Cell-mediated Immunity in Legionnaires' Disease

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ABSTRACT Previous studies from this laboratory have suggested a role for cell-mediated immunity in host defense against *Legionella pneumophila*. In this paper, cell-mediated immunity to *L. pneumophila* in patients recovered from Legionnaires' disease was studied by examining patient mononuclear cell responses to *L. pneumophila* antigens. Patient mononuclear cells were assayed both for their capacity to respond to *L. pneumophila* antigens with the production of cytokines that activate monocytes, as measured by monocyte inhibition of *L. pneumophila* multiplication, and for their capacity to respond with proliferation, as measured by [^{3}H]thymidine incorporation.

Patient mononuclear cells incubated with formalin-killed *L. pneumophila* generated cytokines (supernatants) that were capable of activating in vitro freshly explanted monocytes from a person without historical or serological evidence of *L. pneumophila* infection (nonpatient). Such activated nonpatient monocytes inhibited the intracellular multiplication of *L. pneumophila*, and the degree of inhibition was proportional to the concentration of supernatant added. Patient mononuclear cells incubated with 5×10^6–5×10^8 formalin-killed *L. pneumophila*/ml for 4 d produced maximally potent supernatants; supernatants generated in flat-bottomed wells were equivalent in potency to supernatants generated in cone-shaped wells. Patient *L. pneumophila*-induced mononuclear cell supernatants were less potent than patient concanavalin A-induced mononuclear cell supernatants. Patient mononuclear cells also responded to formalin-killed *L. pneumophila* with proliferation (lymphoproliferation).

Patient mononuclear cells responded more strongly to *L. pneumophila* antigens than mononuclear cells of age- and sex-matched nonpatients, as measured by both assays; responses to concanavalin A were comparable. Mononuclear cells from patients recovered from Legionnaires' disease responded more strongly to *L. pneumophila* than to *Mycobacterium leprae* antigens, whereas mononuclear cells from patients with tuberculoid leprosy responded more strongly to *M. leprae* antigens.

These findings indicate that cell-mediated immunity to *L. pneumophila* develops in patients with Legionnaires' disease and, taken together with previously reported findings, that cell-mediated immunity plays a major role in host defense against *L. pneumophila*.

The monocyte activation assay described in this paper has general applicability for the study of monocyte and mononuclear cell effector functions in selected patients. The assay may be used to study (*a*) the capacity of a patient's monocytes to be activated to an antimicrobial potential by a standard preparation of cytokines and (*b*) the capacity of a patient's mononuclear cells to generate such monocyte-activating cytokines in response to a mitogen or antigen.

INTRODUCTION

Legionella pneumophila, the etiologic agent of Legionnaires' disease, is a facultative intracellular bacterial parasite (1). The bacterium multiplies intracellularly in human monocytes and human alveolar macrophages, and, under tissue culture conditions, multiplication is exclusively intracellular (1, 2).

Previous studies from this laboratory have examined the role of humoral immunity in Legionnaires' disease. These have demonstrated that virulent *L. pneumophila* grown in vivo are resistant to three potential functions of antibody and complement relevant to host defense (3, 4). First, *L. pneumophila* are completely resistant to killing by human complement, even in the presence of high-titer human or rabbit anti-*L. pneumophila* antibody (3). Second, *L. pneumophila* are highly resistant to killing by human phagocytes even in the presence

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of high-titer antibody and complement; although antibody and complement markedly promote the phagocytosis of *L. pneumophila* by human polymorphonuclear leukocytes and monocytes, these phagocytes kill only 0.5 log of an inoculum of *L. pneumophila* under optimal conditions (3, 4). Third, *L. pneumophila* pretreated with antibody and complement and then incubated with monocytes in the presence of these humoral components multiply in monocytes at as rapid a rate as when they enter monocytes in the absence of antibody (4). These studies indicated that humoral immunity does not play a primary role in host defense against *L. pneumophila*.

Another study has demonstrated that activated human monocytes inhibit the intracellular multiplication of *L. pneumophila* (5); the monocytes were activated with cell-free supernatants or cytokines generated by blood mononuclear cells stimulated with the plant mitogen concanavalin A (Con A).[1] This study indicated that cell-mediated immunity likely plays an important role in host defense against Legionnaires' disease.

This study examines the role of cell-mediated immunity in Legionnaires' disease. The study demonstrates (*a*) that patients recovered from Legionnaires' disease have circulating mononuclear cells sensitized to *L. pneumophila* antigens and (*b*) that in response to *L. pneumophila* antigens, patient mononuclear cells both proliferate and generate cytokines capable of activating normal human monocytes such that they inhibit the intracellular multiplication of *L. pneumophila*.

METHODS

Patients and nonpatients. All patients were adults from the New York City area who had had well-documented cases of Legionnaires' disease diagnosed by specialists in infectious diseases (Table I). Patients were studied 4–20 mo after they had fully recovered from Legionnaires' disease. Adults from the New York City area who were not known to have ever had Legionnaires' disease or an *L. pneumophila* infection and who had anti-*L. pneumophila* antibody titers of <1:64 (the threshold level of significance) by the indirect fluorescent antibody (IFA) assay (6) served as controls (nonpatients). In addition, two persons from the New York City area with tuberculoid leprosy were studied; both had no known history of Legionnaires' disease and both had anti-*L. pneumophila* IFA titers of <1:64. Serum IFA titers to *L. pneumophila* Philadelphia 1 were measured on all persons participating in this study by Mr. Austin Swaby, Mrs. Remonia Clarke and Ms. Helen Kravetz at the New York City Department of Health. All patients who participated in this study did so with the approval of their personal physicians. Informed written consent was obtained from all patients and nonpatients. All patients were seen and blood specimens obtained at The Rockefeller University Hospital outpatient clinic (New York).

Six of the seven patients had evidence of infection with serogroup 1 *L. pneumophila* (Table I); all six had significant (≥1:64) serum IFA titers to *L. pneumophila* Philadelphia 1 (a serogroup 1 organism) at the time they were studied. The serum of the seventh patient (patient C) was tested only to combined *L. pneumophila* serogroups 1–4 at the time of his illness; 4 mo later, when he was studied, his serum did not have a significant IFA titer to *L. pneumophila* Philadelphia 1.

Media. Egg yolk buffer, with or without 1% bovine serum albumin, and RPMI 1640 medium were prepared or obtained as described previously (1). No antibiotics were added to any medium in any of the experiments.

Reagents. Con A, three times crystallized and lyophilized, was obtained from Miles-Yeda Ltd., Kankakee, IL; [^{3}H]thymidine, from Schwarz/Mann Division of Becton, Dickinson & Co., Orangeburg, NY.

Serum. Venous blood was obtained, clotted, and serum separated and stored at −70°C until used as described (8). Normal (nonimmune) human serum (type AB) with an IFA anti-*L. pneumophila* titer (6) of <1:64 was obtained from a single nonpatient.

Bacteria. L. pneumophila, Philadelphia 1 strain, were grown in embryonated hens' eggs, harvested, tested for viability and for the presence of contaminating bacteria, stored at −70°C, and partially purified by differential centrifugation just before use as described (1). Formalin-killed *L. pneumophila* were prepared by passing egg yolk grown *L. pneumophila* on CYE agar one time only, collecting the bacteria, incubating them with 2% formalin for 30 min at 4°C and washing the bacteria four times in egg yolk buffer by centrifugation at 12,000 *g* for 10 min at 4°C. In a recently published study of this strain of *L. pneumophila*, IFA titers of human serum obtained against formalin-killed *L. pneumophila* were found to be highly correlated with IFA titers obtained against heat-killed *L. pneumophila*, indicating that antigenic determinants of *L. pneumophila* measured in this assay are well maintained with formalin treatment (9).

Human blood mononuclear cells. Heparinized venous blood was obtained from patients and nonpatients and the blood mononuclear cell fraction was separated by centrifugation over a Ficoll-sodium diatrizoate solution as previously described (1).

Preparation of supernatants. Patient and nonpatient mononuclear cells (6×10^6) were cultured in 35-mm plastic petri dishes in 2 ml RPMI medium containing 25% fresh, nonpatient AB serum. *L. pneumophila* supernatants were prepared by incubating mononuclear cells at 37°C in 5% CO_2-95% air for 4 d (0–10 d in some experiments) with formalin-killed *L. pneumophila* at concentrations ranging from 5×10^3 to 5×10^8 bacterial particles/ml. At the end of the incubation, the mononuclear cell cultures in each petri dish were transferred to individual conical tubes and the leukocytes and bacteria sedimented by centrifugation, first at 450 *g* for 5 min and then 3,000 *g* for 10 min. The supernatants were collected, filtered through 0.2-μm Millipore filters (Millipore Corp., Bedford, MA), and stored at −70°C. Control supernatants were prepared in the same way except that formalin-killed *L. pneumophila* were added at the end of the incubation period rather than at the beginning; these supernatants will be referred to as supernatant controls.

Con A supernatant controls were prepared in the same way except that 15 μg/ml Con A was used instead of formalin-killed *L. pneumophila* and the incubation period was for 2

[1] *Abbreviations used in this paper:* CFU, colony-forming units; Con A, concanavalin A; IFA, indirect fluorescent antibody assay.

TABLE I

Data on Patients Recovered from Legionnaires' Disease

Patient designation	Age/Sex*	Date of illness	Acute IFA	Convalescent IFA	Other diagnostic tests	Interval between illness and study	IFA titer at time of study
		mo/yr	*Titer*			*mo*	
A	45/M	10/79	<1:64‡	1:4,056‡	—	20	1:512‡
B	65/M	1/81	<1:64‡	1:512‡	DFA+§	5	1:128‡
C	47/M	8/81	1:128‖	1:1,024‖	—	4	<1:64‡
D	50/M	9/81	1:128‖	1:1,024‖	—	7	1:64‡
E	56/F	6/81	<1:64‡	1:1,024‡	—	10	1:64‡
F	53/F	8/81	1:512‡	1:2,048‡	—	9	1:512‡
G	57/F	6/81	1:64‖	1:256‖	Culture+¶	11	1:128‡

* The age and sex of nonpatients A, B, D, E, F, and G were A, 25-yr-old female; B, 28-yr-old female; D, 45-yr-old male; E, 51-yr-old female; F, 52-yr-old female; G, 61-yr-old female. Nonpatients D, E, F, and G were age- and sex-matched pairs of patients D, E, F, and G, respectively. The age and sex of the two patients with tuberculoid leprosy were 48-yr-old female and 35-yr-old male.

‡ IFA titer measured to *L. pneumophila* Philadelphia 1.

§ Sputum was positive for *L. pneumophila* by direct immunofluorescent staining using polyvalent anti-*L. pneumophila* serogroups 1–4 antiserum (7).

‖ IFA titer measured to polyvalent *L. pneumophila* serogroups 1–4.

¶ *L. pneumophila* serogroup 1 organism cultured from transtracheal aspirate.

d rather than 4 d because this incubation period yielded maximally potent Con A supernatants (4).

Mycobacterium leprae supernatants and supernatant controls were prepared in the same way as *L. pneumophila* supernatants and supernatant controls except that partially purified armadillo-derived freeze-dried *M. leprae* at 100 μg/ml was used instead of *L. pneumophila*. The *M. leprae* was kindly provided by the Immunology of Leprosy (IMMLEP) project, World Health Organization.

The sediments remaining after supernatants were removed from centrifuged mononuclear cell cultures were all tested for the presence of contaminating microorganisms by resuspending them in 600 μl RPMI medium and culturing aliquots of the suspension on 5% sheep blood tryptic soy broth agar; none of the preparations used in this study were contaminated.

Assay for capacity of supernatants to activate monocytes. The monocyte activating capacity of all supernatants was assayed on the monocytes of a single nonpatient donor. Freshly explanted mononuclear cells (1.5×10^6) from this nonpatient were incubated in 16-mm tissue culture wells (Linbro, 24-well plates, Flow Laboratories, Inc., McLean, VA) in 500 μl of RPMI medium containing 10% fresh nonpatient serum for 1.5 h at 37°C in 5% CO_2-95% air to allow monocytes to adhere. The culture wells were then vigorously washed to remove the nonadherent lymphocyte-enriched fraction of the mononuclear cell population. The monocyte monolayers were then incubated for 24 h in 500 μl of RPMI medium containing 20% fresh nonpatient serum and 0–60% (vol/vol) supernatant or supernatant control prepared as described above. After 24 h, virulent *L. pneumophila* grown in eggs (10^4 colony-forming units [CFU]/ml) were added to the cultures. The cultures were incubated at 37°C in 5% CO_2–95% air on a gyratory shaker for 1 h and under stationary conditions thereafter. CFU of *L. pneumophila* in each culture were determined daily, as described (1).

Assay for thymidine incorporation by mononuclear cells used to generate supernatants. After mononuclear cell cultures used to generate supernatants and supernatant controls were centrifuged and the supernatant fluids removed, the leukocyte pellet was resuspended in 600 μl RPMI medium (1×10^7 of the originally cultured mononuclear cells/ml). Triplicate 50-μl aliquots of this cell suspension were added to microtest wells (Falcon Labware, 96-well tissue culture plate, Div. Becton Dickinson & Co., Oxnard, CA) and mixed with an equal volume of RPMI 1640 containing 2% serum and 5 μCi [^{3}H]thymidine/ml. The cells were incubated for 2 h at 37°C in 5% CO_2–95% air, and harvested on glass fiber filter paper (Whatman Inc., Clifton, NJ) with a cell harvester. The filters were dried, placed in glass vials containing liquid scintillation counting solution (Hydrofluor, National Diagnostics, Somerville, NJ) incubated overnight at 4°C, and counts per minute registered with a liquid scintillation counter (Nuclear Chicago Corp., subsidiary of Searle, G. D., & Co., Skokie, IL).

RESULTS

Mononuclear cells from patients recovered from Legionnaires' disease respond to L. pneumophila antigens with the production of cytokines that activate monocytes. Patients recovered from Legionnaires' disease were studied initially to determine if they have circulating mononuclear cells capable of responding to *L. pneumophila* antigens with the production of cytokines that activate monocytes, as measured by monocyte capacity to inhibit *L. pneumophila* multiplication. *L. pneumophila*-induced mononuclear cell supernatants were prepared by incubating patient mononuclear cells with 5×10^8 formalin-killed *L. pneumophila* for 4 d and supernatant controls were prepared by adding the formalin-killed *L. pneumo-*

phila to the mononuclear cells at the end of the incubation period, as described in Methods. These supernatants were tested by incubating freshly explanted nonpatient monocytes in monolayer culture with the supernatants at concentrations ranging from 0 to 60% for 24 h, infecting the monocytes with virulent *L. pneumophila*, and assaying the cultures daily for CFU of *L. pneumophila*.

Monocytes treated with supernatants inhibited *L. pneumophila* multiplication in comparison with monocytes treated with supernatant controls, and the degree of inhibition was proportional to the concentration of supernatant added (Fig. 1). In experiments of this type

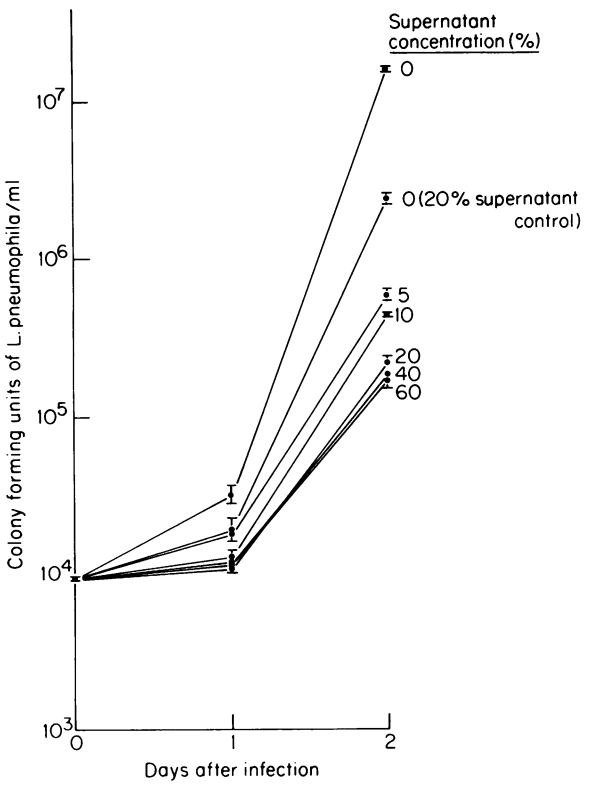

FIGURE 1 Supernatants generated in response to *L. pneumophila* antigens by mononuclear cells of patients recovered from Legionnaires' disease activate nonpatient monocytes such that they inhibit *L. pneumophila* multiplication. Freshly explanted monocytes from a nonpatient were incubated in monolayer culture for 24 h at 37°C in 5% CO_2–95% air in 500 μl RPMI 1640 medium containing 20% fresh nonpatient serum and either 0–60% (vol/vol) cell-free supernatant or 20% supernatant control; the supernatant and supernatant control were prepared from the mononuclear cells of a patient (patient B) recovered from Legionnaires' disease, as described in Methods. After 24 h, *L. pneumophila* (10^4 CFU/ml) were added to the cultures and CFU in each culture determined daily. Each point represents the average for three replicate tissue culture wells±SEM.

with several patients, maximal activation was obtained with 20–40% supernatants.

Optimal conditions for production of maximally potent supernatants. The optimal length of time to incubate patient mononuclear cells with formalin-killed *L. pneumophila* to generate maximally potent supernatants was studied next. Six different supernatants were prepared from each of two patients by incubating their mononuclear cells with 5×10^8 formalin-killed *L. pneumophila* 0–10 d. These supernatants were then tested for their capacity to activate monocytes as described above. In this study, monocytes were incubated with 20% supernatants and supernatant controls (Fig. 2).

Supernatants generated by incubation of patient mononuclear cells with formalin-killed *L. pneumophila* for 4 d were maximally potent, i.e., *L. pneumophila* multiplication was maximally inhibited by monocytes treated with 4-d supernatants. In all subsequent experiments, 4-d supernatants were used.

The optimal number of formalin-killed *L. pneumophila* with which to incubate patient mononuclear cells in order to generate maximally potent supernatants was studied next. Six different sets of supernatants and supernatant controls were prepared from each of two patients by incubating their mononuclear cells with 5×10^3–5×10^8 formalin-killed *L. pneumophila*/ml. Supernatants generated with 5×10^6, 5×10^7, and 5×10^8 formalin-killed *L. pneumophila*/ml were maximally potent (Fig. 3), supernatants generated with 5×10^5 organisms/ml were intermediate in potency, and supernatants generated with 5×10^3 and 5×10^4 organisms/ml were inactive (data not shown). This dose response paralleled that obtained in the mononuclear cell proliferation assay, as measured by [^{3}H]thymidine incorporation, discussed below (Table II).

In other experiments, supernatants generated in flat-bottomed wells were compared with supernatants generated in cone-shaped wells; in cone-shaped wells, leukocytes and bacterial particles settled to the tip of the cone and cell-cell contact was greatly increased. Supernatants generated in flat-bottomed and cone-shaped wells were equivalent in potency (data not shown).

Comparison of patient L. pneumophila- and Con A-induced mononuclear cell supernatants. L. pneumophila-induced supernatants were compared with Con A-induced supernatants prepared from the same patient at the same time. Con A supernatants were collected after 2 d rather than 4 d of incubation with mononuclear cells because, in earlier studies, 2-d Con A supernatants were maximally potent (4).

Con A-induced supernatants resulted in greater inhibition of *L. pneumophila* multiplication in monocytes than *L. pneumophila*-induced supernatants in all

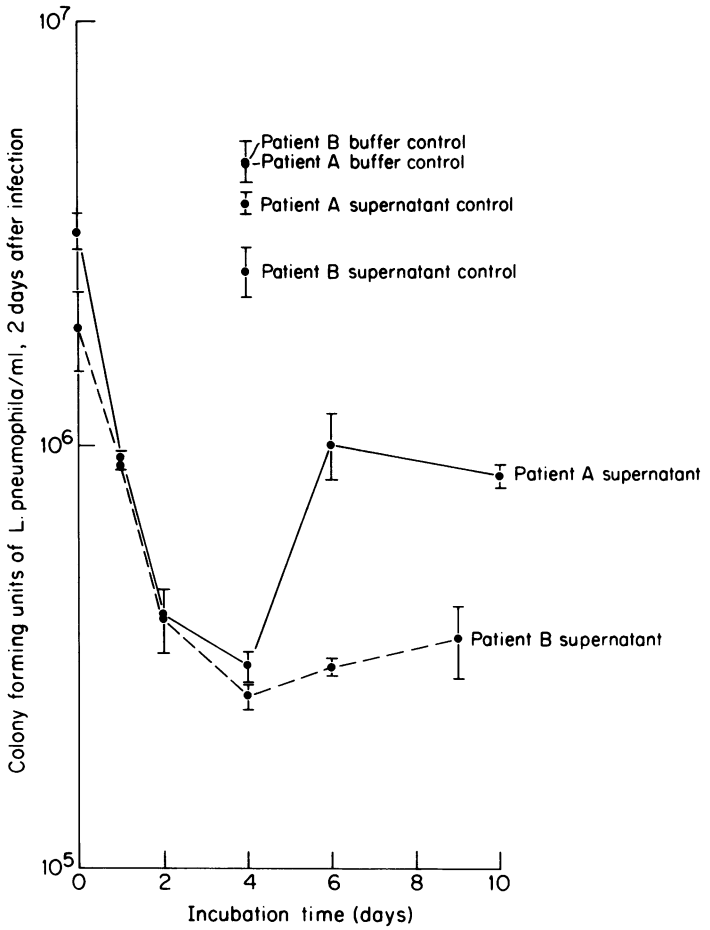

FIGURE 2 4-d *L. pneumophila*-induced mononuclear cell supernatants are maximally potent. Six different supernatants were prepared from each of two patients (patients A and B) by incubating their mononuclear cells for 0–10 d with *L. pneumophila* antigens. A 4-d supernatant control was also prepared from each patient. Freshly explanted nonpatient monocytes were incubated for 24 h with 20% of one of these supernatants, one of the 4-d supernatant controls, or buffer control (RPMI 1640 medium) and infected with *L. pneumophila* as in Fig. 1. CFU in each culture 2 d after infection were determined. Each point on the graph represents the mean CFU/ml 2 d after infection in replicate monocyte cultures treated with one of the six supernatants, two supernatant controls, or buffer control as indicated; the buffer controls are plotted arbitrarily at 4 d. Mononuclear cell supernatant fluids from patient A were tested at a different time than supernatant fluids from patient B. Each point represents the mean for three replicate tissue culture wells±SEM.

seven patients; the results obtained from one patient (patient C), shown in Fig. 3, are representative. These results may indicate that, at the time of the study, the proportion of circulating lymphocytes in these patients sensitized to *L. pneumophila* antigens was less than the proportion of circulating lymphocytes subject to stimulation by the nonspecific mitogen Con A.

Comparison of patient and nonpatient supernatants. The experiments presented thus far show that mononuclear cells from patients recovered from Legionnaires' disease respond to *L. pneumophila* antigens with the production of cytokines capable of activating monocytes. Whether patient mononuclear cells differ in this respect from nonpatient mononuclear cells was now examined by preparing supernatants and supernatant controls from two patients (A and B) and two nonpatients (A and B) and testing them at the same time (Fig. 4).

The patient supernatants resulted in greater monocyte inhibition of *L. pneumophila* multiplication than

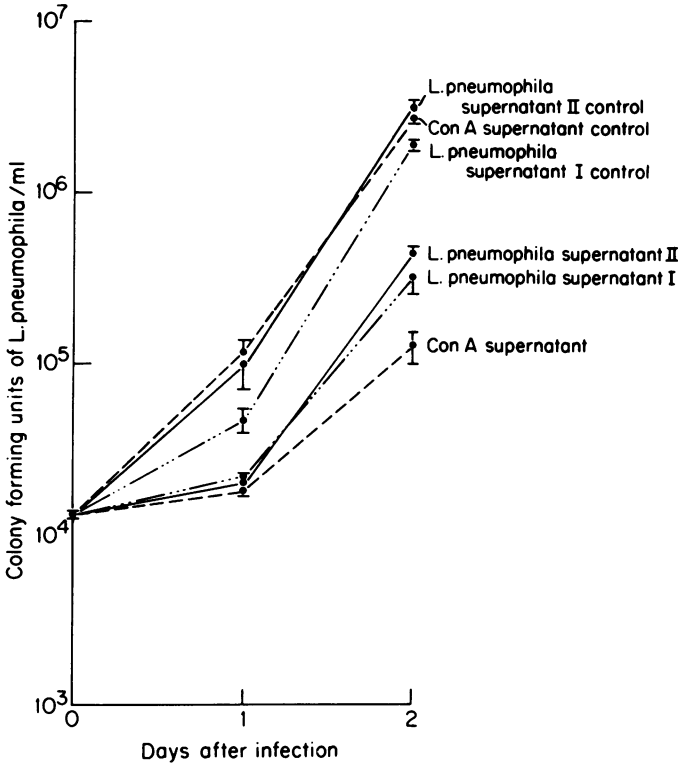

FIGURE 3 Comparison of patient *L. pneumophila*-induced and Con A-induced mononuclear cell supernatants. Two *L. pneumophila*-induced mononuclear cell supernatants and supernatant controls were prepared from a patient (patient C). *L. pneumophila* supernatants I and II were prepared by incubating mononuclear cells for 4 d with 5×10^8 and 5×10^7 formalin-killed *L. pneumophila*/ml, respectively. Monocytes were incubated for 24 h with 40% supernatant or supernatant control, as indicated, and infected with *L. pneumophila* as in Fig. 1. CFU were determined daily. Each point represents the mean for two replicate tissue culture wells±SEM.

the nonpatient supernatants (Fig. 4). However, the nonpatient supernatants reproducibly exhibited a small degree of inhibition in comparison with their supernatant controls.

Patient mononuclear cells respond to L. pneumophila antigens with proliferation. Patient mononuclear cells were examined for their capacity to proliferate in response to *L. pneumophila* antigens. In these experiments, the same mononuclear cells that had been used in the production of *L. pneumophila* supernatants and supernatant controls were assayed for their capacity to incorporate [^{3}H]thymidine. *L. pneumophila* antigen was added to mononuclear cells used to generate supernatant controls immediately before these cells were assayed for their capacity to incorporate [^{3}H]thymidine.

Patient mononuclear cells that had been incubated with *L. pneumophila* antigen for 4 d exhibited enhanced [^{3}H]thymidine incorporation in comparison with control mononuclear cells to which equivalent amounts of *L. pneumophila* antigen were added just before the incorporation assay (Table II). Control mononuclear cells exhibited background levels of [^{3}H]thymidine incorporation. These findings confirmed the impression that was gained by visual inspection of these mononuclear cells using the inverted phase and light microscopes; by microscopy, wells containing mononuclear cells incubated with *L. pneumophila* antigens for 4 d had a higher density of cells (lymphocytes appeared to account for the increase) than wells containing control mononuclear cells, which had about the same cell density at 4 d as at the beginning of the incubation period.

Comparison of mononuclear cells from patients with mononuclear cells from age- and sex-matched nonpatients for capacity to respond to L. pneumophila antigens. Mononuclear cell responses to *L. pneumophila* antigens of patients and nonpatients were further investigated in studies of the mononuclear cells from eight additional persons: four age- (within

TABLE II
Patient Mononuclear Cells Proliferate in Response to L. pneumophila Antigens

Antigen concentration	[^{3}H]Thymidine incorporation by mononuclear cells incubated for 4 d with (+) or without (−) antigen	
	+ Antigen	− Antigen
Formalin-killed L. pneumophila/ml	*cpm/microtest well*	
5×10^8	7,200±224	91±1
5×10^7	7,530±32	108±4
5×10^6	3,666±470	98±5
5×10^5	1,025±30	112±2
5×10^4	322±30	102±1
5×10^3	260±8	112±16

Mononuclear cells (3×10^6/ml) were incubated in petri dishes for 4 d at 37°C with (+ antigen) or without (− antigen) formalin-killed *L. pneumophila* at concentrations ranging from 5×10^8 to 5×10^3/ml as indicated. At the end of the 4-d incubation period, formalin-killed *L. pneumophila* were added to cultures that had been incubated without the bacterial particles (− antigen cultures). The mononuclear cells from each petri dish were distributed into microtest wells and assayed for capacity to incorporate [^{3}H]thymidine, as described in Methods. Each petri dish was assayed in triplicate and the mean counts per minute per microtest well determined. Each datum on the table is the average of the mean counts per minute per microtest well for two petri dishes.

5 yr of each other) and sex-matched patient and nonpatient pairs. Mononuclear cell responses to both *L. pneumophila* antigens and Con A were studied in both of the assays presented in this paper, i.e., the assay for mononuclear cell proliferation, as measured by [^{3}H]thymidine incorporation, and the assay for monocyte-activating cytokines, as measured by monocyte inhibition of *L. pneumophila* multiplication. Mononuclear cells from both members of a pair were obtained on the same day; supernatants and supernatant controls were prepared and tested and the mononuclear cells assayed for proliferation at the same time with the same reagents.

In the mononuclear cell proliferation assay (Table IIIA), patient and nonpatient mononuclear cells responded comparably to Con A. In response to *L. pneumophila* antigens, patient mononuclear cells consistently showed enhanced [^{3}H]thymidine incorporation in comparison with nonpatient mononuclear cells.

The results of the assay for monocyte activating cytokines (Table IIIB) generally paralleled the assay for mononuclear cell proliferation. Patients and nonpatients showed strong mononuclear cell responses to Con A; patient mononuclear cell responses were stronger to Con A than to *L. pneumophila* as mentioned earlier. Patient *L. pneumophila*-induced supernatants were consistently more inhibitory than nonpatient *L. pneumophila*-induced supernatants, as found above (Fig. 4).

Thus, in both assays, patient mononuclear cells showed enhanced responses to *L. pneumophila* antigens in comparison with age- and sex-matched nonpatient controls.

Comparison of mononuclear cells from patients recovered from Legionnaires' disease with mononuclear cells from patients with tuberculoid leprosy for capacity to respond to L. pneumophila and M. leprae antigens. The specificity of mononuclear cell responses to *L. pneumophila* antigens was further examined. Mononuclear cells were obtained from two patients recovered from Legionnaires' disease and from two patients under treatment for tuberculoid leprosy, and the responses of these cells to *L. pneumophila* and *M. leprae* antigens were compared. The patients were not matched for age and sex and the mononuclear cells were not collected on the same day as in the above study; however, in the case of each individual patient, the mononuclear cell supernatants to *L. pneumophila* and *M. leprae* antigens were prepared and tested and the mononuclear cells assayed for proliferation at the same time under the same conditions.

In both the mononuclear proliferation assay (Table IVA) and the assay for monocyte activating cytokines (Table IVB), mononuclear cells from patients recovered from Legionnaires' disease responded more strongly to *L. pneumophila* antigens than to *M. leprae* antigens, whereas mononuclear cells from patients with tuberculoid leprosy responded more strongly to *M. leprae* antigens than to *L. pneumophila* antigens.

DISCUSSION

These experiments show that cell-mediated immunity develops in patients with Legionnaires' disease. Patients recovered from Legionnaire's disease have circulating peripheral blood mononuclear cells sensitized to *L. pneumophila* antigens; remarkably, such cells are present many months and in one case nearly 2 yr after full recovery from Legionnaires' disease. In some cases, mononuclear cell responses to *L. pneumophila* persisted even though antibody titers to the same *L. pneumophila* strain had fallen to or below the threshold level of significance.

The conceptual basis for the experiments in this paper derive in large part from studies of acquired cell-mediated immunity to infection by intracellular bacteria that were conducted by Chase and Lurie in the 1940s, Mackaness in the 1960s, and other investigators

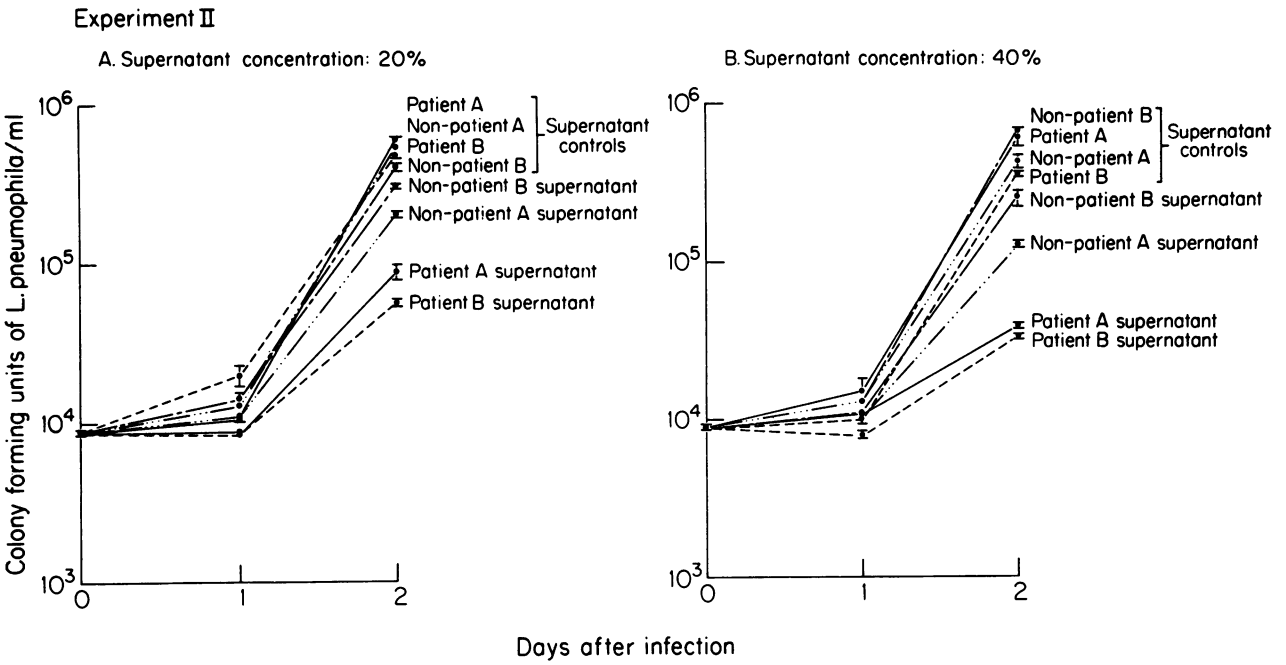

FIGURE 4 Patient supernatants result in greater inhibition of *L. pneumophila* multiplication in monocytes than nonpatient supernatants. Patient and nonpatient *L. pneumophila* supernatants and supernatant controls were prepared as described in Methods. Monocytes were incubated for 24 h with 20% (A) or 40% (B) of these preparations and infected with *L. pneumophila* as in Fig. 1. CFU were determined daily. Each point represents the mean for two replicate tissue culture wells±SEM.

in the 1970s. Chase (10) demonstrated that cutaneous delayed-type hypersensitivity to tuberculin was transferable by specifically sensitized lymphoid cells and not by serum. Lurie (11) pointed out the importance of macrophages in immunity by demonstrating that macrophages harvested from vaccinated animals have an enhanced capacity to inhibit multiplication of tubercle bacilli. Mackaness (12) provided the critical link between the results of Chase and Lurie. He demonstrated that immunity to *Listeria monocytogenes* could be passively transferred to mice with sensitized lymphoid cells and that these cells, in the presence of the sensitizing organism or its antigens, conferred on macrophages the capacity to inhibit the multiplication of intracellular bacteria nonspecifically. In 1971, Simon and Sheagren (13) reproduced Mackaness's result in vitro demonstrating that sensitized lymphocytes in cell culture, in the presence of specific antigen, conferred on mononuclear phagocytes the capacity to inhibit intracellular bacteria nonspecifically. About this time, Lane and Unanue (14) and North (15) showed that the lymphoid cells responsible for transferring immunity to listeriosis and tuberculosis were T cells. Other investigators demonstrated that macrophage function can be altered by soluble products (lymphokines or cytokines) of sensitized lymphocytes and perhaps other mononuclear cells.

Patients with Legionnaires' disease develop both humoral and cell-mediated immunity to *L. pneumophila*. Humoral immunity appears to play a limited role in host defense against *L. pneumophila*, as mentioned in the Introduction. Moreover, under some circumstances, antibody may be counterproductive by enhancing the uptake of *L. pneumophila* by mononuclear phagocytes, in which the bacteria multiply. In contrast, cell-mediated immunity appears to play a major role in host defense against *L. pneumophila;* activated mononuclear phagocytes inhibit the intracellular multiplication of *L. pneumophila* (2, 5).

Nonpatients, i.e., persons with no known history of *L. pneumophila* infection and with antibody levels to the test strain of *L. pneumophila* below the threshold level of significance, had lower mononuclear cell responses to *L. pneumophila* antigens than patients, but responses significantly higher than their own control levels. This may reflect cross-reactivity between *L. pneumophila* and other antigens to which these people were exposed; alternatively, this may reflect subclinical or unrecognized exposure to *L. pneumophila*, which is ubiquitous in the environment.

TABLE IIIA

Mononuclear Cells from Patients Respond to L. pneumophila Antigens with Greater Proliferation and Greater Production of Monocyte-activating Cytokines than Mononuclear Cells from Age- and Sex-matched Nonpatients: Mononuclear Cell Proliferation

	[^{3}H]Thymidine incorporation by mononuclear cells incubated with (+) or without (−) Con A or formalin-killed *L. pneumophila*			
	Con A		*L. pneumophila*	
	+	−	+	−
	cpm/microtest well			
Patient D	12,961±432	143±2	2,214±644	150±26
Nonpatient D	8,222±588	72±5	320±13	90±10
Patient E	12,096±55	140±2	11,832±199	104±4
Nonpatient E	6,058±514	109±4	2,997±99	141±16
Patient F	9,019±288	147±4	3,509±135	326±135
Nonpatient F	14,699±227	153±11	1,864±132	163±4
Patient G	13,120±644	200±11	12,269±1,501	180±13
Nonpatient G	14,295±693	280±26	3,500±139	237±15

Mononuclear cells from four pairs of patient and age- and sex-matched nonpatient were studied. Mononuclear cells from both members of a pair were collected and tested at the same time.

Mononuclear cells (3×10^6/ml) were incubated in petri dishes at 37°C with (+) or without (−) Con A (15 μg/ml) for 2 d or with (+) or without (−) formalin-killed *L. pneumophila* (5×10^8 bacterial particle/ml) for 4 d. At the end of the incubation period, Con A or formalin-killed *L. pneumophila* was added to the appropriate control cultures that had been incubated without them. Then the mononuclear cells from each petri dish were distributed into microtest wells and assayed for capacity to incorporate [^{3}H]thymidine, as described in Methods. Each petri dish was assayed in triplicate and the mean counts per minute per microtest well for that petri dish determined. Each datum on the table is the average of the mean counts per minute per microtest well for three replicate petri dishes±SEM.

TABLE IIIB

Mononuclear Cells from Patients Respond to L. pneumophila Antigens with Greater Proliferation and Greater Production of Monocyte-activating Cytokines than Mononuclear Cells from Age- and Sex-matched Nonpatients: Mononuclear Cell Production of Monocyte-activating Cytokines

	CFU of *L. pneumophila*/ml 2 d after infection in monocyte cultures preincubated with Con A or *L. pneumophila* supernatants or supernatant controls					
	Con A			*L. pneumophila*		
	Supernatant control	Supernatant	Log inhibition*	Supernatant control	Supernatant	Log inhibition*
	CFU/ml			*CFU/ml*		
Patient D	$6.6\pm2.2 \times 10^5$	$1.8\pm0.1 \times 10^4$	1.57	$2.1\pm0.1 \times 10^5$	$7.3\pm1.0 \times 10^4$	0.46
Nonpatient D	$3.2\pm0.3 \times 10^5$	$1.3\pm0.1 \times 10^4$	1.40	$1.7\pm0.2 \times 10^5$	$9.5\pm1.7 \times 10^4$	0.26
Patient E	$6.0\pm0.8 \times 10^5$	$6.8\pm0.8 \times 10^3$	1.95	$2.2\pm0.6 \times 10^5$	$7.0\pm2.3 \times 10^4$	0.49
Nonpatient E	$5.7\pm0.7 \times 10^5$	$3.0\pm0.2 \times 10^4$	1.28	$2.6\pm0.6 \times 10^5$	$1.3\pm0.1 \times 10^5$	0.29
Patient F	$3.4\pm0.2 \times 10^6$	$1.7\pm0.2 \times 10^4$	2.29	$8.9\pm0.1 \times 10^5$	$1.7\pm0.1 \times 10^5$	0.71
Nonpatient F	$1.2\pm0.2 \times 10^6$	$2.8\pm0.5 \times 10^4$	1.64	$1.7\pm0.5 \times 10^6$	$5.7\pm0.3 \times 10^5$	0.47
Patient G	$1.5\pm0.1 \times 10^6$	$2.5\pm0.7 \times 10^4$	1.77	$5.1\pm1.0 \times 10^5$	$1.1\pm0.3 \times 10^5$	0.67
Nonpatient G	$1.7\pm0.1 \times 10^6$	$8.6\pm0.3 \times 10^4$	2.30	$7.9\pm1.4 \times 10^5$	$2.4\pm0.7 \times 10^5$	0.52

Mononuclear cells from four pairs of patient and age- sex-matched nonpatient were studied.

Mononuclear cells from both members of a pair were collected and tested at the same time.

Patient and nonpatient Con A-induced and *L. pneumophila*-induced supernatants and supernatant controls were prepared using 15 μg/ml Con A or 5×10^8 formalin-killed *L. pneumophila*/ml, as described in Methods. Monocytes were incubated for 24 h with 40% supernatant or supernatant control, as indicated, and then infected with *L. pneumophila* (10^4 CFU/ml) as in Figs. 3 and 4. CFU were determined 2 d after infection in replicate monocyte cultures. Data are the mean CFU/ml 2 d after infection for two replicate tissue culture wells±SEM.

* Log inhibition = (log CFU/ml in monocyte cultures treated with supernatant control)-(log CFU/ml in monocyte cultures treated with supernatant).

TABLE IVA

Mononuclear Cells from Patients Recovered from Legionnaires' Disease Respond More Strongly to L. pneumophila than to M. leprae Antigens whereas Mononuclear Cells from Patients with Tuberculoid Leprosy Respond More Strongly to M. leprae Antigens: Mononuclear Cell Proliferation

		[^{3}H]Thymidine incorporation by mononuclear cells incubated with (+) or without (−) *L. pneumophila* or *M. leprae* antigens			
		L. pneumophila		*M. leprae*	
Illness	Patient	+	−	+	−
		cpm/microtest well			
Legionnaires' disease	1.	6,982±233	88±3	2,764±351	107±7
	2.	12,269±1,501	180±13	2,762±117	217±15
Tuberculoid leprosy	3.	5,897±791	436±16	21,023±617	378±20
	4.	2,119±373	111±3	6,251±723	122±10

Mononuclear cells from two patients recovered from Legionnaires' disease (1. and 2.) and from two patients under treatment for tuberculoid leprosy (3. and 4.) were studied. Responses to *L. pneumophila* and *M. leprae* antigens were tested at the same time.

Mononuclear cells were incubated in petri dishes at 37°C for 4 d with (+) or without (−) *L. pneumophila* (5×10^8 bacterial particles/ml) or *M. leprae* (100 μg/ml) antigens. At the end of the incubation period, *L. pneumophila* or *M. leprae* antigen was added to the appropriate control cultures that had been incubated without them. Then mononuclear cells were distributed into microtest wells and assayed for capacity to incorporate [^{3}H]thymidine, as described in Methods. Each petri dish was assayed in triplicate and the mean counts per minute per microtest well for that petri dish determined. Each datum on the table is the average of the mean counts per minute per microtest well for three replicate petri dishes±SEM.

TABLE IVB

Mononuclear Cells from Patients Recovered from Legionnaires' Disease Respond More Strongly to L. pneumophila than to M. leprae Antigens whereas Mononuclear Cells from Patients with Tuberculoid Leprosy Respond More Strongly to M. leprae Antigens: Mononuclear Cell Production of Monocyte-activating Cytokines

		CFU of *L. pneumophila*/ml 2 d after infection in monocyte cultures preincubated with *L. pneumophila* or *M. leprae* supernatants or supernatant controls					
		L. pneumophila			*M. leprae*		
Illness	Patient	Supernatant control	Supernatant	Log inhibition*	Supernatant control	Supernatant	Log inhibition
		CFU/ml			*CFU/ml*		
Legionnaires' disease	1.	$3.8\pm0.3 \times 10^5$	$1.5\pm0.4 \times 10^5$	0.40	$3.1\pm0.5 \times 10^5$	$2.6\pm0.6 \times 10^5$	0.08
	2.	$3.0\pm0.7 \times 10^5$	$2.8\pm0.1 \times 10^4$	1.03	$1.6\pm0.5 \times 10^5$	$1.9\pm0.2 \times 10^6$	0.08
Tuberculoid leprosy	3.	$3.0\pm0.3 \times 10^5$	$9.0\pm2.1 \times 10^4$	0.53	$2.3\pm0.4 \times 10^6$	$2.7\pm0.3 \times 10^5$	0.93
	4.	$3.1\pm0.7 \times 10^4$	$1.0\pm0.1 \times 10^5$	0.45	$3.6\pm0.2 \times 10^5$	$2.8\pm0.2 \times 10^5$	1.11

Mononuclear cells from two patients recovered from Legionnaires' disease (1. and 2.) and from two patients under treatment for tuberculoid leprosy (3. and 4.) were studied. Responses to *L. pneumophila* and *M. leprae* antigens were tested at the same time.

L. pneumophila and *M. leprae* mononuclear cell supernatants and supernatant controls were prepared using 5×10^8 formalin-killed *L. pneumophila*/ml or 100 μg *M. leprae*/ml as described in Methods. Monocytes were incubated for 24 h with 40% supernatant or supernatant control, as indicated, and then infected with *L. pneumophila* (10^4 CFU/ml). CFU were determined 2 d after infection in replicate monocyte cultures. Data are the mean CFU/ml 2 d after infection for three replicate tissue culture wells±SEM.

* Log inhibition is as defined in Table IIIB.

In vitro activated mononuclear phagocytes, whether activated with *L. pneumophila* or mitogen-induced cytokines, inhibit *L. pneumophila* multiplication but do not kill the bacteria (3, 4). This raises the question as to how *L. pneumophila* are eliminated in vivo from the host. Possible explanations include the following. First, *L. pneumophila* unable to multiply in phagocytes may simply die out, as they do slowly in tissue culture medium, and/or they may be expelled mechanically from host lung by normal clearance mechanisms. Second, polymorphonuclear leukocytes, which in vitro kill about half a log of an inoculum of *L. pneumophila* in the presence of antibody and complement, may kill many extracellular bacteria or possibly all of them if repeated encounters between freshly emigrating polymorphonuclear leukocytes and surviving *L. pneumophila* result in further killing of bacteria. Third, in vivo activated mononuclear phagocytes may possess greater antimicrobial capacity than in vitro activated mononuclear phagocytes and be capable of enhanced killing of *L. pneumophila*. Animal studies may help clarify this issue.

In this study, mononuclear cell proliferation in response to antigens correlated with the production of monocyte-activating cytokines in response to these antigens. Both mononuclear cell responses were higher in patients recovered from Legionnaires' disease than in nonpatients (Table III); moreover, in patients with either Legionnaires' disease or tuberculoid leprosy, both mononuclear cell responses were higher to the relevant than irrelevant antigen (Table IV). These two mononuclear cell responses have also been highly correlated in studies of other patients in this laboratory. For example, mononuclear cells from patients with lepromatous leprosy fail to respond to *M. leprae* with either mononuclear cell proliferation or the production of monocyte-activating cytokines, whereas mononuclear cells from patients with tuberculoid leprosy respond strongly in both assays (manuscript in preparation).

The assays described in this paper have general applicability for the study of monocyte and mononuclear cell effector functions in selected patients. These assays may be used to study (*a*) the capacity of a patient's monocytes to be activated to an antimicrobial potential with a standardized preparation of cytokines (e.g., Con A-induced cytokines from normal mononuclear cells) and (*b*) the capacity of a patient's mononuclear cells to generate such monocyte-activating cytokines in response to a mitogen or antigen. The first assay requires only 9×10^6 mononuclear cells to test monocytes to a single cytokine preparation and its control (e.g., Con A-induced supernatant and supernatant control) in triplicate; this allows for six monocyte monolayers (initially from 1.5×10^6 mononuclear cells) in 16-mm diam tissue culture wells. The second assay requires only 12×10^6 mononuclear cells to generate 1.5–2 ml of a single supernatant and supernatant control; this allows for two 35-mm diam petri dishes, each containing 6×10^6 mononuclear cells in 2 ml of medium, or alternatively, eight 16-mm diam tissue culture wells, each containing 1.5×10^6 mononuclear cells in 0.5 ml of medium. Therefore both assays can be conducted with 21×10^6 mononuclear cells, an amount normally obtained from 20 ml of blood or less. In addition, the mononuclear cell proliferation assay can be conducted without any additional cells, as in this study. These assays have proved useful for studying monocyte and mononuclar cell effector functions of patients with a variety of clinical disorders including patients with leprosy and patients with acquired immune deficiency syndrome (AIDS) manifested by opportunistic infections and Kaposi's sarcoma.

L. pneumophila is a particularly convenient test organism for assaying mononuclear phagocyte activation because it multiplies rapidly on solid bacteriologic medium, and it does not multiply extracellularly under tissue culture conditions. Consequently, the inhibitory effect of activated mononuclear phagocytes on *L. pneumophila* multiplication can be accurately quantitated in CFU. In contrast, viruses, rickettsia, toxoplasma, trypanosoma, and leishmania are obligate intracellular organisms and do not multiply on solid medium; *Mycobacterium tuberculosis* multiplies on medium too slowly for practical use in an assay of this type; and *Listeria monocytogenes* can multiply extracellularly under tissue culture conditions and thereby escape the antimicrobial activity of activated mononuclear phagocytes. For use in assays of this type, either egg yolk- or CYE agar-grown *L. pneumophila* can be used and the organisms can be conveniently stored for years at −70°C without significant loss of infectivity or viability.

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