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Blastogenic Responsiveness of Spleen Cells from Guinea Pigs Sensitized to *Legionella pneumophila* Antigens (41685)

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Abstract. An *in vitro* leuckocyte blastogenic assay was utilized to establish an *in vitro* correlate of cell-mediated immunity to *Legionella pneumophila* antigen with spleen cells from sensitized guinea pigs. Incubation of spleen cells from sensitized but not normal guinea pigs with graded amounts of killed whole cell *Legionella* bacteria or sonicate derived from the bacteria resulted in an antigen-induced blast cell proliferation as evidenced by an increased uptake of [^{3}H]-thymidine into spleen cell cultures. Peak responses occurred approximately 4–6 days after incubation of the spleen cells with antigen. Sensitivity of spleen cells from animals immunized with *Legionella* vaccine in adjuvant persisted for at least 150 days, while responses after infection of guinea pigs with viable bacteria persisted about 4–6 weeks. The blastogenic responses of the spleen cells to *Legionella* antigen appeared to be a correlate of cell-mediated immunity.

Legionella pneumophila (*Lp*) is considered an opportunistic intracellular microorganism now known to be the etiologic agent of upper and lower respiratory tract infections in man (1, 2). Guinea pigs have served as a useful *in vivo* infection model for this organism as they develop pulmonary disease symptomatically and histologically similar to that observed in man (3, 4). Since the discovery of this bacterium in 1976, various studies have been performed concerning the bacteriology and pathology of the infectious process in both man and guinea pigs. However, studies concerning the immune response to this organism have largely been limited to examination of antibody responses (5, 6). For example, previous studies in this laboratory showed that serum antibody titers determined by solid phase immunofluorescence assays were detectable as early as 7 days after immunization of guinea pigs with *Lp* in adjuvant and lasted for at least several weeks (7).

Since it has been shown that *Lp* is an intracellular microorganism capable of replicating in guinea pig and human mononuclear cells (8, 9), it seems likely that cell-mediated immunity may play a role in host defense to this organism. Thus, it was of interest to determine whether sensitization of lymphoid cells from animals given *Lp* could be detected *in vitro*. In this regard, lymphocyte blastogenic tests (LBT) have been widely used as *in vitro* correlates of *in vivo* responsiveness to a wide variety of antigens (10). Results of the present study show that spleen cells from guinea pigs immunized with killed *Lp* antigens respond to both whole cell and sonicated extracts derived from this organism. Maximum LBT responsiveness did not occur until about a month or so after initial exposure to this microorganism. The responses, however, persisted for up to 5 months after primary immunization. Guinea pigs injected with a sublethal dose of the bacteria also evinced positive LBT responses, but for a shorter duration.

Methods and Materials. *Animals.* Hartley strain guinea pigs are obtained from Elm Hill Laboratories, Chelmsford, Maine, and weighed 350–400 g at the start of an experiment. They were rested for a minimum of 1 week after receipt before immunization. The animals were housed in pairs in the animal facility and given water and guinea pig pellets *ad libitum.*

Vaccine and antigen preparation. Philadelphia strain 1 *L. pneumophila,* originally a kind gift from Dr. R. McKinney (Centers for Disease Control, Atlanta, Ga.), were grown on CYE agar (Remmel Laboratories, Lenexa, Kans.) and harvested during the log phase of growth, i.e., 24–36 hr at 37°C. The bacteria were killed by suspension in 0.5% formalin diluted with sterile pyrogen-free saline (PFS). Whole cell antigen was prepared by washing the killed bacteria three times in PFS, followed by resuspension to the appropriate concen-

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tration in saline as determined by spectrophotometry at 560 nm with McFarland standards. The whole cell antigen, which also served as the vaccine for animal immunization, was stored at 4°C. Sonicated antigen was prepared from the vaccine by six 30-sec pulses in a sonicator at 20 KHz (Biosonic, Rochester, N.Y.), followed by clarification by centrifugation at 10,000*g*. The sonicate was then filter-sterilized and protein content determined spectrophotometrically using bovine serum albumin standards. One-tenth milliliter of sonicate containing 10 μg protein was derived from 10^6 bacteria. The antigen was stored at −70°C until used.

Immunization. Whole-cell antigen was suspended in complete Freund's adjuvant (CFA), Difco Laboratories, Detroit, Michigan, and 1.0 ml volumes injected in two subcutaneous sites in the nuchal region of the experimental animals. Secondary immunizations were given in incomplete Freund's adjuvant (IFA) in the same volume and site as the primary immunizations. Infected guinea pigs were injected ip with 0.5 ml sterile saline containing 2×10^5 viable *L. pneumophila* obtained after 18 hr growth at 37°C on CYE agar. None of the animals died with this dose, but all showed evidence of fever and illness. Bacteria were recovered up to 7–8 days afterward from the blood of the animals, but not thereafter.

Antigen blastogenic assay. Splenocytes from control or experimental animals were adjusted to a concentration of 1.0×10^6 viable cells/ml in RPMI 1640 medium supplemented with standard concentrations of penicillin–streptomycin antibiotic mixture, L-glutamine (Gibco Laboratories, Grand Island, N.Y.) and 10% fetal calf serum (Rehis, Kanakie, Kans.). The cells were dispensed (0.2 ml) into flat-bottom microtiter plates (Nunc, Gibco), and either whole cell or sonicated antigen added. The plates were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air for 3–7 days followed by a 18- to 24-hr pulse with 0.5 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass.). The cells were then harvested using a multiple automated sample harvester (Brandel, Rockville, Md.) and counts incorporated determined by liquid scintillation spectrometry. As controls, Concanavalin A (Miles, Yeda, Rehovot, Israel) and *E. coli* lipopolysaccharide (Difco) were used to insure that potential T and B cell responsiveness was present in the cell cultures. Stimulation indices (SI) were determined by standard formula and SIs greater than 2.0 considered indicative of a significant antigen blastogenic response (10).

Experimental Results. Skin tests previously performed in this laboratory (data not shown) indicated that delayed type cutaneous hypersensitivity reactions to *Lp* arise in guinea pigs at approximately 30–35 days postimmunization with killed organisms in CFA. Thus, the LBT responsiveness of spleen cells from animals inoculated wth *Lp* was assessed at about this time period by determining uptake of [^{3}H]thymidine after exposure to the *Legionella* antigen *in vitro*. As is evident in Table I, there was only slight uptake of thymidine by spleen cells from normal control guinea pigs incubated *in vitro* for 6 days with graded amounts of either killed intact *Legionella* suspensions or sonicates. On the other hand, incubation of spleen cells from guinea pigs immunized 40 days earlier with *Lp* in CFA resulted in a modest blastogenic response to several of the concentrations of *Lp* whole cell or sonicate antigen. A concentration of 10^5 whole bacteria incubated with spleen cells from immunized but not normal guinea pigs resulted in a two- to threefold enhancement of thymidine uptake. The sonicate at a concentration of 1–10 μg also resulted in a heightened uptake of thymidine. No enhanced uptake of thymidine occurred with spleen cells from either normal or *Lp* immunized guinea pigs when stimulated *in vitro* with 10^5 of 10^7 heat-killed *E. coli* or 10 μg sonicate thereof as controls (Table I).

The peak day of the LBT response occurred approximately 4–6 days after incubation of the spleen cells from sensitized guinea pigs with whole bacterial suspensions (Table II). A concentration of 10^7 bacteria, when added to the spleen cells from sensitized guinea pigs, resulted in the highest (four- to fivefold) increase of thymidine uptake on Day 4. In contrast, the lower doses resulted on peaks on Day 6. Similarly, the sonicate resulted in the highest uptake on Day 6 after incubation. A dose of 1.0 to 10.0 μg sonicate resulted in the highest uptake of thymidine on Day 6. Higher doses, i.e., 20–40 μg, resulted in somewhat lower responses on Day 6 (Table II).

In order to assess the duration of respon-

TABLE I. BLASTOGENIC RESPONSIVENESS OF GUINEA PIG SPLEEN CELLS TO *Legionella* ANTIGEN PREPARATIONS

Bacterial antigens added to cultures[a]	Guinea pig spleen cells tested[b]					
	Lp sensitized			CFA control		
	CPM	SI	*P*	CPM	SI	*P*
None (control)	620 ± 150	—	—	770 ± 60	—	—
Legionella vaccine						
10^4	580 ± 55	0.9	NS	745 ± 190	0.9	NS
10^5	1575 ± 220	2.5	<0.01	560 ± 50	0.7	NS
10^6	1260 ± 180	2.0	<0.01	620 ± 100	0.8	NS
Sonicate						
1.0 μg	1990 ± 95	3.2	<0.01	535 ± 180	0.7	NS
5.0 μg	2150 ± 350	3.5	<0.01	580 ± 130	0.8	NS
10.0 μg	1650 ± 310	2.7	<0.01	630 ± 230	0.8	NS
E. coli vaccine						
10^5	788 ± 215	1.3	NS	730 ± 110	0.9	NS
10^7	740 ± 98	1.2	NS	648 ± 65	0.8	NS
Sonicate						
10.0 μg	760 ± 150	1.2	NS	698 ± 125	0.9	NS

[a] Indicated bacterial preparation added to cultures of 2 × 10^5 viable guinea pig spleen cells 6 days before 18- to 24-hr pulse with [^{3}H]thymidine.

[b] Stimulation indices (SI) and CPM represent means of triplicate cultures; guinea pigs sensitized 40 days earlier by subcutaneous injection with 3 × 10^9 *Lp*/kg in CFA; control animals received CFA alone. *P* determined by Student's *t* test.

siveness observed *in vitro* with spleen cells from sensitized guinea pigs, animals were given *Lp* and then sacrificed at various times thereafter. As is evident in Table III, no response occurred 7 days after sensitization of guinea pigs with *Lp* in CFA. Animals sensitized with *Lp* in CFA for 40 days or longer generally yielded responsive spleen cells. Also, animals sensitized with *Lp* as long as 150 days earlier showed a significant enhancement of thymidine incorporation even when challenged *in vitro* wth a low dose of whole bacterial cells or sonicate. Guinea pigs primed with *Lp* 150 days earlier and then reinjected 40 days before *in vitro* challenge, as well as at -7 and -10 days, showed enhanced responsiveness to the whole bacterial cells, both high and low dose, as well as to the sonicate (Table III). Injection of primed animals 7 days before *in vitro* challenge resulted in a markedly lower response suggesting induction of a refractory period by such treatment (Table III).

Guinea pigs injected with viable *Legionella* also showed evidence of responsiveness when their spleen cells were challenged *in vitro* in the LBT assay with either whole bacterial cell suspension or sonicate. As is evident in Table IV, spleen cells obtained about 4 weeks or longer after sublethal infection with 2 × 10^5

TABLE II. KINETICS OF BLASTOGENIC RESPONSES TO *Legionella* ANTIGEN BY SPLENOCYTES FROM IMMUNIZED GUINEA PIGS

Stimulator *in vitro*[a]	Stimulating index[b] Day after culture initiation				
	3	4	5	6	7
Whole cell					
10^3	1.1	1.2	1.2	2.1	3.2
10^5	1.8	2.2	2.0	3.0	—
10^7	3.1	4.5	2.5	0.8	—
Sonicate					
1 μg	1.3	2.0	2.0	3.7	1.4
5 μg	1.4	1.9	2.9	3.8	1.6
10 μg	1.2	1.6	0.9	2.4	1.7
20 μg	1.1	1.7	0.8	1.9	1.0
40 μg	1.2	1.5	0.7	2.2	1.3

[a] Indicated *Lp* preparation added to cultures of 2 × 10^5 spleen cells from guinea pigs immunized 45 days earlier with 3 × 10^9 *Lp*/kg in CFA.

[b] Mean SI for triplicate cultures assayed on indicated day after *in vitro* stimulation with *Lp* antigen.

TABLE III. BLASTOGENIC RESPONSES OF SPLEEN CELLS FROM GUINEA PIGS AT VRIOUS TIMES AFTER SINGLE OR BOOSTER IMMUNIZATION WITH *Legionella* ANTIGEN

Spleen cell source[a]		*In vitro* stimulation (SI)[b]			
		Whole bacteria		Sonicate	
Primary injection	Secondary	10^3	10^5	1 μg	10 μg
Normal controls	—	0.9	1.5	0.9	1.3
Legionella injected					
−7 days	—	1.1	1.2	0.5	0.8
−25 days	—	0.8	0.7	0.9	1.9
−40 days	—	2.7	3.3	0.7	2.6
−60 days	—	2.1	3.0	3.7	2.4
−150 days	—	2.0	2.8	2.1	3.5
−150 days	−7 days	1.2	1.4	1.3	1.2
−150 days	−40 days	5.5	4.1	2.6	2.2
−150 days	−7 and −10 days	3.4	4.2	3.5	3.8

[a] Guinea pigs immunized on day indicated with 3.0×10^9 *Lp*/kg in CFA for primary and in IFA for secondary injection.

[b] Average response of cultures of 2×10^5 spleen cells tested in triplicate after 6 days of *in vitro* incubation with indicated *Lp* antigen.

Legionella evinced a positive LBT response when tested *in vitro* with 10 μg sonicate. Equivalent responses occurred with the whole bacterial cells as antigens, while no response occurred with *E. coli* as a control (data not shown). Peak responses occurred about 4–5 weeks after infection with the live bacteria. Lower doses of *Legionella* had no effect, while higher doses resulted in death of the guinea pigs, often within 1 week.

Discussion and Conclusion. The results of this study show that blastogenic responsiveness to *Legionella* antigen occurs when splenocytes from guinea pigs immunized with whole cell vaccine or injected with a sublethal dose of the bacteria are incubated with either the killed bacteria or sonicated antigen derived thereof. Readily detectable sensitization was evident by the LBT assay approximately 40 days after primary immunization and such *in vitro* responsiveness persisted for at least 150 days. Very little nonspecific blastogenic responsiveness was observed with spleen cells from control, nonsensitized animals or those sensitized with the mycobacteria containing CFA alone. Spleen cells from such animals failed to show significant blastogenic response except when higher doses of *Lp* antigen such as 50–100 μg or more were used *in vitro* (data not shown). With the doses used for *in vitro* challenge, only spleen cells from sensitized animals showed a significant (SI of 2 or more) increase of thymidine incorporation as compared to control cultures incubated with saline alone. The peak *in vitro* response was between 4 and 6 days with most antigen doses used, either whole cell bacteria or sonicates, regardless of the time after sensitization.

TABLE IV. BLASTOGENIC RESPONSES OF SPLEEN CELLS FROM GUINEA PIGS AFTER INFECTION WITH SUBLETHAL DOSE OF *Legionella*

Time in days after infection[a]	Blastogenic response[b]		
	CPM ± SD	SI	*P*
None (control)	758.5 ± 65.5	—	—
7–10	876 ± 183.6	1.2	NS
15–20	1390 ± 250.5	1.8	<0.05
25–35	2260 ± 310.4	3.0	<0.01
40–60	1530 ± 460.6	2.0	<0.01

[a] Guinea pigs, two to three per group, injected at indicated times before testing with 2×10^5 viable *L. pneumophila.*

[b] Average response of cultures of 2×10^5 spleen cells from indicated guinea pigs assayed in triplicate 6 days after *in vitro* stimulation with 10 μg *L. pneumophila* sonicate.

The question of which cell class responds to the *Legionella* antigen *in vitro* is under active investigation. The kinetics of development of the response suggested that this *in vitro* assay, similar to those reported by others, may

be due to responsiveness of T lymphocytes. Antibody formation was readily detectable as early as 7 days after immunization with the same dose of *Legionella,* with peak responses occurring shortly thereafter (7). Since peak LBT responses did not occur until 40 or more days after sensitization, it appears that this type of response is compatible with the skin reactivity observed previously.

It has been reported that peripheral blood leukocytes from seropositive patients with a history of Legionnaire's Disease responded to *Lp* sonicate with a mean SI of 68 as compared to the responses of the cells incubated without antigen (11). However, there was a very large range of responses reported. Furthermore, peripheral blood cells from seronegative control subjects had a mean SI of 18, indicating a threefold difference between patients and controls. It should be noted that no information was provided as to the clinical severity or length of disease of the patients, nor the time period between overt clinical symptoms and when the tests were performed. No information was provided as to a possible correlation between serum antibody titers to *Legionella* and the LBT responses of the patients or controls. It should be noted that the SIs for both patients and controls were higher than those observed with the guinea pig spleen cells, suggesting that aside from species differences active infection in man may result in a more potent blastogenic response than immunization of guinea pigs with killed antigen. In addition, the relatively high SI of the control subjects suggests that unlike the situation with guinea pigs, *Lp* may be a nonspecific mitogen for human peripheral blood leukocytes. Alternatively, since *Lp* is so ubiquitous, the high SIs for the human control subjects may reflect prior unapparent sensitization. Results of the studies with spleen cells from guinea pigs actively infected with graded numbers of viable *Legionella* revealed that SI of 2.0 to 3.0 develops, indicating that guinea pig spleen cells respond less vigorously than human peripheral blood monocytes to similar *Lp* sonicates even after infection.

Studies in many laboratories over the past decade or so have shown a close correlation between LBT positivity of lymphoid cells to an antigen and skin reactivity to the same antigens, supporting the view that the *in vitro* assay is a correlate of cellular immunity (10). An SI response of 2 or more is considered significant to bacterial antigens. Although this is much lower than the expected response to mitogens, this response, when specific, is thought to be an excellent *in vitro* correlate of CMI. Furthermore, it has recently been reported that human peripheral blood monocytes from patients recovered from Legionellosis showed increased killing of *L. pneumophila in vitro* and also released a soluble factor which mediates enhanced killing by normal monocytes (12, 13). This observation has been interpreted as indicating development of cell-mediated immunity in patients who have recovered from Legionellosis. Thus, the availability of completely *in vitro* assays for determining sensitivity to an organism such as *Legionella* should permit further analysis of the nature and mechanism of immune responses at the cellular level to this microbial pathogen.

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1. Fraser DW, Tsai TR, Orenstein W, Parkin WE, Beecham HJ, Sharran RG, Harris J, Mallison GF, Martin SM, McDade JE, Shepard CC, Brochman PS. Legionnaire's disease: Description of an epidemic of pneumonia. New Engl J Med **297**:1187–1197, 1977.
2. Sanford JP. Legionnaires' disease—the first thousand days. New Engl J Med **300**:654–665, 1979.
3. Pasculle AW, Mycrowitz RL, Rinaldo CR Jr. New bacterial agent of pneumonia isolated from renal transplant recipients. Lancet **2**:58–61, 1979.
4. Berendt RF, Young HW, Allen RG, Knutsen GL. Dose-response of guinea pigs experimentally infected with aerosols of *Legionella pneumophila.* J Infect Dis **141**:186–192, 1980.
5. Klein GC, Jones WL, Feeley JC. Upper limit of serum antibodies to *Legionella pneumophila* in patients with chronic pulmonary disease. J Clin Microbiol **10**:754–755, 1979.
6. Freedman AP, Katz, SM. The prevalence of serum antibodies to *Legionella pneumophila* in patients with chronic pulmonary disease. Amer Rev Respir Dis **123**:238–239, 1981.
7. Vogel FR, Klein TW, Spector SC, Hitchings M, Friedman H. Detection of antibodies to *Legionella pneumophila* in immune guinea pig serum by solid-phase immunofluorescence. J Clin Microbiol **13**:726–727, 1981.

8. Kishimoto RA, White JD, Shirey FG, McGann VG, Berendt RF, Larson EW, Hedlund KW. In vitro response of guinea pig peritoneal macrophages to *Legionella pneumophila.* Infect Immun **31**:1209–1213, 1981.
9. Horwitz MA, Silverstein SC. Interaction of the Legionnaire's disease bacterium with human phagocytes. II. Antibody promotes binding of *L. pneumophila* to monocytes but does not inhibit intracellular multiplication. J Exp Med **153**:398–406, 1981.
10. Varky F, Stjernsward J. Lymphocyte stimulation test for detection of tumor-specific reactivity in human. In: Bloom BR, David JR, eds. *In Vitro* Methods in Cell-Mediated and Tumor Immunity. New York, Academic Press, pp597–606, 1976.
11. Ploufee JF, Baird IM. Lymphocyte transformation to *Legionella pneumophila.* J Clin Lab Immunol **5**:149–152, 1981.
12. Horwitz MA, Silverstein SG. Activated human monocytes inhibit the intracellular multiplication of Legionnaire's Disease Bacteria. J Exp Med **154**:1618–1634, 1981.
13. Horwitz MA. Demonstration of Cell-mediated immunity in Legionnaire's Disease. Clin Res **30**:369A, 1982.

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