniae</i> M10B	PW	48.21	—
<i>E. coli</i> J5	PCP	36.63	—
<i>E. coli</i> K12	PCP	49.90	—
<i>S. minnesota</i> Ra	PCP	31.68	—
<i>S. minnesota</i> Rb	PCP	96.70	—
<i>S. minnesota</i> Rc	PCP	49.90	—
<i>S. minnesota</i> Rd	PCP	78.45	—
<i>S. minnesota</i> Re	PCP	50.48	—

All assays were carried out on at least two occasions and the results presented are the mean of two experiments. All samples were tested in duplicate for each assay. See table III for abbreviations.

salt PW-LPS. Approximately 70 % of native PCP-LPS are more active than sodium salt PCP-LPS. No significant difference was seen between the native and sodium salt form LPS extracted by Triton. LPS from *E. coli* O18:K⁻ grown aerobically was more active than LPS from *E. coli* O18:K⁻ grown anaerobically by a factor of two. *B. fragilis* MPRL 1504 PW-LPS was more active than *E. coli* O18:K⁻ LPS by a factor of 30, *B. fragilis* NCTC 9343 VT PW-LPS by a factor of 10, *B. fragilis* NCTC 9343 PPY PW-LPS by a factor of four and *B. caccae* MPRL 1555 PW-LPS by a factor of four. LPS from all other *Bacteroides* spp. were less active than that from *E. coli* O18:K⁻. A difference in activity for *B. fragilis* NCTC 9343 LPS grown in different media was also seen.

LPS-induced TNF secretion from human buffy coats

The amount of TNF induced by different LPS from human MNL (8×10^6 cells/ml) at several different

concentrations is shown in fig. 2. TNF appears to reach a maximal level at *c.* 160 ng of LPS for all those tested. Based on the data from fig. 2, an LPS concentration of 50 ng/ml was selected to compare a larger panel of LPS from other *Bacteroides* spp. and enterobacteria (table VI). This value was chosen as it was in the middle of the linear part of both the dose response curve and the standard curve of the assay. This would not show the maximum induction capacity of the LPS but was a compromise for comparing a large number of LPS samples. *E. coli* O18:K⁻ LPS stimulated the most TNF production with no difference in TNF production between LPS from *E. coli* O18:K⁻ grown aerobically or anaerobically. On a weight for weight basis, PW-LPS stimulated the greatest TNF production. Comparing PW-LPS, bacteroides LPS induced TNF much less than did *E. coli* O18:K⁻ LPS, with the most active (*B. fragilis* NCTC 9343 VT) being four times less active than *E. coli* O18:K⁻ and the least active (*B. vulgatus* MPRL 1985)

Table VII. TNF concentration from human MNL and endotoxic activity after stimulation with LPS from selected *Bacteroides* spp. and *E. coli* O18:K⁻ LPS relation to KDO content

LPS sample	Extraction method	TNF stimulation (IU/ng of KDO)	LAL activity (EU/ng of KDO)
<i>B. fragilis</i> NCTC 9343 VT	PW	59.38	1583.08
	PCP	7.16	10.69
	Triton	17.3	2784.93
<i>B. fragilis</i> NCTC 9343 PPY	PW	46.11	1191.11
	PCP	35.11	8.44
	Triton	4.90	693.24
<i>B. fragilis</i> MPRL 1504	PW	15.85	5985.54
	PCP	17.42	37.38
	Triton	5.38	3846.99
<i>B. vulgatus</i> MPRL 1985	PW	19.11	115.48
	PCP	3.41	7.93
	Triton	3.00	84.67
<i>B. thetaiotaomicron</i> MPRL 1720	PW	—	—
	PCP	1.55	42.61
	Triton	8.64	867.91
<i>B. ovatus</i> MPRL 1709	PW	30.37	14.98
	PCP	25.89	4.68
	Triton	10.43	385.5
<i>E. coli</i> O18:K ⁻ aerobic	PW	502.16	572.32
<i>E. coli</i> O18:K ⁻ anaerobic	PW	485.60	261.15

See table III for abbreviations.

being 37 times less active than *E. coli* O18:K⁻. TNF production with other enterobacterial LPS tested was of the same level or less than that with *E. coli* O18:K⁻ LPS, all being more active than bacteroides LPS. Sodium salt form *E. coli* O18:K⁻ LPS had half the activity of native form *E. coli* O18:K⁻ LPS. Generally for bacteroides LPS there was no clear distinction in activity between sodium salt and native form LPS.

Reactivity of LPS samples on the basis of KDO concentration

All the above assays were done on a weight for weight basis. However, because endotoxicity is likely to depend on lipid A concentration, it was decided to re-evaluate some of the results of TNF induction and LAL activity relative to KDO concentration in an attempt to compare lipid A levels—on the assumption (which could well be false) that lipid A is proportional to KDO in all species.

The TNF concentration from human buffy coats expressed as IU/ng of KDO and endotoxic activity in LAL expressed as EU/ng of KDO after stimulation with selected bacteroides and *E. coli* O18:K⁻ LPS is presented in table VII. For the TNF concentrations measured, this shows that the low activities seen previously on a weight for weight basis (table VI) were due in part to less KDO being present. However, LAL activity for PCP-extracted LPS was still low when the results were represented/ng of KDO.

Discussion

This study has shown that an aqueous phenol (PW) extract of bacteroides LPS is significantly different both in terms of chemical composition and biological activity from the PCP or Triton products. The three methods were selected in an attempt to produce products of different composition. The classical PW method was likely to select for higher M_r , less hydrophobic material, the PCP method for rough-form, more hydrophobic material, and the Triton-Mg²⁺ method was chosen as a milder, less selective method. It is well accepted that enterobacterial LPS is heterogeneous in M_r even when pure. We acknowledge that the bacteroides "LPS" used in this study may be heterogeneous both in M_r and in molecular composition. The degree of different molecular species of LPS and other co-purifying carbohydrate, due to the probable subpopulation structure of our cultures,³ is by no means certain. The biological activity of enterobacterial LPS is thought to depend on its solubility²² and, for this reason, the native and the standardised, soluble sodium salt forms were prepared from electro-dialysed material.

Our results agree with previous studies⁷ that in a mouse lethality model bacteroides LPS is *c.* 5000-fold less active than LPS from *E. coli*. In past studies,^{7, 23, 24} bacteroides LPS has been quoted as being 100–1000-fold less biologically active *in vitro*, depending on the assay, than LPS from enterobacteria. Our results for bacteroides LPS extracted by the PCP or Triton method agree with this. However, our results for LPS

extracted by the PW method disagree with the accepted view.

Tumour necrosis factor- α (TNF) is considered to be the pivotal cytokine in the host response to endotoxin. In the TNF induction assay, *E. coli* O18:K⁻ LPS was only four-fold more active than *B. fragilis* LPS, seven-fold more than *B. caccae* LPS and up to 50-fold more than the other bacteroides LPS. In the LAL assay, which has questionable value for the in-vivo situation, the same LPS that were the most active in the TNF-induction assay (from *B. fragilis* and *B. caccae*) were 30–40-fold more active than *E. coli* O18:K⁻ LPS, with *E. coli* O18:K⁻ LPS being c. 10-fold more active than the rest of the bacteroides LPS tested. The two-fold difference in activity in the LAL assay for LPS from *E. coli* O18:K⁻ grown aerobically and anaerobically is unclear. Our results also show that solubility (native *versus* sodium salt form LPS) does not play an important role in biological activity as has been described previously for enterobacterial LPS;²² in most cases the sodium salt form was of lower activity. Growth in the defined van Tassel and Wilkins' medium was included because we have found recently that growth in this medium converts several *B. fragilis* strains, including NCTC 9343, from being sensitive to the lytic action of normal human serum to being resistant. This may be due to a change in expression of certain surface polymers such as LPS.²⁵ The results show some differences between the two growth media in respect to both chemical composition and biological activities.

The possibility that some contaminant may be present in the LPS preparations from *Bacteroides* spp. must be considered. In this connection we found no contaminating organisms when culturing the organisms for extraction and the identity of each strain of *Bacteroides* was confirmed before the start of the study. Furthermore, PW-LPS samples from several *Bacteroides* spp. prepared by different individuals at different times in our laboratory were tested alongside our own samples in the LAL and TNF induction assay. They were found to produce nearly identical results (data not shown). Higher biological activity in the PW preparations cannot be due to a biologically active protein as protein levels were negligible after purification (table III). Biological activity also cannot

be due to any contaminating proteinase K as all bacteroides LPS had been treated similarly with proteinase K.

Some PW preparations of bacteroides LPS are clearly more active than others, e.g., *B. fragilis* NCTC 9343 VT/PPY and *B. caccae* MPRL 1555. Chemical analysis does not explain why the LPS from some species are more active than from others. It does show that more carbohydrate and KDO is present in the PW preparations. The amount of KDO destroyed or not released by hydrofluoric acid treatment is not known. Those species that had a low reactivity in the LAL and TNF bioassays (*B. vulgatus* MPRL 1985 and *B. thetaiotaomicron* MPRL 1720) had a smooth LPS profile on PAGE (fig. 1). This is in apparent contradiction to the view that aqueous phenol preferentially extracts smooth-form LPS. Moreover it is surprising that those strains which are obviously smooth (e.g., *B. vulgatus*) are less endotoxic.

In the light of the great variation found in the chemical analyses, PAGE profiles and biological activities of the various preparations, and the knowledge that several different LPS-like molecules have been identified by monoclonal antibodies,²⁶ the suggestion that different extraction methods selectively produce different populations of LPS or perhaps lipid-linked capsular polysaccharides is strong. In nature all of these different molecular species, if expressed, might contribute towards a range of "endotoxic" phenomena. Therefore, it is essential to be able to purify the various components of this heterogeneous "LPS" population and relate them to biological function.

The role of bacteroides LPS in endotoxic shock remains to be defined. We have shown that PW-extracted LPS has a higher biological activity than previously reported.^{7,24} How these chemically extracted "LPS" relate to what is found *in vivo* also needs to be defined. When we consider that *Bacteroides* spp. are in vast excess of *E. coli* in the gut and may have higher biological activity than previously thought, the bacteroides LPS may play an important but neglected role in endotoxic shock.

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References

1. Duerden BI, Drasar BS (eds). Anaerobes in human disease. London, Edward Arnold: 1991.
2. Shah HN, Collins MD. Proposal to restrict the genus *Bacteroides* to *Bacteroides fragilis* and closely related species. *Int J Syst Bacteriol* 1989; **39**: 85–87.
3. Patrick S. The virulence of *Bacteroides fragilis*. *Rev Med Microbiol* 1993; **4**: 40–49.
4. Kasper DL, Weintraub A, Lindberg AA, Lonngrén J. Capsular polysaccharides and lipopolysaccharides from two *Bacteroides fragilis* reference strains: chemical and immunochemical characterization. *J Bacteriol* 1983; **153**: 991–997.
5. Poxton IR, Brown R. Immunochemistry of the surface carbohydrate antigens of *Bacteroides fragilis* and definition of a common antigen. *J Gen Microbiol* 1986; **132**: 2475–2481.
6. Weintraub A, Larsson BE, Lindberg AA. Chemical and immunochemical analyses of *Bacteroides fragilis* lipopolysaccharides. *Infect Immun* 1985; **49**: 197–201.
7. Lindberg AA, Weintraub A, Zähringer U, Rietschel ET. Structure-activity relationships in lipopolysaccharides of *Bacteroides fragilis*. *Rev Infect Dis* 1990; **12** Suppl 2: S133–S141.
8. Maskell JP. The resolution of bacteroides lipopolysaccharides by polyacrylamide gel electrophoresis. *J Med Microbiol* 1991; **34**: 253–257.
9. Beckmann I, van Eijk HG, Meisel-Mikokijczyk F, Wallenburg HC. Detection of 2-keto-3-deoxyoctonate in endotoxins

- isolated from six reference strains of the *Bacteroides fragilis* group. *Int J Biochem* 1989; **21**: 661–666.
10. Runcie C, Ramsay G. Intraabdominal infection: pulmonary failure. *World J Surg* 1990; **14**: 196–203.
 11. Bone RC. Toward an epidemiology and natural history of SIRS (systemic inflammatory response syndrome). *JAMA* 1992; **268**: 3452–3455.
 12. Van Tassell RL, Wilkins TD. Isolation of auxotrophs of *Bacteroides fragilis*. *Can J Microbiol* 1978; **24**: 1619–1621.
 13. Westphal O, Lüderitz O. Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien. *Angew Chem* 1954; **66**: 407–417.
 14. Galanos C, Lüderitz O, Westphal O. A new method for the extraction of R lipopolysaccharides. *Eur J Biochem* 1969; **9**: 245–249.
 15. Uchida K, Mizushima S. A simple method for isolation of lipopolysaccharides from *Pseudomonas aeruginosa* and some other bacterial strains. *Agric Biol Chem* 1987; **51**: 3107–3114.
 16. Hancock IC, Poxton IR (eds). Bacterial cell surface techniques. Chichester, UK, Wiley: 1988.
 17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
 18. Tsai C-M, Frasch CE. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal Biochem* 1982; **119**: 115–119.
 19. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956; **28**: 350–356.
 20. Chen PS, Toribara TY, Warner H. Microdetermination of phosphorus. *Anal Chem* 1956; **28**: 1756–1758.
 21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
 22. Galanos C, Lüderitz O. Lipopolysaccharide: properties of an amphipathic molecule. In: Rietschel ET (ed) Handbook of endotoxin vol 1. Amsterdam, Elsevier. 1984: 46–58.
 23. Loppnow H, Brade H, Dürbaum I *et al.* IL-1 induction-capacity of defined lipopolysaccharide partial structures. *J Immunol* 1988; **142**: 3229–3238.
 24. Fujiwara T, Nishihara T, Koga T, Hamada S. Serological properties and immunobiological activities of lipopolysaccharides from black-pigmented and related oral *Bacteroides* species. *J Gen Microbiol* 1988; **134**: 2867–2876.
 25. Allan E, Poxton IR. The influence of growth medium on serum sensitivity of *Bacteroides*. *J Med Microbiol* 1994; **41**: 45–50.
 26. Lutton DA, Patrick S, Crockard AD *et al.* Flow cytometric analysis of within-strain variation in polysaccharide expression by *Bacteroides fragilis* by use of murine monoclonal antibodies. *J Med Microbiol* 1991; **35**: 229–237.
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