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Legionella pneumophila Growth Restriction and Cytokine Production by Murine Macrophages Activated by a Novel *Pseudomonas* Lipid A

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Peritoneal exudate macrophages from A/J mice activated by purified lipid A preparations from *Pseudomonas vesicularis*, which contain 2,3-diamino-2,3-dideoxy-D-glucose disaccharide phosphomonoester as the lipid A backbone, restricted the growth of *Legionella pneumophila*, an intracellular opportunistic bacteria which readily grows in otherwise permissive macrophages from susceptible A/J mice and induced production of the proinflammatory cytokines interleukin 1 and tumor necrosis factor alpha. Activation of the macrophages was similar to that which occurred after stimulation with more conventional lipid A from other bacteria such as salmonellae. A purified fraction A3 preparation from the *Pseudomonas* lipid A, which lacked only 1 mol of amide-linked fatty acid, in comparison with another fraction (A2), which contained the fatty acid, also markedly activated the usually permissive macrophages from susceptible A/J mice to resist growth of the legionellae. The fraction A3 also induced both interleukin and tumor necrosis factor alpha. These results show that this novel lipid A from *P. vesicularis* can activate macrophages to resist infection with an opportunistic bacterium in a manner similar to that induced by conventional enterobacterial lipid A and that the hydrophobic portion of this *Pseudomonas* molecule may have an important role in activation of macrophages.

It is widely accepted that a major target for bacterial endotoxin is the macrophage, and many macrophage functions are highly sensitive to stimulation with bacterial products. Endotoxin-activated macrophages produce a number of important immunoregulatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α), both of which are mediators of many of the effects attributed to endotoxins. Furthermore, lipopolysaccharide (LPS)-activated macrophages evince enhanced microbicidal activity as well as cytotoxic effects. Such activities by activated macrophages are considered important indicators of the effects of endotoxins in terms of enhanced nonspecific resistance. Recent studies in this laboratory have shown that the facultative intracellular bacterium *Legionella pneumophila* can replicate readily in thioglycolate-induced peritoneal macrophages from A/J mice, although elicited macrophages from other mouse strains are not capable of replicating *L. pneumophila* in vitro and are normally resistant (23). However, growth of legionellae in macrophages from otherwise permissive A/J mice was found to be inhibited by first pretreating the macrophages with endotoxin (5). Specifically, we reported that endotoxins from *Escherichia coli* or *Salmonella typhimurium* induced permissive macrophages from A/J mice to resist *Legionella* growth.

Recently we found that pseudomonads contain a highly unusual LPS in terms of structure (10). This endotoxin contains 2,3-diamino-2,3-dideoxy-D-glucose (DAG) as the amino sugar component of the lipid A backbone. Furthermore, the LPS from pseudomonads is nontoxic or has very little toxicity. We reported that the lipid A from *Pseudomonas diminuta* JCM 2788 and *Pseudomonas vesicularis* JCM 1477 contains DAG disaccharide phosphomonoester as its backbone. These lipid A's and a major component, i.e., the A3 fraction, induced a local Shwartzman reaction, pyrogenicity, and *Limulus* lysate activity similar to activities induced by conventional enterobacterial lipid A's (10–12, 15). Moreover, we found these *Pseudomonas* lipid A's contained D-glucuronic acids as the major constituent of the reducing portion of the DAG disaccharide. In the present study, we investigated the effects of the lipid A preparation from *P. vesicularis* in activating macrophages from A/J mice to resist growth of *L. pneumophila* as well as to produce the immunoregulatory cytokines IL-1 and TNF-α, considered important in resistance to bacterial infection, including that caused by legionellae, compared with the effects of lipid A preparations from standard enterobacterial endotoxin.

For these experiments, inbred female strain A mice purchased from Jackson Laboratory, Bar Harbor, Maine, were used. They were 8 to 10 weeks of age at the initiation of an experiment and were fed commercial mouse chow and water ad libitum. The LPS was obtained from *P. vesicularis* JCM 1477 by the phenol-chloroform-petroleum ether extraction procedure as described previously (9). Free lipid A and its purified fractions A3 and A2 were prepared as described previously (4, 8, 10). Stock solutions of these endotoxin preparations were prepared by dissolving 200 μg of each in 1.0 ml of pyrogen-free 0.05% triethylamine solution.

Peritoneal exudate cells were obtained from the A/J mice 4 days after intraperitoneal injection of thioglycolate broth (GIBCO Laboratories, Detroit, Mich.). The cells were resuspended in RPMI medium supplemented with 10% fetal calf serum and allowed to adhere to 96-well Costar tissue culture plates. For use in *Legionella* growth assays, the macrophage monolayers were incubated with various concentrations of endotoxin or lipid A preparations for 24 h (5, 7). There was no effect by the endotoxin or lipid A treatment of the macrophages at the concentrations used on cell number or viability,

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TABLE 1. Effects of endotoxin on growth of legionellae in elicited peritoneal macrophages

Endotoxin (ng/ml)[a]	No. of bacteria (mean CFU [10^3] ± SE)[b]	
	Pseudomonas prepn	*Salmonella* prepn
None (control)	1,200 ± 400	
0.1	910 ± 50	970 ± 51
1.0	120 ± 7[c]	94 ± 11[c]
10.0	35 ± 11[c]	18 ± 3[c]
100.0	19 ± 3[c]	5 ± 2[c]

[a] Endotoxin from the indicated bacteria was added to 10^5 peritoneal macrophages cultured for 48 h with 10^7 *L. pneumophila*.
[b] Results for triplicate cultures in four experiments.
[c] Statistically significant from the control value ($P < 0.001$).

TABLE 2. Effects of lipid A preparations on growth of legionellae in elicited peritoneal macrophages from A/J mice

Stimulator concn (ng/ml)[a]	No. of bacteria (mean CFU [10^3] ± SE) in stimulated macrophages[b]			
	Pseudomonas prepn			*Salmonella* lipid A
	Lipid A	A2	A3	
None (control)	1,350 ± 420			
1.0	720 ± 210	NT[c]	740 ± 50	550 ± 14
10.0	88 ± 18	1,100 ± 60[d]	53 ± 23	41 ± 9
100.0	25 ± 9	510 ± 250[d]	9 ± 2	7 ± 4
1,000.0	7 ± 1	180 ± 24[d]	4 ± 1	4 ± 1

[a] The indicated concentration of lipid A or fraction thereof was added to 10^6 macrophages.
[b] Results for triplicate cultures in four experiments.
[c] NT, not tested.
[d] Statistically different from the value for growth restriction induced by similar concentrations of *Pseudomonas* lipid A ($P < 0.005$).

determined by the standard trypan blue dye exclusion test over the 48- to 72-h culture period (7). However, there was a marked effect on the ability of the treated macrophages 24 h later to phagocytize formalin-killed *Saccharomyces cerevisiae* yeast suspensions in standard phagocytic assays. There was a three- to fivefold increase in phagocytosis of the yeast particles by the LPS-treated macrophage cultures compared with control cultures without similar LPS treatment. Control monolayers were incubated with fresh medium alone, the medium was removed, and the monolayers were infected with legionellae at a concentration of 20 bacteria per macrophage for 30 min. After being washed with buffered saline three times, the monolayers were incubated further for 48 h in medium. The number of viable bacteria in 1% saponin cell lysates was determined by using buffered charcoal yeast extract agar as described previously (21, 23).

For induction and assay of monokines, the macrophage monolayers were incubated in medium only or with various concentrations of either endotoxin or the lipid A preparations for 24 h in 5% CO_2 and air at 37°C. Cell-free supernatants of the cultures were then tested by the cytolysis assay with WEHI 164 cells to determine TNF-α activity as described previously (5–7, 17). The number of units of TNF-α present in 1 ml of a test supernatant was calculated in comparison with a standard recombinant murine TNF-α (Genzyme Co., Boston, Mass.). IL-1 activity in the supernatants was assayed by the standard blastogenic response of thymocytes from C3H/HeJ mice in the presence of concanavalin A (0.2 μg) as described previously (13, 21). The specificity of the TNF-α or IL-1 bioassay was confirmed by neutralization procedures with a specific monoclonal antibody (Pharmingen, San Diego, Calif.). For this neutralization test, the culture supernatant, in a 0.1-ml volume, was first incubated at 37°C for 30 min with an equal volume of the specific antibody before the assay for cytokine activity.

The *Pseudomonas* endotoxin preparation was found to be equivalent to endotoxin from the standard *Salmonella* preparation in activating the elicited macrophages from otherwise permissive A/J mice to inhibit the growth of legionellae (Table 1). For example, when the macrophage monolayers from the A/J mice were first incubated with various concentrations of the endotoxin for 24 h, the monolayers evinced a marked resistance to subsequent *Legionella* infection. This nonpermissiveness of the LPS-treated macrophages infected with legionellae was readily apparent at 48 h of culture, the time of peak growth of the bacteria in normal macrophages. Inhibition of growth was also observed at 24 h of culture. The legionellae did not replicate at a greater rate in the LPS-stimulated macrophages at the earlier time, and the decreased *Legionella* growth at 48 h of culture could not be due to a more rapid growth of the bacteria in the monolayers at 24 h, which might have had a deleterious effect on the macrophages. There was little, if any, difference in the number or viability of the macrophages in the LPS-treated monolayers after *Legionella* infection at any time of culture as compared with control cultures (data not shown). LPS stimulation of the macrophage cultures, however, increased phagocytosis of the legionellae. This increased phagocytosis, as determined by microscopic examination, was about two- to threefold greater than in non-LPS-treated cultures. However, such increased phagocytosis did not appear to result in altered toxicity of the monolayers or a greater loss of the phagocytes after infection in comparison with the non-LPS-treated cells (data not shown). Thus, altered phagocytosis did not appear to result in an altered growth rate of the legionellae in the cultures.

The LPS-induced resistance of the macrophage cultures was induced by as little as 1 ng of endotoxin per ml and was dose dependent. Macrophages treated with 100 ng of either *Pseudomonas* or *Salmonella* endotoxin per ml evinced more than 100-fold-reduced growth of legionellae compared with untreated control macrophage cultures. Furthermore, the *Pseudomonas* and *Salmonella* lipid A preparations similarly activated the macrophages to resist *Legionella* growth (Table 2). The A3 preparation was as effective, or even more so, in inducing growth inhibition as the unfractionated lipid A. However, it was found that the A2 fraction from the *Pseudomonas* lipid A was deficient in inducing such resistance to legionellae. The difference was statistically significant ($P < 0.005$), as determined by Student's t test (compared with the effect of *Pseudomonas* lipid A or fraction A).

As shown in Table 3, the TNF-α-inducing activity of the endotoxin, as well as the *Pseudomonas* lipid A, was equivalent to that of similar *Salmonella* preparations. A dose as low as 0.01 μg of endotoxin per ml induced detectable amounts of TNF-α, and larger doses were even more effective. The A3 fraction, the major component of the *Pseudomonas* lipid A, was also a strong inducer of TNF-α. However, the A2 fraction induced only minimal amounts of TNF-α, even at a dose as high as 1 μg/ml (statistically significant; $P < 0.005$). The macrophage monolayers, before stimulation with the endotoxin preparations, produced no detectable cytokines (data not shown). Furthermore, as is evident in Table 4, the IL-1-inducing activity of the *Pseudomonas* endotoxin and lipid A preparation was essentially equivalent to that of the *Salmonella* endotoxin and lipid A fraction. Doses as low as 1 μg/ml induced this monokine, but higher doses, such as 10 μg/ml,

TABLE 3. TNF-α-inducing activities of endotoxin preparations

Stimulus[a]	TNF-α activity (mean U/ml ± SE) in supernatants[b]		
	1,000 ng/ml[c]	100 ng/ml	10 ng/ml
P. vesicularis			
LPS	1,846 ± 251	916 ± 128	342 ± 62
Lipid A	1,377 ± 256	497 ± 128	286 ± 49
A2	82 ± 10[d]	<64[d]	<64[d]
A3	1,264 ± 158	575 ± 192	179 ± 13
S. minnesota R595			
LPS	1,656 ± 400	717 ± 231	460 ± 57
Lipid A	1,161 ± 160	781 ± 282	397 ± 71

[a] The indicated endotoxin was added to 10^6 peritoneal macrophages from A/J mice, and TNF-α activity was detected at 24 h in WEHI 164 supernatants.
[b] Results for three to four cultures in three experiments.
[c] Concentration for stimulation of macrophages.
[d] Statistically different from activity induced by *Pseudomonas* lipid A ($P < 0.001$).

were needed for maximum induction. In contrast to TNF-α bioactivity, both the purified A2 and A3 fractions from the *Pseudomonas* lipid A preparation were essentially similar in the ability to stimulate IL-1 induction.

Previous studies in this laboratory demonstrated that the lipid A preparations derived from *P. vesicularis* and from *P. diminuta*, both of which are different from the conventional lipid A derived from enterobacteriaceae, contains a DAG disaccharide as the backbone but can induce various endotoxic activities (1–4). In the present study, it was found that this novel lipid A induced activities similar those induced by conventional endotoxin from enterobacteriaceae in that the compound activated normally permissive macrophages to restrict the growth of an important intracellular opportunistic bacterium, i.e., *L. pneumophila*, as well as to secrete the monokines IL-1 and TNF-α. This activity induced by the *Pseudomonas* endotoxin was equal to that induced by more classic enterobacterial endotoxins, such as *Salmonella minnesota* R595 lipid A. It is of interest that the unusual lipid A from pseudomonads, which does not have the β-1-6-linked glycocyamine disaccharide as the backbone and also does not have the classic glucosidically linked phosphate group which plays an important role in many endotoxic activities, displays strong biological activities comparable to those of enterobacterial lipid A. The novel chemical structure of the *Pseudomonas* lipid A suggests that equivalent biological activities of *Pseudomonas* and *Salmonella* lipid A's are due to a similarity of overall structure. Indeed, the hydrophilic and lipophilic balances of both lipid A molecules are generally similar, although there are differences in the backbone components, such as DAG instead of D-glucosamine and D-glucuronic acid instead of a glucosidically linked phosphate group.

TABLE 4. IL-1-inducing activities of endotoxin preparations

Stimulus[a]	IL-1 activity (mean cpm ± SE) in supernatants[b]		
	10,000 ng/ml[c]	1,000 ng/ml	100 ng/ml
P. vesicularis			
LPS	25,002 ± 4,960	3,788 ± 1,162	1,876 ± 762
Lipid A	11,591 ± 2,148	2,936 ± 928	1,340 ± 816
A2	10,138 ± 1,170	2,148 ± 278	774 ± 430
A3	10,902 ± 2,709	2,157 ± 479	1,187 ± 218
S. minnesota R595			
LPS	24,919 ± 4,500	2,076 ± 956	1,132 ± 536
Lipid A	10,678 ± 1,595	1,705 ± 485	1,216 ± 247

[a] The indicated endotoxin preparation was added to 10^6 peritoneal macrophages, and IL-1 activity was detected at 24 h in thymocytes from C3H/HeJ mice.
[b] Results for three to four cultures per group in three experiments.
[c] Concentration for stimulation of macrophages.

It should be noted that in a recent serological study in this laboratory (1), the presence of a common epitope was suggested for *Pseudomonas* and *Salmonella* lipid A's, using an enzyme-linked immunosorbent assay (ELISA) with a cross-reactive monoclonal antibody (Sm 161M). It was found that this monoclonal antibody could recognize the nonreducing region of these lipid A molecules by the ELISA, using many synthetic lipid A analogs, including both disaccharide and monosaccharide types (3, 12). Such results suggested that the reducing portion of the *Pseudomonas* lipid A was serologically similar to that of the *Salmonella* lipid A. Thus, the conformation of the overall structures of both lipid A's might have an important role in the expression of equivalent biological activities.

Studies of synthetic analogs of partial structures of lipid A have shown that the structure of the hydrophobic portion of the lipid A molecule, including type, chain length, and the nature of that portion of the linkage of fatty acids, as well as backbone structure, influences biological activities (13, 16, 18–20). A similar relation was observed between A2 and A3 fraction preparations. For example, the A2 preparation, which lacks only 1 mol of amide-linked fatty acid compared with the A3 preparation, had reduced macrophage activation ability, including TNF-α-inducing activity, as well as the ability to induce restrictive activity in *Legionella*-infected permissive A/J macrophages. However, the IL-1-inducing activity was similarly present in both the A2 and A3 preparations.

Previous studies showed that the A2 fraction from either the *P. vesicularis* or the *P. diminuta* lipid A was deficient in inducing the local Shwartzman reaction, decreasing body weight, or inducing TNF-α in *Mycobacterium bovis* BCG-primed mice (10, 12, 15). Nakaya et al. reported that the A3 preparation could induce differentiation of mouse leukemia M1 cells, while the A2 preparation could not (17). Moreover, treatment of *P. vesicularis* lipid A3 with triethylamine, which removes some ester-linked fatty acid, also resulted in a marked decrease in local Shwartzman activity (11). Such observations suggest that the hydrophobic portion of the *Pseudomonas* lipid A molecule plays an important role in these activities. Furthermore, the difference in structural requirements regarding TNF-α- and IL-1-inducing activities was also reported (14, 19). Thus, it seemed important to examine several indicators of macrophage function to study the structure-activity relationship of such lipid A.

In this study, the growth restriction assay for legionellae was used as one indicator of macrophage activation by the endotoxin or lipid A. The mechanism of restriction of growth of this intracellular bacteria in macrophages is not yet clear. However, it is interesting that in this study, TNF-α induction was more closely coupled than IL-1 induction in terms of growth restriction. For example, the A3 fraction at a concentration of 100 to 1,000 ng/ml caused significant IL-1 and TNF-α induction as well as *Legionella* growth restriction, while the A2 fraction at the same dose induced high IL-1 levels but was relatively deficient in inducing both TNF-α and growth restriction. The possibility that TNF-α acts in an autocrine fashion or in events associated with TNF-α induction may be important in *Legionella* growth restriction by macrophages. Regardless of the mechanisms involved, however, the results of this study demonstrated that this novel lipid A from *P. vesicularis* endotoxin can activate macrophages to restrict the growth of legionellae as well as to produce TNF-α and IL-1. This effect was similar

to the activities of conventional enterobacterial lipid A, and the hydrophobic portion of the lipid A molecule appeared to have an important role in such macrophage activation.

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