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Agar Microdroplet Assay for Delayed Hypersensitivity to *Legionella pneumophila* Serogroup 1

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An agarose microdroplet technique was utilized to assess the cellular immunity of guinea pig lymphoid cells to *Legionella pneumophila* antigen in vitro. Both direct and indirect migration inhibition procedures were shown to be capable of detecting sensitization of guinea pigs to *L. pneumophila* antigens. Animals injected with adjuvant alone or unrelated antigens did not yield spleen cells responsive to *L. pneumophila*, indicating the specificity of the response. Migration inhibition factor induction by *Legionella* antigen in vitro correlated well with skin test responses in vivo. The positive reaction detected by migration inhibition occurred at times similar to that of skin reactivity but later than that of the earliest serum antibody titers. The assay appears to be useful for monitoring sensitization to *Legionella* and may be applicable to the study of cell-mediated immunity to this bacterium in infected individuals.

Legionella pneumophila is now considered the etiological agent of many atypical bacterial pneumonias of humans (5, 6, 22). Since the discovery of this class of organisms in 1976 in Philadelphia, Pa., during a regional American Legion convention, many studies have been performed concerning the nature and characteristics of these bacteria. However, much less is known concerning the immune response to these microorganisms. Specific antibody to *Legionella* can be detected, and this antibody, from both humans and experimental animals, has been used to identify the organisms by a variety of serological procedures (2, 6, 18, 19, 21). In addition, such serological tests have been utilized to demonstrate a rise in serum antibodies to the organism in infected patients (2, 4, 13, 20). Among these tests are the indirect and direct immune fluorescence procedures for serological diagnosis of infection. However, individuals without disease symptoms or clinical history of infection by this organism also have antibodies to *Legionella* demonstrable by the fluorescent-antibody technique. Recently, other serological techniques, including microagglutination, counterimmunoelectrophoresis, and enzyme-linked immunosorbent assay procedures have been developed to detect serum antibody to the organism, as well as antigen (4, 7, 19).

The involvement of cell-mediated immunity (CMI) or humoral immunity in resistance to and pathogenesis of Legionnaires disease is not clear at this time. Earlier studies in this and other laboratories have shown that cellular immune reactions to *Legionella* antigens can be detected in experimental animals. Delayed skin reactivity occurs in guinea pigs hyperimmunized to the organism in adjuvant (23). Furthermore, there have been some reports that greater levels of blastogenic responses to *Legionella* antigen by peripheral blood leukocytes occur in patients who have recovered from infection with *Legionella* as compared with noninfected individuals when their leukocytes were cultured in vitro with sonic extracts prepared from these bacteria (16). Other investigators have demonstrated that mononuclear phagocytes from human or animal sources ingest virulent legionellae but fail to kill the bacteria which eventually grow within the phagocyte (8–12). Horwitz and Silverstein recently demonstrated that human monocytes activated in vitro with lymphokines were capable of killing legionellae in vitro, suggesting a possible role for CMI in resistance to infection (10). In the present study, a migration inhibition assay as a correlate for CMI (1, 3) was utilized with spleen cells from guinea pigs sensitized to *Legionella* antigen. A microdroplet agar procedure was used to detect CMI, and positive reactions were found to correlate with development of sensitization as determined by skin test reactions to *Legionella* as a function of time after infection. An indirect migration inhibition factor (MIF) assay in which mouse peritoneal exudate

cells were used as the indicator was also developed, showing that indirect assays may be useful for studying CMI to *Legionella*.

METHODS AND MATERIALS

***Legionella* antigen.** *Legionella pneumophila*, serogroup 1, Philadelphia 1 strain, was originally obtained through the courtesy of Roger McKinney, Centers for Disease Control, Atlanta, Ga. The bacteria were cultured on charcoal-yeast extract agar plates (REMEL; Lenexa, Kans.) and harvested with saline during the log phase of growth (24 to 36 h) (15) with a glass rod scraper. The bacteria were suspended in pyrogen-free saline and washed three times by centrifugation (10,000 × *g*, 30 min) at 4°C. For vaccine preparation, the organisms were suspended at approximately 3×10^9 bacteria per ml (by comparison with MacFarland standards) in 0.5% Formalin for 24 h at room temperature. Killing of the bacteria was confirmed by subculture to charcoal-yeast extract plates. Killed bacteria for whole-cell preparations were washed three times by centrifugation and resuspended in phosphate-buffered saline at 3×10^9 bacteria per ml and stored at 4°C until use. Sonic extracts were prepared by treating suspensions of 10^{10} organisms per ml (compared with a MacFarland standard) with six 1-min pulses with a biosonic sonicator (Bronwill Scientific, Rochester, N.Y.), using an intensity of 25. Cellular debris was removed by centrifugation for 30 min at 10,000 × *g* at 4°C, and after filter sterilization (0.22 μm filter), the sonic extracts were adjusted to 1 mg/ml by determination of absorbancy at 280 nm. Sonic extracts were stored in 1-ml volumes at −70°C and used only once after thawing. Large batches of both whole-cell and sonic-extract preparations were prepared, and the same batch was used in any one series of experiments.

Immunization. Groups of Hartley strain guinea pigs, weighing approximately 400 g each, were immunized with *Legionella* vaccine in complete Freund adjuvant (CFA) so that each animal received 1.0 ml of the adjuvant containing approximately 10^9 killed bacteria in two sites in the nuchal region. Blood was obtained from the animals by cardiac puncture, and the serum was collected and stored at −70°C until titrated for antibody activity. At various times after sensitization, guinea pigs were skin tested by intradermally injecting 100 μg of sonic extract in 100 μl of saline. Skin tests were read at 24 and 48 h. This dose of sonic extract gave no induration at 48 h when injected into either normal guinea pigs or guinea pigs receiving previous injections with CFA but no *Legionella*. Animals injected with *Legionella* in CFA consistently responded with areas of induration of 5 mm or more 48 h after intradermal injection of 100 μg of sonicate. Therefore, reactions of >5 mm of induration were considered positive for delayed hypersensitivity to *L. pneumophila*. Animals with demonstrable skin reactions were sacrificed at various times, their spleens were removed, and cell suspensions were prepared for the assays described below.

Serological tests. Serum specimens were serially diluted in saline and tested by microagglutination procedures exactly as described previously (19). In brief, serum dilutions were incubated in microtiter plates with 0.25-ml suspensions of safranine-stained, Formalin-killed *L. pneumophila*. Titers were recorded as the reciprocal of the highest serum dilution resulting in a shield, rather than a button, pattern.

MIF procedure. Direct and indirect migration inhibition (MIF) assays were performed by a modification of the technique described by McCoy et al. (14). For the direct migration inhibition assays, approximately 2×10^7 guinea pig splenocytes were mixed with 0.1 ml of 0.2% agarose in RPMI 1640 medium containing 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). Individual droplets of approximately 2 μl each were placed in wells of a flat-bottomed, 96-well microtiter plate (Nunc; GIBCO) and placed in a refrigerator for 5 min to allow the droplets to solidify. Medium (200 μl) with or without *Legionella* antigen was added carefully to each well, and the diameters of the droplet were measured, taking two readings at right angles with an ocular micrometer on an inverted tissue culture microscope. The plates were incubated for 24 h at 37°C in a humidified incubator containing 5% CO_2 in air and the diameters were measured again. The differences in diameter of cell migration from 0 to 24 h served as an indicator of migratory activity and the percent migration inhibition was calculated by the following formula: $\{1 - [(\text{diameter change in test})/(\text{diameter change in control})]\} \times 100$. All assays were performed at least in triplicate, with the mean changes in diameter used in the calculation. A percent migration inhibition of ≥20% was considered significant (14).

The indirect assay was performed by incubating guinea pig splenocytes for 24 to 48 h with *Legionella* whole-cell or sonic-extract antigens, followed by centrifugation to remove the splenocytes. Controls received equivalent doses of antigen just before centrifugation. The remaining supernatant fluids were collected, filter sterilized, and stored in small volumes at −70°C until tested. The fluids were tested for MIF activity with peritoneal macrophages from BALB/c mice that were injected intraperitoneally 3 days previously with 1 ml of 0.5% dextran. The peritoneal cells were prepared in agarose as described for the guinea pig splenocytes in direct assays. The supernatants were diluted 1:2 in medium, and 200 μl was added to the wells. Migration in wells receiving test supernatants was compared with that in cultures receiving control fluids. Supernatant fluids from guinea pig splenocytes that had been treated with concanavalin A, which is known to induce MIF production by guinea pig spleen cells (17), were used as a control for response of the mouse macrophages to guinea pig MIF.

RESULTS

Previous studies in this laboratory indicated that immunization of guinea pigs by injections with 3×10^9 *L. pneumophila* organisms per kg in CFA led to the development of positive cutaneous hypersensitivity within 25 to 40 days after injection. Thus, it was of interest to determine whether skin reactivity could be found to correlate with MIF production by splenocytes in vitro. Therefore, skin test-positive guinea pigs which had been sensitized 4 weeks earlier were sacrificed, and their splenocytes were assayed in both direct and indirect assays for MIF produc-

TABLE 1. Direct and indirect MIF assays with lymphoid cells from guinea pigs sensitized with *L. pneumophila* antigens

Migration assay[a]	Source of guinea pig cells[b]	% Migration inhibition with[c]:					
		Whole bacteria (no./ml)			Sonicate (μg)		
		10^3	10^5	10^7	1.0	10.0	20.0
Direct	Normal (control)	4	3	5	4	3	4
	Injected	2	4	6	31	25	21
Indirect	Normal (control)		4			2	1
	Injected		39			57	

[a] Migration assays with spleen cells from normal guinea pigs or guinea pigs sensitized 4 weeks earlier with *L. pneumophila* vaccine (10^9 bacteria). The direct assay employed guinea pig splenocytes from either control or *L. pneumophila*-injected animals. Indirect assays employed supernatants from control or *L. pneumophila*-injected guinea pig cells combined with mouse peritoneal exudate cells.

[b] Sensitized animals showed positive skin test to *L. pneumophila*.

[c] Percent migration inhibition for three to five cultures per group with indicated antigen, as compared with migration areas without antigen.

tion (Table 1). In the direct MIF assay, sonicated or whole-cell preparations of *Legionella* antigen were added to droplets containing spleen cells from sensitized guinea pigs or normal guinea pigs used as controls. A consistent percent migration inhibition (>20%) occurred with the spleen cells from the sensitized animals, but this was evident only when sonic extract was used as the antigen. Little significant inhibition occurred with intact, killed bacteria. In other experiments (data not shown), increasing the concentrations of bacteria per well failed to result in migration inhibition of spleen cells from sensitized animals. In no instance were the spleen cells from normal animals inhibited in migration with either sonic extract or whole cells.

Cell-free supernatant fluids from splenocyte cultures incubated with either whole-cell or sonicated *Legionella* preparations resulted in significant inhibition of migration of mouse peritoneal macrophages (Table 1). The supernatant fluids obtained 24 to 48 h after incubation of 10^6 spleen cells from guinea pigs sensitized 4 weeks previously with *Legionella* resulted in a consistent percent cell migration inhibition of ≥20%. The percent migration inhibition was usually in the range of 40 to 60%. This was evident, however, only when *Legionella* antigen, either whole cell or sonic extract, was added to spleen cells from the specifically sensitized guinea pigs but not from normal guinea pigs. Furthermore, addition of antigen alone, either whole bacteria or sonic extract, directly to the peritoneal cells from normal mice failed to influence the expected migration pattern, indicating that, as with guinea pig lymphoid cells, the *Legionella* antigen had no effect on normal cell populations, at least in terms of migration in agar.

In additional studies, the migration procedure was used to monitor the development of sensitivity of the guinea pigs to the antigen. For this purpose, groups of guinea pigs were sensitized with *Legionella* antigen in CFA. One or two guinea pigs were sacrificed at various times thereafter, and washed spleen cell suspensions were prepared and used for indirect MIF assay with *Legionella* sonic-extract antigen. Antibody activity was detected as early as 7 days after immunization (19) and reached high titers by 40 to 60 days. Skin reactivity to *Legionella* became evident about 25 to 40 days after sensitization, when the first significant (induration of 5 mm or greater) delayed cutaneous reactions occurred. MIF reactivity, as determined by indirect assay with mouse peritoneal cells as the indicator, paralleled the development of skin reactivity. By 25 days after immunization, reactions became positive for all sensitized guinea pigs. Spleen cells obtained 25 to 150 days after sensitization readily showed MIF activity upon challenge in vitro with *Legionella* sonic extracts (Table 2). It is important to note that, in control experiments, all animals injected with complete adjuvant and saline alone, without *Legionella*, failed to evince either skin or MIF reactivity to the *Legionella* antigen. These animals also did not develop serum antibody to these bacteria.

TABLE 2. Comparison of indirect migration inhibition assay results with serum antibody titers and skin reactions of guinea pigs sensitized with *Legionella* antigen

Time after sensitization (days)[a]	% Migration inhibition[b]	Serum antibody titer[c]	Skin reactions[d]
None (control)	3.5	1:10	−
25–40	24.4	>1:1,280	+
50–60	41.2	>1:1,280	+
150	42.1	1:640	+

[a] Guinea pigs were sensitized by intradermal injection with 10^9 legionellae in CFA.

[b] Average percent migration inhibition by indirect assay with 10 μg of sonic extract as antigen and mouse PE cells as indicator.

[c] Average serum titer for two to four guinea pigs per time period tested as determined by the microagglutination test.

[d] Induration of 5 mm or greater 48 h after intradermal injection with 100 μg of *L. pneumophila* sonic extract.

DISCUSSION

The results of this study show that a microdroplet MIF assay may be utilized to evaluate sensitization of spleen cells of immunized animals to *L. pneumophila* antigens. In the direct migration inhibition procedures, spleen cells from sensitized guinea pigs were found to be inhibited in their expected migration when incubated in vitro with *Legionella* sonic extracts but not whole-cell antigen, possibly owing to the inability of the larger particulate nature of the whole-cell antigen to gain access to the sensitized spleen cells in the agarose droplets. In contrast, the intact bacterial preparations were effective in inducing MIF reactions in the indirect assay. In the direct assay with spleen cells, inhibition of both polymorphonuclear cells and macrophages occurs, due to the action of leukocyte migration inhibitory factor and MIF, respectively, released by sensitized lymphocytes in response to antigen.

In the indirect assay, peritoneal exudate cells are used as the indicators of these factors, which are present in supernatants of test splenocytes exposed to *Legionella* antigens in vitro. The dextran-elicited murine PE cell preparations, consisting of 85% macrophages with very few polymorphonuclear leukocytes, as determined by microscopic examination, served as an indicator of lymphokine activities. A number of other investigators have demonstrated that both guinea pig and human lymphokines (17) are capable of inhibiting the migration of mouse macrophages in vitro. Thus, the indirect MIF assay has several advantages over the direct assay for clinical laboratory studies. First, fewer sensitized lymphocytes are needed for MIF production for indirect assays, as compared with the relatively large number of cells necessary for direct procedures. Second, the assay portion (i.e., migration) of the direct procedure is a more technically difficult procedure. Third, for the indirect assay, there is no need to obtain large numbers of peripheral blood leukocytes from a patient for direct incorporation into agar. The blood leukocytes are used merely for stimulating MIF release into culture supernatants. Fourth, it is widely recognized that supernatant fluids can be readily stored at −70°C or even lower freezer temperatures with little or no loss of MIF activity. Fifth, direct comparison of activity with preparations obtained at various times after infection or sensitization is possible, as well as titration of activity by performing tests with serial dilutions of cell supernatants at selected time intervals. Finally, activity of cell supernatants generated in the presence of bacterial antigens such as those from *Legionella* may be compared with that of supernatants generated by exposure of the same cells to mitogens such as concanavalin A or phytohemagglutinin, both of which are known to induce MIF production by human T cells (17).

Studies are currently in progress to assess MIF and skin test responses in guinea pigs after infection with viable *L. pneumophila* organisms. Other studies also have been designed to study human peripheral blood lymphocytes in regard to MIF production in response to incubation with *Legionella* antigens, using donors with or without anti-*Legionella* antibody in their sera. It is noteworthy that a recent report has shown that the blastogenic responses to *Legionella* antigens is evident with peripheral blood leukocytes from patients who have antibody titers to the organism and a history of infection (16). The blastogenic test in vitro is also considered a correlate of CMI, although it is known that B lymphocytes also respond to a wide variety of antigens and mitogens. In that study, however, it was apparent that, although the blastogenic response was quite high to *Legionella* sonic extracts, there was also an extremely high level of responses of leukocytes from patients without a history of exposure to *Legionella* or without *Legionella* antibodies in their sera. The blastogenic test may be too nonspecific to use as a correlate of cellular immunity, or even sensitization to *Legionella*, or *Legionella* antigen may be mitogenic for humoral peripheral blood leukocytes.

It is noteworthy that, in the present study, in which guinea pig splenocytes were used, sensitization to the bacterial antigens detected by both the direct and the indirect MIF procedures correlated well with skin test reactivity, suggesting that the MIF assay, like this assay with other antigens, reflects cellular immunity. Regardless of the actual mechanisms involved in the MIF response to *Legionella* antigen in vitro, it is apparent that this procedure is a rapid and effective immunological method for the detection of specific sensitization to this antigen. Further studies with the MIF assay and lymphoid cells from patients recovering from infection with *Legionella*, as well as from experimental animals infected with the organisms, should provide additional information concerning the value of this method for detecting cellular immunity as compared with humoral antibody tests now available for detecting and assaying immune responses to *Legionella* antigens.

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