Biological properties of lipopolysaccharides from *Bordetella* species

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Biological activities of lipopolysaccharides (LPS) extracted from *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* were compared with those of *Escherichia coli* LPS. The LPS preparations from *B. pertussis* showed biological activities comparable to those of *E. coli* LPS in terms of lethal toxicity in galactosamine-sensitized mice, pyrogenicity in rabbits, mitogenicity in C3H/He spleen cell cultures, macrophage activation, and induction of tumour necrosis factor. All the activities of LPS preparations from *B. parapertussis*, except mitogenicity, were lower than those of *E. coli* LPS. LPS from *B. parapertussis* gave the greatest mitogenic action of all those tested. Biological activities stronger than or comparable to those of *E. coli* LPS were observed for LPS from *B. bronchiseptica*.

Introduction

Lipopolysaccharides (LPS) extracted from Gram-negative bacteria are potent molecules which elicit in animals a range of characteristic responses. These are often due to stimulation of host mediators such as interleukin-1 and tumour necrosis factor (TNF) (Haeflner-Cavaillon *et al.*, 1984; Nowotny, 1985). Among these responses are the induction of the Shwartzman reaction (Movat & Burrowes, 1985), antitumour effects (Freinman *et al.*, 1987) and adjuvant activity (McGhee *et al.*, 1980).

LPS from *Bordetella pertussis* also exhibits the biological activities described above (Chaby & Caroff, 1988), although lipid A derived from this LPS is non-toxic and does not induce the Shwartzman reaction (Ayme *et al.*, 1980). There is little information on the biochemical, immunological and biological properties of LPS from the other two species of *Bordetella*, namely *B. parapertussis* and *B. bronchiseptica*. In the preceding paper (Amano *et al.*, 1990) we described the biochemical and immunological properties of preparations of LPS from the three species of *Bordetella*, and in the present paper we give some of their biological activities, i.e. lethal toxicity in

galactosamine-sensitized mice, pyrogenicity, mitogenicity, macrophage activation and TNF-inducing activity. These activities of *Bordetella* LPS were compared to those of *Escherichia coli* LPS.

Methods

Organisms and extraction of LPS fractions. *B. pertussis* strains Tohama [phase I, BpI(Tohama)] and AK-168 [phase I, BpI(AK-168)], *B. parapertussis* strains 21815 [Bpp(21815)] and AK-167 [Bpp(AK-167)], and *B. bronchiseptica* strains L3 [Bbs(L3)] and H-214 [Bbs(H-214)] were obtained from our laboratory stock. Cultivation and purification of *Bordetella* strains, extraction of LPS with hot phenol/water, and purification by ultracentrifugation and by treatment with proteolytic enzymes were carried out as described previously (Amano *et al.*, 1990). LPS from *E. coli* O55:B5 was purchased from Difco.

Determination of lethal toxicity. This was done by the method described by Galanos *et al.* (1979). Various concentrations of LPS in 0.2 ml water were injected intravenously (i.v.) into male 10-week-old C57Bl/6 mice (Shizuoka Animal Center, Shizuoka, Japan) immediately after intraperitoneal (i.p.) administration in 0.5 ml of pyrogen-free saline containing 10 mg D-galactosamine (Sigma).

Determination of pyrogenicity. Pyrogenicity was determined in Japanese white rabbits (2.1–2.4 kg) (Gokita Breeding Service, Tokyo, Japan) of the same sex in groups of three for each dose to be tested, as described by Kumazawa *et al.* (1988). The sample was injected i.v. into three rabbits. Rectal temperature was monitored continuously for 3 h, and the maximal increase in temperature was recorded for each rabbit.

Measurement of limulus amoebocyte lysate (LAL) gelation. LAL gelation activity was tested using Pregel-S reagent (Seikagaku Kogyo Co.).

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Abbreviations: LPS, lipopolysaccharide(s); TNF, tumour necrosis factor; i.v., intravenously; i.p., intraperitoneal(ly); LAL, limulus amoebocyte lysate.

Measurement of macrophage activation. This was done by measuring phagocytic, cellular lysosomal enzyme (acid phosphatase), and cytostatic activities of macrophages obtained from the peritoneal cavity of ICR mice (Shizuoka Animal Center) which had been injected i.p. with 1 µg of test sample 4 d earlier, as described by Kumazawa *et al.* (1988).

Phagocytic activity was assessed by measuring the radioactivity of ⁵¹Cr-labelled, antibody-sensitized sheep erythrocytes which were phagocytosed by *in vivo*-stimulated peritoneal macrophages. Acid phosphatase activity was measured as follows. Peritoneal macrophages stimulated *in vivo* were lysed in 96-well microplates with 0.1% Triton X-100, and 20 µl 0.2 M-sodium acetate buffer (pH 5.0) plus 20 µl 24 mM-*p*-nitrophenyl phosphate were added to the macrophage lysates. After incubation at 37 °C for 30 min, 200 µl 0.2 M sodium carbonate was added to the reaction mixture, and the *A*₄₀₅ was measured. Enzyme activity was calculated from a standard curve made using known amounts of potato acid phosphatase. Cytostasis-inducing activity was assessed by measuring the growth-inhibitory action against EL-4 lymphoma cells. The activity was calculated as the percentage inhibition of [³H]thymidine incorporation into EL-4 cells.

Assay of TNF-inducing activity. This was done by the method described by Kumazawa *et al.* (1988). Briefly, 10 µg LPS was administered i.v. into ICR mice (Charles River Japan, Atsugi, Japan) primed with *Propionibacterium acnes*. Sera were separated from blood 1.5 h after administration of LPS and treated at 56 °C for 30 min before use. The TNF activity in the sera was determined by measuring the percentage inhibition of [³H]thymidine uptake into L929 cells.

Determination of mitogenic activity. Spleen cells, obtained from C3H/He and C3H/HeJ mice (Charles River Japan), were suspended in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum, 25 mM-HEPES, 2 mM-L-glutamine, 100 units penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹, and incubated in triplicate at 37 °C for 48 h in the presence or absence of LPS preparations (Kumazawa *et al.*, 1988). The cells were given [³H]thymidine 4 h before harvest, and mitogenicity was assayed by determining the radioactivity incorporated into the cells.

Results

Toxicity, pyrogenicity and LAL gelation activity

Toxicity in galactosamine-sensitized mice is shown in Table 1. Among the preparations of LPS tested, Bbs(L3) was the most toxic, while Bbs(H-214) LPS and two samples of *B. pertussis* LPS possessed toxicity similar to that of *E. coli* LPS. Weak toxic activity was shown for two preparations from *B. paraptussis*.

The smallest dose of LPS that consistently evoked a rise in body temperature (over 0.6 °C per rabbit) was 1 ng kg⁻¹ for Bbs(L3), and 10 ng kg⁻¹ for BpI(TOHAMA), Bbs(H-214) and *E. coli* LPS; for BpI(AK-168), Bpp(21815) and Bpp(AK-167) a dose of 100 ng kg⁻¹ was required to induce a fever response (Table 1).

In the LAL gelation test, all the LPS preparations from *Bordetella* species showed activity the same as [BpI(AK-168), Bpp(21815), Bpp(AK-167) and Bbs(H-214)] or stronger than [BpI(TOHAMA) and Bbs(L3)] the LPS from *E. coli* (Table 1).

Macrophage activation

This was determined by measuring phagocytosis of ⁵¹Cr-labelled antibody-sensitized sheep erythrocytes (Fig. 1a), increased level of cellular lysosomal enzyme (acid phosphatase) (Fig. 1b), and induction of cytostasis (Fig. 1c). All the LPS preparations from *Bordetella* species induced significant macrophage activation at a dose of 1 µg per mouse. At this dose level, the most active LPS was Bbs(L3), which had an activity comparable to that of *E. coli* LPS. The two LPS preparations from *B. pertussis* and that from Bbs(H-214) were weaker than the LPS from *E. coli*, and the activities of the two preparations from *B. paraptussis* were low.

TNF-inducing activity

The two preparations of LPS from *B. pertussis* and the two from *B. bronchiseptica* exhibited TNF-inducing activity comparable to that of LPS from *E. coli* (Fig. 2). However, the two preparations from *B. paraptussis* were less active than LPS from *E. coli*.

Mitogenic activity

The LPS preparations from BpI(TOHAMA), two strains of *B. paraptussis* and Bbs(H-214) showed much higher mitogenic activity than *E. coli* LPS in C3H/He spleen cell cultures at a concentration of 10 ng per 0.1 ml per well (Fig. 3). The mitogenic activity of LPS from BpI(AK-168) and Bbs(L3) was comparable to that of LPS from *E. coli*. None of the LPS preparations from *Bordetella* species showed significant activity in C3H/HeJ mice at the same concentration as used in C3H/He mice (data not shown).

Discussion

LPS from *B. pertussis* is known to have activities similar to those of the LPS from Gram-negative enterobacteria. The endotoxic activities of LPS, such as fever, Schwartzman reactivity and depression of blood pressure leading to shock and death, are produced by the endotoxin from *B. pertussis* (Finger *et al.*, 1976; Ayme *et al.*, 1980). Our data demonstrate the toxicity of LPS from other *Bordetella* species. The LPS preparations from *B. bronchiseptica* were similar in toxicity to LPS from *B. pertussis* and *E. coli*, whereas LPS from *B. paraptussis* had lower toxicity.

Amano *et al.* (1990) showed molecular mass differences of LPS from three species of *Bordetella* by SDS-PAGE: LPS from *B. pertussis* contained two low molecular mass bands, LPS from *B. paraptussis*

Fig. 1. Potency of peritoneal macrophages activated *in vivo* with LPS from *Bordetella* species or *E. coli*. Peritoneal macrophages were obtained from ICR mice injected i.p. with 1 μ g LPS 4 d before. Control experiments were done with macrophages from unstimulated mice. The results are means $\pm$ standard deviation of three separate experiments. (a) Phagocytic activity measured using ^{51}Cr -labelled, antibody-sensitized sheep erythrocytes. (b) Acid phosphatase activity of lysates of 1×10^5 cells determined as A_{405} using *p*-nitrophenyl phosphate as substrate. (c) Cytostatic activity measured using macrophages co-cultivated for 40 h with target EL-4 lymphoma cells in an effector:target cell ratio of 10:1. The activity was assessed by the degree of inhibition of [^3H]thymidine uptake into the target cells.

Fig. 2. TNF-inducing activity of LPS from *Bordetella* species or *E. coli*. LPS was administered i.v. at a dose of 1 μ g into ICR mice primed with *Propionibacterium acnes*. Sera were obtained 1.5 h after administration of LPS. TNF activity was expressed as the percentage inhibition of [^3H]thymidine uptake into L929 cells. (a) $\circ$, BpI (TOHAMA) LPS; $\triangle$, Bpp (21815) LPS; $\square$, Bbs (L3) LPS; $\bullet$, *E. coli* LPS. (b) $\circ$, BpI (AK-168) LPS; $\triangle$, Bpp (AK-167) LPS; $\square$, Bbs (H-214) LPS; $\bullet$, *E. coli* LPS. The results are from a single experiment.

contained ladder structural bands in the high molecular mass area and a single low molecular mass band, and LPS from *B. bronchiseptica* contained ladder structural high molecular mass bands and two low molecular mass bands. Peppler (1984) and Li *et al.* (1988) also demonstrated the presence of two different LPS components in *B. pertussis*, by SDS-PAGE and immunoblotting. These physical differences could be reflected in the difference of the toxicity among *Bordetella* LPS.

Lehmann *et al.* (1987) reported that TNF may be a mediator of toxicity of LPS from Gram-negative bacteria in galactosamine-sensitized mice. Our data, showing that the TNF-inducing activity of *Bordetella* LPS

correlates with toxicity, support this idea. The macrophage activation ability of the various preparations of *Bordetella* LPS also correlated with their toxicity. Lasfargues *et al.* (1986) also reported that *B. pertussis* endotoxin is able to induce in mouse peritoneal macrophages cytostatic activity against tumour cells, and that this activity was due to the lipid region of the endotoxin.

Endotoxins from *B. pertussis* are known to be potent mitogens and polyclonal activators for murine B lymphocytes (Girard *et al.*, 1981; Haeflner-Cavaillon *et al.*, 1982). Girard *et al.* (1981) reported that *B. pertussis* LPS induces a mitogenic response in C3H/HeJ B lymphocytes (which are unresponsive to enterbacterial LPS) as

Fig. 3. Mitogenic activity of LPS from *Bordetella* species or *E. coli*. C3H/He spleen cells were stimulated for 48 h with the indicated dose of LPS. Mitogenic activity is expressed as mean [^3H]thymidine uptake of triplicate cultures. (a) $\circ$, BpI(TOHAMA) LPS; $\square$, BpI(AK-168) LPS; $\bullet$, *E. coli* LPS. (b) $\circ$, Bpp(21815) LPS; $\square$, Bpp(AK-167) LPS; $\bullet$, *E. coli* LPS. (c) $\circ$, Bbs(H-214) LPS; $\square$, Bbs(L3) LPS; $\bullet$, *E. coli* LPS.

Table 1. Endotoxic activities of LPS preparations from *Bordetella* species and *E. coli*

LPS	Lethal toxicity MLD (ng)*	LAL gelation activity (ng ml ⁻¹)	Pyrogenicity MED (ng kg ⁻¹)†
BpI(TOHAMA)	1	0.01	10
BpI(AK-168)	1	0.1	100
Bpp(21815)	10	0.1	100
Bpp(AK-167)	10	0.1	100
Bbs(L3)	0.1	0.01	1
Bbs(H-214)	1	0.1	10
<i>E. coli</i>	1	0.1	10

* The minimal lethal dose (MLD) was determined by using six galactosamine-sensitized mice for each dose of LPS preparation.

† The minimal effective dose (MED) causing pyrogenicity was determined by using three Japanese white rabbits for each dose of LPS preparation.

well as in C3H/He B lymphocytes. We also demonstrated mitogenic activity in LPS preparations of *B. parapertussis*, *B. bronchiseptica* and *B. pertussis*, although at the LPS concentrations tested, only cells from C3H/He mice, and not C3H/HeJ mice, responded. The mitogenicity of two LPS preparations from *B. parapertussis* was much stronger than that of *E. coli*, while the LPS from Bbs(L3) showed comparable mitogenicity to LPS from *E. coli*. The results on mitogenic activity do not correlate with the other activities of LPS tested, which may indicate that a different mechanism is involved in the induction of mitogenicity and the other biological activities of LPS tested in this work.

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