

Legionella pneumophila-Induced Immunostimulation and Blastogenesis
of Normal Mouse Spleen Cells *in Vitro* (41727)

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Abstract. Cell-free sonic extracts prepared from *Legionella pneumophila* serogroup 1 were found to enhance the uptake of [³H]thymidine by normal mouse spleen cell cultures *in vitro* and also stimulate an enhanced antibody response to sheep erythrocytes, both in immunized and nonimmunized cultures. Increased background antibody responses to other erythrocyte species also occurred, indicating that the *Legionella* antigen was a polyclonal B cell activator. A purified cell wall component with physicochemical properties relatively similar to endotoxin, but without toxicity for mice, was found to have mitogenic activity for normal mouse spleen cells and immunostimulatory properties for anti-erythrocyte antibody response. Heating the sonicate or the purified somatic antigen for 10 min diminished immunoenhancing activity but had little effect on mitogenic properties. These results point to the complex effects of *Legionella*-derived antigens on normal lymphoid cell function and indicate that antigens derived from *Legionella* have marked immunomodulatory properties.

Bacteria may markedly influence nonspecific immune responses, both *in vivo* and *in vitro*. Cell wall derived components from many Gram-negative or Gram-positive bacteria are known to stimulate the antibody response of normal lymphoid cells *in vitro* (1-3). In the present study, cell wall components isolated from *Legionella pneumophila*, including a crude cell-free sonicate and purified endotoxin-like somatic antigen, were found to be markedly mitogenic for normal murine spleen cells *in vitro* and also markedly enhanced the antibody response to sheep erythrocytes.

Materials and Methods. *Animals.* Inbred male BDF₁ hybrid mice obtained from Jackson Laboratories Supply Company, Indianapolis, Indiana, were used for these studies. They were approximately 6-8 weeks of age and housed in groups of 6-10 in plastic mouse cages and fed Purina mouse pellets and water *ad libitum*.

Bacterial preparations. Philadelphia Strain 1 *L. pneumophila* serogroup 1 was obtained initially from the Centers for Disease Control through the courtesy of Dr. Roger McKinney. The bacteria were grown on CYE agar for 24-36 hr and harvested with a rubber policeman into sterile saline containing 0.5% Formalin. After overnight incubation at room temper-

ature, the killed bacteria were sedimented by centrifugation at 10,000g and washed several times with sterile saline. They were resuspended to a concentration of 3×10^9 bacteria/ml saline and sonicates prepared with a Raytheon sonicator using three pulses at 60 sec each. The sonicates were clarified by centrifugation at 10,000g and frozen in small volumes at -70°C. A somatic antigen preparation, designated F₁, was prepared exactly as described previously (4, 5). In brief, the F₁ fraction purified from the *Legionella* was obtained by Sepharose 6B filtration as the eluate from the void volume. This material is a high-molecular-weight complex (4×10^6 Da or greater) composed of 35% carbohydrates, 26% protein, 1.8% phospholipid, and 1% 2-keto-3-deoxyoctonate. This material has many of the physicochemical properties of endotoxin and gave a biphasic fever response when injected into rabbits at a dose of 1.0 µg and also induced localized Schwartzman reactions. However, unlike conventional LPS, it had an LD₅₀ of greater than 500 µg for mice.

Mitogenic assay. Dispersed cell suspensions from normal mice were prepared by teasing individual spleens into sterile RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) in sterile petri plates. After several washings with saline, cell suspensions con-

sisting of 2.5×10^6 viable nucleated cells per milliliter were resuspended in 0.2 ml sterile Medium 1640 (Gibco, Grand Island, N.Y.) containing 10% fetal calf serum plus penicillin and streptomycin (100 μg each/ml medium). A volume of 0.25 ml of each cell mixture was added to individual wells of 96-well microtiter plates (Nunc). Cell cultures with or without addition of graded concentrations of *Legionella* preparations were incubated for various lengths of time at 37°C in a humidified incubator with 95% air and 5% CO₂. The blastogenic response of the splenocytes was determined by adding [³H]thymidine (0.5 μCi 2.0 c/mM, New England Nuclear, Boston, Mass.) per well. The amount of thymidine taken into the cells was determined 18 hr later by standard scintillation counting in a Packard liquid scintillation spectrometer.

Immune response to sheep erythrocytes. The number of antibody plaque forming cells (PFC) to sheep red blood cells (SRBCs) was determined for cultures of mouse spleen cells incubated with or without the bacterial preparations using a standard microtiter technique exactly as described previously (6). In brief, suspensions of 5×10^6 viable nucleated spleen cells prepared from mice exactly as for the blastogenic assay were cultured in individual wells in 96-well microtiter plates in enriched medium. Individual cultures were immunized by addition of 0.25 ml medium containing 2×10^6 sheep erythrocytes. To various cultures were added, in graded amounts, either sonicates or the purified F₁ antigen. Following incubation at 37°C for 5 days in a humidified chamber with 95% air and 5% CO₂, the numbers of PFCs per culture were determined by the microplaque assay of Yamada-Yamada exactly as described previously (6). The effects of the *Legionella* preparations on the SRBC-induced PFC response or the background PFC response was always determined in comparison to responses of similar cultures in the same experiments which were not treated with *Legionella* antigen.

Experimental Results. As is evident in Table I, both the *Legionella* sonicate and the purified F₁ antigen markedly affected the uptake of [³H]thymidine by the normal mouse spleen cell cultures. Blastogenesis occurred with 100 μg sonicate added per culture. Ten micrograms had much less effect, whereas 1.0 μg did not

TABLE I. EFFECT OF *Legionella* ANTIGENS ON BLASTOGENIC RESPONSE OF NORMAL MOUSE SPLEEN CELL CULTURES

<i>In vitro</i> stimulator ($\mu\text{g}/\text{culture}$) ^a	Blastogenic responses ^b		
	cpm	SI	P
None (control)	290 \pm 70	—	—
<i>Legionella</i> sonicate			
1.0	410 \pm 30	1.4	NS
10.0	930 \pm 40	3.2	<0.01
50.0	1580 \pm 160	5.5	<0.01
100.0	1790 \pm 90	6.2	<0.01
<i>Legionella</i> F ₁ Ag			
1.0	37 \pm 60	1.3	NS
10.0	1130 \pm 110	3.9	<0.01
50.0	2400 \pm 170	8.3	<0.01
100.0	1560 \pm 380	5.4	<0.01
<i>E. coli</i> LPS			
10.0	5860 \pm 1580	20.2	<0.01
100.0	4310 \pm 760	14.9	<0.01

^a Indicated concentration of antigen preparation added to cultures of normal mouse spleen cells.

^b Average cpm, \pm SD, for triplicate cultures 72 hr after culture initiation; SI indicates stimulation index and P determined by Student's *t* test.

increase thymidine uptake. Similarly, the F₁ antigen enhanced the blastogenic response in a dose-dependent manner, with optimal enhancement occurring with the 10- and 50- μg doses of antigen. The 1- μg dose had no significant effect while the 100- μg dose of F₁ antigen slightly decreased the blastogenic response.

As is evident in Table II, addition of graded amounts of sonicate or F₁ antigen to normal mouse spleen cells *in vitro* being immunized with sheep RBCs resulted in a dose-dependent stimulation of the number of PFCs being formed. A two- to four-fold increase in the PFC response occurred in cultures containing 10 or 50 μg sonicate. One microgram of sonicate had a slight to moderate effect on the PFC response, whereas 100 μg also had a slight to moderate effect, but proportionally less than the 10- or 50- μg doses. The F₁ antigen, at a dose of 10 or 100 μg , increased the response about twofold.

The altered number of PFCs was not related to alteration in the viability or total number of cells in the cultures treated with the sonicate or somatic antigen as compared to control

TABLE II. EFFECT OF *Legionella* ANTIGEN ON ANTIBODY RESPONSE OF NORMAL MOUSE SPLEEN CELLS TO SHEEP ERYTHROCYTES

<i>In vitro</i> addition ($\mu\text{g}/\text{culture}$) ^a	PFC/ 10^6 spleen cells ^b	Percentage of control	P
None (control)	560 \pm 110	—	—
<i>Legionella</i> sonicate			
1.0	955 \pm 150	171	<0.05
10.0	1840 \pm 420	329	<0.01
50.0	4050 \pm 360	723	<0.01
100.0	6500 \pm 580	1161	<0.01
<i>Legionella</i> F ₁ Ag			
1.0	1160 \pm 210	207	<0.01
10.0	3580 \pm 420	639	<0.01
50.0	4100 \pm 480	732	<0.01
<i>E. coli</i> LPS			
10.0	940 \pm 110	168	<0.01
100.0	1280 \pm 195	229	<0.01

^a Indicated concentration of *Legionella* antigen added to cultures of 5×10^6 spleen cells from normal mice immunized on day of culture initiation with 2×10^6 SRBCs.

^b Average PFC response, \pm SD, for three to five cultures per group 5 days after culture initiation.

cultures, since there was no significant change in the total number of viable cells per culture at the time of assay. Also, the increased numbers of PFCs was not due to a shift in the peak day of the immune response. PFCs were assayed at various times after culture initiation for spleen cells treated with either the sonicate or the F₁ antigen and compared to control cultures (Table III). The peak day of the re-

sponse occurred on Day 5 after culture initiation regardless of whether or not bacterial antigen had been added to the cultures. Similar increases occurred with *E. coli* LPS, a known positive stimulator. Furthermore, those cultures containing the bacterial antigens showed enhanced numbers of antibody formation throughout the 7-day period of observation so that essentially similar enhancement was observed on all days tested.

The *Legionella* preparations also induced a heightened antibody response to RBCs other than those from sheep. The background response to horse and guinea pig erythrocytes were stimulated in unimmunized cultures, indicating that these bacterial preparations had a polyclonal B cell activation effect, similar to that known to occur with other bacterial immunomodulators such as *E. coli* LPS (Table IV). Heating the somatic or F₁ antigen to 56°C for 30 min had no effect on either immunostimulating or mitogenic properties, but boiling at 100°C for 10 min abolished the immunostimulatory activity of the sonicate and F₁ antigen but only had a marginal or no effect on mitogenicity (data not shown).

Discussion. *Legionella pneumophila* is a newly described Gram-negative opportunistic pathogen first found to be the etiologic agent of Legionnaire's Disease in Philadelphia in 1976. These microorganisms are considered an important cause of bacterial pneumonias as well as other systemic and subclinical infections (7-9). Over the last few years at least 12

TABLE III. CYTOKINETICS OF ANTIBODY RESPONSE BY MOUSE SPLEEN CELLS IMMUNIZED *IN VITRO* WITH SHEEP ERYTHROCYTES AND TREATED WITH BACTERIAL PREPARATIONS

<i>In vitro</i> stimulator ^a	Heat treated ^b	PFC/ 10^6 spleen cells on Day ^c				
		+3	+4	+5	+6	+7
None (control)		325	418	780	610	336
<i>Legionella</i> sonicate	-	630	940	2860	1130	710
	+	310	530	1050	740	530
<i>Legionella</i> F ₁ Ag	-	520	795	2430	1060	610
	+	285	—	1100	—	450
<i>E. coli</i> LPS	-	480	—	1560	—	570
	+	390	—	1930	—	400

^a Cultures of 5×10^6 spleen cells from normal mice incubated *in vitro* with 50.0 μg of indicated bacterial preparation.

^b Heat-treated bacterial preparations boiled at 100°C for 10 min prior to addition to spleen cell cultures.

^c Average number of PFC for three to four cultures per group tested on indicated day after *in vitro* immunization with 2×10^6 SRBC.

TABLE IV. STIMULATION OF "BACKGROUND" ANTIBODY RESPONSES TO ERYTHROCYTES BY NONIMMUNIZED MOUSE SPLEEN CELL CULTURES TREATED WITH BACTERIAL ANTIGENS

<i>In vitro</i> stimulation ($\mu\text{g}/\text{culture}$) ^a	PFC response per 10^6 spleen cells ^b		
	SRBC	HRBC	G.P. RBC
None (control)	38 \pm 8	5 \pm 2	10 \pm 3
<i>Legionella</i> sonicate			
10.0	140 \pm 30	10 \pm 3	18 \pm 7
50.0	210 \pm 46	28 \pm 6	39 \pm 10
<i>Legionella</i> F ₁ Ag			
10.0	97 \pm 10	16 \pm 5	35 \pm 6
50.0	340 \pm 65	37 \pm 10	86 \pm 12
<i>E. coli</i> LPS			
100.0	125 \pm 30	40 \pm 9	55 \pm 10

^a Indicated bacterial preparation added to cultures of 2×10^6 normal mouse spleen cells.

^b Average PFC response, \pm SD, to sheep, horse, or guinea pig RBCs for three to four cultures per group 4 days after culture initiation.

other species of *Legionella* or *Legionella*-like organisms have been isolated and identified. A number of studies have been concerned with purification of antigens derived from these organisms, both for epidemiologic purposes and serologic identification of the organism. Studies concerning the nature of a possible protective immunogen to be used as a vaccine have also been reported (5).

As is evident from the results of the present study, a crude soluble sonicate consisting of solubilized antigens present in the bacteria were found to have marked effects on the *in vitro* responsiveness of normal mouse spleen cells as assessed by the blastogenic test and antibody plaque formation *in vitro*. A recent report based on crossed immunoelectrophoresis indicates that similar cell preparations reacted in two-dimensional precipitin reactions with hyperimmune rabbit sera and gave evidence of at least 82 distinct antigenic moieties (10). Thus, it is difficult to determine which, if any, of the various antigenic components present in the sonicate may be responsible for the immunoenhancing and/or mitogenic effects. Heating the sonicate to 100°C reduced much of the immunostimulatory effects but did not alter mitogenic effects, suggesting that different components

may be responsible for the two different responses.

Previous studies in this and other laboratories have shown that bacterial adjuvants do not necessarily have to be mitogenic (11). Experiments with the boiled sonicate suggest that the reverse is also true, i.e., mitogenicity may be present after 100°C treatment but the immunostimulatory effect is inhibited, suggesting that a heat-labile material is the immunomodulator, at least for the *in vitro* response to the sheep erythrocytes. Additional studies with the purified F₁ antigen supports the possibility that a non-LPS moiety may be responsible for both effects.

Previous studies have shown that the F₁ antigen is the serogroup specific antigen of *L. pneumophila* and is free of many of the other antigenic determinants, including flagellar and cross-reactive determinants, etc. (5). As shown in this study, the F₁ antigen, in relatively small concentrations, enhanced the thymidine uptake of mouse spleen cell cultures and also had similar immunoenhancing effects on both the antigen specific and nonspecific PFC response to sheep and other erythrocytes. Boiling the F₁ antigen removed most of the mitogenic activity but only moderately suppressed the immunostimulatory activity.

The significance of the immunoenhancing and mitogenic properties of the sonicate and the purified antigen is not clear, but it is important to know that many other bacterial cell wall components have similar properties, both for murine lymphoid cells as well as lymphoid cells from other species, including man (2, 3). Indeed, many of the altered immune responses noted in bacterial infections have been attributed to effects of cell wall components. Immunoderegulation often accompanies infection with Gram-negative as well as Gram-positive bacteria. The results of the present study indicate that similar modifications of immune responses may be related to exposure of normal lymphoid cells to *Legionella* antigens. The observation that a heat-labile immunostimulation component in the *Legionella* sonicate is noteworthy and indicates that the active material cannot be LPS or peptidoglycans. Further analysis of purified component of *Legionella* in terms of immunostimulatory and mitogenic properties appears warranted.

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1. Schwab JH. Suppression of immune responses of microorganisms. *Bact Rev* **39**:121-143, 1974.
 2. Nowotny A. Beneficial Effects of Endotoxins, New York, Plenum Press, 1983.
 3. Friedman H, Klein T, Szentivanyi A. Immunomodulation by Bacteria and Their Products, New York, Plenum, 1981.
 4. Johnson W, Elliott JA, Helms CM, Renner ER. A high molecular weight antigen in Legionnaire's Disease Bacteria: Isolation and partial characterization. *Ann Intern Med* **90**:638-641, 1979.
 5. Elliott JA, Johnson W, Helms CM. Ultrastructural localization and protective activity of a high molecular weight antigen isolated from *Legionella pneumophila*. *Inf Immun* **31**:822-824, 1981.
 6. Kano I, Pan S-H, Friedman H. A simplified procedure for in vitro immunization of dispersed spleen cells. *J Immunol Methods* **11**:55-62, 1976.
 7. McDade JE, Shephard CC, Fraser DW, Tsou TR, Reches MA, *et al.* Legionnaire's Disease: Isolation of a bacterium and demonstration of its role in other respiratory diseases. *N Engl J Med* **297**:1197-1203, 1977.
 8. Meyer RD, Finegold SM. Legionnaire's Disease. *Annu Rev Med* **31**:219-232, 1980.
 9. David GS, Winn WC, Beaty HN. Legionnaire's Disease: Infection caused by *Legionella pneumophila* and Legionella-like organisms. *Clin Chest Med* **2**:145-166, 1981.
 10. Collins MT, Cho S-N, Hoiby N, Espersen F, Black L, Reef VS. Crossed immunoelectrophoretic analysis of *Legionella pneumophila* serogroup 1 antigens. *Inf Immun* **39**:1428-1440, 1983.
 11. Frank S, Specter S, Nowotny A, Friedman H. Immunocyte stimulation in vitro by nontoxic bacterial lipopolysaccharide derivatives. *J Immunol* **119**:855-860, 1977.
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