BACTERIAL PATHOGENICITY

Biological activities of lipopolysaccharides of *Proteus* spp. and their interactions with polymyxin B and an 18-kDa cationic antimicrobial protein (CAP18)-derived peptide

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The saccharide constituents of lipopolysaccharides (LPS) of *Proteus* spp. vary with the strain and contain unique components about which little is known. The biological activities of LPS and lipid A from S- and R-forms of 10 *Proteus* strains were examined. LPS from all S-form *Proteus* strains was lethal to D-(+)-galactosamine (GalN)-loaded, LPS-responsive, C3H/HeN mice, but not to LPS-hypo-responsive C3H/HeJ mice. *P. vulgaris* O25 LPS evoked strong anaphylactoid reactions in N-acetylmuramyl-L-alanyl-D-isoglutamine (MDP)-primed C3H/HeJ mice. LPS from S- and R-form *Proteus* strains induced production of nitric oxide (NO) and tumour necrosis factor (TNF) by macrophages isolated from C3H/HeN but not C3H/HeJ mice. Lipid A from *Proteus* strains also induced NO and TNF production, although lipid A was less potent than LPS. The effects of LPS were mainly dependent on CD14; LPS-induced NO and TNF production in CD14⁺ J774.1 cells was significantly greater than in CD14⁻ J7.DEF.3 cells. All LPS from *Proteus* strains, and especially from *P. vulgaris* O25, exhibited higher anti-complementary activity than LPS from *Escherichia coli* or *Pseudomonas aeruginosa*. Polymyxin B inactivated *Proteus* LPS in a dose-dependent manner, but these LPS preparations were more resistant to polymyxin B than *E. coli* LPS. CAP18_{109–135}, a granulocyte-derived peptide, inhibited *Proteus* LPS endotoxicity only when the LPS:CAP18_{109–135} ratio was appropriate, which suggests that CAP18_{109–135} acts through a different mechanism than polymyxin B. The results indicate that LPS from *Proteus* spp. are potently endotoxic, but that the toxicity is different from that of LPS from *E. coli* or *Salmonella* spp. and even varies among different *Proteus* strains. The variation in biological activities among *Proteus* LPS may be due to unique components within the respective LPS.

Introduction

Bacteria belonging to the genus *Proteus*, especially *P. mirabilis*, are the second most common cause of urinary tract infections, and a major cause of nosocomial infections [1]; these bacteria also cause wound infection, pneumonia and septicaemia. Several

pathogenic factors associated with *Proteus* spp. have been described, including fimbriae, flagella, urease, IgA and IgG proteases, haemolysins and outer-membrane lipopolysaccharide (LPS) [2]. Chemical and serological studies of LPS from *Proteus* strains have demonstrated that it has uncommon constituents, such as 4-amino-4-deoxy-L-arabinosyl residues (AraN) in the 3-deoxy-octulosonic acid (KDO) and lipid A regions [3, 4], D-galacturonic acid in the core region, and amino and uronic acids and unusual sugars in the O-polysaccharide domain. These constituents are not present in other

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members of the Enterobacteriaceae [5]. Although chemical analyses have yielded substantial information about proteus LPS, there is as yet little information available about its biological activities. Proteus-type lipid A has been synthesised, and it is similar to synthetic *Escherichia coli* (compound 506) and *Salmonella minnesota* R595 lipid A analogues with respect to B cell mitogenic activity, lethality and induction of a local Shwartzman reaction [6]. However, the actions of the uncommon constituents in proteus LPS have not yet been investigated. In this study, the extent to which unique constituents from the polysaccharide, KDO and lipid A regions of proteus LPS influence the biological activities of the bacteria was investigated.

Materials and methods

Bacterial strains

P. mirabilis strains of serotypes O10 (PrK 19/57), O13 (PrK26/57), O23 (PrK41/57), O26 (PrK49/57) and O43 (PrK69/57) and *P. vulgaris* strain PrK48/57 of serotype O25 were obtained from the Czech National Collection of Type Cultures, Prague, Czechoslovakia. *P. vulgaris* strain OX19 of serotype O1 was a kind gift from Dr S. Kaluzewski, National Institute of Hygiene, Warsaw, Poland. *P. mirabilis* strains S1959 (O3), R45/S1959 (Re-chemotype) and R110/S1959 (Ra-chemotype) were obtained from the Institute of Microbiology and Immunology, University of Lodz, Poland.

LPS and lipid A

R-chemotype LPS was prepared from *P. mirabilis* strains R45 and R110 by the phenol-chloroform, petroleum-ether, extraction procedure [7]. S-form proteus LPS was prepared by the phenol-water method with modification [8] and purified as reported previously [9]. *Klebsiella pneumoniae* O3 LPS was prepared from *K. pneumoniae* strain LEN-1 (O3:K1⁻) by the phenol-water method and purified as described previously [10]. *P. mirabilis* lipid A was obtained after hydrolysis of *P. mirabilis* S1959 (O3) LPS with acetic acid 1%. *E. coli* F515 LPS (Re-chemotype) was kindly provided by E. Th. Rietschel, Forschungszentrum, Borstel, Germany. *S. minnesota* R595 LPS (Re-chemotype) was generously provided by K. Hisatsune, Josai University, Sakado, Japan. *Pseudomonas aeruginosa* LPS was a gift from Y. A. Knirel, N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, Moscow, Russia. *E. coli* O55 LPS and *S. minnesota* R60 (Ra-chemotype) LPS were obtained from List Biological Laboratories, Campbell, CA, USA. Synthetic *E. coli*-type lipid A (compound 506) [11] was provided by Daiichi Pharmaceutical, Tokyo, Japan.

Stock solutions of LPS and lipid A were prepared by dissolving the compounds in endotoxin-free water or

triethylamine 0.025% (1 mg/ml), respectively; stocks were stored at 4°C. Immediately before use, stock solutions were diluted appropriately in endotoxin-free distilled water, endotoxin-free saline or RPMI-1640 medium.

The chemical structures of several proteus LPS, especially those of the O-polysaccharide repeating unit, have been well characterised [3, 12–25]. The O-polysaccharide structures of S-form LPS used in the experiments are shown in Table 1. The calculated mol. wts of the test samples were: *P. mirabilis* lipid A, 2211; *E. coli* lipid A (506), 2001; *P. mirabilis* R45 LPS, 2782; *S. minnesota* R595 LPS, 2731; *E. coli* F515 LPS, 2459; *P. mirabilis* R110 LPS, 4588; and *S. minnesota* R60 LPS, 4780.

Reagents

Synthetic muramyl dipeptide (MDP) [26] was provided by Daiichi Pharmaceutical, Tokyo, Japan. Murine recombinant interferon-gamma (INF- γ) and human recombinant tumour necrosis factor- α (TNF- α) were provided by Dainippon Pharmaceutical and Shionogi Pharmaceutical, Osaka, Japan, respectively. Polymyxin B (PmxB; mol. wt 1190) and D(+)-galactosamine (D-GalN) were purchased from Sigma. Human CAP18 is a 140 amino acid protein [27]; CAP18_{109–135} is a 27 amino acid segment (FRKSKEKIGKEFKRIVQRIKDFLRNLV) in the C-terminal region of CAP18 which has been identified as the LPS-neutralising and antimicrobial domain [28]. CAP18_{109–135} peptide (mol. wt 3480) was synthesised by Chiron Mimotopes Peptide Systems San Diego, CA, USA. The peptide was subjected to fast atom bombardment mass spectrometry and yielded the expected parent M + H ions within limits (1 unit of mass). CAP18_{109–135} was dissolved in saline, divided into aliquots and stored at –80°C; samples were thawed only immediately before use.

Animals

LPS-responsive C3H/HeN mice and LPS-hypo-responsive C3H/HeJ mice were obtained from Nippon Clea, Tokyo, Japan and maintained in the animal facility at the Jichi Medical School under standard conditions. Female C3H/HeN mice were used at 10–15 weeks of age, and female or male C3H/HeJ mice were used at 8–12 weeks of age.

Lethal toxicity in galactosamine-loaded mice

The lethal toxicity of LPS for mice was determined as described previously [29]. C3H/HeN and C3H/HeJ mice were inoculated intraperitoneally (i.p.) with 0.2 ml of LPS solution (10–10 000 ng of LPS/mouse) together with 0.5 ml of D-GalN solution (18 mg/mouse). Mortality was scored 48 h after the challenge and the LD50 was calculated.

Table 1. The O-saccharide repeating unit structures of LPS of *Proteus* spp.

O antigen	Structure of repeating unit	Ref.
O23	$\rightarrow 4)-\alpha\text{-D-Gal}_p\text{A}-(1\rightarrow 3)-\beta\text{-D-Glc}_p\text{NAc}-(1\rightarrow 2)-\beta\text{-Gal}_p\text{A}-(1\rightarrow 3)-\alpha\text{-D-Gal}_p\text{NAc}-(1\rightarrow$	14
O43	$\rightarrow 3)-\alpha\text{-D-Gal}_p\text{A}-(1\rightarrow 3)-\alpha\text{-D-Glc}_p\text{NAc}-(1\rightarrow 4)-\alpha\text{-D-Glc}_p-(1\rightarrow 4)-\alpha\text{-D-Gal}_p\text{A}-(1\rightarrow$	13
O13	$\rightarrow 3)-\beta\text{-D-Glc}_p\text{NAc}-(1\rightarrow 3)-\alpha\text{-D-Gal}_p-(1\rightarrow$ $\uparrow 1,4$ $\alpha\text{-D-Gal}_p\text{A}$ $\uparrow 6$ AlaLys	21
O26	$\rightarrow 4)-\alpha\text{-D-Gal}_p\text{A}-(1\rightarrow 4)-\alpha\text{-D-Gal}-(1\rightarrow 3)-\beta\text{-D-Gal}_p\text{A}-(1\rightarrow 3)-\beta\text{-D-Glc}_p\text{NAc}-(1\rightarrow$ $\uparrow 6$ AlaLys	19, 20
O3	$\rightarrow 4)-\beta\text{-D-Glc}_p\text{A}-(1\rightarrow 3)-\beta\text{-D-Gal}_p\text{NAc}-(1\rightarrow 6)-\beta\text{-Gal}_p\text{NAc}-(1\rightarrow$ $\uparrow 1,2$ $\alpha\text{-D-Glc}_p$	15
O10	$\rightarrow 4)-\alpha\text{-D-Gal}_p\text{NAc}-(1\rightarrow 3)-\alpha\text{-D-Gal}_p\text{A}-(1\rightarrow 3)-\alpha\text{-D-Glc}_p\text{NAc}-(1\rightarrow$ $\uparrow 1,3$ $\alpha\text{-L-Alt}_p\text{A}$	18, 24
O25	$\rightarrow 4)-\alpha\text{-D-Gal}_p\text{NAc}-(1\rightarrow 3)-\beta\text{-D-Glc}_p\text{NAc}-(1\rightarrow 2)-\alpha\text{-L-Rha}_p-(1\rightarrow 2)-\beta\text{-D-Ribf}-(1\rightarrow$ $\uparrow 3$ $\alpha\text{-D-Glc}_p-3(\text{R-Lac})$	5
O1	$\rightarrow 4)-\alpha\text{-L-Qui}_p\text{NAc}-(1\rightarrow 3)-\beta\text{-D-Glc}_p\text{NAc}-(1\rightarrow 4)-\alpha\text{-Gal}_p\text{NAc}-(1\rightarrow 4)-\alpha\text{-D-Gal}_p\rightarrow 1\text{-P}-(\text{O}\rightarrow$ $\uparrow 1,3$ $\alpha\text{-L-Qui}_p\text{NAc}$	17, 25

AlaLys, 1,9-dicarboxy-2,8-diaminononane;
AltA, alturonic acid.

Induction of anaphylactoid and lethal reactions in muramyl dipeptide (MDP)-primed mice

Assays for anaphylactoid and lethal reactions were performed as described previously [30]. Female or male C3H/HeJ mice were inoculated intravenously (i.v.) with 100 μg of MDP dissolved in 0.2 ml of pyrogen-free saline. Then, 4 h later, mice were challenged by i.v. injection with LPS (6–100 μg of LPS in pyrogen-free saline/mouse), observed for 1 h, and the incidence of anaphylactoid reactions and death was recorded. Anaphylaxis was characterised by rolling, jumping, severe convulsions and unconsciousness 5–20 min after injection of LPS and by occasional deaths 10–30 min after the injection. Mice were monitored further every 24 h for 3 days, although all deaths occurred within 1 h of injection.

Murine macrophages

Mouse peritoneal exudate cells (PECs) were harvested by peritoneal lavage 4 days after i.p. injection of 1.5 ml of Brewer thioglycolate 3% broth (Difco Laboratories, Detroit, MI, USA) as described previously [31]. The PECs were washed with serum-free RPMI 1640 medium (ICN Biomedicals, Costa Mesa, CA, USA) containing 4 mM L-glutamine, penicillin 100 U/ml and streptomycin 100 $\mu\text{g}/\text{ml}$ and seeded to a concentration of 2×10^5 cells/well in 96-well plates (Nunc, Roskilde, Denmark). The cells were incubated for 3 h at 37°C in air 95%/CO₂ 5%, and then washed with serum-free RPMI 1640 medium to remove non-adherent cells. The remaining adherent cells consisted of > 95% macrophages as determined by morphology and non-specific esterase staining. These cells were

cultured in the presence of various concentrations of test preparation in 200 μl of RPMI 1640 medium containing heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA) 5% with and without IFN- γ 0.5 U/ml for C3H/HeJ-derived and C3H/HeN-derived cells, respectively. The culture supernates were collected after 3.5 h for the TNF assay and after 48 h for the NO assay.

Murine macrophage-like cells

Mouse macrophage-like J774.1 cells were kindly provided by Dr T. Suzuki, University of Kansas Medical Center. An LPS-resistant, membrane CD14[−] mutant cell line, J7.DEF.3, was derived from J774.1 cells as described previously [32]. These cells were grown in flasks (Nunc) with RPMI 1640 containing FBS 5%, 4 mM L-glutamine, penicillin 100 U/ml and streptomycin 100 $\mu\text{g}/\text{ml}$ at 37°C, in air 95%/CO₂ 5%. Once the cells reached confluence, fresh culture medium was applied and the cells were maintained for a further 24 h. Cells were then collected by vigorous pipetting and were centrifuged. The cells were washed once with the culture medium and seeded into 96-well culture plates at 2×10^5 cells/well in 180 μl of RPMI 1640 medium containing FBS 5% and INF- γ (1 or 2 U/ml) for 1 h. Various test preparations (20 μl) were then added to each well and the cells were cultured for a further 48 h, after which culture supernates were collected for NO assay.

TNF assay

TNF activity was determined as described previously [33] with a functional cytotoxic assay in TNF-sensitive

L929 cells (donated by Dr M. J. Parmely, University of Kansas Medical Center).

Nitric oxide (NO) assay

NO formation was measured as the stable end product, nitrite (NO_2^-), in the supernates of macrophage or macrophage-like cell cultures by the Griess reagent method as described previously [33].

Assay for LPS anti-complementary activity

Various amounts of LPS were dissolved in distilled water (12.5 μl) and fresh human serum (50 μl) was added to the LPS solution as a source of complement. The mixture was incubated for 30 min at 37°C, and LPS anti-complementary activity in the mixture was determined with a liposome-based assay for measuring total complement activity (C3H/HA test; Wako Chemical Industries, Osaka, Japan) [34]. The kit contained dinitrophenyl (DNP)-labelled liposomes containing entrapped glucose-6-phosphate dehydrogenase, anti-DNP antibody and substrate (NAD^+). Liposome lysis caused by complement activity was detected spectrophotometrically by determination of the activity of released glucose-6-phosphate dehydrogenase. Complement activity in the samples was quantified as a function of the rate of absorbance increase ($\Delta\text{A}/\text{min}$).

Assay for LPS sensitivity to polymyxin B or CAP18₁₀₉₋₁₃₅

Polymyxin B and CAP₁₀₉₋₁₃₅ are capable of neutralising the endotoxicity of LPS and lipid A. LPS and lipid A preparations were mixed with polymyxin B or CAP₁₀₉₋₁₃₅, or neither as control, and incubated at

37°C for 30 min. The solutions were added to J774.1 cell cultures which were maintained for a further 48 h. The culture supernates were then collected and NO in the supernates was assessed. Attenuation of LPS endotoxicity by polymyxin B or CAP₁₀₉₋₁₃₅ was indicated by a decline in the NO yield. CAP₁₈₁₀₉₋₁₃₅ alone (0.1–10 $\mu\text{g}/\text{ml}$) did not induce any detectable NO production in J774.1 cells.

Results

Lethal toxicity of proteus LPS in GalN-loaded mice

GalN-loaded mice are markedly susceptible to LPS endotoxicity [29]. The lethal effects of LPS from *P. mirabilis*, *P. vulgaris*, *E. coli* O55 and *K. pneumoniae* O3 were compared in GalN-loaded C3H/HeN and C3H/HeJ mice (Table 2). All proteus LPS preparations were toxic to C3H/HeN mice to a degree similar to that of LPS from other members of the Enterobacteriaceae. The LD₅₀ values of LPS from *P. mirabilis* O23 and O26 were each c. 52 ng, which was similar to the values for *E. coli* O55 LPS and *K. pneumoniae* O3 LPS. LPS from *P. vulgaris* O25 was significantly less lethal than LPS from the other strains (LD₅₀ versus *E. coli* O55 LPS; $p < 0.05$). In contrast, LPS preparations were not lethal in C3H/HeJ mice.

Anaphylactoid and lethal reactions evoked by proteus LPS in MDP-primed C3H/HeJ mice

S-form LPS preparations from some strains of bacteria, including *Salmonella* spp., *Porphyromonas gingivalis*, *Prevotella intermedia* and *K. pneumoniae* O3 [30, 35; personal communication by H. Takada, Tohoku Uni-

Table 2. Lethal toxicity of enterobacterial LPS in D-GalN-loaded C3H/HeN and C3H/HeJ mice

LPS source	Dose (ng/mouse)	Number of survivors/total number of mice (LD ₅₀)*	
		C3H/HeN	C3H/HeJ
<i>E. coli</i> O55	10 000	ND (40)	3/4 (>1000)
	1000	0/4	ND
	100	2/8	ND
	10	4/4	ND
<i>K. pneumoniae</i> O3	10 000	ND (100)	4/4 (>1000)
	1000	0/4	ND
	100	2/4	ND
	10	4/4	ND
<i>P. mirabilis</i> O23	10 000	ND (52)	4/4 (>1000)
	1000	0/4	ND
	100	1/4	ND
	10	4/4	ND
<i>P. mirabilis</i> O26	10 000	ND (52)	4/4 (>1000)
	1000	0/4	ND
	100	1/4	ND
	10	4/4	ND
<i>P. vulgaris</i> O25	10 000	ND (470)	4/4 (>1000)
	1000	0/4	ND
	100	8/8	ND

C3H/HeN and C3H/HeJ mice were inoculated i.p. with LPS at indicated doses together with 18 mg of GalN. Lethality was scored on day 2. ND, not determined.

*Lethal dose for 50% of mice (ng LPS/mouse).

versity, Sendai, Japan] induce lethal anaphylactoid reactions in MDP-primed mice. The polysaccharide portion of LPS apparently plays a key role in the induction of anaphylaxis, because lipid A and R-chemotype LPS from *E. coli* and *Salmonella* spp. do not elicit the reaction. LPS and lipid A preparations from *Proteus* spp. were examined for their ability to induce lethal anaphylaxis in MDP-primed C3H/HeJ mice (Table 3). *K. pneumoniae* O3 LPS elicited anaphylactic reactions that killed all mice within 20 min after injection. *E. coli* O55 LPS did not induce such reactions. Of eight S-form LPS, Re-chemotype LPS and lipid A preparations from *Proteus* spp., only *P. vulgaris* O25 LPS evoked severe anaphylaxis similar to that seen with *K. pneumoniae* O3 LPS. *P. mirabilis* O43 and O10 LPS showed less activity, whereas *P. mirabilis* O23, O13, O26 and O3 LPS and *P. vulgaris* O1 LPS had no effect in the mice.

TNF and NO production in C3H/HeN and C3H/HeJ macrophages

Enterobacterial LPS and *Ps. aeruginosa* LPS activate peritoneal C3H/HeN mouse macrophages inducing TNF and NO production, whereas their effects on C3H/HeJ macrophages are minimal [33, 36]. S-form LPS from *P. mirabilis* O23 and O26 and from *P. vulgaris* O25 dose-dependently induced both TNF and NO production in C3H/HeN macrophages (Fig. 1a). The threshold concentration was 0.1–1 ng/ml and maximal induction occurred at 10 ng/ml; these dose-response relationships were similar to that of *E. coli* O55 LPS. The dose-response relationships of other S-

form LPS from *P. mirabilis* O3, O10, O13, O43 and from *P. vulgaris* O1 were similar (data not shown). Thus, differences in the sugar components in O-polysaccharides among proteus LPS had little effect on TNF or NO production. Ra- and Re-chemotype LPS of *Proteus* strains induced production of TNF and NO in C3H/HeN macrophages, and the effect was similar to that of salmonella Ra- and Re-chemotype LPS (Fig. 1b). *Proteus* lipid A induced TNF and NO production in a manner that was qualitatively similar to synthetic *E. coli* lipid A, although its potency was c. 100 times less than Re-chemotype LPS (Fig. 1c). The endotoxicity of lipid A was thus augmented when O-polysaccharide or R-core oligosaccharide was present. In contrast, C3H/HeJ macrophages produced little or no TNF and NO in response to any of the endotoxic preparations tested (Fig. 1 and other data not shown).

CD14-dependent macrophage activation by proteus LPS

Macrophage activation in response to enterobacterial LPS and *Ps. aeruginosa* LPS is mediated mainly by the cell-surface form of CD14 (mCD14) [33, 36, 37]. However, an alternative CD14-independent pathway has been suggested [32]. The CD14 dependence of proteus LPS-induced macrophage activation was examined with CD14⁺, murine macrophage-like J774.1 cells [32, 38] and their CD14⁻ mutant, J7.DEF3 cells [32, 33]. All LPS preparations from *Proteus* strains tested induced dose-dependent increases in TNF and NO production in both J774.1 and J7.DEF3 cells (Fig. 2). However, dose-response curves obtained from the

Table 3. Incidence of anaphylactoid reaction and death in muramyl-dipeptide (MDP)-primed C3H/HeJ mice after injection of LPS*

Stimulants	Dose (μ g/mouse)	Number of mice			
		Total	Anaphylactoid reaction [†]	Dead [‡]	Survival (%)
S-form LPS					
P. mirabilis O23	100	4	0	0	100
P. mirabilis O43	100	8	3	1	88
P. mirabilis O13	100	4	0	0	100
P. mirabilis O26	100	4	0	0	100
P. mirabilis S1959 (O3)	100	4	0	0	100
P. mirabilis O10	100	8	3	1	88
P. vulgaris O25	100	12	12	12	0
	50	4	4	1	75
	25	4	3	0	100
P. vulgaris OX19(O1)	100	8	0	0	100
K. pneumoniae O3	100	12	12	12	0
	50	4	4	4	0
	25	4	4	3	25
	13	4	4	2	50
	6	4	0	0	100
E. coli O55	100	4	0	0	100
Re-chemotype LPS					
P. mirabilis R45	100	4	0	0	100
Lipid A					
P. mirabilis	100	4	0	0	100

*C3H/HeJ mice were inoculated i.v. with 100 μ g of MDP and then challenged by i.v. injection of the indicated doses of LPS 4 h later.

[†]When the mouse showed a severe convulsion, unconsciousness, or rolling and jumping within 1 h after LPS injection.

[‡]Mice were monitored and death was scored on day 3.

Fig. 1. *Proteus* LPS- and lipid A-induced TNF and NO production in macrophages obtained from C3H/HeN and C3H/HeJ mice. Macrophages from C3H/HeN (closed symbols) or C3H/HeJ mice (open symbols) were cultured with S-form LPS (a), R-form LPS (b), or lipid A (c). The culture supernates were collected for TNF assay after 3.5 h (left panels) and after 48 h for NO assay (right panels). Each point represents the mean (bar SE) for three samples and the data represent one of two experiments with similar results. a, *P. mirabilis* O23 (●, ○); *P. mirabilis* O26 (■, □); *P. vulgaris* O25 (▲, △); *E. coli* O55 (◆, ◇). b, *P. mirabilis* Re-chemotype (●, ○); *P. mirabilis* Ra-chemotype (▲, △); *S. minnesota* Re-chemotype (■, □); *S. minnesota* Ra-chemotype (◆, ◇). c, *P. mirabilis* lipid A (●, ○) and *E. coli* synthetic lipid A (■, □).

CD14⁻ mutant cells were shifted to concentrations 10–1000 times higher than those of CD14⁺ parental cells. This confirmed that proteus LPS-induced macrophage activation was largely dependent on CD14.

Anti-complementary activity of proteus LPS

Because only *K. pneumoniae* O3 and *P. vulgaris* O25 LPS potently induced lethal anaphylaxis in MDP-primed C3H/HeJ mice, and LPS from *K. pneumoniae* O3 was known to have extremely high anti-complementary activity [39], it was considered worthwhile to examine proteus LPS, especially *P. vulgaris* O25 LPS, for anti-complementary activity. All LPS preparations tested exhibited concentration-dependent anti-complementary activity (Fig. 3). LPS from *K. pneumoniae* O3 and *P. vulgaris* O25 were the most effective; they

elicited c. 60% inhibition of complement at an LPS concentration of 1 μ g/ml. The inhibitory effects of *P. mirabilis* O23, O43, O13, O26, O3 and O10 LPS and *P. vulgaris* OX19(O1) LPS were also relatively strong, whereas the effects of *E. coli* O55 LPS and *Ps. aeruginosa* LPS were weak.

Sensitivity of proteus LPS to polymyxin B

Proteus spp. are naturally resistant to polymyxin B. Polymyxin B neutralises many of the biological activities of LPS by binding to its lipid A domain. Therefore this study examined whether the endotoxicity of proteus LPS and lipid A was affected by treatment with polymyxin B. The magnitude of the effect was estimated from the change in LPS-evoked NO production by J774.1 cells in the presence of polymyxin B. As

Fig. 2. CD14 dependence of proteus LPS-induced production of TNF and NO. CD14⁺J774.1 (closed symbols) and CD14⁻J7.DEF3 (open symbols) cells were cultured in the presence of *P. mirabilis* O23 (●, ○), *P. mirabilis* O26 (■, □) and *P. vulgaris* O25 (▲, △) LPS. The culture supernates were collected after 3.5 h for TNF assay (a), and after 48 h for NO assay (b). Each point represents the mean (bar SE); the data represent one of two experiments with similar results.

shown in Fig. 4a, polymyxin B inactivated LPS from *P. mirabilis* O23, O26 and *P. vulgaris* O25 dose-dependently, but proteus LPS were obviously more resistant to polymyxin B than LPS from *E. coli* O55. Similarly, LPS from S-form *P. mirabilis* O43, O13, O3, O10 and *P. vulgaris* O1 were also resistant to polymyxin B (data not shown).

A low concentration (1 μg/ml) of polymyxin B potently neutralised *S. minnesota* Ra-chemotype LPS, whereas a substantially higher concentration (100 μg/ml) was necessary to neutralise *P. mirabilis* Ra-chemotype LPS (Fig. 4b). Both *S. minnesota* and *P. mirabilis* Re-chemotype LPS were more resistant to polymyxin B than *E. coli* Re-chemotype LPS (Fig. 4c). Moreover, a relatively low concentration of polymyxin B neutralised *P. mirabilis* lipid A as well as *E. coli* lipid A (Fig. 4d). To evaluate these results more precisely, the relative potency of polymyxin B in neutralising test preparations was calculated as the ID₅₀/stimulant concentration value (Table 4). In the cases of R-chemotype LPS and lipid A, the values were also calculated on a molecular basis. The neutralising values for S-form LPS from *Proteus* spp. were 6–500-fold higher than that for *E. coli* O55 LPS, indicating that proteus LPS were much more resistant to polymyxin B than *E. coli* O55 LPS.

Sensitivity of proteus LPS to CAP18_{109–135}

The C-terminal 27 amino-acid fragment of CAP18, designated CAP18_{109–135}, has the ability to neutralise

Fig. 3. Anti-complementary activity of proteus LPS. Increasing doses of LPS were mixed with fresh human sera and incubated for 30 min at 37°C. Residual complement activity in the sera was determined by a liposome immunoassay as described in Materials and Methods. The rate of the increase in absorbance for serum without LPS was 0.190 ± 0.031 ΔA/min ($n = 13$); this value was defined as 100% complement activity. The ΔA/min for heat-inactivated serum was 0.009 ± 0.001 ($n = 3$). ●^{1–6}, *P. mirabilis* O23¹, O43², O13³, O26⁴, S1959 (O3)⁵, O10⁶; □¹, *P. vulgaris* O25; ●⁷, *P. vulgaris* OX19(O1); □², *K. pneumoniae* O3; O¹, *E. coli* O55; O², *Ps. aeruginosa* Fisher 2.

Fig. 4. Sensitivity of proteus LPS to polymyxin B. LPS or lipid A (10 ng/ml in panel a, 100 ng/ml in panels b and c or 1000 ng/ml in panel d) were mixed with selected concentrations of polymyxin B (abscissa) and incubated at 37°C for 30 min. The mixtures were then added to J774.1 cells which were then cultured for 48 h and NO in the culture supernates was assayed. Each point represents the mean (bar SE); the data represent one of two experiments with similar results. a, *P. mirabilis* O23 (●); *P. mirabilis* O26 (▲); *P. vulgaris* O25 (■); *E. coli* O55 (○) LPS. b, Ra-chemotype LPS of *P. mirabilis* R110 (●) and *S. minnesota* R60 (▲). c, Re-chemotype LPS of *P. mirabilis* R45 (●); *S. minnesota* R595 (▲); *E. coli* F515 (■). d, *P. mirabilis* lipid A (●) and *E. coli* lipid A (▲).

LPS endotoxicity [28, 40]. Therefore, the sensitivity of proteus LPS to CAP18₁₀₉₋₁₃₅ was examined with the same protocol that was used to characterise the effects of polymyxin B. The activities of *P. mirabilis* O23 LPS were dose-dependently affected by CAP18₁₀₉₋₁₃₅ between 0.1 and 1.3 μg/ml (Fig. 5a). Although the effects of *P. mirabilis* Ra- and Re-chemotype LPS and lipid A on NO synthesis were also reduced by treatment with CAP18₁₀₉₋₁₃₅, the optimal LPS: CAP18₁₀₉₋₁₃₅ ratios were observed within that range (Fig. 5b, c and d). Higher concentrations of CAP18₁₀₉₋₁₃₅ neutralised LPS less effectively. The endotoxicity of LPS from *P. mirabilis* O43, O13, O26, O3 and O10, *P. vulgaris* O25 and O1, *E. coli* O55, *S. minnesota* R60 and R595, as well as synthetic *E. coli* lipid A, was also reduced by CAP18₁₀₉₋₁₃₅ treatment (data not shown). The relative potency of CAP18₁₀₉₋₁₃₅ in neutralising LPS and lipid A was calculated as ID50 stimulant concentration values (Table 4). These neutralising values for S-form LPS from *Proteus* spp. were similar to that for *E. coli* O55 LPS.

Discussion

Although *Proteus* is a genus of the family Enterobacteriaceae, the chemical constituents of LPS from *Proteus* spp. are different from LPS of other enterobacteria. For instance, the fatty acid composition of lipid A from *Proteus* spp. differs from that of *E. coli* and *S. minnesota* lipid A, and the AraN residue is linked to the phosphate residue of the glucosamine backbone at the 4' position [3]. Uncommon constituents that are not detected in other enterobacterial LPS are found in the polysaccharide chains of proteus LPS; these include D-galacturonic acid in the core region as well as uronic acids and unusual sugars and amino acids in the O-polysaccharide portion of LPS. Furthermore, N-acetylglucosamine and mannose – which are recognised by mannose-binding protein (MBP) – are found in the polysaccharide chains of proteus LPS. O-polysaccharide structures vary among LPS from *Proteus* spp. and this might be expected to cause variation in the endotoxic actions of the respective *Proteus* strains.

Table 4. Inhibition of LPS- and lipid A-induced NO production in J774.1 cells by polymyxin B and CAP₁₀₉₋₁₃₅

Stimulant	Dose of stimulant (ng/ml)	Neutralising values (ID50/dose of stimulant)*	
		Polymyxin B	CAP18 _{109–135}
S-form LPS			
P. mirabilis O23	10	1300	54
P. mirabilis O43	10	>10 000	124
P. mirabilis O13	10	2500	62
P. mirabilis O26	10	500	54
P. mirabilis S1959(O3)	10	500	100
P. mirabilis O10	10	800	101
P. vulgaris O25	10	120	26
P. vulgaris OX19(O1)	10	400	98
E. coli O55	10	20	60
Ra-chemotype LPS			
P. mirabilis R110	100	400 (105) [†]	ND
	10	ND	14 (11)
S. minnesota R60	100	4.0 (1.0)	ND
	10	ND	400 (291)
Re-chemotype LPS			
P. mirabilis R45	100	48 (20)	4.2 (5.2)
S. minnesota R595	100	90 (39)	ND
	10	ND	122 (155)
E. coli F515	100	3.2 (1.5)	ND
Lipid A			
P. mirabilis	1000	0.48 (0.26)	0.48 (0.75)
E. coli (compound 506)	1000	1.1 (0.65)	2.0 (3.5)

ND, not determined.

*ID50 was calculated as the dose of tested agent that reduced NO production in J774.1 cells by 50% in the absence of any inhibitors. Values were calculated from the ID50/stimulant concentration.

[†]The values in parentheses are the values calculated on a molecular basis.

LPS from *Proteus* strains were potently endotoxic, exhibiting properties similar to other enterobacterial LPS. *Proteus* LPS killed GalN-sensitised C3H/HeN mice but not C3H/HeJ mice, activated C3H/HeN but not C3H/HeJ macrophages, and were highly dependent on CD14 for activation of C3H/HeN macrophages. *Proteus* LPS were comparatively resistant to the action of polymyxin B which neutralises the toxicity of LPS from *E. coli* and *Salmonella* spp. However, there was obvious variability in endotoxic potency among *proteus* LPS with respect to anaphylactoid reactions, anti-complementary activity and sensitivity to polymyxin B.

Lipid A is the principal endotoxic moiety of LPS. Shimizu et al. [6] reported that the endotoxicities of synthetic *Proteus*- and *E. coli*-type lipid A were similar, based on their mitogenicity in murine splenic cells and lethal toxicity in GalN-sensitised mice; however, the capacity of *proteus* lipid A to induce a local Shwartzman reaction was weak.

In the present study, the abilities of *P. mirabilis* lipid A and synthetic *E. coli* lipid A to induce TNF and NO production in murine macrophages were approximately equal. The endotoxic potency of lipid A was substantially lower than that of S-form or Re-chemotype LPS. To obtain maximal production of TNF and NO by macrophages, it was necessary to use concentrations of lipid A 10–100 times higher than were used with R-chemotype LPS. Thus, the presence of the polysac-

charide domain of LPS apparently enhances the endotoxicity of lipid A. LPS polysaccharide-induced modulation of endotoxicity – including modulation of local skin responses [41], complement activation [39, 42], haemagglutination [43], macrophage arachidonic acid metabolism [44], activation of B cells and macrophages [45, 46] and induction of TNF/NO production [32, 47] – has also been reported with LPS from *E. coli*, *Salmonella* spp. and *K. pneumoniae*. Despite the unique components in their polysaccharide chains, *proteus* LPS did not exhibit any special endotoxicity.

TNF and NO are thought to be powerful mediators of endotoxic shock. It is known that the soluble and surface forms of CD14 and serum factors such as lipopolysaccharide-binding protein (LBP) [48] and septin [49] participate in macrophage activation by LPS [37]. The binding of LPS–LBP complexes to mCD14 stimulates macrophages to produce large quantities of active molecules such as IL-1, TNF, IL-6 and NO [50]. However, it has been suggested that CD14-independent pathways may also be present [32, 33, 51–53]. The finding that CD14⁺ J774.1 cells produced significantly more TNF and NO in response to *proteus* LPS than CD14[−] J7.DEF cells suggests that *proteus* LPS recognition by macrophages is primarily mediated by CD14.

The induction of anaphylaxis by LPS depends upon the

Fig. 5. Sensitivity of proteus LPS to CAP18₁₀₉₋₁₃₅. LPS or lipid A (10 ng/ml) were mixed with selected concentrations of CAP18₁₀₉₋₁₃₅ (abscissa) and incubated at 37°C for 30 min. The mixtures were then added to J774.1 cells which were then cultured for 48 h and NO in the culture supernates was assayed. Each point represents the mean (bar SE); the data represent one of two experiments with similar results. a, *P. mirabilis* O23 LPS; b, *P. mirabilis* R110 (Ra-chemotype) LPS; c, *P. mirabilis* R45 (Re-chemotype) LPS; d, *P. mirabilis* lipid A.

presence of the polysaccharide chains; moreover, the structural requirements of the chain are, apparently, very specific. Neither *S. minnesota* R-chemotype LPS nor lipid A induced anaphylaxis, and only a few LPS preparations from strains of *Salmonella*, oral black-pigmented bacteria and *K. pneumoniae* were capable of eliciting the response [30, 35; personal communication from H. Takada]. The accumulation of platelets secreting serotonin, but not histamine, in the lung has been suggested to play a role in anaphylactoid reactions [30, 54, 55]. TNF production was apparently not essential for induction of anaphylaxis, as the reaction was elicited in C3H/HeJ mice in the absence of detectable TNF production [35]. In the present study, only *P. vulgaris* O25 LPS induced strong anaphylactoid reactions like those evoked by *K. pneumoniae* O3 LPS, and little or no TNF or NO production was detected, confirming that TNF and NO do not participate in LPS-induced anaphylaxis.

The ability to induce anaphylaxis is believed to be associated with potent activation of complement. It was observed that LPS from *P. vulgaris* O25 and *K. pneumoniae* O3 possessed strong anti-complementary activity. *K. pneumoniae* O3 LPS has a mannose

homopolymer in its O-polysaccharide chain that binds to MBP and strongly activates complement via the lectin pathway [39, 55, 56]. Unlike mannose, other sugars – including N-acetylglucosamine (GlcNAc), N-acetylmannosamine, rhamnose and fucose – have an equatorial conformation at the 3- and 4-OH groups or at the 2- and 3-OH groups, which can form hydrogen bonds with lectins [57]. Because the O-polysaccharide chains of *P. vulgaris* O25 LPS contain N-acetylglucosamine, rhamnose, ribose and an unusual acidic component – an ether of glucose with (R)-lactic acid (3-O-[(R)-1-carboxyethyl]-D-glucose) – they may be able to form a centre for hydrogen bonding leading to complement activation via the lectin pathway. All proteus LPS with the exception of *P. mirabilis* O3 LPS, which has GlcNAc in its core oligosaccharide [58], contain GlcNAc in their polysaccharide moieties. Consequently, proteus LPS-induced complement activation may be generally mediated by the lectin pathway.

E. coli O55 LPS also contains a GlcNAc residue, but its GlcNAc residue binds to repeating units of O-polysaccharide at position 3, and GlcNAc at position 3 lacks the ability to bind MBP. *Ps. aeruginosa* Fisher 2 O-polysaccharide does not contain GlcNAc. Consistent

with the discussion above, LPS from *E. coli* O55 and *Ps. aeruginosa* Fisher 2 possessed little if any anti-complementary activity. It is known that enterobacterial LPS is sensitive to polymyxin B. Nevertheless, like the living organisms, proteus LPS were relatively resistant to polymyxin B-induced inactivation. Most proteus LPS possess two AraN residues in the KDO/lipid A region [3, 4, 59], which may provide them with their resistance to polymyxin B. *P. mirabilis* R110 LPS contains AraN, and Re-chemotype LPS from *P. mirabilis* R45 has two AraN residues, one bound to KDO and the other bound to the ester-linked phosphate group of lipid A [4]. *S. minnesota* R595 LPS has one lipid A-bound AraN residue [60]. The study found that proteus LPS with AraN residues were resistant to polymyxin B. *E. coli* S-form LPS and lipid A (compound 506) do not contain AraN [61], nor do *E. coli* F515 (Re-chemotype) and *S. minnesota* R60 (Ra-chemotype) LPS [62, 63]. *P. mirabilis* lipid A probably loses its AraN residues during the process of extraction with mild acid hydrolysis. These LPS and lipid A were sensitive to polymyxin B which supports the idea that the presence of AraN residues in proteus LPS is associated with resistance to polymyxin B-evoked inactivation. The O-polysaccharide chain in LPS may also protect LPS from the destructive action of polymyxin B, because S-form LPS were more resistant than lipid A or R-chemotype LPS.

Treatment of proteus LPS and lipid A with CAP18₁₀₉₋₁₃₅ together with polymyxin B weakened their endotoxicity. However, the profiles of CAP18₁₀₉₋₁₃₅-induced inhibition were quite different from those of polymyxin B, suggesting that the mode of action of the two substances was different. Polymyxin B dose-dependently attenuated the activity of LPS, whereas the suppressive action of CAP18₁₀₉₋₁₃₅ was not dose-dependent. Instead, as with the zones of precipitation in antigen-antibody reactions, optimal CAP18₁₀₉₋₁₃₅: LPS ratios were required to obtain suppression. CAP18₁₀₉₋₁₃₅ peptide may bind LPS polyvalently, thereby hindering access of LPS to target cells. Another indicator of the differences between CAP18₁₀₉₋₁₃₅ and polymyxin B was their spectra of sensitivity among Proteus LPS. Proteus S-form and R-chemotype LPS were resistant to polymyxin B, whereas they were comparatively sensitive to the CAP18₁₀₉₋₁₃₅, which suggests that AraN residues do not affect CAP18₁₀₉₋₁₃₅ binding to LPS.

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