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Immunogenicity and Adjuvanticity of Lipopolysaccharide from *Legionella pneumophila* (42466)

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Abstract. Lipopolysaccharide isolated from *Legionella pneumophila* was found to be a potent antigen and inducer of antibody with strong adjuvant activity for related and unrelated antigens such as sheep erythrocytes by *in vivo* and *in vitro* systems. The LPS was also a potent stimulator of blastogenic responses by spleen cells from normal mice as well as from mice immunized with inactivated whole cells of *Legionella.* It strongly stimulated production of interferon and interleukin 1. These results indicate that the LPS of *Legionella* may be an important immune regulator in the host reponse.

Legionella pneumophila and related species are gram-negative, opportunistic, facultative intracellular bacteria. They are etiologic agents of Legionnaire's disease, as well as other respiratory and systemic infections. Previous studies using *in vivo* and *in vitro* model systems have shown that these organisms induced marked cellular and humoral immune responses in host animals, and that cell sonicates and lipopolysaccharide (LPS) were associated with these immune responses (1–6). Lipopolysaccharide of *Legionella* has been shown to have unique biochemical and biological characteristics. LPS from these organisms has been charcterized by the presence of large amounts of branched-chain fatty acids unique for the species from which they were isolated and contains little or no hydroxy fatty acids commonly associated with lipids of endotoxin (7, 8). The LPS of *Legionella* species was relatively low in endotoxicity in animals and associated with the serogroup (somatic) antigens (7–9). It is immunoprotective in a guinea pig model by intraperitoneal challenge and is a potent adjuvant in inducing enhanced antibody formation and delayed hyprsensitivity to intrinsic and extrinsic antigens (4, 6, 8, 10, 11).

In the present investigation, the immunogenic and adjuvant activities of *Legionella* LPS were studied by *in vivo* and *in vitro* systems using a preparation of LPS isolated and purified by a mild physicochemical procedure designed to preserve its chemical and biological activities. The mouse model was chosen for this study since this rodent mimics man more closely in natural resistance to *Legionella* than do highly suspectible guinea pigs which have been studied previously.

Materials and Methods. *Experimental animals.* Inbred BDF/1 mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were 8–10 weeks of age at the initiation of each experiment. The animals were kept in groups of six to eight in plastic mouse cages in a pathogen-free animal facility and fed mouse pellets and water *ad libitum.* One group of animals was given killed *Legionella* vaccine (10^8 cells/mouse) ip, followed 30 days later with an additional ip injection of vaccine. Seven days later, these mice were used as *Legionella*-sensitized spleen donors.

Legionella organisms and LPS. Strains of serogroup 1 *L. pneumophila* were used as described previously (1, 2) and LPS was prepared as described (8). Briefly, LPS was extracted from the bacteria with a solution of 0.5 *M* lithium acetate and 0.2 *M* ethylene-diamine tetraacetic acid, pH 7.2, containing lysozyme (10 mg/g packed cells), deoxyribonuclease (30 μg/g cells), and ribonuclease (30 μg/g cells). Extraction was performed at 36°C overnight with vigorous stirring. The supernatant was separated from the cell debris by centrifugation and concentrated in an Amicon concentrator (Amicon Corp., Lexington, MA) equipped with a PM-10 filter. The buffer was changed to 0.05 *M* Tris–HCl and 0.1 *M* NaCl at pH 8 during concentration. The extract was then chromatographed on DEAE-cellulose with a buffer gradient of 0.05 *M* Tris–HCl in 0.1 *M* NaCl and 0.05 *M* Tris–HCl in 0.4 *M* NaCl at

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pH 8. The fractions from the first peak at 280 nm were pooled and concentrated and the LPS was separated from other bacterial components by filtration on Sephadex G-200 (Pharmacia Laboratories, Piscataway, NJ) with 0.01 *M* Tris–HCl and 0.2 *M* NaCl, pH 8. The LPS fraction was eluted in the first peak and was further treated wtih Pronase (Calbiochem–Behrling, La Jolla, CA) at 37°C overnight. The purified LPS was separated from the digestion mixture with a Sephadex G-50 column, dialyzed against buffered saline, pH 7.2, and concentrated on a PM-10 filter. Chemical and biological assays for the isolated LPS were performed as described previously (7, 8). The isolated LPS yielded fatty acid profiles identical with *L. pneumophila* as determined by gas–liquid chromatography (GLC), and consisted of approximately 22% carbohydrate assayed as hexose, 2% protein, and 76% fatty acids and other constituents. Content of 3-deoxy-D-manno-2-octulosonic acid (KDO) in the LPS was determined to be 0.61% by the microcolorimetric thiobarbituric acid method as described previously (7, 8), and was qualitatively confirmed by GLC (personal communication, Dr. G. G. Guerrant, Centers for Disease Control, Atlanta, GA). The LPS possessed the biological and serological activities as well as the SDS–polyacrylamide gel electrophoresis profiles as described previously (6–8).

Antibody tests. Mice immunized with either the inactivated whole cell vaccine (1) or the purified LPS were tested for antibody responsiveness to *Legionella* cells by the microagglutination procedure as described previously (1, 12). In addition, for study of adjuvanticity, the serum of immunized as well as normal mice was tested for antibody responsiveness to an antigen unrelated to *Legionella,* i.e., sheep erythrocytes (SRBC), by microhemagglutination and the number of antibody plaque-forming cells (PFC) to the SRBC was determined with spleen cell suspensions from normal and immunized mice as described (12). Induction of PFC *in vitro* to SRBC also was determined as described (12).

Blastogenic assay. Spleen cells from *Legionella*-sensitized mice and from normal mice were assessed for their responsiveness to the *Legionella* LPS by the lymphocyte blastogenic assay as described (1, 3). In brief, suspensions of 2×10^5 washed spleen cells were placed in microwells in 96-well microtiter plates and incubated either without stimulator as controls or with graded amounts of killed *Legionella* organisms or LPS for 48 hr in an atmosphere of 95% air and 5% CO_2 at 37°C. To each well then was added 0.5 μCi [^{3}H]thymidine and the plates were incubated for an additional 18 hr. The tritiated thymidine uptake was determined using standard liquid scintillation techniques. The Δcpm was calculated as the mean cpm $\pm$ SD for triplicate cultures incubated with a stimulator minus the cpm $\pm$ SD of cultures incubated without a stimulator.

Mediator assay. Interleukin 1 (IL-1) was determined by the standard assay in which cell-free culture supernatants, from adhered spleen cells incubated with LPS for 24 hr, were tested for their ability to stimulate thymidine incorporation of C_3H/HeJ thymocytes (1.5×10^6 cells per culture). Interferon (IFN) was determined by the ability of the supernatants from spleen cells (10^7) cultured for 24 hr to protect L929 cells from infection with a standard amount of vesicular stomatitis virus as described (14–17). The interferon activity was characterized as to class by prior treatment with monoclonal anti-IFN-γ. All immunological assays were done in RPMI 1640 with 10% fetal calf serum, antibiotic, and 2-mercaptoethanol (5×10^{-5} *M*).

Results. Table I shows that the purified *Legionella* LPS was by itself a potent antigen in mice as determined by microagglutination with *Legionella* cells. Serum antibody titers in mice immunized with 10 μg LPS were comparable to the titers of those animals that had received 10^8 inactivated *Legionella* whole cells, with an average titer of 256 for both groups. Likewise, injection of *Legionella* had an enhancing effect on antibody responses to unrelated antigens, i.e., SRBC. This was demonstrated by *in vivo* and by *in vitro* systems (Table II). An intraperitoneal injection of either *Legionella* LPS or whole cells plus SRBC into mice or adding either the LPS or the whole bacterial cells to cultured mouse spleen cells immunized *in vitro* with SRBC resulted in marked increases in the PFC response to the SRBC. These results indicate that the LPS contained much if not all of the adjuvant activity of the *Legionella* whole cells for the antibody response in mice to SRBC.

TABLE I. EFFECT OF *Legionella* WHOLE CELL VACCINE OR PURIFIED *Legionella* LPS ON ANTIBODY RESPONSE TO *Legionella* AND TO SHEEP ERYTHROCYTES (SRBC)

Legionella preparation[a]	Anti-*Legionella* titer[b]	Background antibody to SRBC[c]	
		Serum titer	PFC/spleen (±SE)
None (control)	4	4	56 ± 8
Whole cell vaccine			
10^6 cells	64	32	283 ± 17
10^8 cells	256	96	396 ± 34
Legionella LPS (μg)			
1.0	24	16	136 ± 18
5.0	198	48	240 ± 43
10.0	256	64	315 ± 36

[a] *Legionella* preparation injected ip into groups of five to eight mice each.

[b] Average titer determined by microagglutination of individual sera 10 days after immunization of eight mice per experiment with indicated *Legionella* preparation.

[c] Antibody to SRBC response determined 10 days after immunization of normal mice with indicated *Legionella* preparation; serum titer assayed by direct microhemagglutination and PFCs determined with whole spleen cell suspensions and averages calculated from individual determinations in triplicate for each animal.

Table III summarizes the results of experiments on blastogenic activity of spleen cells from normal mice and from mice immunized with inactivated *Legionella* whole cells in reponse to challenge with *Legionella* LPS or the whole bacterium. Spleen cells from normal and sensitized mice responded equally well to stimulation with LPS. However, addition of vaccine to the cultures resulted in an increased proliferation response of cells from sensitized animals.

The *Legionella* LPS was also a potent inducer of interleukin 1 and interferon in spleen cell cultures from normal mice (Table IV). It induced as high or a higher level of IL-1 and interferon as did the whole cells. Treatment of the interferon-containing culture supernatants with monoclonal anti-IFN-γ decreased the titer by only about 50%, indicating that not only IFN-γ but also IFN-α/β was induced by the *Legionella* LPS (data not shown). Interestingly, although neither the LPS nor the vaccine induced increased amounts of IL-1 in sensitized cells, the vaccine did result in increased induction of interferon. Very little if any IL-2 was present in the spleen cell cultures from normal or immunized mice upon *in vitro* challenge with LPS or whole cells.

Discussion. Previous studies have shown that inactivated *Legionella* whole cells or a soluble sonicate extract stimulated blastogenic responses by spleen cells from both normal and *Legionella*-sensitized mice. Spleen cells from mice which had been immunized with graded doses of *Legionella* showed significantly heightened blastogenic activities (1, 3). Purified serogroup-specific antigens of *Legionella* were found to act as adjuvant in inducing delayed-type hypersensitivity to the

TABLE II. EFFECT OF *Legionella* PREPARATION ON ANTIBODY RESPONSE OF MICE TO SHEEP ERYTHROCYTES (SRBC)

Legionella preparation[a]	PFC responses of 10^6 spleen cells[b]			
	In vivo[c]	Percentage of control	*In vitro*[d]	Percentage of control
None (control)	798 ± 64	—	630 ± 52	—
Vaccine, 10^8 cells	1964 ± 243	246	1731 ± 134	275
LPS (μg)				
1.0	1132 ± 108	147	975 ± 38	155
5.0	1848 ± 276	232	1563 ± 241	248
10.0	2132 ± 213	268	1875 ± 331	298

[a] Preparation of *Legionella*, either killed whole cell vaccine or purified LPS, injected ip into groups of five to six mice each or added to cultures of normal spleen cells.

[b] Average plaque-forming cell (PFC) response to SRBC per 10^6 spleen cells determined in triplicate for individual animals.

[c] Mice injected with *Legionella* preparations and 4×10^8 SRBC 4 days earlier.

[d] Cultures of spleen cells (1×10^7) from mice immunized *in vitro* with 2×10^6 SRBC 5 days earlier were treated *in vitro* with indicated *Legionella* preparation.

TABLE III. BLASTOGENIC RESPONSE OF SPLEEN CELLS FROM NORMAL AND *Legionella*-SENSITIZED MICE STIMULATED *in Vitro* WITH *Legionella* WHOLE CELL VACCINE OR LPS

In Vitro stimulator[a]	Normal mice Δcpm × 10^{-3} ± SD[b]	*Legionella*-sensitized mice[c] Δcpm × 10^{-3} ± SD[b]
Vaccine 10^8 cells	12.2 ± 1.3	18.6 ± 0.9
LPS (μg/ml)		
10	33.3 ± 0.7	30.7 ± 1.6
5	17.6 ± 0.9	—
1	6.4 ± 0.8	—

[a] *Legionella* preparation, either killed whole cell vaccine or purified LPS, added to cultures of 2 × 10^5 spleen cells.

[b] Mean of three to four cultures.

[c] Mice injected ip with 10^8 killed *Legionella* bacteria 30 days earlier, and given a boost of vaccine 7 days prior to setting up assay.

protein cross-reacting antigens in guinea pigs (8), and heat-inactivated *Legionella* cells were as potent as mycobacteria in an oil emulsion adjuvant in inducing antibody and delayed hypersensitivity (10, 11). The lipopolysaccharide isolated and purified from *Legionella* species by a mild physicochemical procedure designed to preserve its molecular integrity and biological activities was found to be the active principle of adjuvanticity for guinea pigs in an aqueous mixture with antigen as well as in an oil emulsion (8). Other studies also showed that *Legionella* cells and crude cellular extracts rich in LPS but also containing other components were potent enhancers of antibody responses to sheep erythrocytes in mice and induced soluble mediators of immunity such as interleukins and interferons (1–3, 17). Heating the LPS-rich preparations diminished the activity somewhat, suggesting that materials other than the heat-resistant LPS also might be active.

In the present study, we tested the antigenicity and adjuvanticity of purified *Legionella* LPS in the mouse model by *in vivo* and *in vitro* systems. The mouse model has advantages over guinea pig models in that rodents, like man, are much more resistant to *Legionella* infection than guinea pigs. Epidemiologic studies have shown that only a small number of individuals exposed to *Legionella* species develop the disease and, in most cases, individuals who came down with the disease appeared to have genetic or acquired immune dysfunction or other precipitating factors. Mice treated with cyclophosphamide, a potent immunosuppressive agent, became highly susceptible to *Legionella* infection, and their resistance was enchanced by immunization with inactivated *Legionella* cells (18).

Our results using the mouse model demonstrated that purified LPS of *Legionella* itself was a potent antigen, eliciting strong antibody responses. As the LPS antigens were identical with the serogroup-specific antigens as assayed by immunofluorescence and immunodiffusion (4, 8, 9), detection of a serogroup-specific antibody in serum or body fluids has been an

TABLE IV. EFFECT OF *Legionella* WHOLE CELL VACCINE OR PURIFIED *Legionella* LPS ON PRODUCTION OF IL-1 AND/OR INTERFERON BY MOUSE SPLEEN CELLS

Legionella preparation for stimulation[a]	Normal mice IL-1[a] (cpm × 10^{-3})	Normal mice IFN[b] (U/ml)	*Legionella*-immunized mice[b] IL-1[a] (cpm × 10^{-3})	*Legionella*-immunized mice[b] IFN[b] (U/ml)
None	—	10	—	10
Vaccine 10^8 cells[c]	22.2 ± 3.4	150 ± 10	27.2 ± 2.9	200 ± 20
LPS (μg)				
5.0	16.7 ± 2.1	150 ± 10	22.7 ± 3.1	200 ± 10
10.0	24.5 ± 2.9	250 ± 10	26.6 ± 3.8	250 ± 20

[a] *Legionella* preparation used to stimulate mouse spleen cells.

[b] Cell-free supernatants obtained from cultures of 5 × 10^6 mouse spleen cells for four to five mice per group after stimulation *in vitro* with indicated *Legionella* preparation and averages determined for triplicate cultures per mouse per group.

[c] Mice sensitized by ip injection of 10^8 heat-killed *Legionella* bacteria 30–40 days earlier.

effective tool in diagnosis of legionellosis in man.

The adjuvant activity of *Legionella* LPS was demonstrated by both *in vitro* and *in vivo* systems. In other experiments (data not shown) boiling the purified LPS for 30 min at 100°C failed to reduce its adjuvant activity, indicating that a heat-labile component, such as protein or peptide, could not account for the activities induced by the purified LPS.

Consistent with previous reports using other animal models (6, 8, 10, 11), injection of the LPS in aqueous solution without oil into mice significantly enhanced antibody responses to sheep erythrocytes. It also heightened the anti-sheep erythrocyte response when added to culture of spleen cells from normal mice immunized *in vitro* with sheep erythrocytes, and induced blastogenic responses of spleen cells from normal mice as well as from mice immunized with killed intact *Legionella* bacteria. In addition, the *Legionella* LPS as well as the whole cell vaccine were inducers of interleukin 1 and interferons. Thus, it is apparent that the adjuvantic principle of *L. pneumophila* is attributable, at least in part if not entirely, to the LPS moiety.

The LPS of *Legionella* has unique biochemical characteristics as compared with endotoxins present in the cell walls of other gram-negative bacteria (7, 8). Thus LPS has similar immunoenhancing activities as many more "classical" LPS derivatives from a wide variety of gram-negative bacteria. Previous studies in this laboratory had shown that LPS derived from *Serratia, Salmonella,* and *Escherichia coli* have similar immunopotentiating activities (19–22). The LPS from these bacteria readily induce a blastogenic response in normal murine spleen cells and also enhance antibody responses to sheep RBCs, both *in vivo* and *in vitro.* Such endotoxins also stimulate soluble mediators, including interleukin 2 and interferons. However, the magnitude of the responses induced by these different endotoxins varies according to the preparations used. Nevertheless, it is evident that *Legionella* LPS and other bacterial endotoxins have generally similar immunoenhancing activities despite distinct biochemical characteristics. The results of the present study indicate that *Legionella* LPS by itself is a potent immunostimulator and acts as an adjuvant in the absence of an oil emulsion. Although the host–parasite relationship in *Legionella* infection remains to be elucidated, the properties of the LPS moiety in *Legionella* seems to be related to the strong immune reactivity of these organisms in experimental animals such as mice, and may be significant in the immune responses of humans to these opportunistic pathogens.

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