Lipopolysaccharide-induced non-specific resistance to systemic *Escherichia coli* infection in mice

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Summary. A high degree of non-specific resistance to a lethal systemic *Escherichia coli* infection was induced in mice by pretreatment with a small dose ($<5 \mu\text{g}/\text{mouse}$) of the homologous lipopolysaccharide (LPS) or with heterologous rough-type LPS from *E. coli* K-12. The route of LPS administration, intraperitoneally or subcutaneously, did not influence the development of resistance, suggesting that a systemic cell activation was responsible for the effect. The enhanced elimination of bacteria was similar to that in mice recovering from a sublethal *E. coli* infection. In the LPS-treated mice, elimination of the challenge bacteria from the peritoneal cavity and the blood started 3–4 h after challenge whereas, in controls, the bacterial numbers continued to increase until the mice died. The detoxified LPS derivative, monophosphoryl lipid A (MPL), also increased the survival of mice infected with *E. coli* O18:K1. However, the dose of MPL required for optimal infection resistance was 100-fold greater than that of native, *E. coli* K-12 LPS, corresponding to the 100-fold reduced toxicity of MPL for mice and rabbits in lethality and pyrogenicity assays.

Introduction

Although mice are usually quite resistant to *Escherichia coli* infection, the mouse virulence of an *E. coli* O18:K1 strain, isolated from a case of neonatal meningitis, was high ($\text{LD}_{50} 4 \times 10^5$ bacteria/mouse) (Vuopio-Varkila *et al.*, 1987). An intraperitoneal injection of this strain resulted in peritonitis, followed by increasing numbers of bacteria in the blood. The duration of the infection was short. If the mice survived, the bacterial counts in the mice started to decrease by 24 h, and the animals were free from the infection within 48 h (Vuopio-Varkila, 1987). Furthermore, if the convalescent mice were challenged after 48 h with another lethal dose of *E. coli*, they were highly resistant to the infection.

This rapid development of resistance suggested that non-specific resistance mechanisms were important. Many previous studies have shown that immunostimulation with bacterial cell-wall components, such as lipopolysaccharide (LPS), can cause a non-specific increase in infection resistance as well as bactericidal and tumoricidal capabilities (Rowley, 1955; Landy, 1956; Abernathy, 1957; Nowotny, 1985; Chase *et al.*, 1986). LPS is a potent stimulator of many cell types, especially macrophages.

To learn more about the development of resist-

ance, the course of *E. coli* O18:K1 infection in convalescent mice has been compared with that in LPS-pretreated mice. Because of the toxicity of native LPS, we also studied the possibility of eliciting resistance to infection with a LPS derivative, monophosphoryl lipid A, which is reported to be of reduced toxicity (Ribi, 1984; Ribi *et al.*, 1986).

Materials and methods

Bacterial strains

For the standard mouse challenge, we used *E. coli* IH 3080 (serotype O18:K1:H7) (Vuopio-Varkila *et al.*, 1987), an isolate from a case of human neonatal meningitis. In some experiments other challenge strains were used: *E. coli* IH 23265 (O18:K5), isolated from neonatal meningitis, and *E. coli* IH 3136 (O1:K1), isolated from neonatal septicaemia. All strains were stored at -70°C in skimmed milk in small volumes until used. The inoculum for injection was prepared by growing the bacteria overnight in Luria broth (Miller, 1972) at 37°C on a rotatory shaker, then diluting the culture 10-fold in fresh broth and growing for a further 120 min in the same conditions.

Experimental infection

(CBA $\times$ C57BL/6) F1 hybrid mice (8–10 weeks old) bred at this Institute were used in most assays. C3H/HeJ mice were obtained from the breeding unit of the Department of Bacteriology and Immunology, University

of Helsinki and used at 8–16 weeks old. The bacterial challenge (10-fold dilutions of exponentially-growing bacteria in 0.2 ml of saline) was injected intraperitoneally (ip). The LD50 was determined from the day 5 survival data of groups of 6 mice (Reed and Muench, 1938).

Tissue counts of bacteria

Samples were taken from the bacterial inoculum as well as the peritoneal cavity and blood at different times (three mice for each time point) for measuring the number of viable bacteria (cfu) by plating out a series of dilutions. The blood samples (100 µl) were obtained from the retro-orbital plexus of mice anaesthetised with ether. Thereafter the mice were killed by cervical dislocation, and the peritoneal fluid was harvested by injecting 2 ml of saline ip and withdrawing 0.5 ml after gentle massaging. The dilution factor was included in all calculations. Each point in the figures represents the number of bacteria (cfu)/total volume of blood or peritoneal fluid and is the geometric mean of results from three mice.

LPS preparations

E. coli O18 LPS was isolated from strain EH 817 (serotype O18:K1⁻), a spontaneous non-capsulate mutant of strain IH 3080 (Vaara *et al.*, 1984), by the phenol-water method (Westphal and Jann, 1965). *E. coli* K-12 LPS was isolated by the phenol-chloroform-petroleum ether method (Galanos *et al.*, 1969) from strain W3350 received from Dr M. Brammer, University of Leicester. Both LPSs were diluted in water, stored at -70°C in small volumes and sonicated briefly before use. Detoxified endotoxin (monophosphoryl lipid A; MPL) from *Salmonella typhimurium* ReG30/C21 was purchased from Ribi Immunochemicals Research Inc., MT., USA and dissolved in triethylamine 0.1% v/v to a concentration of 2 mg/ml.

LPS pretreatment

LPS preparations (0.1-ml volumes) were injected subcutaneously (sc) or ip into mice at different times before bacterial challenge.

Assays for LPS toxicity

Pyrogenicity was determined in New Zealand White rabbits by intravenous (iv) injection of the LPS preparations and by monitoring the rectal temperature change according to the European Pharmacopoeia (1971).

Lethality was determined in 12-week-old, F1 CBA × C57BL/6 mice by ip injection of 12.5 mg of galactosamine diluted in sterile water followed by iv injection of LPS within 1 h. The lethality in mice was recorded after 72 h. (Galanos *et al.*, 1979).

Results

E. coli O18:K1 infection in convalescent mice

When mice were given a sublethal dose of live *E. coli* O18:K1 by ip injection (0.1 LD50 = 4×10^4 cfu/mouse, which they were able to clear in 24 h) and challenged ip 48 h later with a second bacterial dose, they effectively eliminated an otherwise lethal (10 LD50) *E. coli* O18:K1 challenge (fig. 1). Thus, by 4 h after challenge, the bacteria had already grown rapidly in the peritoneal cavities of untreated mice (fig. 2A), whereas in convalescent mice the numbers were already reduced (5.5×10^5 cfu compared with 7.9×10^7 cfu in the controls; fig. 1). After 24 h, the inoculum was completely cleared in the convalescent mice and all of the control animals were dead. Correspondingly, the LD50 of the *E. coli* O18:K1 strain was at least 50-fold higher in the convalescent mice than in normal mice ($> 2 \times 10^7$ and 4×10^5 cfu/mouse, respectively).

E. coli O18:K1 infection in mice pretreated with LPS

To determine whether comparable resistance to infection could be induced by LPS, *E. coli* O18 LPS was isolated from a non-capsulate mutant of the challenge strain and injected ip 24 h before chal-

Fig. 1. Viable bacteria in the peritoneal cavity 4 h after an ip challenge with 4×10^6 cfu of *E. coli* O18:K1. Mice had been given a sublethal *E. coli* O18:K1 ip infection 48 h before challenge (convalescent), or 5 µg of LPS ip 24 h before challenge (K-12 LPS or O18 LPS) or no pretreatment (control). The challenge dose is indicated by an arrow. Each point represents the geometric mean of results from three mice; the vertical lines show the scatter between the values.

Fig. 2. Time course of *E. coli* O18:K1 infection in normal (—) and K-12 LPS-treated (5 μ g of LPS sc 24 h before challenge) (---) mice: **A** peritoneal cavity; **B** blood. The challenge dose is indicated by an arrow. Each point represents the geometric mean of results from three mice; the vertical lines show the scatter between the values.

lence. The mice survived, showing that LPS indeed had a protective effect. We then proceeded to characterise the LPS effect in a systematic way.

The LPS effect could be seen by 4 h after challenge as a reduction in the number of bacteria in the peritoneal cavity (6.7×10^6 cfu compared with 7.9×10^7 cfu in the control mice; fig. 1). The specificity of the LPS was not important; LPS purified from the rough *E. coli* K-12 gave the same result as the homologous LPS (fig. 1).

The number of viable *E. coli* in the peritoneal cavity of the LPS-treated mice increased as in control mice for the first 3 h after challenge (fig. 2A). Thereafter, the number started to decrease in the LPS-treated, but not in the control, mice. The bacterial counts in the blood paralleled the bacterial growth in the peritoneal cavity (fig. 2B). By 10 h after challenge, the LPS-treated mice had cleared most of the injected bacteria; all these mice survived the infection that was lethal to control animals. On the basis of these experiments, 4 h after challenge was considered a suitable time for demonstrating the LPS effect on bacterial growth.

The route of LPS administration was not important for the development of resistance; both ip and sc injections resulted in a similar resistance in the mice (fig. 3). Subsequently, the sc route was used to avoid causing a local inflammatory reaction in the peritoneal cavity.

In the LPS-hyporesponsive mouse strain, C3H/HeJ, which was 1000-fold more susceptible to the *E. coli* O18:K1 peritonitis, we could not show any

LPS-induced increase in resistance (data not shown).

If the interval between the injections of LPS and bacteria was 24 h instead of 2 h, the LPS treatment was more effective (fig. 3). The increase in resistance lasted for 4 days from the LPS injection (data not shown) and two separate LPS injections ($5 \mu\text{g}/\text{injection}$) within 24 h did not further increase the efficacy of the LPS treatment as estimated by the number of viable *E. coli* in the peritoneal cavity at 4 h (fig. 3). Furthermore, if the mice were given four injections of LPS within 14 days ($5 \mu\text{g}/\text{injection}$, sc), the growth of a lethal *E. coli* challenge in the peritoneal cavity was prevented if the last injection was given on day 1, but not if it was given on day 7 or day 14 before infection (data not shown).

The dose-response range for the LPS-induced resistance was rather wide (table I). Even an LPS dose of $0.5 \mu\text{g}/\text{mouse}$ resulted in a 100-fold increase in LD50. All these doses are $\leq 5\%$ of the lethal dose which is reported to be *c.* 1 mg/mouse for the K-12 LPS (Galanos *et al.*, 1979).

Effect of a detoxified LPS preparation on the E. coli O18:K1 infection

Because of the many adverse effects of LPS, it was of interest to determine whether a less toxic LPS derivative was still effective in inducing infection resistance. When $10 \mu\text{g}$ of MPL was injected sc 24 h before challenge with 2.5 LD50 of

Fig. 3. The effect of timing and route of LPS administration ($5 \mu\text{g}$ *E. coli* K-12 LPS per injection) on the numbers of viable bacteria found in the peritoneal cavities of the mice 4 h after challenge with 2.5 LD50 of *E. coli* O18:K1. The challenge dose is indicated by an arrow. Each point represents the geometric mean of results from three mice; the vertical lines show the scatter between the values.

Table I. Effect of LPS pretreatment 24 h before challenge on mortality from *E. coli* O18:K1 infection

Pretreatment preparation	Dose (μ g)	Number of mice dead/total on day 5 after challenge with	
		100 LD50	10 LD50
K-12 LPS injected sc	50	0/6	0/6
	5	0/6	0/6
	0.5	1/5	0/6
	0.05	nt	5/6
	0.005	nt	6/6
MPL injected sc	50	nt	0/6
	5	nt	6/6
	0.5	nt	6/6
none		6/6	6/6

nt = not tested.

E. coli O18:K1 the mice survived and decreased numbers of viable bacteria were detected at 4 h in the peritoneal cavities (fig. 4). With this rather low challenge dose the effect of MPL on the early course of the infection was thus similar to that of K-12 LPS. However, when the challenge dose was increased to 10 LD50, 100 times more MPL (50 μ g)

Fig. 4. The effect of different LPS preparations on the numbers of viable *E. coli* O18:K1 found in the peritoneal cavities of mice 4 h after a 2.5 LD50 challenge. The LPS preparations were injected sc 24 h before bacterial challenge. The challenge dose is indicated by an arrow. Each point represents the geometric mean of results with three mice; the vertical lines show the scatter between the values.

than *E. coli* K-12 LPS (0.5 μ g) was required to allow 100% survival of the mice (table I).

To test whether the reduced ability of MPL to induce resistance to infection was associated with a similar reduction in toxicity, we compared activities of MPL and *E. coli* K-12 LPS in two separate toxicity assays. In galactosamine-treated mice, which are 10⁵-fold more sensitive than normal mice to the lethal effects of LPS (Galanos *et al.*, 1979), an intravenous injection of MPL was 100-fold less toxic (LD50 = 100 ng) than *E. coli* K-12 LPS (LD50 = 1 ng) (table II). In the rabbit pyrogenicity assay also, a 100-fold larger dose of MPL than K-12 LPS was required to induce a significant fever response (200 ng/kg and 2 ng/kg of MPL and LPS, respectively, iv) (table III).

Effect of E. coli K-12 LPS pretreatment on systemic infections with other *E. coli* strains

Because LPS pretreatment protected mice against the standard *E. coli* O18:K1 infection, the role of non-specific infection resistance against two other capsulate *E. coli* strains was investigated. These challenge strains were clinical isolates from neonatal septicaemia and in both cases a pre-injection of rough-type *E. coli* K-12 LPS provided significant protection from a lethal bacterial challenge (table IV).

Discussion

Mice that had recovered from a sublethal *E. coli* O18:K1 infection possessed a greatly increased resistance to a subsequent challenge with a lethal dose at a time, 1–2 days after the first infection, when an antibody response would hardly have had time to develop. A similar increase in resistance

Table II. Mortality of galactosamine-sensitised mice due to LPS or MPL

Galactosamine dose/mouse	Number of mice dead/total 48 h after injection* of	
	MPL	K-12 LPS
10 μ g	5/5	nt
1 μ g	4/5	nt
100 ng	3/6	4/6
10 ng	0/3	5/6
1 ng	nt	3/5

* LPS or MPL was injected iv in a volume of 0.2 ml, 20–60 min after galactosamine injection.

nt = not tested.

Table III. Pyrogenicity of LPS and MPL preparations in rabbits

Preparation	Dose (ng/kg)	Sum of increases of temperatures in 3 rabbits (°C)	Pyrogenicity*
K-12 LPS	2	3.20	+
	0.2	1.19	+
MPL	200	2.52	+
	20	0.59	—
	2	0.29	—

* A preparation was considered to be pyrogenic if the sum was $> 1.15^{\circ}\text{C}$ for 3 rabbits.

was achieved by pretreating the mice with a small dose of the homologous LPS or heterologous rough-type LPS. This suggested that LPS was the bacterial component responsible for the development of resistance.

The observation that minute LPS doses can increase the host's resistance to infection is not new (Rowley, 1955; Landy, 1956; Abernathy, 1957; Cluff, 1970; Parant, 1983). Although the exact mechanisms leading to restriction of bacterial proliferation and survival of the animals are still not known, the activation of long-living phagocytic cells (macrophages) has been considered to be important (Galelli *et al.*, 1977). This view is also supported by studies demonstrating that LPS can induce non-specific resistance to infection in neutropenic, immunosuppressed mice, in which macrophages are believed to be the main functional phagocytic cells (Tegtmeier and Andersen, 1983; Vuopio-Varkila, 1987). From *in-vitro* studies it is known that LPS stimulation increases the production of interleukin 1 (IL-1) by mononuclear cells (Gery and Waksman, 1972). IL-1 release is, on the

Table IV. Effect of LPS pretreatment on mortality from different *E. coli* infections

Pretreatment	Number of mice dead/total on day 5 after challenge with <i>E. coli</i> strain and dose* (cfu/mouse)		
	018:K5 (3.6×10^7)	01:K1 (2×10^6)	018:K1 (4×10^6)
K-12 LPS 5 μg /mouse sc 24 h before challenge	0/5	0/6	0/6
None	6/6	6/6	6/6

* Challenge doses were c. $10 \times \text{LD}_{50}$.

other hand, required for maximal macrophage activation.

To learn more of the mechanism of the LPS-induced infection resistance a well-characterised *E. coli* O18:K1 infection model was used. Further evidence of the non-specific nature of the LPS effect was provided by two other *E. coli* strains against which the rough K-12 LPS was also shown to be protective. LPS pretreatment has a similar effect on experimental infections with *Klebsiella* sp., group B streptococci and *Listeria monocytogenes* infections in the mice (Vuopio-Varkila, unpublished observations). The finding that the LPS can be injected in a site different from that of the bacterial challenge site suggests that a systemic, non-specific cell activation was responsible for the effect. In the C3H/HeJ (LPS-hyporesponsive) mice, LPS pretreatment failed to increase resistance to *E. coli* O18:K1 peritonitis. Similar results have been reported in an experimental klebsiella infection model (Chedid *et al.*, 1976). This mouse strain is known to be defective in many B-cell and macrophage responses to LPS as well as in induction of acute-phase serum proteins after LPS administration (Rosenstreich, 1985). Also, IL-1 release from macrophages is deficient in these mice (Rosenstreich, 1985). Thus, this finding gives further evidence for the important role of macrophages in LPS-induced infection resistance.

In normal mice, *E. coli* O18:K1 multiplies rapidly in the peritoneal cavity and, with a high challenge dose, this leads to death in 24–48 h. In LPS-treated mice, the challenge organisms multiplied in a similar manner for the first 3–4 h but thereafter the numbers of viable bacteria started to decline and the mice survived. The decrease in bacterial numbers, and resulting recovery, were similar to events in control mice challenged with a smaller inoculum, but occurred much earlier.

The known adverse effects of LPS, such as fever, hypotension, the local Shwartzman reaction, are ascribed to the lipid A part of endotoxin (Luderitz *et al.*, 1978). Recently, attention has been directed to the possible use of LPS derivatives with less toxic effects such as the chemically-modified, monophosphoryl lipid A (MPL; Ribi, 1984). It has also become evident that detoxification of bacterial LPSs may take place *in vivo* by the enzymic activity of human neutrophils (Munford and Hall, 1986).

Our studies with MPL revealed that although it was 100-fold less toxic for rabbits and mice, as measured by fever induction or lethal toxicity, 100-fold larger doses were required for optimal resistance to infection compared to native rough-type LPS. No such relationship has been previously

reported for MPL but in most other studies its effects have not been quantitated (Chase *et al.*, 1986; Madonna *et al.*, 1986; Masihi *et al.*, 1986). In fact, the pyrogenic and mitogenic actions of IL-1 appeared to be related (Duff and Durum, 1983), suggesting that if fever induction were reduced then the macrophage activation step also would be

limited. However, a recent report has described a short peptide fragment of human IL-1 devoid of pyrogenic activity but still potent in immunostimulation (Antoni *et al.*, 1986).

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