INFECTION AND IMMUNITY, Dec. 1993, p. 5056–5061
0019-9567/93/125056-06$02.00/0

Vol. 61, No. 12

# *Legionella pneumophila* Growth Restriction in Permissive Macrophages Cocultured with Nonpermissive Lipopolysaccharide-Activated Macrophages

SATORU ARATA,† CATHERINE NEWTON, THOMAS W. KLEIN, AND HERMAN FRIEDMAN*

*Department of Medical Microbiology and Immunology, University of South Florida College of Medicine, 12901 Bruce B. Downs Boulevard, Tampa, Florida 33612*

Received 23 July 1993/Accepted 10 September 1993

**Macrophages can be activated by lipopolysaccharides (LPS) from gram-negative bacteria to evince a number of biological activities, including increased resistance to intracellular infection by opportunistic bacteria. In the present study, intraperitoneal injection of LPS into A/J mice activated peritoneal macrophages so that they resisted subsequent in vitro infection with *Legionella pneumophila*. Coculture of these macrophages with those from nontreated A/J mice converted the entire population of cells from permissive to nonpermissive. This effect did not appear to be mediated by soluble factors released from the LPS-treated macrophages, since the levels of interleukins-1 and -6 and tumor necrosis factor alpha produced by the macrophages were not found to be markedly elevated at the time when the macrophages from the LPS-treated mice were most effective in converting normal macrophages to nonpermissiveness. Furthermore, macrophages from mice injected intraperitoneally with either interferon or tumor necrosis factor alpha did not evince nonpermissiveness and also did not have the ability to convert normal spleen cells to nonpermissiveness. Polymyxin B, a known inactivator of LPS activity, did not inhibit the macrophages from the LPS-treated mice from inducing this resistance. It seemed unlikely that free LPS released from the macrophages mediated this effect. The results of this study thus showed that macrophages activated by LPS in vivo can evince nonpermissiveness for *Legionella* growth in vitro and also can induce macrophages from normal, permissive mice to become nonpermissive for *Legionella* growth in vitro.**

Lipopolysaccharide (LPS; or endotoxin), an outer membrane component of gram-negative bacteria, activates macrophages, resulting in production and release of several cytokines, including interleukin-1α (IL-1α), IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) (15). While these cytokines under certain conditions are toxic to the host, resulting in septic shock (4), they are also involved in a cascade of events which protect against bacterial, especially intracellular, infection (5, 6, 9, 14, 17). The mechanisms involved in the interaction of LPS with surface receptors, thereby activating secondary signals and ultimately activating the macrophage, are currently being investigated by many groups. Several LPS-binding receptors have been identified, including CD14 (22), CD11-CD18 (21), and a 73-kDa protein (13). Additionally, a protein in serum has been shown to bind LPS prior to interaction with a receptor on the cell (18). However, there are still many questions concerning the mechanism of LPS binding to macrophages, including signal transduction, duration of macrophage activation, and effects of metabolism or detoxification of LPS bound to the macrophages. We have reported that thioglycolate-elicited macrophages from A/J mice (TG-Mϕ) are permissive for intracellular growth of *Legionella pneumophila*, an opportunistic pathogen (23), but upon in vitro LPS treatment become resistant to the organism (7). In the present study, it was found that coculturing of limited numbers of nonpermissive macrophages from LPS-treated mice with permissive macrophages from normal A/J mice resulted in conversion of the entire population of macrophages to a state of nonpermissiveness for *Legionella* growth. Such conversion occurred with as few as 1% macrophages from the LPS-treated mice.

* Corresponding author.
† Present address: Department of Chemistry, Showa University Medical School, Tokyo, Japan.

## MATERIALS AND METHODS

**Animals.** Inbred female A/J mice were purchased from Jackson Laboratories, Bar Harbor, Maine. They were housed in groups of 8 to 10 and fed commercial mouse chow and water ad libitum.

**Bacteria and endotoxin.** *L. pneumophila*, serogroup 1, was originally obtained from a case of legionellosis at Tampa General Hospital and maintained as frozen stock at −70°C. Prior to use, the frozen organisms were cultured on buffered charcoal-yeast extract agar for 48 h. The LPS used was prepared by phenol-chloroform extraction of *Salmonella minnesota* R595 (11).

**Macrophages.** Macrophages were elicited from A/J mice by intraperitoneal injection of 3 ml of thioglycolate (TG) medium (Difco Laboratories, Detroit, Mich.) 4 days prior to harvesting. For LPS-activated macrophages (LPS-Mϕ), TG-injected mice were given an intraperitoneal injection of LPS (30 μg per mouse) at various times during the last 24 h of the TG priming. In other experiments, TG-elicited mice were injected intraperitoneally with either recombinant murine gamma interferon (IFN-γ, 2,000 U; Pharmingen, San Diego, Calif.) or TNF-α (1,000 U; Genzyme, Cambridge, Mass.) during the last 24 h of TG priming. Macrophages were collected by peritoneal lavage, using 5 ml of phosphate-buffered saline. The macrophages were then washed in Hanks' balanced salt solution (Sigma, St. Louis, Mo.) and resuspended in RPMI 1640 medium (Sigma) plus 10% fetal calf serum (Hyclone, Logan, Utah).

***Legionella* CFU assay.** TG-Mϕ, LPS-Mϕ, IFN-γ-Mϕ,

TNF-α-Mϕ, or a mixture of these at a total of $10^5$ cells per well was adhered to 96-well tissue culture plates (Costar, Cambridge, Mass.) for 2 h at 37°C to remove nonadherent cells. The macrophages were then washed and incubated for 24 h prior to infection with legionellae (20:1) for 30 min at 37°C. The extra legionellae were washed out with Hanks' balanced salt solution. After additional incubation for either 0, 24, or 48 h, the macrophages were lysed with 0.1% Saponin. The resulting lysates were diluted and plated on buffered charcoal-yeast extract agar plates to determine the CFU, as described previously (23). In other experiments, LPS-Mϕ were cultured for 1 to 4 days prior to the addition of TG-Mϕ and the continuation of the *Legionella* assay. In another series of experiments, TG-Mϕ were incubated with supernatants (1:3 dilution) generated from LPS-Mϕ of mice treated with the endotoxin for 0.5, 2, 4, or 12 h and cultured for 24 h.

**Cytokine supernatants.** Macrophage cultures, TG-Mϕ, LPS-Mϕ, or TG-Mϕ plus 10% LPS-Mϕ, were incubated in 24-well plates (Costar). After 24 h, supernatants were collected and tested for TNF or IL-1 activity.

**TNF assay.** TNF was determined with a $^{51}Cr$ release assay (1, 3, 8). WEHI-164 cells were labelled with sodium $^{51}$chromate (100 μCi per $10^6$ cells) for 1 h. Serial dilutions of serum samples and a TNF-α standard (Genzyme) were incubated in 96-well plates (Costar) with the labelled WEHI cells ($5 \times 10^3$ per well) for 18 h at 37°C. Supernatants were collected and counted in a Packard Cobra Gamma Counter (Packard, Sterling, Va.). Percent cytotoxicity was determined by the following formula: (cpm − spontaneous release)/(total − spontaneous release). The total units of TNF-α per milliliter were calculated from a standard curve, using recombinant TNF-α provided as described previously.

**IL-1 assay.** Supernatants (100 μl) were incubated with C3H/HeJ thymocytes ($1.5 \times 10^6$) in 100 μl of fetal calf serum-RPMI supplemented with concanavalin A (0.2 μg/ml) in 96-well plates (Costar). After 48 h, the cultures were pulsed with [$^3$H]thymidine (0.5 μCi per well) for 18 h (1, 3, 8). The cells were then harvested onto glass fiber filters and counted in a liquid scintillation counter. In addition, an enzyme-linked immunosorbent assay (ELISA), using monoclonal antibody to murine IL-1, was utilized exactly as described previously, and the amount of IL-1 protein was quantitated (8). The amount of IL-6 protein in the supernatants was also determined by ELISA, using monoclonal antibody to murine IL-6 (1).

**Polymyxin B pretreatment.** TG-Mϕ plus LPS (10 or 100 ng/ml), TG-Mϕ mixed with 1 or 10% LPS-Mϕ, and TG-Mϕ were incubated with 1 or 10 μg of polymyxin B sulfate (7,900 U/mg; Sigma) for 24 h prior to infection with *L. pneumophila*. Also, polymyxin B solutions, at the same concentrations, were added to cultures after the *L. pneumophila* was washed out.

**Statistical methods.** The data are presented as the arithmetic means ± standard errors of the means (SEM). One-way analysis of variance was used to evaluate the significance of differences between the means. Specific comparisons were conducted by the Student-Newman-Keuls method. A $P$ value of $<0.05$ was considered significant.

## RESULTS

**In vivo LPS treatment.** *L. pneumophila* has been shown to grow intracellularly in TG-Mϕ from A/J mice, i.e., permissive for *L. pneumophila* (23). In preliminary studies, macrophages were obtained from A/J mice injected with LPS doses of 1.0 to 200 μg per mouse 24 h before the TG-elicited cells were obtained. The macrophages were then infected in vitro with *L. pneumophila*. Macrophages from A/J mice injected with saline only as a control showed rapid replication of the *L. pneumophila* in vitro. Cells from mice given 1 μg of LPS showed about a 20 to 40% restriction of growth of *L. pneumophila*, while macrophages from mice given a 10- or 20-μg dose of LPS showed a 50 to 60% suppression of *Legionella* growth. Animals injected with 30 μg of LPS showed nearly a 90 to 95% inhibition of *Legionella* growth in the cells. The 50-, 100-, and 200-μg doses of LPS had essentially similar effects in converting macrophages from the A/J mice to a nonpermissive state. Thus, the 30-μg dose of LPS was used for all further studies. Macrophages from the A/J mice injected with this dose of LPS during the final 24 h of TG priming showed marked nonpermissiveness for in vitro growth of *L. pneumophila*. As indicated in Fig. 1, such resistance was most evident when the macrophages were obtained 30 min after LPS injection into the mice, and the macrophages showed progressively less nonpermissiveness with time, although even at 24 h there was still a five- to tenfold inhibition of their ability to replicate *L. pneumophila* compared with TG-elicited macrophages from control mice.

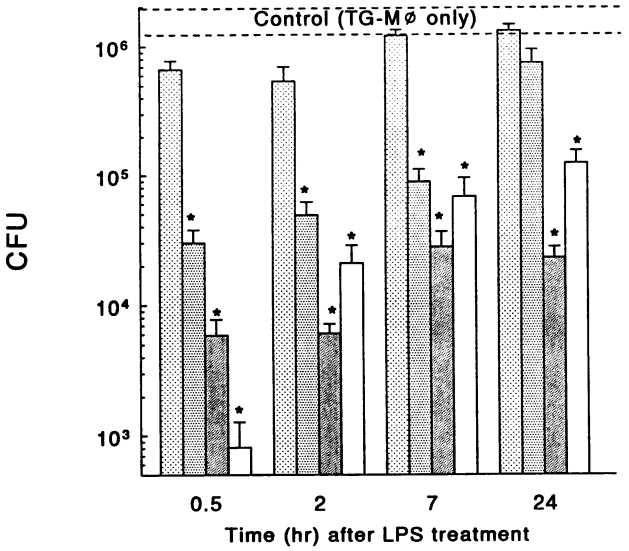


FIG. 1. Mice were injected with thioglycolate and then with LPS intraperitoneally (30 μg per mouse); peritoneal exudate cell macrophages were harvested 0.5 to 24 h after LPS treatment. These macrophages, either alone or combined at the indicated ratio with macrophages from thioglycolate-injected mice, were plated at a total of $10^5$ cells per well, cultured in vitro for 24 h, and infected with *L. pneumophila* (20:1); CFU were determined 48 h later. Each point represents CFU ± SEM for three to seven experiments. The asterisk indicates a significant difference from the TG-Mϕ control at $P < 0.05$. Symbols: ▨, 0.1% LPS-Mϕ; ▦, 1% LPS-Mϕ; ▩, 10% LPS-Mϕ; □, LPS-Mϕ only.

**LPS-Mϕ and TG-Mϕ cocultures.** Coculturing these resistant LPS-Mϕ in limited amounts with the permissive TG-Mϕ appeared to convert the cultures to nonpermissiveness. The addition of 0.1% LPS-Mϕ to normal cells had little effect on growth restriction (Fig. 1). However, the addition of 1 or 10% LPS-Mϕ to the cultures significantly inhibited the growth and replication of *L. pneumophila* (Fig. 1). While this restriction was effective for up to 24 h after LPS treatment of the donor mice for 10% LPS-Mϕ and up to 7 h for 1%

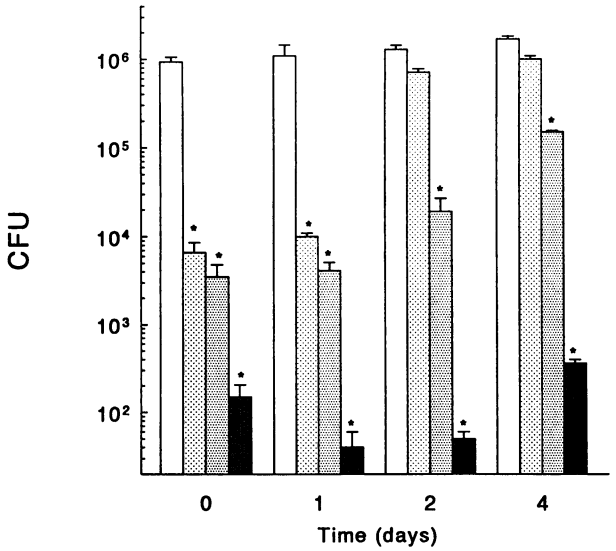


FIG. 2. Indicated numbers of macrophages (0 to $10^5$) from mice injected with LPS 0.5 h earlier were cultured with various numbers of TG-elicited macrophages for a total of $10^5$ cells per well for 1 to 4 days. The resulting cultures were then infected with *L. pneumophila* (20:1) and washed, and CFU were determined at 48 h. Results are expressed as mean CFU ± SEM for three separate experiments. The asterisk indicates a significant difference from the TG-Mϕ control at $P < 0.05$. Symbols: □, TG-Mϕ; ▨, +1% LPS-Mϕ; ▩, +10% LPS-Mϕ; ■, LPS-Mϕ.

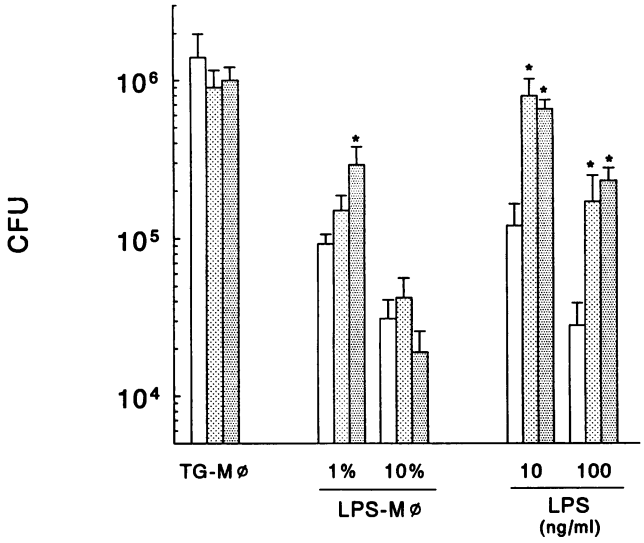


FIG. 4. TG-Mϕ, LPS-Mϕ and TG-Mϕ mixture, or TG-Mϕ plus LPS was treated with the indicated concentrations (□, none; ▨, 1 μg/ml; ▩, 10 μg/ml) of polymyxin B for 24 h. The cells were infected and washed, and the same concentrations of polymyxin B were added back. At 48 h, CFU were determined. Results are expressed as the mean CFU ± SEM for five separate experiments. The asterisk indicates a significant difference from the polymyxin B zero control at $P < 0.05$.

LPS-Mϕ, a 2-h or less posttreatment was optimal. While in vitro culturing of the LPS-Mϕ maintained their resistance for up to 4 days, their ability to confer this resistance to TG-Mϕ began to wane by day 2 (Fig. 2). When a time course of the resistance was performed with 2-h LPS-Mϕ, it was shown that the growth was restricted throughout the 48-h culture period (Fig. 3).

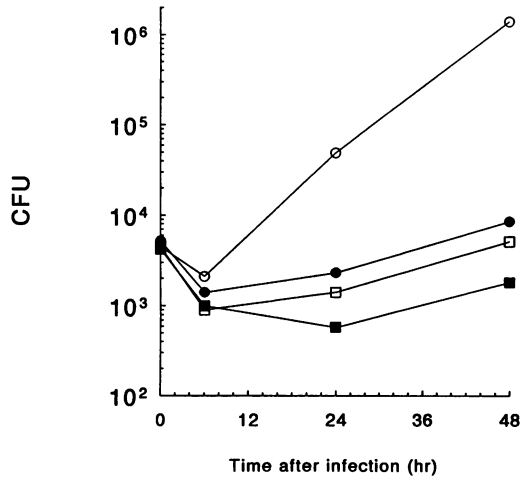


FIG. 3. Thioglycolate-elicited macrophages from LPS-injected mice (2 h preinjection) were cultured or cocultured (at the indicated ratio) for 24 h in vitro. Cultures were then infected with *L. pneumophila*, and CFU were determined 0, 6, 24, or 48 h later. Each point represents mean CFU of three to four cultures per point for a representative experiment from five separate experiments. Symbols: ○, TG-Mϕ only; □, +1% LPS-Mϕ; ■, +10% LPS-Mϕ; ●, LPS-Mϕ only.

**Polymyxin B treatment to eliminate the possibility of carryover of free LPS.** Polymyxin B treatment of the macrophages from LPS-injected mice was performed. This treatment by itself did not alter the activation of macrophages for growth restriction of *L. pneumophila* (Fig. 4). However, polymyxin B had an inhibitory effect on the ability of LPS to activate macrophages in vitro. It was noted, however, that the polymyxin B treatment of the LPS inhibited only partially the ability of the endotoxin to activate macrophages from A/J mice to resist *Legionella* growth in vitro. Although inhibition was statistically significant, it was apparent that macrophages treated with the 100-μg/ml dose of LPS and polymyxin B (either 1 or 10 μg/ml) did not result in *Legionella* growth that was as good as that in stimulated TG-elicited control macrophages. For example, there was less growth of *L. pneumophila* in macrophages activated with polymyxin B-treated LPS. Nevertheless, it is apparent that following incubation of LPS with polymyxin, the ability of the endotoxins to activate the macrophages to resist *Legionella* growth was still marked. However, polymyxin B at a concentration of either 1.0 or 10.0 μg/ml was more effective in inhibiting the 10-μg/ml dose of LPS, since growth of *L. pneumophila* was essentially normal after macrophages were cultured with this dose of LPS treated with polymyxin. However, it was evident that similar doses of polymyxin had either no or only limited effects on the LPS-macrophage cocultures in terms of inducing resistance to *Legionella* growth. In addition, the standard *Limulus* lysate test for endotoxin, which could measure as little as 0.01 ng of LPS, was negative for culture supernatants of 10% macrophages from mice injected 2 h earlier with 30 μg of LPS (unpublished data). Thus, it appeared unlikely from these experiments that free LPS was the major factor responsible for the effect noted.

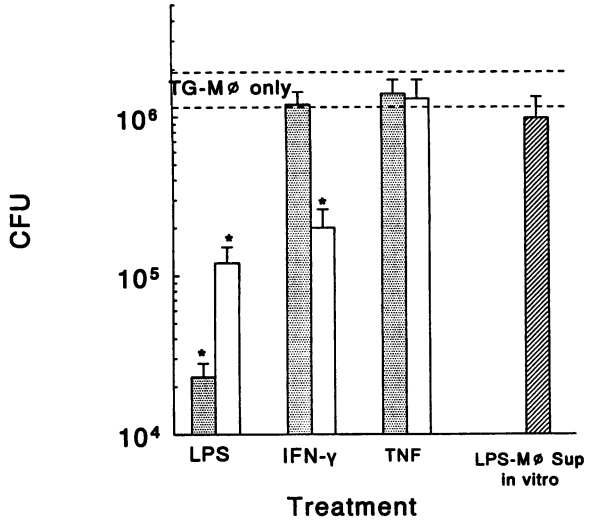


FIG. 5. Mice were injected with thioglycolate plus recombinant murine IFN-γ (2,000 U), recombinant murine TNF-α (1,000 U), or LPS (30 μg), and peritoneal macrophages were harvested 24 h later. Macrophages, alone or mixed with TG-elicited macrophages, were plated and cultured for 24 h. In some experiments, TG-elicited macrophages were cultured for 24 h with a 1:3 dilution of a 24-h supernatant from 2-h LPS-ϕ. Each culture was then infected with *L. pneumophila*, and CFU ± SEM were determined at 48 h. Results are expressed as the mean CFU ± SEM for three to four different experiments. The asterisk indicates a significant difference from the TG-Mϕ control at $P < 0.05$. Symbols: ▥, 10% treated Mϕ plus TG-Mϕ; □, treated Mϕ only; ▨, TG-Mϕ plus supernatant.

**Soluble factors.** Soluble factors released into the supernatants of LPS-Mϕ cultures did not appear to be involved in converting TG-elicited macrophages from normal A/J mice to nonpermissiveness. As shown in Fig. 5, supernatants obtained 24 h after culture of macrophages from mice injected 2 h earlier with LPS had no effect on normal macrophages in terms of altered growth inhibition of *L. pneumophila*. Similarly, supernatants obtained from macrophages 30 min or 2, 4, or 12 h after injection of the donor mice with LPS also had no effect (data not shown). In addition, injection of the recombinant cytokine TNF-α, rather than LPS, into mice 24 h earlier did not induce the macrophages from these mice either to restrict the growth of *L. pneumophila* or to convert normal macrophages to nonpermissiveness (Fig. 5). Similarly, when TNF-α was injected into the mice 4, 8, or 12 h prior to obtaining the macrophages from the donors, there was no effect on the ability of the cells to replicate *L. pneumophila* and also no effect on the ability of the cells to convert normal TG-elicited macrophages to a state of nonpermissiveness (data not shown). Recombinant IFN-γ had some effect, but it was less than that of LPS injection (Fig. 5). There was, however, increased production of the cytokines IL-1, IL-6, and TNF-α when macrophages were obtained from LPS-injected mice and cultured in vitro for 24 h. Similarly, when 10% macrophages from these LPS-injected mice were cocultured with normal TG-elicited macrophages for 24 h, there was also an increase in cytokine production (Table 1). However, it did not appear that this increase was directly related to the increased nonpermissiveness of the macrophage populations to replicate *L. pneumophila*. In additional experiments, it was found that after separation of the macrophages from the LPS-infected mice from cells from the normal mice by a cell-impermeable membrane in a standard microwell culture chamber (Costar Transwell plates), there was no alteration of the permissiveness of the normal macrophages for *Legionella* growth, although the macrophages from the LPS-infected mice still restricted the growth of *L. pneumophila* in the other compartment of the same culture well (Table 2). This occurred even when equal numbers of macrophages from both sources were used (a ratio of 1:1). Thus, it seemed unlikely that a cell factor mediated the effect.

TABLE 1. Production of TNF, IL-1, and IL-6 by macrophages after LPS stimulation

| Macrophages (mouse source) | Cytokine activity | | | |
|---|---|---|---|---|
| | TNF (U/ml)[a] | IL-1 bioassay (cpm, $10^3$)[b] | IL-1 protein (pg/ml)[c] | IL-6 protein (pg/ml)[c] |
| Normal | 11.7 ± 3.2 | 2.1 ± 0.9 | 9.8 ± 0.6 | 1.5 ± 0.8 |
| LPS treated | 157.8 ± 17.6 | 3.0 ± 1.1 | 68.9 ± 29.3 | 28.6 ± 2.9 |
| Normal plus LPS treated (10%) | 452.6 ± 32.1 | 5.3 ± 2.0 | 72.5 ± 8.5 | 21.1 ± 2.3 |

[a] Bioassay with $5 \times 10^3$ WEHI cells.
[b] Comitogenic bioassay with C3H/HeJ thymocytes.
[c] ELISA for secreted protein.

## DISCUSSION

Previous studies in this laboratory showed that *Legionella*-permissive thioglycolate-elicited macrophages from A/J mice could readily be activated in vitro by LPS to restrict the growth of *L. pneumophila* (7). The results of the present study show further that macrophages from LPS-injected mice also restricted the growth of *L. pneumophila*. Additionally, these LPS-treated macrophages were capable of activating normal TG-Mϕ to inhibit *Legionella* growth when as few as 1% LPS-Mϕ were added to TG-Mϕ cultures from non-LPS-treated mice. Such conferred resistance did not appear to be a function merely of carryover of free LPS, since polymyxin B treatment was essentially unable to inhibit this resistance. There was little, if any, effect of polymyxin B treatment of the macrophages from the LPS-treated mice in terms of suppression of their ability to convert normal macrophages to nonpermissiveness. However, the polymyxin B experiments do not completely rule out the possibility that free or bound LPS on the macrophages has a role. Similarly, in other pilot studies it was found that an antiserum to the LPS capable of neutralizing

TABLE 2. Growth of legionellae in mixed macrophage culture separated by cell-impermeable membrane

| Macrophage culture | *Legionella* growth (CFU)[a] | | |
|---|---|---|---|
| | 24 h | 48 h | 72 h |
| TG-Mϕ (normal control)[b] | $2.3 \times 10^4$ | $1.3 \times 10^6$ | $3.8 \times 10^5$ |
| LPS-Mϕ (LPS-treated donors)[c] | $7.6 \times 10^2$ | $1.8 \times 10^3$ | $1.4 \times 10^3$ |
| 10% LPS-Mϕ + TG-Mϕ[d] | $5.8 \times 10^2$ | $1.7 \times 10^3$ | $1.7 \times 10^3$ |
| 50% LPS-Mϕ + TG-Mϕ[d] | $2.9 \times 10^2$ | $1.2 \times 10^3$ | $1.5 \times 10^3$ |
| LPS-Mϕ (outer chamber)[e] | $3.9 \times 10^2$ | $1.9 \times 10^3$ | $1.6 \times 10^3$ |
| TG-Mϕ (inner chamber)[e] | $2.9 \times 10^4$ | $3.8 \times 10^6$ | $5.7 \times 10^4$ |

[a] Growth in three to five cultures at indicated times; SEM, <5%.
[b] Macrophages from peritonea of TG-elicited A/J mice.
[c] Mice injected with LPS (30 μg) 2 h before Mϕ were obtained.
[d] Mixed cocultures of LPS-Mϕ and macrophages from normal mice.
[e] Microcultures with cell-impermeable membrane between outer and inner chambers (Costar Transwell).

endotoxin activity at a relatively high concentration also failed to affect the ability of the macrophages from the LPS-treated mice to convert normal macrophages to nonpermissiveness (unpublished data). Thus, it appeared unlikely that free LPS released by the macrophages from the endotoxin-treated mice after LPS injection was directly involved in converting macrophages from normal mice to resist the growth of *L. pneumophila*. Although the ability of macrophages from the endotoxin-treated mice to do so continued for at least 24 h after injection of LPS into the donors, the macrophages were less capable of such conversion than those obtained at an earlier time. Nevertheless, it was noted that even after 4 days in culture the mixture of macrophages from LPS-treated donors with macrophages from normal mice still showed restriction of *Legionella* growth. Thus, if free LPS mediated this effect, it is noteworthy that nonpermissiveness of the cocultured cells was retained for at least 2 to 4 days.

Cytokines alone did not appear sufficient to mediate this effect, since supernatants (i.e., soluble factors) from LPS-treated macrophages had little, if any, effect in converting TG-Mϕ cultures from normal A/J mice to nonpermissiveness. Furthermore, while LPS-Mϕ and normal TG-Mϕ cocultures were found to produce elevated levels of IL-1 and TNF, as well as IL-6, which is known to contribute to the apparent IL-1-like bioactivity of the IL-1 detected by the comitogenic bioassay, treatment of the mice for up to 24 h with recombinant TNF-α or IFN-γ failed to induce a similar state of nonpermissiveness in the macrophages obtained from these mice or macrophages from such mice cocultured with normal TG-Mϕ. Furthermore, as shown in additional studies, incubation of the normal TG-Mϕ with LPS-Mϕ in microwell plates in which a cell-impermeable membrane separated the inner from the outer culture (Costar Transwell plates) did not affect the growth of *L. pneumophila* in the normal TG-Mϕ-containing compartment. Thus, such data suggest a requirement for cell-to-cell contact. Jirillo et al. showed that lipid A-activated monocytes evince increased phagocytosis of autologous polymorphonuclear cells by a cell-to-cell mechanism (10). While their data implied an involvement of membrane-associated cytokines, we do not believe that this is the mechanism in our system. IFN-γ has been shown to activate macrophages and stimulate the release of TNF and IL-1 (2, 3, 12, 16). As would be expected, we found that IFN-γ treatment reduced the permissiveness of macrophages to *Legionella* growth (12). However, such IFN-γ-treated macrophages were not able to affect TG-elicited macrophages to control *Legionella* growth. Therefore, while LPS and IFN-γ can activate thioglycolate-elicited macrophages to resist growth, only LPS treatment of the donor mice could affect the macrophages to confer resistance to naive TG-Mϕ. Such results, plus previous studies with cytokines in this laboratory, appear to argue against cytokine release by LPS-Mϕ as an activating factor for TG-Mϕ.

The results of coculture experiments with membrane-separated culture wells appeared also to argue against the possibility that soluble factors released by macrophages from the LPS-treated mice affect normal macrophages to restrict the growth of *L. pneumophila*. The membranes in the microwells are large enough to permit the passage of cytokines such as IL-1, TNF, and even IFN. It is possible, however, that other soluble factors, such as NO, may be involved in this effect. However, NO is very short-lived and difficult to detect. Furthermore, other studies in this as well as other laboratories have indicated that NO is not produced by macrophages unless IFN-γ is used to activate the cells. It is possible, but seems highly unlikely, that LPS induces the macrophages to produce IFN-γ and this, in turn, activates the macrophages to release NO. Experiments, however, are in progress to attempt to determine whether NO is being produced by these macrophages and whether macrophage production of NO has an effect on normal macrophages to resist the growth of *L. pneumophila*.

While the mechanism by which LPS-ϕ activate normal TG-elicited macrophages to restrict *Legionella* growth is uncertain, the data suggest a requirement for cell-to-cell contact. An LPS-binding protein which increases by as much as 100-fold during acute-phase response has been identified (20). This protein has been shown to bind LPS; then the LPS–LPS-binding protein complex binds to a cell receptor, thereby enhancing the ability of LPS to activate macrophages (22). We postulate that the LPS-ϕ may be functioning in a similar manner. That is, the LPS-treated macrophages present the LPS in some enhanced manner to normal TG-elicited macrophages, allowing the naive macrophages to be activated by a much lower concentration of LPS.

It would be of interest to determine whether LPS-treated macrophages from non-*Legionella*-infected mice could transfer in vivo resistance to *Legionella* infection to naive A/J recipient mice. Previous studies in this laboratory, as well as studies by others, had shown that spleen cells can adoptively transfer immune resistance from immunized animals to naive or immunocompromised recipients. It is not known, however, whether macrophage-enriched cell populations can do so similarly. Over a half-century ago, it was first shown that cell-mediated immunity from skin-reactive guinea pigs sensitized to *Mycobacterium tuberculosis* could be transferred to naive recipient guinea pigs by peritoneal cell suspensions rich in macrophages. However, lymphocytes in the peritoneal exudate apparently transferred this cell-mediated immune response. Thus, it is possible, but unlikely, that in the present study the TG-elicited peritoneal exudate cells from the donor mice contained some lymphocytes, if they were not all removed by the adherence procedure. Such lymphocytes, even if present in very small numbers, might have a role in the transfer of resistance to *L. pneumophila*, even though the bacteria grow only in macrophages. It is possible that LPS somehow affected such cells to participate in resistance to the growth of the bacteria in vitro. Experiments are in progress to determine whether lymphocytes from *Legionella*-immunized mice, as well as lymphocytes from mice stimulated with immunomodulators such as LPS, have the ability to interact with macrophages to induce resistance to *L. pneumophila*. Regardless of the mechanisms involved, however, the results obtained in this study show that small numbers of macrophage-rich populations from LPS-treated mice have the ability to transfer to a larger macrophage population the resistance to this important intracellular opportunistic bacterium. Further studies appear to be warranted to determine the mechanisms involved.

## ACKNOWLEDGMENT

This study was supported in part by a grant from the Public Health Service National Institute of Allergy and Infectious Diseases, AI 16618.

## REFERENCES

1. **Arata, S., C. Newton, T. W. Klein, and H. Friedman.** 1993. *Legionella pneumophila*-induced tumor necrosis factor (TNF)

production in permissive vs. nonpermissive macrophages. Proc. Soc. Exp. Biol. Med. **203:**26–29.
2. **Bhardwaj, N., T. W. Nash, and M. A. Horwitz.** 1986. Interferon-gamma-activated human monocytes inhibit the intracellular multiplication of *Legionella pneumophila*. J. Immunol. **137:** 2662–2669.
3. **Blanchard, D. K., H. Friedman, T. W. Klein, and J. Y. Djeu.** 1989. Induction of interferon-gamma and tumor necrosis factor by *Legionella pneumophila*: augmentation of human neutrophil bactericidal activity. J. Leukocyte Biol. **45:**538–545.
4. **Bone, R. C.** 1991. The pathogenesis of sepsis. Ann. Intern. Med. **115:**457–469.
5. **Denis, M.** 1991. Involvement of cytokines in determining resistance and acquired immunity in murine tuberculosis. J. Leukocyte Biol. **50:**495–501.
6. **Dinarello, C.** 1992. Role of interleukin-1 in infectious diseases. Immunol. Rev. **127:**119–139.
7. **Egawa, K., T. W. Klein, Y. Yamamoto, C. A. Newton, and H. Friedman.** 1992. Enhanced growth restriction of *Legionella pneumophila* in endotoxin-treated macrophages. Proc. Soc. Exp. Biol. Med. **200:**338–342.
8. **Friedman, H., C. Newton, R. Widen, T. W. Klein, and J. A. Spitzer.** 1992. Continuous endotoxin infusion suppresses rat spleen cell production of cytokines. Proc. Soc. Exp. Biol. Med. **199:**360–364.
9. **Havell, E. A., L. L. Moldawer, D. Helfgott, P. L. Killan, and R. B. Sehgal.** 1992. Type I IL-1 receptor blockade exacerbates murine listeriosis. J. Immunol. **148:**1486–1492.
10. **Jirillo, E., P. Descandia, M. R. Ribaud, B. Cannuscio, C. D. Simmone, and S. Antonaci.** 1992. Enhancement of polymorphonuclear cell phagocytosis by lipid A-activated monocytes via cell-to-cell contact. A possible role for membrane-associated cytokines. Immunopharmacol. Immunotoxicol. **14:**343–354.
11. **Kasai, N., S. Arata, J. I. Mashimo, K. Okuda, Y. Aihara, S. Kotani, H. Takada, S. Kusumoto, M. Imoto, H. Yoshimura, and T. Shimamoto.** 1986. Synthetic *Salmonella*-type lipid A antigen with high serological specificity. Infect. Immun. **51:**43–48.
12. **Klein, T. W., Y. Yamamoto, H. K. Brown, and H. Friedman.** 1991. Interferonγ induced resistance to *Legionella pneumophila* in susceptible A/J mouse macrophages. J. Leukocyte Biol. **9:**98–103.
13. **Lei, M. G., S. A. Stimpson, and D. C. Morrison.** 1991. Specific endotoxic lipopolysaccharide-binding receptors on murine splenocytes. III. Binding specificity and characterization. J. Immunol. **147:**1925–1932.
14. **Liu, Z., R. J. Simpson, and C. Cheers.** 1992. Recombinant interleukin-6 protects mice against experimental bacterial infection. Infect. Immun. **60:**4402–4406.
15. **Lynn, W. A., and D. T. Golenbock.** 1992. Lipopolysaccharide antagonists. Immunol. Today **13:**271–276.
16. **Nash, T. W., D. M. Libby, and M. A. Horwitz.** 1988. IFNγ-activated human alveolar macrophages inhibit the intracellular multiplication of *Legionella pneumophila*. J. Immunol. **140:** 3978–3981.
17. **Newman, G. W., H. X. Gan, P. L. McCarthy, and H. G. Remold.** 1991. Survival of human macrophages infected with *Mycobacterium avium intracellulare* correlates with increased production of tumor necrosis factor-α and IL-6. J. Immunol. **147:**3942–3948.
18. **Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. T. Tobias, and R. J. Ulevitch.** 1990. Structure and function of lipopolysaccharide binding protein. Science **249:**1429–1431.
19. **Skerrett, S. J., and T. R. Martin.** 1992. Recombinant murine interferon-γ reversibly activates rat alveolar macrophages to kill *Legionella pneumophila*. J. Infect. Dis. **166:**1354–1361.
20. **Tobias, P. S., K. Soldau, and R. J. Ulevich.** 1989. Identification of a lipid A binding site in the acute phase reactant lipopolysaccharide binding protein. J. Biol. Chem. **264:**10867–10871.
21. **Wright, S. D., S. M. Levin, M. T. C. Jong, Z. Chad, and L. G. Kabbash.** 1989. CR3 (CD11b/CD18) expresses one binding site for arg-gly-asp-containing peptides and a second site for bacterial lipopolysaccharide. J. Exp. Med. **169:**175–183.
22. **Wright, S. D., R. F. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison.** 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science **249:**1431–1433.
23. **Yamamoto, Y., T. W. Klein, C. A. Newton, R. Widen, and H. Friedman.** 1988. Growth of *Legionella pneumophila* in thioglycolate-elicited peritoneal macrophages from A/J mice. Infect. Immun. **56:**370–375.