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Cellular Immunity to *Legionella pneumophila* in Guinea Pigs Assessed by Direct and Indirect Migration Inhibition Reactions In Vitro

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Spleen cell cultures from guinea pigs given *Legionella pneumophila* vaccine in complete Freund adjuvant or as a sublethal infection were inhibited in their migration activity in vitro when incubated with specific antigen. Both direct and indirect migration inhibition assays revealed sensitization of the guinea pigs to the bacterium, with demonstrable reactivity 25 to 40 days or more after sensitization. No consistent reactions occurred when the guinea pigs were given the killed *Legionella* vaccine in incomplete Freund adjuvant in saline. However, spleen cells from guinea pigs injected with sublethal doses of the *Legionella* vaccine 3 to 4 weeks earlier showed positive migration inhibition factor reactivity. Cutaneous hypersensitivity and lymphocyte blastogenic responsiveness in vitro also developed in guinea pigs sensitized with killed *Legionella* vaccine in complete adjuvant or given a sublethal infection with the bacterium. These results indicate that in vitro assays for migration inhibitory activity may be utilized to monitor the development of the sensitization of guinea pigs to *L. pneumophila*, and such reactions correlate with skin reactivity and in vitro lymphocyte blastogenic responses.

The exposure of individuals to many microbial agents, especially intracellular microbial pathogens, including opportunistic bacteria, viruses, and fungi, results in the development of serum antibodies, as well as cell-mediated immunity (CMI) readily detectable by various in vivo and in vitro assays. Recent studies have shown that *Legionella pneumophila*, the etiological agent of many atypical bacterial pneumonias in humans (4, 14, 16, 21), readily elicits antibody responses detectable by a wide variety of serological techniques, including immunofluorescence, microagglutination, and enzyme-linked immunoassays (3, 5, 12, 17, 19).

Since *L. pneumophila* is considered an intracellular microorganism which infects macrophages and perhaps other lymphoid cells (7, 9–11; H. Friedman and T. W. Klein *in* C. Krassman, ed., *New Infectious Agents*, in press; M. A. Horwitz, Clin. Res. **30**:369, 1982) it seems likely that these organisms may also affect CMI or induce such responses. Recent reports have indicated that the incubation of human peripheral blood leukocytes from patients who had recovered from *L. pneumophila* infection with sonic extracts derived from these bacteria resulted in blastogenic responses considered a correlate of CMI (15). Increased levels of [^{3}H]thymidine were incorporated into such cells, with an average stimulation index of about 50. However, similar tests with noninfected control patients also resulted in positive reactions, but at a reduced level (an average stimulation index [SI] of about 20). Recent similar studies in this laboratory have shown that guinea pigs and mice develop sensitized lymphoid cell populations capable of responding by blast cell transformation to either sonic extracts or killed suspensions of *L. pneumophila* after active immunization or challenge infection. Such responses appear to be a correlate of CMI, especially since these animals also develop skin test reactivity at the same time as their lymphoid cells respond in vitro to the *Legionella* antigen.

In the present study, spleen cells from guinea pigs actively sensitized with *Legionella* antigen, either by immunization or subclinical infection, were inhibited in their normal in vitro migration when cultured together with soluble sonic extracts from the bacteria, but not with intact *L. pneumophila*. In addition, we performed an indirect migration inhibition assay by stimulating supernatants from spleen cells in vitro with either intact killed bacteria or sonic extracts thereof. The inhibition of the migration of mouse peritoneal exudate cells occurred, indicating

that soluble lymphokines mediating migration inhibition reactions could be utilized in vitro to assess reactivity to *Legionella* antigens.

MATERIALS AND METHODS

Experimental animals. Hartley guinea pigs, approximately 300 to 400 g in weight, were purchased from Cantell Laboratories, Lenex, Kans. They were housed in groups of two in the animal facility and fed guinea pig pellets and water ad libitum. Mouse peritoneal exudate (PE) cells were obtained from BALB/c animals maintained in plastic mouse cages in groups of 6 to 10. The mice were purchased from Jackson Memorial Laboratories, Bar Harbor, Maine, and fed Purina mouse pellets and water ad libitum.

***Legionella* antigen.** *L. pneumophila* Philadelphia strain 1, serogroup 1, originally obtained through the courtesy of Roger McKinney, Centers for Disease Control, Atlanta, Ga., was cultured for 24 to 36 h on charcoal-yeast extract agar plates and harvested with saline, using a rubber policeman. The bacteria were washed by centrifugation in sterile saline at 4°C and then suspended to the appropriate concentration. For vaccine preparation, the bacteria were killed with 0.5% Formalin in sterile saline at 37°C for 18 to 24 h. Sonic extracts were prepared by treating killed suspensions of 3×10^9 bacteria per ml with six 1-min pulses with a Raytheon biosonic sonicator. The sonic extracts were clarified by centrifugation at 10,000 × *g* and frozen at −70°C until used.

Immunization and infection. Groups of guinea pigs were injected with *L. pneumophila* in either complete Freund adjuvant (CFA) or incomplete adjuvant (IFA) obtained from Difco Laboratories, Detroit, Mich. For this purpose, the animals were sensitized by injecting 3×10^9 bacteria in 1.0 ml of adjuvant in two sites in the nuchal region. Control animals were injected with the same amount of adjuvant without bacteria or with bacteria in saline only. For experiments with living bacteria, fresh overnight cultures of *L. pneumophila* were washed twice with sterile saline, and graded numbers were injected intraperitoneally in 1.0 ml of sterile saline.

Skin tests. Guinea pigs were injected intradermally with 0.1 ml of sonic extract containing 100 μg of protein as determined by the absorbance at 280 nm, using bovine serum albumin as the standard. The area of induration plus erythema was measured at 4, 24, and 48 h.

Migration inhibition assay. For assay of the migration inhibition factor (MIF), both direct and indirect microdroplet procedures were utilized essentially as described previously (7, 13, 20). In the direct assay, a suspension of freshly washed spleen cells, at a concentration of 1×10^7 viable nucleated cells per ml of medium, was mixed with 0.4% agarose in RPMI 1640 medium with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) and placed as microdrops in individual wells in 96-well microtiter plates. To each drop was added 0.2 ml of medium with or without the addition of graded amounts of either killed *L. pneumophila* or sonic extracts thereof. For the indirect assay, 2×10^6 spleen cells per ml of medium were incubated for 24 h at 37°C with *Legionella* antigen, either whole cell vaccine or sonic extract. The cells were then separated by centrifugation at 1,000 × *g* for 30 min to obtain the supernatant, and a 0.2 volume was added to agarose microdroplet cultures of 1×10^7 PE cells prepared from BALB/c mice injected intraperitoneally 72 h earlier with sterile dextran. The microdroplet cultures, both for the direct and indirect assays, were then incubated for 24 h at 37°C, and the degree of migration of the lymphoid cells was determined by microscopy, using a calibrated lens insert. The percent migration inhibition was calculated as the area of migration of the cells in the agarose droplets containing antigen as compared with that of the cells incubated in droplets containing medium without antigen. An inhibition of migration of 20% or more is considered significant (7).

Blastogenic test. For the lymphocyte blastogenic test, 2×10^5 guinea pig spleen cells were incubated in microwells in Nunc microtiter plates in RPMI 1640 medium containing 10% fetal calf serum (GIBCO) exactly as described elsewhere (18). To each test well was added a graded amount of *Legionella* sonic extract or, as a control, purified protein derivative, lipopolysaccharide, or concanavalin A to assure that the spleen cells were responding. After 4 to 6 days of incubation at 37°C in 5% CO_2 under 95% air, the cells were pulsed by an 18-h exposure to 0.5 μCi of [^{3}H]thymidine (Schwartz Laboratories, Boston, Mass.). The amount of isotope in the cell cultures was determined by standard scintillation counting, using a MASH apparatus to transfer the cells and a Packard scintillation counter. The SI was calculated from the counts per minute of the cell cultures treated in vitro with antigen in comparison with that of untreated cell cultures. An SI of 2.0 or more is considered significant (18).

RESULTS

The migration inhibition of leukocytes in the agarose drops was observed in both the direct and indirect assays using spleen cells from guinea pigs sensitized 30 days earlier with killed *L. pneumophila* in CFA (Table 1). Spleen cells from sensitized guinea pigs directly incubated in microdroplets with graded amounts of *Legionella* sonic extracts showed positive inhibition of migration. In contrast, the incubation of the spleen cells in agarose drops with graded amounts of *Legionella* whole cell suspension failed to reveal direct migration inhibition, regardless of the dose used (Table 1). However, the indirect assay, with mouse PE cells as the indicator, readily revealed the inhibition of cell migration in cultures containing supernatants from the same spleen cells from sensitized animals incubated with either sonic extracts or intact killed bacteria. Migration inhibition occurred with supernatants from cultures of spleen cells from guinea pigs sensitized with *Legionella* vaccine 30 days earlier that were treated in vitro with 10^5 or 10^7 intact killed bacteria, as well as with cultures treated with 10 or 20 μg of sonic extract. Spleen cells from normal guinea pigs not sensitized with *L. pneumophila* or from control

TABLE 1. Determination of migration inhibition of lymphoid cells by direct and indirect assays

Test antigen[a]	Concn	% Migration inhibition[b] ± SD by:			
		Direct assay	P	Indirect[c] assay	P
L. pneumophila whole cells	10^3	2.1 ± 0.5	NS	38.6 ± 9.4	0.01
	10^5	4.2 ± 0.7	NS	57.5 ± 11.3	0.01
	10^7	4.5 ± 0.3	NS	—[d]	
L. pneumophila sonic extracts	1 μg	31.5 ± 6.8	<0.01	21.4 ± 10.6	<0.05
	10 μg	25.8 ± 7.3	<0.01	30.3 ± 5.9	<0.01
	20 μg	21.5 ± 6.0	<0.05	39.2 ± 8.3	<0.01
	40 μg	21.3 ± 4.9	<0.05	35.5 ± 7.6	<0.01

[a] The indicated antigen preparation, either killed whole cell vaccine or sonic extracts thereof, was used for the migration inhibition assays.

[b] The average percent inhibition response for five to six microdrop cultures of spleen cells from 30-day *L. pneumophila*-sensitized guinea pigs tested in triplicate with the indicated concentration of antigen was compared with that of controls; *P* was determined by Student's *t* test. NS, Not significant.

[c] In the indirect assay, 0.1-ml supernatants from *L. pneumophila*-stimulated spleen cells were added to cultures of normal mouse PE cells.

[d] —, None.

animals injected with CFA only did not show MIF reactivity when incubated directly with *Legionella* antigen in a direct test or develop supernatants capable of an indirect MIF reaction with mouse PE cells as the indicator.

The killed *L. pneumophila* in CFA consistently resulted in the sensitization of the animals as shown by the positive MIF reactivity of their spleen cells in vitro. Guinea pigs injected with the same numbers of *L. pneumophila* in IFA or in saline alone failed to develop such reactivity (Table 2). MIF activity also was evident when guinea pigs were immunized with *L. pneumophila* in CFA and given a booster, either in CFA or even in IFA. Spleen cells from guinea pigs given a single injection of *L. pneumophila* in CFA, with or without a second immunization in IFA, showed marked MIF activity in the indirect assay in vitro (Table 2). In contrast, spleen cells from animals given either a single injection or two injections of *L. pneumophila* in IFA failed to respond to the *Legionella* sonic extracts in vitro. In one of three experiments, we observed some inhibitory activity (11%), but this was not considered significant. The injection of guinea pigs with the same dose of *L. pneumophila* (3×10^9 bacteria) in saline 70 days previously, or 30 and 70 days previously, also failed to induce significant migration inhibition activity. Thus, *L. pneumophila* appeared to sensitize guinea pigs for MIF reactivity in vitro only when administered in CFA initially. Little, if any, significant increase in reactivity occurred when such guinea pigs were given a second injection of *L. pneumophila* in IFA or saline.

Since the injection of guinea pigs with killed *L. pneumophila* in CFA induced MIF reactivity in vitro, it was of interest to determine whether the infection of animals with viable *L. pneumophila* would do the same. Therefore, graded numbers of viable bacteria, ranging in concentration from 10^4 to 10^8 bacteria per guinea pig, were used in attempts to sensitize the animals. The largest numbers of *L. pneumophila* killed the guinea pigs within 3 to 5 days. The animals given lower doses showed signs of illness, including fever. The survivors were tested at various times thereafter for the ability of their spleen cells to produce MIF activity by the indirect assay. The animals injected with the lower numbers of viable *L. pneumophila* 2 to 3 weeks earlier showed evidence of reactivity (Table 3). A consistent response occurred about week 3 or 4 after infection. Little, if any, significant reaction was obtained with spleen cells less than 2 weeks after infection (Table 3).

The comparison of the MIF reactivity of spleen cell cultures with the skin reactivity of the animals to *L. pneumophila* and spleen cell blastogenesis showed a good correlation among the three assays. None of the control animals were skin test positive when injected intradermally with 100 μg of *Legionella* antigen. Also, none showed evidence of blastogenic cell transformation in vitro (an SI of less than 2). In contrast, guinea pigs which had been sensitized for 40 days with *Legionella* antigen in CFA showed consistent skin reactivity and blastogenic responsiveness, as well as MIF reactivity (Table 4). Animals injected 25 days earlier with *L. pneumophila* showed moderate to marked MIF activity in vitro but little, if any, blastogenic reactivity. However, the skin tests were generally positive, although the area of induration after skin testing was generally smaller than that observed in the 40-day-sensitized guinea pigs. The animals given viable *L. pneumophila* 3 to 4 weeks earlier and positive by the MIF assay

TABLE 2. Production of MIF by guinea pig spleen cells in response to *L. pneumophila* antigen assessed by indirect assay

Treatment[a] at:		% Migration inhibition[b] in:		
Primary immunization	Secondary immunization	Expt 1	Expt 2	Expt 3
None (controls)	−	−6.6	−12.8	−15.2
Immunization with *L. pneumophila* in CFA	−	45.5	47.3[c]	54.1[c]
	+	44.4[c]	49.3[c]	62.1[c]
Immunization with *L. pneumophila* in IFA	−	−20.9	−13.5	−13.6
	+	−17.4	11.0	−22.1
Immunization with *L. pneumophila* in saline	−	−5.5	8.6	4.6
	+	4.2	9.2	−9.5

[a] Spleen cells from guinea pigs treated as indicated were incubated in the presence or absence of 10 μg of *Legionella* sonic extracts. The guinea pigs had been sensitized with 3×10^9 *L. pneumophila* in CFA, IFA, or saline 70 days earlier, and some received a secondary injection of 3×10^9 *L. pneumophila* in IFA 30 days before testing.

[b] The percent inhibition of in vitro migration of dextran-elicited mouse PE cells incubated in supernatants of antigen-stimulated guinea pig spleen cells or control supernatants was determined; each experiment represents the results of two to three triplicate cultures.

[c] A significant *P* value of 0.01 was calculated by Student's *t* test.

were generally reactive by skin tests (induration of 5 mm or larger upon the injection of 100 μg of sonic extracts) and showed blastogenic reactivity with an SI of 2.5 to 3.

DISCUSSION

The results of this study show that the leukocyte migration inhibition reaction, both direct and indirect, may reveal immunological sensitization of guinea pigs to *L. pneumophila* whole cell vaccine. The animals injected with killed *L. pneumophila* in CFA became sensitized as evidenced by migration inhibition of their spleen cells incubated in vitro with *Legionella* sonic extracts. However, intact bacteria did not reveal migration inhibition, possibly because the bacteria could not penetrate into the agarose droplets, as could the solubilized sonic extracts. In the indirect assay, culture supernatants of guinea pig spleen cells incubated either with the sonic extracts or whole cell vaccine, inhibited the migration of normal mouse PE cells in the same agarose microdroplet procedure.

It is now widely accepted that cellular immunity may be readily detected in vitro when lymphoid cells from sensitized subjects, either humans or experimental animals, are incubated with the appropriate specific antigen, resulting in the inhibition of the expected migration of a variety of target cells, including macrophages,

TABLE 3. Determination by indirect assay of migration inhibition reactivity induced by supernatants from guinea pig spleen cells stimulated with *Legionella* antigen

Guinea pig treatment[a]	Time after infection (days)	% Migration inhibition[b] ± SD	*P*[c]
None (control)	—[d]	5.6 ± 1.3	—
Viable *L. pneumophila* (10^4)	7–10	4.5 ± 3.2	NS
	14–18	11.6 ± 4.5	NS
	21–30	22.9 ± 7.3	<0.01
	40–60	14.5 ± 5.2	NS
Viable *L. pneumophila* (10^5)	7–10	8.3 ± 2.1	NS
	14–18	16.5 ± 4.9	NS
	21–30	28.6 ± 8.3	<0.01
	40–60	22.2 ± 5.6	<0.05

[a] Guinea pigs, in groups of three to four, were injected intraperitoneally with the indicated dose of viable *L. pneumophila* at the indicated time before testing.

[b] The average percent migration inhibition for four to six microdrop cultures of mouse PE cells with supernatants from the indicated guinea pig spleen cells stimulated in vitro with 10 μg of *Legionella* sonic extracts for 24 h was determined.

[c] *P* was determined by Student's *t* test. NS, Not significant.

[d] —, None.

TABLE 4. Comparative responses of guinea pigs to *Legionella* antigen determined by in vitro and in vivo assays

Guinea pig treatment[a]	Time of testing (days after infection)	Indirect MIF reactions (% inhibition ± SD)[b]	Blastogenic reaction[c]		Skin test reactions (mm^2)[d]
			cpm (± SD)	SI	
None (controls)		5 ± 3.8	570 ± 62	0.8	<5.0
Injection of *L. pneumophila*	7	7.0 ± 5.2	877 ± 52	1.2	<5.0
	25	36.5 ± 18.5[e]	1,316 ± 195[e]	1.8	8.3 ± 1.2
	40	52.3 ± 12.6[e]	2,339 ± 360[e]	3.2	10.4 ± 2.5[e]
	70	38.5 ± 15.9[e]	2,267 ± 270[e]	3.1	11.5 ± 2.9[e]
Infection with *L. pneumophila*	10	3.5 ± 4.5	658 ± 120	0.9	<5.0
	30–40	38.6 ± 18.5[e]	1,907 ± 2.0[e]	2.6	9.6 ± 2.8[e]

[a] The control guinea pigs were given CFA only at −40 days, and *L. pneumophila*-treated animals were given 3×10^9 killed bacteria in CFA or 2×10^5 viable bacteria intraperitoneally on the day indicated.

[b] The average percent migration inhibition for mouse PE cells in triplicate cultures with supernatants from the indicated guinea pig spleen cell cultures treated for 24 h in vitro with 10.0 μg of sonic extracts was compared with that of controls without antigen.

[c] The average reaction for three to six cultures of spleen cells from the indicated guinea pig group 6 days after stimulation with 10.0 μg of sonic extracts was compared with that of nonstimulated controls.

[d] Skin reactions in square millimeters induration were determined at 24 to 48 h after skin testing with 100 μg of sonic extracts.

[e] Significantly positive as determined by Student's *t* test ($P < 0.01$).

granulocytes, other leukocytes, etc. (1, 2, 13). The indirect assay has been utilized extensively to study the mechanism of such reactions (7, 13).

In the present study, it was apparent that MIF activity could be induced in cultures of guinea pig spleen cells 25 to 40 days after the administration of killed *L. pneumophila* in CFA. This is about the same time that MIF activity develops to mycobacterial antigens after the administration of tubercle bacilli in adjuvant. The injection of the same dose of killed *L. pneumophila* in saline or in IFA without mycobacteria failed to elicit a similar reaction. However, it is noteworthy that the injection of viable *L. pneumophila* in a sublethal dose resulted in positive MIF reactivity several weeks later. All of the animals evincing such MIF activity by their spleen cells cultured in vitro with *Legionella* sonic extracts had shown some degree of infection, such as fever, the first few days after infection. Also, *L. pneumophila* could be recovered from the blood of these animals up to 7 days after infection, but not thereafter.

It is noteworthy that earlier studies had shown that serum antibody responses to *L. pneumophila* become apparent as early as 7 to 10 days after immunization (19). Such antibody activity was readily detected by immunofluorescence and microagglutination procedures. Furthermore, sera obtained from animals used in the present study, including those obtained within the first week after infection or 7 days after immunization with bacteria in adjuvant, demonstrated antibody activity by the microagglutination procedure (data not shown). Thus, although the MIF assay appeared to be a sensitive in vitro correlate for CMI reactions, it may not reveal sensitization as readily as serological tests. Indeed, it is widely acknowledged that in many microbial infections cellular immunity may not develop until late after infection, whereas serum antibodies develop as an early response. In this regard, it is not clear which, if either, form of immunity is protective of legionellosis. Recently, it has been reported that rats, generally considered insensitive to infection by *L. pneumophila*, develop antibodies after vaccination and that such antibodies may passively transfer protective immunity to other rats (8). Furthermore, sheep serum containing antibodies to *L. pneumophila* was also found to be protective. No similar reports concerning protective immunity in guinea pigs, considered to be a sensitive animal model for infectious studies with legionellae, have been published.

A correlation was observed between the MIF reactivity of spleen cells from sensitized animals and positive skin reactions, as well as in vitro lymphocyte blastogenesis. Earlier, Wong et al. showed that guinea pigs, sensitized by immunization with *L. pneumophila* in CFA, develop skin reactivity as shown by subsequent tests with purified cross-reactive antigen derived from *L. pneumophila* (22). The skin reactions were measured by determining the size of erythema only, and no information was presented as to the duration of sensitization or nature of the antigen used for sensitization. It was also noted in that report that normal guinea pigs, skin tested with the same group-specific antigens,

showed a significant degree of nonspecific cutaneous hypersensitivity. In the present study, sonic extracts derived from the homologous *Legionella* serotype elicited a positive skin reaction only in sensitized animals, not in control animals.

Lymphocyte blastogenic reactions have been observed with blood leukocytes from patients with legionellosis incubated in vitro with sonic extracts of *L. pneumophila* (15). In that situation, it is also noteworthy that sonic extracts induced a significant leukocyte stimulation from control subjects without evidence of legionellosis. Although the response of the control subjects was lesser, it was still evident that the sonic extracts induced a marked reaction with blood monocytes from normal patients. In contrast, the results of the present study show that sonic extracts used for in vitro testing did not produce a nonspecific blastogenic response by splenocytes from nonsensitized guinea pigs. Similarly, the sonic extracts did not induce a nonspecific MIF activity, either direct or indirect, with spleen cells from nonsensitized guinea pigs. Thus, sonic extracts, at the doses used in the present study, did not induce nonspecific effects in vitro or in vivo.

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