Enhanced Growth Restriction of *Legionella pneumophila* in Endotoxin-Treated Macrophages (43439)

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Abstract. **Macrophages from A/J mice are permissive for growth of *Legionella pneumophila*, an intracellular opportunistic pathogen that grows preferentially in macrophages. Macrophages from other mouse strains are highly resistant to growth of Legionella. In the present study, it was found that macrophages from A/J mice are readily activated by pretreatment with lipopolysaccharide (LPS), so that the cells do not permit Legionella to replicate *in vitro*, as occurs when untreated macrophages from A/J mice are cultured with these organisms for 48 hr. The augmentation of Legionella growth inhibition by LPS-activated macrophages from nonpermissive BDF_1 mice also occurred. After *in vitro* infection, there was a 1000-fold increase in the number of Legionella in A/J macrophages and approximately a 10-fold increase in BDF_1 macrophages, but LPS treatment of macrophages from either strain resulted in marked growth restrictions. This suppression was both dose dependent as well as dependent upon the time of addition of the LPS to the macrophages. Furthermore, the lipid A component of LPS was found to be as effective as the intact LPS in activating macrophages to inhibit the intracellular growth of Legionella. Further studies concerning the mechanisms involved are clearly warranted and in progress.** [P.S.E.B.M. 1992, Vol 200]

Legionella pneumophila is an opportunistic intracellular pathogen and replicates in blood monocytes or macrophages from humans or guinea pigs and also causes disease in man and guinea pigs. These bacteria, however, do not infect most strains of mice as readily as guinea pigs (1–3). Recently, we reported that thioglycollate-induced macrophages from A/J mice support the growth of Legionella *in vitro* (4). This strain of mice is much more permissive for Legionella infection than other mouse strains. However, the mechanism of the differences in growth of Legionella in macrophages from permissive A/J mice as compared with replication of the bacteria in macrophages from other mice which are nonpermissive is still unclear.

Bacterial endotoxins are known to be among the most potent activators of macrophages, including macrophages from mice (5–9). In the present study, we examined the effects of endotoxin treatment of macrophages from permissive A/J mouse macrophages versus effects on macrophages from a nonpermissive mouse strain in regard to intracellular growth of Legionella. We found that treatment of macrophages from A/J mice *in vitro* with endotoxin for 24 hr resulted in marked inhibition of growth of Legionella subsequently infecting the cells *in vitro*. This growth inhibition was similar to the normal inhibition occurring in macrophages from nonpermissive mice. The effect observed was dose dependent as well as dependent upon the time of addition of the endotoxin to the macrophage cultures. A purified lipid A preparation from intact endotoxin was also effective in inducing anti-Legionella activity and it seemed likely that this component of lipopolysaccharide (LPS) was important in the response.

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Materials and Methods

Mice. Inbred female A/J, BDF_1, and C_3H/HeJ mice were used for these studies. They were purchased from Jackson Laboratories, Bar Harbor, ME, and were used at approximately 7–10 weeks of age.

Legionella. A virulent strain of *L. pneumophila*, Serogroup 1, was used for these experiments. The organism was obtained at autopsy from a case of fatal legionellosis at Tampa General Hospital and cultured on buffered charcoal yeast extract agar exactly as described previously (4).

Endotoxin. Lipopolysaccharide (LPS) from *Escherichia coli* 011:B4 was used for these studies as the macrophage activator. The entotoxin that was prepared by the phenol water extraction procedure was obtained from Sigma Chemical Co., St. Louis, MO. Monophosphoryl lipid A (MLA) derived from *E. coli* was purchased from Ribi Immunochemical Co., Hamilton, MT. Legionella LPS was prepared by boiling an overnight suspension of bacteria and extraction with phenol water as described previously (10, 11).

Macrophages. Elicited peritoneal macrophages were obtained from individual mice 3–4 days after intraperitoneal injection of 3% thioglycollate obtained from Difco Laboratories, Detroit, MI (4). Elicited macrophages were collected with 5 ml of Hanks' balanced salt solution and suspended in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum. Cell numbers were counted with a hemocytometer and the macrophages were allowed to adhere to tissue culture plates (24-well plates; Costar, Cambridge, MA) for 2 hr in 5% CO_2 at 37°C. The resulting monolayers were washed with Hanks' balanced salt solution.

Legionella Growth. The macrophage monolayers (approximately 1×10^6 cells/well) were incubated for 24 hr with RPMI 1640 medium which contained 10% fetal calf serum in the presence or absence of the endotoxin. The monolayers were then infected with Legionella (1×10^6 bacteria/well) for 30 min at 37°C in an atmosphere of 5% CO_2, washed to remove nonphagocytized bacteria, and then reincubated with medium for 48 hr (4, 12). The number of viable bacteria in lysates of the macrophage cultures after lysing with sterile distilled water was determined in buffered charcoal yeast extract agar by standard plate counting.

Statistical Analysis. Comparisons between experimental groups were performed according to Student's *t* test.

Results

As is apparent in Figure 1, the Legionella grew in cultures of macrophages from A/J mice as compared with the minimal growth in macrophage cultures from nonpermissive BDF_1 mice. There was approximately a 100-fold difference in growth of the bacteria in the macrophages between permissive A/J and nonpermissive BDF_1 mouse strains. The number of Legionella 48 hr after infection of macrophages from A/J mice represented an approximately 1000-fold increase, whereas the much smaller number of Legionella in macrophages from the BDF_1 mice showed only a maximum 10-fold increase as compared with the number at the time of culture infection 48 hr earlier. Addition of *E. coli* LPS at a concentration of 5 μg/ml markedly suppressed growth of Legionella in the macrophages from the permissive A/J mice. The number of Legionella after 48 hr of culture in LPS-treated macrophages from A/J mice was reduced almost 100-fold. However, the LPS-pretreated macrophages bound the same numbers of Legionella as the untreated macrophages. Only the growth of the Legionella in the cells was affected. Furthermore, the number of Legionella growing in LPS-treated cells from these permissive mice was almost the same as the number that grew in macrophages from nonpermissive BDF_1 mice. It is noteworthy, however, that even when nonpermissive macrophages from BDF_1 mice were treated with LPS for 24 hr, the number of bacteria was lower than the initial number at zero time in untreated macrophages.

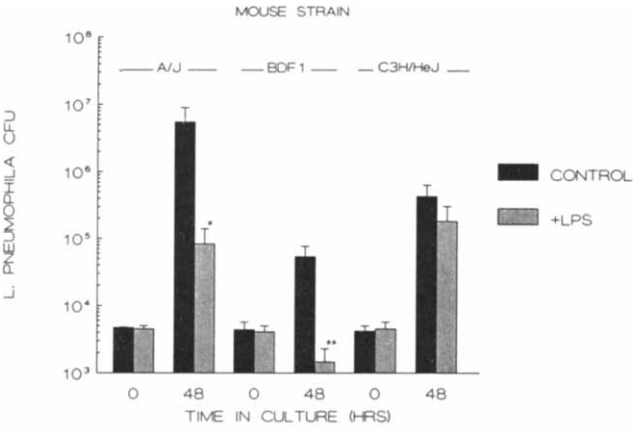

Figure 1. Effect of LPS pretreatment of macrophage cultures on resistance to *L. pneumophila in vitro*. Macrophages were obtained from indicated mouse strain and treated with 5.0 μg/ml of *E. coli* LPS for 24 hr before challenge infection with Legionella. Infection occurred with 10^6 bacteria for 30 min and macrophages were then washed to remove nonphagocytized bacteria. The number of viable bacteria (colony-forming units) in macrophage lysates was determined 0 and 48 hr after infection. Data represent mean ± SD for three experiments. * $P < 0.05$; ** $P < 0.01$.

C_3H/HeJ mice are well known as low-responders to LPS. The macrophages from this mouse strain showed an intermediate position in regard to permissiveness for Legionella growth. Figure 1 shows the partial permissiveness of C_3H/HeJ mouse macrophages to the growth of Legionella. However, the growth of the bacteria in macrophages from these mice was not significantly altered by their treatment with LPS. Thus, unlike the effect of LPS on macrophages from both nonpermissive BDF_1 and permissive A/J mice, LPS

treatment of macrophages from this mouse strain had little effect.

Inhibition of Legionella growth in macrophages from A/J mice only occurred when LPS was used to pretreat the cultures prior to infection with Legionella. Little or no effect was noted when LPS was added at the time of challenge infection with Legionella or 24 hr afterward. Furthermore, when LPS was added 4, 8, or 12 hr before challenge infection, little, if any, effect on subsequent infection with Legionella was noted.

Inhibition of Legionella growth in LPS-treated macrophages was dose dependent. As is apparent from Table I, marked suppression occurred when as little as 0.5 μg of LPS/10^6 macrophages was added 24 hr before infection. There was almost an 85–95% decrease in Legionella growth with this dose. One microgram or 5 μg LPS/ml resulted in greater suppression of Legionella growth by the macrophages. A larger dose of LPS resulted in macrophage loss and appeared toxic for the cultures.

A major component of LPS is considered to be the lipid A moiety. MLA is relatively nontoxic as compared with whole LPS and was found effective in inducing resistance of macrophages to infection with Legionella. As is apparent in Table II, this component of *E. coli* LPS was essentially as effective as the same amount of intact LPS in inducing resistance of the macrophages from the permissive A/J mice. Similarly, the nonpermissive macrophages from BDF_1 mice supported the growth of Legionella even less when pretreated with MLA as compared with the *E. coli* LPS (data not shown). It is of interest that LPS from the homologous Legionella species was as effective as the LPS from *E. coli* in inducing the macrophages from A/J mice to evince resistance to subsequent infection with Legionella. Similar results were observed with the macrophages from nonpermissive BDF_1 mice.

Table I. Effect of LPS Concentration on Resistance of A/J Mouse Macrophages to Infection *In Vitro* by *L. pneumophila*

LPS concentration[a] (μg/ml)	Legionella growth[b]	
	Colony-forming units (× 10^4)	Percentage of control
None (control)	710 ± 40	100
0.5	43 ± 20[c]	6.0
1.0	6.2 ± 2.1[d]	<1.0
5.0	4.0 ± 1.8[d]	<1.0

[a] Macrophage cultures were treated with indicated concentration of *E. coli* LPS for 24 hr before challenge infection with Legionella. Monolayers were washed to remove nonphagocytized bacteria 30 min after infection and incubated in fresh medium without LPS for 48 hr.
[b] Average number of colony-forming units ± SD for three triplicate cultures after 48 hr of infection.
[c] $P < 0.07$.
[d] $P < 0.03$.

Table II. Effect of LPS Preparations on Resistance of A/J Mouse Macrophages to Infection *In Vitro* by *L. pneumophila*

Macrophage treatment[a]	Legionella growth[b]	
	Colony-forming units (× 10^4)	Percentage of control
None (control)	360.0 ± 26	100
E. coli		
LPS	3.4 ± 1.5[c]	<1.0
MLA	4.7 ± 0.8[c]	1.3
Legionella		
LPS	3.0 ± 1.2[c]	<1.0

[a] Elicited macrophages were treated *in vitro* with *E. coli* or Legionella LPS (5.0 μg) or MLA (5.0 μg) for 24 hr before infections with *L. pneumophila in vitro*, and then washed to remove nonphagocytized bacteria 30 min after infection and incubated with new media.
[b] Average number of colony-forming units ± SD 48 hr after infection of three or more cultures per group for three experiments.
[c] $P < 0.0001$.

Discussion

Infection of permissive A/J mouse macrophages with Legionella occurs *in vitro* as compared with nonpermissiveness of macrophages from other mouse strains, including BDF_1 mice (4). The mechanisms of such differences between A/J mouse macrophages and those from other mouse strains in terms of infection with Legionella is not yet understood. A/J mice have been reported to be deficient in the killing capacity of intracellular microorganisms such as Chlamydia (13), as well as tumor cell killing (14). The lack of ability of the macrophages from these animals to evince cytolytic activity against targets as diverse as Chlamydia and tumor cells suggests that there may be a defect in the functional activity of macrophages from these animals. In the present study, it was found that endotoxin treatment of macrophages from A/J mice for 24 hr was effective in altering the permissiveness of the cells to intracellular growth of Legionella *in vitro*. Similar results with A/J mouse macrophages have been reported by this laboratory (15), i.e., treatment of A/J macrophages with recombinant γ-interferon leads to an inhibition of Legionella growth. Such results suggest that A/J mouse macrophages are suitably responsive to cytokines and LPS in restricting the growth of Legionella. LPS-treated nonpermissive macrophages obtained from BDF_1 mice showed more restriction of the Legionella growth. In contrast, macrophages from C_3H/HeJ mice were partially permissive but were not able to be converted to nonpermissiveness by treatment with LPS. This clearly supports the view that activation of macrophages to induce nonpermissiveness for the growth of Legionella is induced by LPS.

It has been widely recognized that endotoxins are not only potent activators of macrophages, but also nonspecifically enhance resistance of mice to a variety

of infectious agents, including *Mycobacterium tuberculosis* and parasites (5, 16–18). These effects induced by endotoxin may be mediated through enhanced levels of cytokines produced by macrophages, since it is well known that LPS is a potent stimulator of macrophage production of interleukin 1 as well as tumor necrosis factor (19–21). Preliminary studies have shown that infection of mice with Legionella results in induction of relatively high levels of interleukin 1 and tumor necrosis factor, but there seems to be no difference in production of these cytokines by macrophages from permissive A/J mice as compared with nonpermissive mice (4).

It is well known that a wide variety of microbicidal metabolites, including various enzymes and anions, have effects on intracellular bacteria and fungi. In other studies in this laboratory, it was found that LPS treatment of macrophages can induce the production of such metabolites, but there is essentially no difference in activation of peritoneal macrophages from A/J mice versus other mouse strains, such as BDF_1, in terms of metabolite induction by LPS. Thus, there seems to be no correlation between the ability of macrophages to respond to LPS by production of cytokines or antimicrobial metabolites and resistance to Legionella infection. However, it is clear from this study that an activator of macrophages such as LPS has a potent effect in inducing macrophages to resist infection with Legionella. Activation of macrophages by killed bacteria, including Legionella or *E. coli* vaccine, or other known macrophage activators such as muramyl dipeptides, also decreases the permissiveness of macrophages from A/J mice for *in vitro* infection with Legionella (unpublished data).

Other studies have shown no difference in binding of Legionella *in vitro* to macrophages from A/J or BDF_1 mice with or without pretreatment with LPS. Thus, the initial interaction of the bacteria with macrophages is not affected by activation of the cells with LPS (unpublished data). However, the ability of the cells to permit replication of Legionella is markedly altered following treatment with LPS. It is not clear whether this is due to killing of the bacteria by the activated macrophages or the inability of the bacteria to grow and/or replicate in the activated macrophages. However, the latter situation seems to be most likely, since there is little evidence of enhanced killing of Legionella by A/J macrophages versus macrophages from nonpermissive BDF_1 mice with or without treatment with activators such as cytokines, interferon, or even LPS.

Further studies are warranted to examine in greater detail the mechanisms whereby LPS activates macrophages from a permissive mouse strain such as A/J to inhibit the replication of an important opportunistic pathogen like Legionella. It is apparent, however, that intact LPS is not necessary for activation of the macrophages since an important component of the lipopolysaccharide, i.e., the lipid A moiety, is sufficient for activation. It is widely accepted that lipid A is the major biologic component of LPS that accounts not only for the toxicity of the whole molecule, but also for the ability of LPS to induce a wide variety of host factors attributed to stimulation of macrophages (22). Thus, it seems likely from this study that the lipid A component of LPS-treated macrophages is as effective as its intact LPS. This effect is similar to the many observations that lipid A can stimulate antibacterial immunity both *in vivo* and *in vitro* and that a nontoxic derivative, i.e., MLA, has similar effects.

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