Cell-mediated Immunity in Legionnaires’ Disease

MARCUS A. HORWITZ, Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021

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 [3H]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 x 10⁶–5 x 10⁸ 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 of...
of high-titer antibody and complement; although anti-
obody and complement markedly promote the phagocytosis of *L. pneumophila* by human polymorphonu-
clear leukocytes and monocytes, these phagocytes kill
only 0.5 log of an inoculum of *L. pneumophila* under
optimal conditions (3, 4). Third, *L. pneumophila* pre-
treated with antibody and complement and then in-
cubated with monocytes in the presence of these hu-
moral 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 im-
munity does not play a primary role in host defense
against *L. pneumophila*.

Another study has demonstrated that activated hu-
man 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). 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 im-
munity in Legionnaires’ disease. The study demon-
strates (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 ac-
tivating normal human monocytes such that they in-
hbit the intracellular multiplication of *L. pneumo-
phila*.

**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 fluores-
cent antibody (IFA) assay (6) served as controls (nonpatients).
In addition, two persons from the New York City area with
tuberculous 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 nonpa-

dients. 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* Phila-
delphia 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;
[NH₂]thyminidine, 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 vi-
ability 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. pneumo-
phila* were prepared by passing egg yolk grown *L. pneu-
phila* on CYE agar one time only, collecting the bacteria,
inoculating them with 2% formalin for 30 min at 4°C and
washing the bacteria four times in egg yolk buffer by cen-
trifugation at 12,000 g for 10 min at 4°C. In a recent published
study of this strain of *L. pneumophila*, IFA titers of
human serum obtained against formalin-killed *L. pneumo-
phila* were found to be highly correlated with IFA titers ob-
tained 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 centrifu-
gation over a Ficoll-sodium diatrizoate solution as previously
described (1).

**Preparation of supernatants.** Patient and nonpatient
mononuclear cells (6 × 10⁶) 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% 
CO₂–95% air for 4 d (0–10 d in some experiments) with for-
malin-killed *L. pneumophila* at concentrations ranging from
5 × 10⁶ to 5 × 10⁸ 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 leu-
kocytes 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. Con-

*Abbreviations used in this paper: CFU, colony-forming
units; Con A, concanavalin A; IFA, indirect fluorescent anti-
body assay.*
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 × 10⁶) 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% CO₂-95% 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 (10⁴ colony-forming units [CFU]/ml) were added to the cultures. The cultures were incubated at 37°C in 5% CO₂-95% 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 × 10⁵ 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 [³H]thymidine/ml. The cells were incubated for 2 h at 37°C in 5% CO₂-95% air, and harvested on glass fiber filter paper (Whatman Inc., Clifton, 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 × 10⁶ formalin-killed *L. pneumophila* for 4 d and supernatant controls were prepared by adding the formalin-killed *L. pneumo-
pneumophila 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 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 $5 \times 10^6$ 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 $5 \times 10^3$–$5 \times 10^6$ formalin-killed L. pneumophila/ml. Supernatants generated with $5 \times 10^6$, $5 \times 10^5$, and $5 \times 10^4$ formalin-killed L. pneumophila/ml were maximally potent (Fig. 3), supernatants generated with $5 \times 10^4$ organisms/ml were intermediate in potency, and supernatants generated with $5 \times 10^3$ and $5 \times 10^5$ organisms/ml were inactive (data not shown). This dose response paralleled that obtained in the mononuclear cell proliferation assay, as measured by [3H]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
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...
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-
Mononuclear cells (3 x 10⁶/ml) were incubated in petri dishes for 4 d at 37°C with (+ antigen) or without (− antigen) formalin-killed \textit{L. pneumophila} at concentrations ranging from 5 x 10⁶ to 5 x 10⁷/ml as indicated. At the end of the 4-d incubation period, formalin-killed \textit{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 microtiter wells and assayed for capacity to incorporate [³H]thymidine, as described in Methods. Each petri dish was assayed in triplicate and the mean counts per minute per microtiter well determined. Each datum on the table is the average of the mean counts per minute per microtiter well for two petri dishes.

5 yr of each other) and sex-matched patient and nonpatient pairs. Mononuclear cell responses to both \textit{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 [³H]thymidine incorporation, and the assay for monocyte-activating cytokines, as measured by monocyte inhibition of \textit{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 II A), patient and nonpatient mononuclear cells responded comparably to Con A. In response to \textit{L. pneumophila} antigens, patient mononuclear cells consistently showed enhanced [³H]thymidine incorporation in comparison with nonpatient mononuclear cells.

The results of the assay for monocyte activating cytokines (Table II B) 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 \textit{L. pneumophila} as mentioned earlier. Patient \textit{L. pneumophila}-induced supernatants were consistently more inhibitory than nonpatient \textit{L. pneumophila}-induced supernatants, as found above (Fig. 4).

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

\textbf{Comparison of mononuclear cells from patients recovered from Legionnaires' disease with mononuclear cells from patients with tuberculoid leprosy for capacity to respond to \textit{L. pneumophila} and \textit{M. leprae} antigens.} The specificity of mononuclear cell responses to \textit{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 \textit{L. pneumophila} and \textit{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 \textit{L. pneumophila} and \textit{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 II A) and the assay for monocyte activating cytokines (Table II B), mononuclear cells from patients recovered from Legionnaires' disease responded more strongly to \textit{L. pneumophila} antigens than to \textit{M. leprae} antigens, whereas mononuclear cells from patients with tuberculoid leprosy responded more strongly to \textit{M. leprae} antigens than to \textit{L. pneumophila} antigens.

\textbf{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 \textit{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 \textit{L. pneumophila} persisted even though antibody titers to the same \textit{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.
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

[3H]Thymidine incorporation by mononuclear cells incubated with (+) or without (−) Con A or formalin-killed L. pneumophila

<table>
<thead>
<tr>
<th></th>
<th>Con A</th>
<th>L. pneumophila</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>cpm/microtest well</td>
<td></td>
</tr>
<tr>
<td>Patient D</td>
<td>12,961±432</td>
<td>143±2</td>
</tr>
<tr>
<td>Nonpatient D</td>
<td>8,222±558</td>
<td>72±5</td>
</tr>
<tr>
<td>Patient E</td>
<td>12,096±55</td>
<td>140±2</td>
</tr>
<tr>
<td>Nonpatient E</td>
<td>6,058±514</td>
<td>109±4</td>
</tr>
<tr>
<td>Patient F</td>
<td>9,019±288</td>
<td>147±4</td>
</tr>
<tr>
<td>Nonpatient F</td>
<td>14,699±227</td>
<td>153±11</td>
</tr>
<tr>
<td>Patient G</td>
<td>13,120±644</td>
<td>200±11</td>
</tr>
<tr>
<td>Nonpatient G</td>
<td>14,295±693</td>
<td>280±26</td>
</tr>
</tbody>
</table>

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 × 10⁴/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 × 10⁴ 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 [3H]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

<table>
<thead>
<tr>
<th></th>
<th>Con A</th>
<th>L. pneumophila</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant control</td>
<td>Supernatant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFU/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient D</td>
<td>6.6±2.2 × 10⁶</td>
<td>1.8±0.1 × 10⁴</td>
</tr>
<tr>
<td>Nonpatient D</td>
<td>3.2±0.3 × 10⁵</td>
<td>1.3±0.1 × 10⁴</td>
</tr>
<tr>
<td>Patient E</td>
<td>6.0±0.8 × 10⁵</td>
<td>6.8±0.8 × 10⁴</td>
</tr>
<tr>
<td>Nonpatient E</td>
<td>5.7±0.7 × 10⁵</td>
<td>3.0±0.2 × 10⁴</td>
</tr>
<tr>
<td>Patient F</td>
<td>3.4±0.2 × 10⁶</td>
<td>1.7±0.2 × 10⁴</td>
</tr>
<tr>
<td>Nonpatient F</td>
<td>1.2±0.2 × 10⁶</td>
<td>2.8±0.5 × 10⁶</td>
</tr>
<tr>
<td>Patient G</td>
<td>1.5±0.1 × 10⁶</td>
<td>2.5±0.7 × 10⁴</td>
</tr>
<tr>
<td>Nonpatient G</td>
<td>1.7±0.1 × 10⁶</td>
<td>8.6±0.3 × 10⁴</td>
</tr>
</tbody>
</table>

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 × 10⁴ 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 (10⁴ 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

<table>
<thead>
<tr>
<th>Illness</th>
<th>Patient</th>
<th>L. pneumophila</th>
<th>M. leprae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm/microtest well</td>
<td></td>
</tr>
<tr>
<td>Legionnaires’</td>
<td>1.</td>
<td>6,982±233</td>
<td>88±3</td>
</tr>
<tr>
<td>disease</td>
<td>2.</td>
<td>12,269±1,501</td>
<td>180±13</td>
</tr>
<tr>
<td>Tuberculoid</td>
<td>3.</td>
<td>5,897±791</td>
<td>436±16</td>
</tr>
<tr>
<td>leprosy</td>
<td>4.</td>
<td>2,119±373</td>
<td>113±3</td>
</tr>
</tbody>
</table>

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 × 10^6 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 [3H]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                                         | Log inhibition | Log inhibition |
|                         |                                                  | CFU/ml   | CFU/ml         |               |
|                         |                                                  | Supernatant |              | Supernatant    |              |
|                         |                                                  | control   | Supernatant | control       | Supernatant  |
| Legionnaires’            | 1.                                              | 3.8±0.3 X 10^5 | 1.5±0.4 X 10^5 | 0.40          | 3.1±0.5 X 10^5 | 2.6±0.6 X 10^5 | 0.08 |
| disease                  | 2.                                              | 3.0±0.7 X 10^5 | 2.8±0.1 X 10^4 | 1.03          | 1.6±0.5 X 10^5 | 1.9±0.2 X 10^6 | 0.08 |
| Tuberculoid              | 3.                                              | 3.0±0.3 X 10^4 | 9.0±2.1 X 10^4 | 0.53          | 2.3±0.4 X 10^4 | 2.7±0.3 X 10^4 | 0.93 |
| leprosy                  | 4.                                              | 3.1±0.7 X 10^4 | 1.0±0.1 X 10^4 | 0.45          | 3.6±0.2 X 10^5 | 2.8±0.2 X 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 × 10^6 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 IIIIB.
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, polymophonuclear 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 polymophonuclear 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 tuberculous 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 × 10⁶ 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 × 10⁹ mononuclear cells) in 16-mm diam tissue culture wells. The second assay requires only 12 × 10⁶ 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 × 10⁶ mononuclear cells in 2 ml of medium, or alternatively, eight 16-mm diam tissue culture wells, each containing 1.5 × 10⁶ mononuclear cells in 0.5 ml of medium. Therefore both assays can be conducted with 21 × 10⁶ 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 mononuclear 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.

ACKNOWLEDGMENTS

I am grateful to Ms. Diane Chodkowski for her excellent and resourceful technical assistance. I thank Ms. Helen Kravetz, Drs. Barry Hartman, Mark Kaplan, William Levis, and David Sencer for their invaluable assistance in locating or referring patients recovered from Legionnaires’ disease or under treatment for tuberculoid leprosy.

This work was supported by grant AI 17254 and Biomedical Research support grant SO7 RR07065 from the National Institutes of Health and a grant from the Heiser Program for Research in Leprosy.

REFERENCES


