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Lipopolysaccharide of *Legionella* as Adjuvant for Intrinsic and Extrinsic Antigens (41975)

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Abstract. Lipopolysaccharide (LPS) isolated from *Legionella* species was found to be a potent adjuvant. When *Legionella* LPS was injected into animals as an aqueous mixture or oil emulsion with protein antigens, it potentiated humoral antibody titers to these antigens by four- to sixfold. The LPS also acted as an intrinsic adjuvant to induce delayed hypersensitivity to the cross-reacting protein antigens present in cells of *Legionella* species, providing a potentially useful means for detecting legionellosis by skin test. The adjuvanticity of *Legionella* LPS was comparable in potency to *Mycobacterium tuberculosis* H37Ra in Freund's complete adjuvant. However, *Legionella* LPS caused much less tissue inflammation and appeared to function differently in some aspects. © 1984 Society for Experimental Biology and Medicine.

As the etiologic agents of Legionnaires' disease and Pontiac fever, *Legionella* species are opportunistic facultative intracellular pathogens. They are gram-negative bacteria with unique fatty acid profiles, characterized by the presence of large amounts of branched-chain fatty acids (1). The bacterial cells of *Legionella* species were relatively low in "endotoxicity" as determined by induction of pyrogenicity, serum heparin-precipitable protein, Shwartzman reaction, weight loss, and lethality in animals but were highly reactive in gelating limulus lysate (2). Heat-inactivated *Legionella* cells were found to be a potent adjuvant in an oil emulsion, potentiating antibody synthesis and hypersensitivity in immunized animals (3, 4). The present report describes a mild procedure for isolating immunologically highly active lipopolysaccharide (LPS) from *Legionella* species and demonstrates that it was the active principle of adjuvant activities of *Legionella* with unique characteristics. It also acted as an intrinsic factor to induce delayed hypersensitivity in animals to the proteinaceous cross-reacting antigens of *Legionella* species, raising the possibility of detecting *Legionella* infection by skin test or other *in vitro* diagnostic methods (e.g., lymphocyte stimulation) based on immunologic memory of host cells.

Materials and Methods. *Isolation of lipopolysaccharide. Legionella pneumophila* strains Knoxville 1, Philadelphia 1, Togus 1, Bellingham 1, *L. bozemanii,* and *L. dumoffii* were grown at 36°C on Mueller–Hinton agar supplemented with 1% dehydrated hemoglobins and 0.05% cysteine hydrochloride. The bacteria were harvested and washed twice with phosphate-buffered saline (PBS), pH 7.2. Lipopolysaccharide was extracted from each strain with an aqueous solution of 0.5 *M* lithium acetate and 0.2 *M* ethylenediamine tetraacetic acid, pH 7.2, containing lysozyme (10 mg/g packed cells, Sigma Chemical Co., St. Louis, Mo.), deoxyribonuclease (30 μg/g cells, Sigma), and ribonuclease (30 μg/g cells, Sigma). Extraction was effected at 36°C overnight with vigorous stirring. The solubilized cellular material was separated from the cell debris by centrifugation and concentrated with a PM-10 filter pad in an Amicon[1] concentrator (Amicon Corp., Lexington, Mass.). The buffer was changed to 0.05 *M* Tris·HCl, pH 8, and 0.1 *M* NaCl in the concentrator. The extract was then clarified by centrifugation and chromatographed on diethylaminoethyl cellulose (Whatman Biochemical Ltd., Maidstone, Kent, England) 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 pH 8. Fractions from the first peak as monitored 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, N.J.) with 0.01 *M* Tris·HCl and

[1] Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

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0.2 *M* NaCl, pH 8. The LPS fraction which was eluted as the first peak from the Sephadex G-200 column was further treated with Pronase (Calbiochem-Behring, La Jolla, Calif.) at 37°C overnight, and the purified LPS was separated from the digestion mixture with a Sephadex G-50 column. The LPS fractions were pooled, dialyzed against buffered saline, pH 7.2, and concentrated on a PM-10 filter to an opalescent solution.

Chemical and biological assays for lipopolysaccharide. The microassay method of Karkhanis *et al.* (5) was used to detect the presence of 2-keto-3-deoxyoctonate, and fatty acids were determined by gas–liquid chromatography as described by Moss *et al.* (1). Protein was determined by the method of Lowry *et al.*(6). Total carbohydrate was determined as hexose by the phenol–sulfuric acid method (7), and the diphenylamine reaction of Burton (8) was used to detect residual deoxyribonucleic acid in the LPS preparations. For determining dry weight, a preparation of LPS was dialyzed in distilled water for 48 hr. An aliquot of the preparation was then heated at 60°C in a drying oven until a constant weight was reached.

Endotoxicity of *Legionella* LPS was assayed by pyrogenicity test in rabbits (9), mouse weight gain test (10), and Shwartzman reaction. Young adult rabbits (New Zealand strain) were sensitized intradermally with 25 μg of *L. pneumophila* LPS in 0.1 ml of pyrogen-free saline and were tested for Shwartzman reaction 24 hr later by intravenous injection of 0.1 ml of the same *L. pneumophila* LPS solution. Endotoxin lot EC-2 (Food and Drug Administration, Rockville, Md.) was used as positive control.

Serologic activities of LPS were assayed by gel diffusion as previously described (11).

Antigens for detecting adjuvanticity. Neisseria gonorrhoeae strain V1 (12) was grown on GC agar supplemented with 1% IsoVitalex (Difco, Detroit, Mich.) in candle jars for 48 hr at 36°C. Gonococcal outer membrane protein (GOMP) was extracted from the bacteria by the method of Johnston *et al.* (13). The GOMP preparation was further purified by treatment with 2% sodium deoxycholate to remove contaminating LPS, and the GOMP antigen was separated from the reaction mixture by column chromatography and ultrafiltration on a PM-10 filter as described by Frasch (14).

The proteinaceous cross-reacting antigens of *Legionella* were isolated from *L. pneumophila* strain Knoxville 1 as previously described (15). Bovine serum albumin (BSA) was purchased from Sigma Chemical Company, and was essentially free of low-molecular-weight substances.

Immunization and antibody assay. Purified *Legionella* LPS in saline or Freund's incomplete adjuvant, and Freund's complete adjuvant H37Ra (Difco) were mixed with protein antigens for immunizing and sensitizing animals. Male Hartley strain guinea pigs weighing about 350 to 450 g were used in all experiments.

For antibody study, the guinea pigs were divided at random into four groups with 10 animals per group for each of the three test antigens, namely GOMP, BSA, and cross-reacting antigens of *Legionella.* Each group of animals received intradermally 1 ml of one of the following antigen mixtures at four to six sites in the nuchal and posterior scapular area: (a) GOMP, BSA, or *Legionella* cross-reacting antigens in PBS, (b) test antigens mixed with 0.5 mg (dry wt) of *Legionella* LPS in saline, (c) test antigens emulsified with 0.5 mg of *Legionella* LPS in Freund's incomplete adjuvant, and (d) test antigens emulsified in Fruend's complete adjuvant. The final antigen concentration was 2 mg of protein per dose. Because of the low antigenicity of purified GOMP, the animals that were given the GOMP antigen in PBS received two additional weekly boosters of 0.5 mg protein per dose beginning 7 days after the first injection. All other groups of animals were immunized with one single dose. The animals were bled 4 weeks after the first injection for antibody assay.

Antibodies to the test antigens in sera from immunized animals were determined by the enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates were coated by adding to each well 0.1 ml of a solution of the respective test antigen in PBS. After incubation for 2 hr at 37°C, excess antigen solution was aspirated, and the plates were washed three times with PBS. One-tenth of

a milliliter of a 1:10 dilution of serum was added to the first well, and serial twofold dilutions were made by adding PBS containing 0.05% Tween 80 (J. T. Baker Chemical Co., N.J.) (PBST). The final volume of each well was made up to 0.1 ml with PBST, and the plate was incubated at 37°C for 1 hr. The plates were then washed three times with PBS, and 0.1 ml of the appropriate dilution of peroxidase-labeled, anti-guinea pig serum was added. After incubation for 1 hr, the plates were washed twice with PBS, and to each well was added 0.1 ml of substrate containing 10 mg of orthophenylenediamine and 1 ml of 3% hydrogen peroxide per 100 ml of substrate solution in distilled water. The plates were incubated for 1 hr, and the enzyme reaction was terminated by adding 0.1 ml of 6 *N* sulfuric acid.

Dermal hypersensitivity. Groups of guinea pigs were sensitized by intradermal injection of 1 ml of one of the following antigen mixtures: (a) GOMP or cross-reacting antigens (test antigens) from *L. pneumophila* in saline, (b) test antigens mixed with 0.5 mg (dry wt) of *Legionella* LPS in saline, (c) test antigens emulsified in Freund's incomplete adjuvant containing 0.5 mg of *Legionella* LPS, (d) test antigens emulsified in Freund's complete adjuvant, and (e) heat-killed *L. pneumophila* emulsified in Freund's incomplete adjuvant. Each sensitizing dose contained 2 mg of the respective test antigen determined as protein. Normal control animals were given saline with no adjuvant. Skin tests were performed 4 to 6 weeks after sensitization with the respective test antigens at 100 μg protein per 0.1-ml dose. Skin lesions were measured at 24 and 48 hr after injection as the diameters of erythema and induration.

Results. *Legionella lipopolysaccharide.* The LPS isolated from strains of *L. pneumophila* yielded fatty acid profiles characteristic of *L. pneumophila,* with the saturated, branched-chain 16-carbon acid (i-16:0) as the major component. Other major fatty acids were a saturated, 17-carbon branched-chain acid (a-17:0), a monounsaturated 16-carbon straight-chain acid (16:1), a 15-carbon branched-chain acid (a-15:0), and a saturated 14-carbon branched-chain acid (i-14:0) in proportion typical of *L. pneumophila* as described by Moss *et al.* (1), who used digest of whole bacterial cells for their tests. Little or no hydroxy fatty acids were found in *L. pneumophila* LPS. The LPS preparations from *L. bozemanii* and *L. dumoffii* also yielded fatty acid profiles typical of the respective species as previously described for whole bacterial cells (16, 17). The presence of 2-keto-3-deoxyoctonate was detected by the microassay procedure (9) and was later confirmed by gas-liquid chromatography (18) (personal communication, Dr. G. Guerrant, Centers for Disease Control, Atlanta, Ga.). Chemically, *Legionella* LPS contained approximately 2% protein, 22.5% carbohydrate determined as hexose, and 75.5% fatty acids, and other constituents.

The serotypic antigens which are responsible for serogroup specificity (11) of *Legionella* were an integral part of the LPS. Isolated LPS possessed the serogroup specificity of the parent strains as assayed by immunodiffusion using purified LPS as antigen. The LPS from serogroup 1 strains Knoxville 1, Philadelphia 1, and Bellingham 1 reacted specifically with any of the antisera against whole bacterial cells of these serogroup 1 strains but did not cross-react with the serogroup 2 antiserum against strain Togus 1. The serological activities of *Legionella* LPS were not affected by heating at 100°C for 30 min and by treatment with Pronase, consistent with the properties of LPS antigens. Extraction of the LPS preparations with chloroform:methanol (1:3) without prior saponification reduced the overall fatty acid contents but did not affect its serological activities. However, repeated extraction with the organic solvents followed by treatment with Pronase caused degradation of the LPS and loss of serological activities.

Endotoxicity of *Legionella* LPS was demonstrated in animals. Intravenous injection of 5 μg (dry wt) of LPS from *L. pneumophila* strain Knoxville 1 in pyrogen-free saline into rabbits induced a biphasic temperature response characteristic of gram-negative bacterial endotoxin. Intraperitoneal inoculation of mice with a dose of 200 μg of the LPS preparation per animal produced overt symptoms and a weight change curve typical

of endotoxemia. Shwartzman reaction was provoked in sensitized rabbits with an average diameter of 21 mm from five tests.

Adjuvant activities of L. pneumophila lipopolysaccharide. Isolated *L. pneumophila* LPS was as effective as *Mycobacterium tuberculosis* H37Ra in potentiating antibody responses and in inducing delayed hypersensitivity to intrinsic and extrinsic protein antigens. The effect of oil emulsion on the adjuvant activities of *L. pneumophila* LPS appeared to be minimal. When the LPS of *L. pneumophila* was injected with protein antigens with or without oil, it potentiated the titers of humoral antibody to the protein antigens by four- to sixfold (Table I). The adjuvant effect of *L. pneumophila* LPS was most pronounced on the purified GOMP antigen, which, after its separation from the gonococcal LPS-protein complex by treatment with sodium deoxycholate, was a weak antigen, and induced low titers of antibody (0–160) in animals after a series of 3 weekly injections. In comparison, one single injection of the GOMP mixed with *L. pneumophila* LPS or Freund's complete adjuvant yielded antibody five- to sixfold higher in titer. Antibody to BSA or cross-reacting antigens of *Legionella* was potentiated by about four- to sixfold by *L. pneumophila* LPS or Freund's complete adjuvant.

When injected intradermally with protein antigens, the LPS of *L. pneumophila* induced delayed hypersensitivity in host guinea pigs to the protein antigens (Table II). The overt symptoms and time sequence of skin reactions in animals sensitized with *L. pneumophila* LPS as adjuvant were not different from the reactions induced with Freund's complete adjuvant, the visible and histopathologic characteristics of which were previously described (15, 19). In the present experiments, skin lesions of soft subcutaneous swelling and erythema were evident within 2 to 3 hr after injection of skin test antigens. This early reaction was followed by the development of induration and diffuse erythema which continued to increase in size and intensity until the reaction reached the peak between 24 and 48 hr. The lesion was pink in color and firm and stiff in composition with the erythema and induration identical in size. The lesion then subsided slowly over the next 48 to 72 hr. In comparison, the animals immunized with the isolated proteinaceous cross-reacting antigens in PBS showed a pre-

TABLE I. EFFECTS OF *Legionella lipopolysaccharide* (LLPS) AS ADJUVANT ON ANTIBODY RESPONSES

	Antibody titer and potentiation factor (PF)					
	Legionella cross-reacting antigen		Gonococcal outer membrane protein		Bovine serum albumin	
Adjuvant	Antibody	PF	Antibody	PF	Antibody	PF
None	102 (20–160)	1	46* (0–160)	1	280 (80–640)	1
LLPS in saline	640 (320–1280)	6.3	228 (40–640)	4.9	1376 (320–2560)	4.9
LLPS in oil emulsion	512 (160–1280)	5.0	296 (40–640)	6.4	1216 (320–2560)	4.3
Mycobacteria H37Ra (Freund's complete adjuvant)	622* (160–1280)	6.1	264 (40–1280)	5.7	1344* (320–5120)	4.8

Note. Antibody titers are averages of 9 (*) or 10 animals. The range of titers in individual animals is given in parentheses. The "potentiation factor" is the ratio of average antibody titers between an adjuvant test and the control with no adjuvant for the particular antigen. $P < 0.01$.

TABLE II. EFFECTS OF *Legionella lipopolysaccharide* (LLPS) ON INDUCTION OF SKIN HYPERSENSITIVITY TO PROTEIN ANTIGENS

	Diameter of skin reaction (mm)	
Sensitization	24 hr	48 hr
Legionella cross-reacting antigens (LCR) in saline	20 (13–26)	7 (5–12)
LCR mixed with LLPS	24 (18–27)	20 (16–24)
LCR mixed with LLPS in Freund's incomplete adjuvant	23 (19–26)	20 (18–23)
LCR in Freund's complete adjuvant*	26 (21–28)	23 (16–25)
Heat-killed *Legionella* in Freund's incomplete adjuvant	26 (23–29)	24 (19–26)
Nonsensitized control for *Legionella* LCR	4 (0–8)	3 (0–4)
Gonococcal outer membrane protein (GOMP) in saline	6 (0–11)	3 (0–8)
GOMP mixed with LLPS*	10 (8–15)	8 (6–12)
GOMP mixed with LLPS in Freund's incomplete adjuvant	14 (11–17)	11 (8–15)
GOMP in Freund's complete adjuvant	13 (11–15)	9 (8–12)
Nonsensitized control for GOMP	3 (0–5)	2 (0–4)

Note. Reaction diameters are means of seven (*) or eight tests with range of individual reactions in parentheses.

dominantly immediate type skin reaction which faded rapidly in 10 to 24 hr after skin test.

It was also observed in these experiments that inactivated *Legionella* bacteria or isolated *Legionella* LPS in oil emulsion caused much less tissue inflammation than mycobacteria. The *Legionella* bacteria or LPS water-in-oil adjuvant rarely caused open abscesses, which happened quite frequently with the oil-base adjuvant containing mycobacteria. Inactivated *Legionella* bacteria or isolated *Legionella* LPS in aqueous mixture with protein antigens were well tolerated by the animals, causing little overt tissue damage at the injection sites.

Discussion. We previously reported that heat-inactivated bacteria of *Legionella* in oil emulsion were a potent adjuvant, and their adjuvanticity was comparable to that of *M. tuberculosis* H37Rv in Freund's complete adjuvant (3, 4). In the present study, the LPS isolated from *Legionella* was demonstrated as the active principle of adjuvanticity with unique chemical and biological properties. The results also indicate that structural and chemical differences may exist between the *Legionella* LPS and the classical gram-negative bacterial endotoxin.

As adjuvant, the *Legionella* LPS was approximately as potent as *M. tuberculosis* H37Ra in Freund's complete adjuvant in potentiating antibody production and inducing delayed hypersensitivity. Emulsification in oil was not essential for manifestation of adjuvant activities for *Legionella* LPS. In their study on typhoid immunity, Johnson and co-workers (20) demonstrated that simultaneous injection of endotoxins from *Salmonella typhosa* and other gram-negative bacteria with protein antigens into rabbits resulted in enhanced antibody responses to the protein antigens. Their findings were later expanded by other investigators (21, 22) to show that endotoxin inhibited immunologic unresponsiveness to protein antigens. It appears that oil emulsion is not a requirement for adjuvant activities of bacterial lipopolysaccharide. Recently, Friedman and co-workers (23) reported that *L. pneumophila* whole cells as well as cell extracts enhanced the uptake of labeled thymidine by normal spleen cells, and heightened antibody responses to sheep erythrocytes in cell cultures. Their results indicate that *Legionella* may act as a polyclonal B-cell activator, and enhance antibody response helper factors.

There are other indications that *Legionella* LPS and *M. tuberculosis* differ in their adjuvant properties. McMaster and co-workers (24) have been able to separate delayed hypersensitivity from other disease components in experimental allergic thyroiditis and aspermatogenesis in guinea pigs by using *L. pneumophila* and *M. tuberculosis* as adjuvant. Animals were immunized with extracts of

guinea pig testis, thyroid, and rabbit spinal cord emulsified in oil containing either *L. pneumophila* or *M. tuberculosis.* After 30 days, thyroiditis and orchitis were observed in the animals that had received injections with *M. tuberculosis,* but no thyroiditis and little, if any, orchitis were detected in those sensitized with *L. pneumophila* adjuvant. However, both *M. tuberculosis* adjuvant and *L. pneumophila* adjuvant induced allergic encephalomyelitis and induced the same degree of delayed hypersensitivity to the tissue extracts as assayed by skin test. Their results indicate that delayed hypersensitivity to the tissue antigens was not, by itself, the sole cause of thyroiditis and orchitis, and the apparently different adjuvant properties of *M. tuberculosis* and *L. pneumophila* may provide a useful tool to elucidate the pathogenesis of some autoimmune diseases.

The mechanism of adjuvanticity of *Legionella* and its role in the host–parasite relationship remain to be elucidated. However, the adjuvant activities of *Legionella* LPS may explain the hypersensitivity observed in immunized guinea pigs when the animals were skin-tested with the proteinaceous cross-reacting antigens (15, 19), and may provide a means for detecting present or past *Legionella* infection based on the immunologic memory of host cells. The *Legionella* LPS isolated by the procedure described in this report were the most reactive cellular fractions in the *in vitro* cell culture system as an immunostimulatory agent (personal communication, Dr. H. Friedman, University of South Florida, Tampa, Fla.). Further investigation with highly reactive LPS preparations will shed light on these recently observed biological activities of *Legionella* species.

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