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Interaction between the Legionnaires' Disease Bacterium (*Legionella pneumophila*) and Human Alveolar Macrophages

Influence of Antibody, Lymphokines, and Hydrocortisone

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Abstract. We have studied the interaction between virulent *Legionella pneumophila* and human alveolar macrophages, the resident phagocytes at the site of infection in Legionnaires' disease. *L. pneumophila* multiplied 2.5–5 logs within 3 d, as measured by colony forming units, when incubated with freshly explanted alveolar macrophages in monolayer culture. At the peak of bacterial multiplication, the alveolar macrophage monolayers were destroyed. *L. pneumophila* multiplied more rapidly in 4-d-old than in freshly explanted alveolar macrophages. Inside alveolar macrophages, *L. pneumophila* were located within membrane-bound vacuoles whose cytoplasmic sides were studded with ribosomes.

Alveolar macrophages that were incubated with concanavalin A (Con A) stimulated human mononuclear cell supernatants (cytokines), inhibited *L. pneumophila* multiplication, and the degree of inhibition was proportional to the concentration of Con A supernatant added.

Anti-*L. pneumophila* antibody in conjunction with complement promoted phagocytosis of *L. pneumophila* by alveolar macrophages. By electron microscopy, most (75%) of the phagocytized *L. pneumophila* were intracellular. However, freshly explanted alveolar macrophages were able to kill only 0–10% of an innoculum of *L. pneumophila* even in the presence of antibody and complement. At the same time, alveolar macrophages also killed opsonized *Escherichia coli* poorly. Increasing the ratio of macrophages to bacteria, adhering the macrophages to microcarrier beads, or preincubating the macrophages for 24 or 48 h with Con A supernatants failed to augment alveolar macrophage killing of opsonized *E. coli*.

Corticosteroids appear to increase patient susceptibility to Legionnaires' disease. However, pretreatment of alveolar macrophages and monocytes with hydrocortisone had no influence on intracellular multiplication of *L. pneumophila* or on the inhibition of that multiplication by activated alveolar macrophages or monocytes. Hydrocortisone did impair cytokine-induced aggregation of alveolar macrophages.

These findings demonstrate that *L. pneumophila* multiplies in human alveolar macrophages and that they do so within a ribosome-lined phagosome; that freshly explanted alveolar macrophages kill few *L. pneumophila* even in the presence of antibody and complement; that activated alveolar macrophages inhibit *L. pneumophila* multiplication; and that steroids do not exert a direct suppressive effect on the anti-*L. pneumophila* activity of activated or nonactivated alveolar macrophages. Our findings indicate that alveolar macrophages may play a central role in both the pathogenesis of Legionnaires' disease and in host defense against it. This paper shows that a human resident macrophage can be activated to a higher state of antimicrobial capacity and that the human alveolar macrophage can serve as an effector cell in cell-mediated immunity.

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Introduction

Inhaled *Legionella pneumophila* cause Legionnaires' disease, which is a serious and often fatal form of pneumonia. Upon

reaching the terminal air spaces of the lung (alveoli), *L. pneumophila* encounter alveolar macrophages, which are the resident phagocytic cells. There are several reasons to believe that alveolar macrophages play a critical role in both the pathogenesis of Legionnaires' disease and in host defense against *L. pneumophila.* Alveolar macrophages are a prominent feature of the alveolar exudate in lung biopsy specimens from patients with Legionnaires' disease, and those cells frequently contain large numbers of intracellular *L. pneumophila* (1, 2). This finding, coupled with previous observations from this laboratory that *L. pneumophila* is a facultative intracellular pathogen that multiplies within human monocytes (3), suggests that *L. pneumophila* may multiply within alveolar macrophages. This intracellular multiplication may be instrumental in the pathogenesis of Legionnaires' disease. Regarding host defense, alveolar macrophages are considered to be important contributors to host resistance against pulmonary pathogens (4–6). Moreover, alveolar macrophages are the most abundant potential effector cells in the lung for cell-mediated immunity. Previous studies from this laboratory have indicated that cell-mediated immunity plays a major role in host defense against *L. pneumophila,* whereas humoral immunity plays a minor role (7–10). Although human alveolar macrophages are likely to be of central importance to Legionnaires' disease, their interaction with *L. pneumophila* has not been investigated in in vitro studies.

We have investigated the interaction between virulent *L. pneumophila* and human alveolar macrophages that were obtained by bronchoalveolar lavage from volunteers. We shall demonstrate that *L. pneumophila* multiplies intracellularly in human alveolar macrophages; that *L. pneumophila* multiplies within a ribosome-lined phagosome in these macrophages; that alveolar macrophages ingest but do not kill antibody and complement-coated *L. pneumophila;* that activated alveolar macrophages inhibit the intracellular multiplication of *L. pneumophila;* and that corticosteroids, which are associated with increased incidence and severity of Legionnaires' disease in patients receiving these drugs (10–13), do not influence *L. pneumophila* multiplication in activated or nonactivated alveolar macrophages.

Methods

Media. Egg yolk buffer with 1% bovine serum albumin was prepared as described previously (3). RPMI-1640, Dulbecco's phosphate-buffered saline (PBS), and Hank's balanced salt solution (HBSS) were obtained from Gibco Laboratories, Grand Island, NY.

Reagents. Concanavalin A (Con A) was obtained from Miles-Yeda Ltd., Kankakee, IL. Penicillin-streptomycin, lyophilized, was obtained from Gibco Laboratories and reconstituted with normal saline.

Agar. Modified charcoal yeast extract agar was prepared in 100 × 15-mm bacteriologic petri dishes as described (3).

Serum. Venous blood was obtained and allowed to clot. The serum was separated and stored at −70°C until used as described (14). Nonimmune human serum (type AB) with an indirect fluorescent antibody anti-*L. pneumophila* titer (15) of <1:64 was obtained from an adult donor who was not known to have ever had Legionnaires' disease. Immune human serum with an indirect fluorescent antibody anti-*L. pneumophila* titer of 1:4,096 was collected from an adult donor who had recently recovered from Legionnaires' disease. Where indicated, serum was incubated at 56°C for 30 min just before use to inactivate complement (heat-inactivated serum).

Cytodex microcarrier beads. Cytodex microcarrier beads were obtained from Pharmacia Fine Chemicals, Piscataway, NJ and prepared as per manufacturer's instructions. The beads were first swollen in PBS (100 ml PBS/g beads) in plastic tubes and then autoclaved for 20 min at 120°C. The final concentration was adjusted with RPMI to 1.25×10^5 microcarriers per milliliter. In experiments, an alveolar macrophage to microcarrier ratio of 50:1 was used.

Hydrocortisone. Hydrocortisone (cortisol), obtained from Sigma Chemical Co., St. Louis, MO, was first dissolved in 100% ethyl alcohol to 14 mg/ml and then diluted with 0.9% normal saline (Abbott Laboratories, Chicago, IL) to a concentration of 0.3 mg/ml (final concentration of alcohol equals 2.1%). A solution of 2.1% absolute ethyl alcohol (v/v) in normal saline served as a hydrocortisone control solution for this concentration of hydrocortisone. All further dilutions of hydrocortisone and hydrocortisone control solutions were made in RPMI. In some experiments, hydrocortisone hemisuccinate (Sigma Chemical Co.) was used instead of hydrocortisone. Concentrations of hydrocortisone in some cultures were kindly assayed chromatographically by Dr. Ralph Peterson (Cornell University Medical College, New York, NY).

Bacteria. L. pneumophila, Philadelphia 1 strain, was 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 (3). In some experiments, *L. pneumophila* that had been passed one time on charcoal yeast extract agar were used.

Escherichia coli, serotype 09:K29:H^-, strain Bi 161-42, an encapsulated, serum-resistant strain, and *E. coli* serotype 09:$K29^-$:H^-, an unencapsulated serum-resistant mutant of strain Bi 161-42, were cultured and harvested for experiments as described (14).

Human blood mononuclear cells. Human peripheral blood mononuclear cells were obtained from a normal adult donor who was not known to have ever had Legionnaires' disease and whose serum had an indirect fluorescent antibody anti-*L. pneumophila* titer of <1:64 (15). The blood mononuclear cell fraction was obtained by centrifugation over a Ficoll-sodium diatrizoate solution and the adherent subpopulation (containing 90% monocytes) was prepared as described (15). Mononuclear cells that were used in the production of Con A-induced supernatants were obtained from the buffy coat of blood donated at the New York Blood Center (7), New York City.

Human alveolar macrophages. Alveolar macrophages were obtained by bronchoalveolar lavage (performed by D.L.) from normal adult volunteers or from volunteer patients who were undergoing bronchoscopy for reasons unrelated to this study (e.g., solitary nodule, remote hemoptysis). Bronchoscopy in the patients was done in a normal segment of the lung contralateral to the segment with suspected pathology. None of the volunteers or patients had a pulmonary infection at the time of bronchoscopy and none was receiving antibiotics or immunosuppressive medications. Informed consent was obtained from all subjects before bronchoscopy.

1. *Abbreviations used in this paper:* CFU, colony-forming units; Con A, concanavalin A; HBSS, Hank's balanced salt solution.

Bronchoscopy was performed using a Pentax bronchoscope (F8190, Precision Instrument Corp., Norwood, NJ). Before the procedure, subjects received 0.5 mg of intramuscular atropine, and their nasopharynx was anesthetized with 2% topical viscous lidocaine (Astra Pharmaceutical Products, Inc., Worcester, MA). During passage of the bronchoscope past the larynx and trachea, a variable amount (2–10 ml) of 1% lidocaine (Elkins-Sinn, Cherry Hill, NJ) was instilled to suppress coughing. Bronchoalveolar lavage was performed by instilling sterile 0.9% saline in 50-ml aliquots into a segment of normal lung and then retrieving this fluid by gentle suction. This procedure was repeated 2–5 times (maximum of 300 ml instilled). Grossly bloody specimens were discarded.

The lavage fluid was transferred to the laboratory on ice and then centrifuged at 325 *g* for 10 min at 4°C to pellet the alveolar leukocytes. The leukocytes were resuspended and washed twice in RPMI by centrifugation at 225 *g* for 10 min, counted in a hemocytometer, adjusted to a concentration of 2.0×10^6 cells/ml, and stored in plastic tubes on ice until use.

Bronchoalveolar lavage was performed on 10 patients and 14 volunteers without any significant complications. The cell yield ranged from 1 to 12.5×10^7. The cells were >95% viable by trypan blue exclusion. Examination of a stained cytocentrifuged sample from each lavage revealed that ~90–95% of the cells were macrophages of various sizes; the remaining cells were monocytes, lymphocytes, eosinophils, polymorphonuclear leukocytes, and a few bronchial or oral epithelial cells. As it was difficult to distinguish between monocytes and small alveolar macrophages, additional cytocentrifuged samples were stained for myeloperoxidase (16, 17). On the average, 95% of these cells were myeloperoxidase negative (alveolar macrophages) and 5% myeloperoxidase positive (monocytes, granulocytes). In any one experiment, alveolar macrophages from only one donor were used.

Preparation of alveolar macrophage monolayers. The alveolar macrophages were inevitably contaminated during bronchoscopy with small numbers of bacteria that are normally resident in the nasopharynx. Therefore, in all experiments requiring long-term culture it was necessary first to treat the macrophage cultures briefly with antibiotics. Alveolar macrophages (5×10^5 cells/ml) were incubated in 16-mm diam plastic tissue culture wells (Linbro, Flow Laboratories, Inc., McLean, VA) in 500 μl RPMI that contained 20% fresh normal human serum and 100 μg/ml penicillin and streptomycin for 2 h at 37°C in 5% CO_2-95% air. During this time, the alveolar macrophages adhered to the tissue culture wells. Afterwards, the alveolar macrophage monolayers were washed vigorously 4–5 times with warm RPMI to remove nonadherent leukocytes and killed bacteria. The monolayers were then cultured in RPMI that contained 20% fresh normal human serum and incubated at 37°C in 5% CO_2-95% air. Viability of these adherent cells was >95% by trypan blue exclusion.

Preparation of supernatants of Con A-stimulated human mononuclear cells. Human peripheral blood mononuclear cells (3×10^6/ml) were cultured in plastic petri dishes in RPMI medium that contained 25% fresh normal human serum and incubated at 37°C in 5% CO_2-95% air with Con A (15 μg/ml) for 2 d. At the end of this incubation period, the cultures were transferred to conical centrifuge tubes and centrifuged at 200 *g* for 10 min at 4°C to pellet the leukocytes. The supernatant was removed, filtered through 0.2-μM millipore filters (Millipore Corp., Bedford, MA) and stored at −70°C. Control supernatants were prepared in the same way as supernatants except that Con A (15 μg/ml) was added at the end rather than at the beginning of the incubation period (supernatant control).

Assay for growth of L. pneumophila in alveolar macrophages in monolayer culture. L. pneumophila (5×10^3 colony-forming units [CFU]) were added to alveolar macrophage monolayers such that the final concentration of bacteria was $\sim 10^4$ CFU/ml. The cells were incubated at 37°C in 5% CO_2-95% air, first on a gyratory shaker at 200 rpm for 1 h and then under stationary conditions for the remainder of the experiment. Then, CFU of *L. pneumophila* per milliliter were determined daily as previously described (3).

Electron microscopy of alveolar macrophages in monolayer culture. Alveolar macrophage monolayers were prepared as described above except that 4×10^6 macrophages were cultured in 35-mm plastic petri dishes. Some monolayers were infected with *L. pneumophila* (10^4 CFU/ml) and incubated 1–3 d. Infected and uninfected monolayers were fixed and processed for electron microscopy as previously described (3).

Assay for activation of alveolar macrophages. Alveolar macrophage monolayers were incubated in 500 μl RPMI that contained 20% fresh normal human serum and 10–70% (vol/vol) Con A supernatant or supernatant control at 37°C in 5% CO_2-95% air for 24 h before infection with *L. pneumophila.* The alveolar macrophages were then infected with *L. pneumophila* and the cultures assayed for CFU daily as described in the preceeding assay.

Assay for alveolar macrophage killing of L. pneumophila or E. coli. L. pneumophila (5×10^5 CFU/ml) in 250 μl HBSS were preincubated at 22°C for 10 min with 300 μl of a serum preparation that contained anti-*L. pneumophila* antibody (200 μl heat-inactivated immune serum plus 100 μl heat-inactivated normal serum), complement (100 μl normal serum plus 200 μl heat-inactivated normal serum), both antibody and complement (200 μl heat-inactivated immune serum plus 100 μl normal serum), or neither antibody nor complement (300 μl heat-inactivated normal serum). *E. coli* (5.0×10^5 or 3.5×10^6 CFU/ml) were preincubated with similar serum preparations except that fresh normal human serum (containing high titer antibody to *E. coli*) or rabbit anti-*E. coli* antibody were used as the antibody source (14). Then 5.0×10^5 alveolar macrophages or 1.5×10^6 mononuclear cells (containing $\sim 5 \times 10^5$ monocytes), or HBSS alone were added to each tube, bringing the final volume to 900 μl. The tubes were gassed with 5% CO_2-95% air to achieve pH 7.4 (as judged by the color of the phenol red dye), capped, sealed with parafilm, and incubated for 1 or 2 h at 37°C on a gyratory shaker at 300 rpm. At the end of the incubation period, the tubes were placed in an ice water bath and the contents of each tube were sonicated under sterile conditions for 10 s continuously with a microtip that was attached to a sonicator (Heat-Systems Ultrasonics, Inc., Plainview, NY) set at the 2.75 position. This amount of sonic energy was sufficient to lyse alveolar macrophages or monocytes completely but did not decrease the viability of *L. pneumophila* or *E. coli.* CFU/ml of *L. pneumophila* or *E. coli* were determined by serially diluting the contents of each tube and plating on charcoal yeast extract or blood agar, respectively.

In some experiments, the killing capacity of alveolar macrophages was assayed after 24 or 48 h in culture. In these experiments, it was necessary to treat the macrophages in suspension culture briefly with antibiotics to eliminate contaminating bacteria, as with macrophages in monolayer culture. Freshly explanted alveolar macrophages (5×10^6/ml) were incubated in 2 ml RPMI that contained 10% fresh normal human serum and 100 μg/ml penicillin-streptomycin in polypropylene tubes (1063, 12 × 75 mm, Falcon, Oxnard, CA) for 2 h at 37°C in 5% CO_2-95% air. The cells were then washed twice by centrifugation at 225 *g* at 4°C for 10 min, resuspended in RPMI, and counted. This procedure resulted in a loss of ~30% of the original

bronchoalveolar lavage yield. The remaining cells were >85% viable by trypan blue exclusion. The alveolar macrophages were then adjusted to 5×10^6 cells/ml and incubated in polypropylene tubes in 1 ml RPMI that contained 10% fresh normal human serum at 37°C in 5% CO_2-95% air until used in the killing assay. In other experiments, the macrophages were also incubated with 35% Con A supernatant or supernatant control for 24 h before the killing assay. In some experiments, blood mononuclear cells (containing 35% monocytes) were studied in the same assay.

The killing capacity of adherent alveolar macrophages was studied by allowing the macrophages to adhere either to the side of polystyrene tubes (2057, 17 × 100 mm, Falcon) or to microcarrier beads before incubating the cells with bacteria. In experiments with microcarrier beads, 5×10^5 alveolar macrophages were incubated in polypropylene tubes in 1 ml RPMI that contained 10% fresh normal human serum and 1×10^4 microcarrier beads for 2 h at 37°C in 5% CO_2-95% air to allow the alveolar macrophages to adhere to the beads. By phase-contrast microscopy, most beads had numerous (30–40) adherent macrophages by this time. *E. coli* opsonized with the serum preparations described above were then added to the tubes and the cells were centrifuged at 900 *g* for 5 min at 22°C to promote contact between the bacteria and macrophages. The tubes were then placed on a gyratory shaker and the killing assay carried out as described above.

Fluorescence and electron microscopy assays of phagocytosis of L. pneumophila by alveolar macrophages. Alveolar macrophages (5×10^5/ml) were incubated in plastic tubes with 5×10^6 CFU *L. pneumophila* (preincubated with various serum preparations as in the killing assay) in 1.0 ml HBSS in 5% CO_2-95% air on a gyratory shaker at 300 rpm for 1 h at 37°C. The cells were then prepared for examination by fluorescence and electron microscopy. For fluorescence microscopy, the cells were cooled in an ice bath, cytocentrifuged onto glass slides, fixed with Diff-Quik fixative (Harleco, American Hospital Supply Corp., Gibbstown, NJ), and stained with fluorescein-conjugated rabbit anti-*L. pneumophila* antibody (3). For electron microscopy, the cells were fixed and processed as described (9).

The fluorescence microscopy assay was used to determine the number of macrophage-associated *L. pneumophila.* The number of fluorescent bacterial particles bound or ingested by ≥180 consecutive alveolar macrophages and the percentage of macrophages with ≥1 cell-associated bacterial particle were enumerated. From this data, the average number of bacterial particles/alveolar macrophage was calculated. The electron microscopy assay was used to determine whether cell-associated bacteria were intracellular or attached to the outside of the macrophage. The percentage of 50 consecutive macrophage-associated bacteria that were within or on the macrophage was determined.

Assay for the influence of corticosteroids on L. pneumophila multiplication in alveolar macrophages on monocytes. Alveolar macrophage monolayers, prepared as previously described, were incubated with 0.0001–100 μg/ml hydrocortisone or hydrocortisone control and 20% fresh human serum in 500 μl RPMI at 37°C in 5% CO_2-95% air for 20 min, 24, 48, or 96 h before infection with *L. pneumophila.* Colony forming units of *L. pneumophila*/ml were determined daily. In two experiments, alveolar macrophage monolayers were incubated with hydrocortisone or hydrocortisone control as above and then assessed for viability with trypan blue exclusion at 1, 24, 48, and 96 h after the onset of incubation.

In other experiments, alveolar macrophage monolayers were first incubated with 0.001–50 μg/ml hydrocortisone or hydrocortisone control with 20% serum in 350 μl RPMI at 37°C in 5% CO_2-95% air for 20 min, 24, or 72 h and then with 150 μl of Con A supernatant or supernatant control for an additional 24 h. All cultures were subsequently infected with *L. pneumophila* and CFU of *L. pneumophila* per milliliter were determined daily.

In other experiments, human peripheral blood monocytes were incubated with the same concentrations of hydrocortisone or hydrocortisone control solutions for 20 min, 48 h, or 96 h. Afterwards, these cultures were either immediately infected with *L. pneumophila* or first incubated for 24 h with Con A supernatant or supernatant control and then infected with *L. pneumophila.*

Results

Human alveolar macrophages support the multiplication of L. pneumophila. We infected freshly explanted human alveolar macrophages in monolayer culture with virulent *L. pneumophila* and assayed the cultures daily for CFU of *L. pneumophila*/ml as described in Methods. By 72 h after infection, CFU of *L. pneumophila* per milliliter in all cultures increased 2.5–5 logs (Fig. 1). *L. pneumophila* multiplied more rapidly in alveolar macrophages that were incubated for 4 d before infection than in freshly explanted macrophages. In these 4-d cultures, CFU of *L. pneumophila* per milliliter increased 3–5 logs by 48 h (e.g., see control cultures, Fig. 5 *B*), i.e., ~24 h sooner than in freshly explanted macrophages.

L. pneumophila did not multiply in tissue culture medium (RPMI that contained 20% fresh human serum) lacking alveolar macrophages or in tissue culture medium conditioned for 3 d

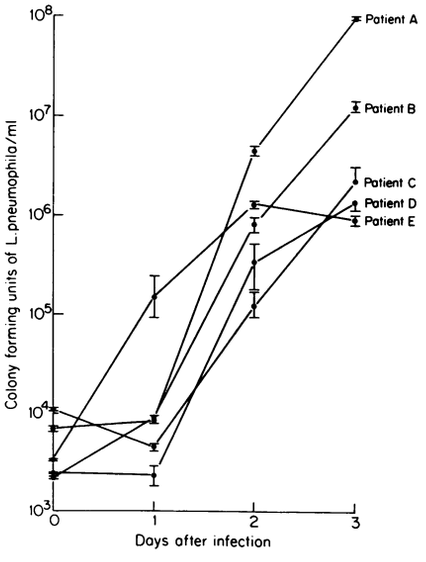

Figure 1. L. pneumophila multiplies in alveolar macrophages. In separate, consecutive experiments, freshly explanted alveolar macrophages (5×10^5/ml) from five subjects (A–E) were cultured in plastic tissue culture wells in RPMI medium that contained 20% fresh human serum and were infected with *L. pneumophila.* CFU of *L. pneumophila* per milliliter were determined daily. Each point represents the mean for three replicate wells±SEM.

by viable human alveolar macrophages. Moreover, when we incubated *L. pneumophila* with intact alveolar macrophages or an equivalent number of sonically disrupted alveolar macrophages, the bacteria multiplied only in cultures containing intact alveolar macrophages. Thus, as previously demonstrated with monocytes (3), under tissue culture conditions, *L. pneumophila* multiplies only in the presence of intact host cells, in this case alveolar macrophages.

During the course of infection, we inspected the alveolar macrophage monolayers daily by phase-contract microscopy. The majority of the alveolar macrophages remained adherent until the peak of infection. At this point, the monolayer was disrupted and most cells were either nonadherent or destroyed. Thereafter, CFU of *L. pneumophila* per milliliter in the culture declined.

Electron microscopy of alveolar macrophages infected with L. pneumophila. We examined alveolar macrophages before and 48 h after infection with *L. pneumophila.* Both uninfected and infected alveolar macrophages contained numerous membrane-bound cytoplasmic inclusions that were characteristic of these cells (Fig. 2). In infected alveolar macrophages, *L. pneumophila* were in membrane-bound cytoplasmic vacuoles (Fig. 2 *A*). These vacuoles contained single or multiple bacteria. The cytoplasmic sides of these vacuoles were studded with ribosomes (Fig. 2 *B*), which were previously described for *L. pneumophila* vacuoles in monocytes (3, 18).

Alveolar macrophages activated by Con A-stimulated mononuclear cell supernatant inhibit the multiplication of L. pneumophila. We incubated alveolar macrophages in monolayer culture for 24 h in medium that contained 10, 30, or 70% Con A-stimulated mononuclear cell supernatants (supernatant) or with 0, 10, 30, or 70% supernatant control. We then infected the monolayers with *L. pneumophila.*

Alveolar macrophages that were treated with supernatant consistently inhibited *L. pneumophila* multiplication by 1–2 logs in comparison with alveolar macrophages that were treated with supernatant control (Table I). The degree of inhibition was dependent upon the concentration of supernatant (Fig. 3). By phase-contrast microscopy, alveolar macrophages appeared activated within 24 h of incubation with supernatant. The activated macrophages exhibited more extensive spreading and aggregation compared with alveolar macrophages that were treated with supernatant control (Fig. 4). By 48 h after infection, monolayers of activated alveolar macrophages were intact, but monolayers of nonactivated macrophages were disrupted.

Alveolar macrophages fail to kill L. pneumophila. We examined the capacity of alveolar macrophages from six different subjects to kill virulent *L. pneumophila* in the killing

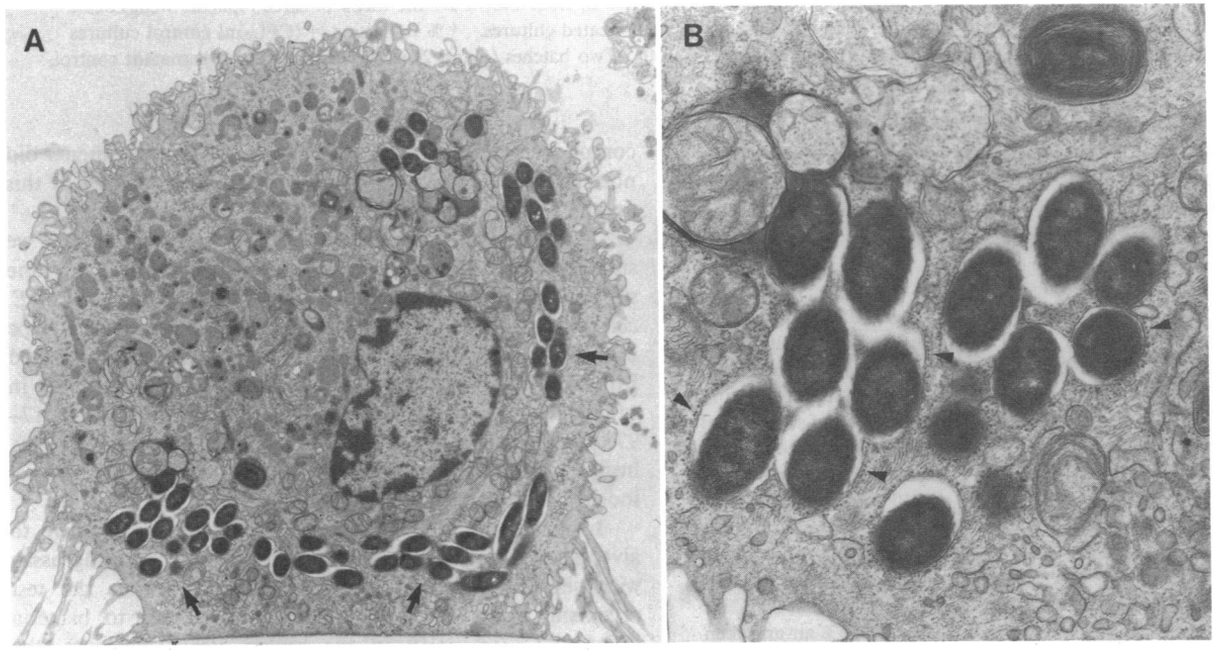

Figure 2. Electron micrographs of alveolar macrophages. Alveolar macrophages in monolayer cultures were infected with *L. pneumophila,* incubated in RPMI medium that contained 10% serum for 24 h at 37°C in 5% CO_2-95% air, and processed for electron microscopy. (*A*) The alveolar macrophage, which is loaded with cytoplasmic inclusions characteristic of these cells, is heavily infected with *L. pneumophila* (arrows). × 5,700. (*B*) This is a higher magnification micrograph of part of the cell shown in *A*. The *L. pneumophila* bacteria are located in membrane-bound vacuoles studded with ribosomes (arrow-heads) that appear separated from the vacuolar membrane by a gap of ~100 Å. × 21,500.

Table I. Activated Alveolar Macrophages Inhibit L. Pneumophila Intracellular Multiplication

Experiment	Supernatant preparation	CFU of *L. pneumophila*/ml 48 h after infection	Inhibition of *L. pneumophila* multiplication by activated alveolar macrophages	
			Log inhibition*	% Inhibition†
1	Buffer control	$1.2\pm0.5 \times 10^5$	1.57	97%
	Supernatant 1 (30%)	$3.3\pm1.68 \times 10^3$		
2	Supernatant control 2 (30%)	$1.68\ (\pm0.21) \times 10^6$	1.12	92.7%
	Supernatant 2 (30%)	$1.22\ (\pm0.12) \times 10^5$		
3	Supernatant control 2 (30%)	$1.78\ (\pm0.21) \times 10^6$	0.83	85.3%
	Supernatant 2 (30%)	$2.61\ (\pm0.81) \times 10^5$		
4A	Supernatant control 1 (70%)	$1.18\ (\pm0.37) \times 10^6$	1.57	97.3%
	Supernatant 1 (70%)	$3.17\ (\pm0.75) \times 10^4$		
B	Buffer control	$1.24\ (\pm0.13) \times 10^6$	1.78	98.3%
	Supernatant 2 (30%)	$2.06\ (\pm1.05) \times 10^4$		
C	(2.5×10^5 macrophages/well)			
	Supernatant control 1 (70%)	$4.1\ (\pm1.99) \times 10^3$	2.09	99.1%
	Supernatant 1 (70%)	$3.3\ (\pm2.33) \times 10^1$		

In separate experiments, 5×10^5 alveolar macrophages (except in experiment 4 C where 2.5×10^5 macrophages were used) from four subjects were incubated with Con A supernatant (30%, 70%), supernatant control (30%, 70%), or buffer control at 37°C for 24 h in 95% air-5% CO_2 and then infected with *L. pneumophila*, as described in Methods. CFU of *L. pneumophila* per millimeter were determined 24 and 48 h after infection. Maximal inhibition of *L. pneumophila* multiplication was observed at 48 h. Data shown are the mean of three replicate wells±SEM.
* Log inhibition = log CFU/ml control cultures − log CFU/ml supernatant-treated cultures. † % Inhibition = (CFU/ml control cultures − CFU/ml supernatant-treated cultures)/(CFU/ml control cultures) × 100. Two batches (1 and 2) of supernatant and supernatant control, prepared from different donors on different days, were used.

assay described in Methods. Freshly explanted alveolar macrophages from all subjects were not able to kill >0–10% of the original inoculum of *L. pneumophila*. Incubating the *L. pneumophila* with high-titer anti-*L. pneumophila* antibody and/or complement for 10 min at 22°C before the experiments did not significantly enhance alveolar macrophage killing of this organism (data not shown).

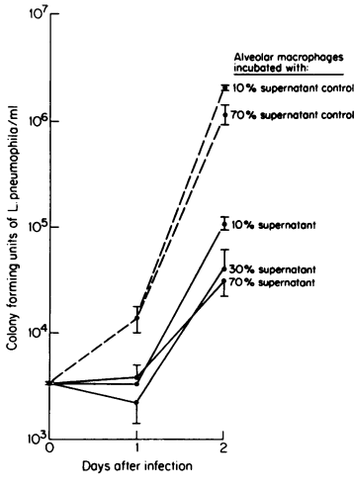

Figure 3. Inhibition of *L. pneumophila* multiplication by alveolar macrophages treated with different concentrations of supernatant or supernatant control. Alveolar macrophages (5×10^5/ml) from one subject were incubated with supernatant (10, 30, 70%) or supernatant control (10%, 70%) for 24 h at 37°C in 5% CO_2-95% air and then infected with *L. pneumophila*. The CFU of *L. pneumophila* per milliliter were determined daily. Each point represents the mean of three replicate wells±SEM.

At the same time and under the same conditions we studied the capacity of alveolar macrophages from the same subjects to kill encapsulated and unencapsulated strains of *E. coli*. At the end of the assay, CFU per milliliter of either type of *E. coli* in tubes that contained macrophages were unchanged from the beginning of the assay, whereas CFU per milliliter in tubes without macrophages had increased ~50% (range 17–97%) as a result of bacterial multiplication. In the same assay, human peripheral blood monocytes killed 99% of the original inoculum of the encapsulated *E. coli*.

In an attempt to identify conditions more conducive to alveolar macrophage killing, we carried out the killing assay with several modifications, employing *E. coli* as the test organism. First, we used different macrophage to bacteria ratios (1:1, 1:7, 1:10) in the killing assay. However, the proportion of the bacterial inoculum killed remained constant over the range of ratios tested. Second, we used alveolar macrophages that had been maintained in culture for 24–48 h before the killing assay. However, these macrophages killed fewer opsonized encapsulated *E. coli* than freshly explanted alveolar macrophages. Third, we used alveolar macrophages

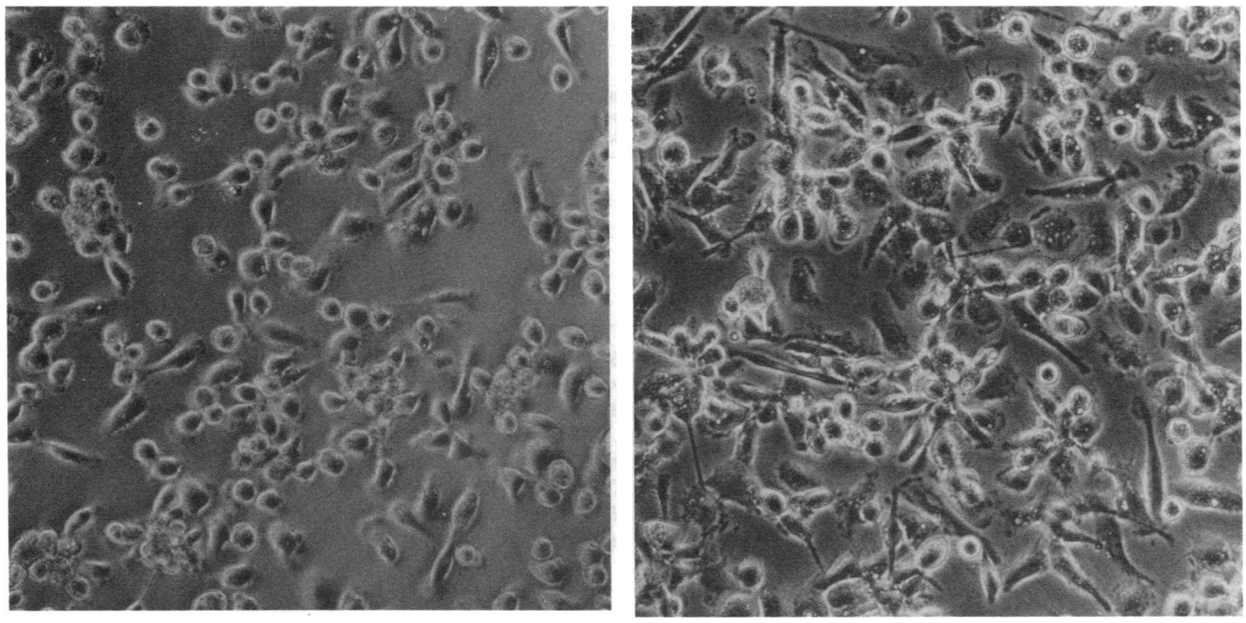

Figure 4. Influence of cytokines on morphology of alveolar macrophages. Alveolar macrophages in monolayer culture were incubated in RPMI medium that contained 10% serum and either supernatant (*left*) or supernatant control (*right*) for 24 h at 37°C in 5% CO_2-95% air. The cells were photographed at the same magnification using a phase-contrast microscope. × 200.

that had been treated with Con A supernatant for 24 h before the killing assay to activate them. However, supernatant-treated alveolar macrophages failed to kill opsonized *E. coli.* Fourth, we used adherent alveolar macrophages rather than macrophages in suspension culture. However, alveolar macrophages adherent to microcarrier beads or to the sides of plastic tubes killed approximately the same proportion of an inoculum of opsonized *E. coli* as alveolar macrophages in suspension.

Thus, none of the modifications resulted in enhanced alveolar macrophage bactericidal activity against *E. coli.* We conclude that under the conditions of this assay, alveolar macrophages, in comparison with blood monocytes, exert little bactericidal activity against opsonized *E. coli.* In addition, alveolar macrophages kill an insignificant proportion (0–10%) of an inoculum of opsonized *L. pneumophila.*

Phagocytosis of L. pneumophila by alveolar macrophages. The poor killing of *L. pneumophila* by alveolar macrophages prompted us to examine whether these macrophages are capable of phagocytizing *L. pneumophila.* We incubated alveolar macrophages with *L. pneumophila* in suspension and examined the macrophages for cell-associated bacteria by fluorescence microscopy and for intracellular bacteria by electron microscopy. By fluorescence microscopy, alveolar macrophages from two subjects, although incapable of killing *L. pneumophila* in the killing assay, were able to bind or ingest *L. pneumophila.* Most alveolar macrophages had at least one cell-associated bacterium. Alveolar macrophages bound or ingested more *L. pneumophila* in the presence of both anti-*L. pneumophila* antibody and complement than they did in the presence of complement alone (Table II). By electron microscopy, we examined alveolar macrophages incubated with *L. pneumophila* that had been preopsonized with antibody and complement. Most (75% in experiment 1 and 74% in experiment 2) of the cell-associated *L. pneumophila* were inside the macrophages in cytoplasmic, membrane-bound vacuoles (Table II). Thus, alveolar macrophages phagocytize opsonized *L. pneumophila.*

Lack of hydrocortisone effect on the interaction between L. pneumophila and alveolar macrophage or monocytes. As corticosteroids are associated with an increased incidence and severity of Legionnaires' disease (11–13), we investigated the effect of a corticosteroid (hydrocortisone) on the interactions of *L. pneumophila* with alveolar macrophages and blood monocytes. We first examined the influence of hydrocortisone on *L. pneumophila* multiplication in alveolar macrophages. We incubated alveolar macrophages in monolayer culture with a wide range of hydrocortisone concentrations (0.0001–100 μg/ml) for 20 min, 48 h, and 96 h before infection with *L. pneumophila.* Except at the highest concentrations (50 or 100 μg/ml), hydrocortisone had no significant effect on *L. pneumophila* multiplication in alveolar macrophages (Fig. 5). In macrophages incubated with 50 or 100 μg/ml hydrocortisone, *L. pneumophila* multiplication was slightly but consistently decreased, but this decrease undoubtedly reflected diminished viability of these macrophages. After 24 h incubation, >95% of macrophages treated with 0.001–10 μg/ml hydrocortisone

Table II. Capacity of Alveolar Macrophages to Phagocytize L. pneumophila

Experiment	Serum ligands	Percentage of macrophages binding or ingesting ≥1 *L. pneumophila*	Average number *L. pneumophila*/macrophage with ≥1 associated bacteria	Index of macrophage-*L. pneumophila* association*	Percentage of macrophage-associated *L. pneumophila* that are intracellular
1	Antibody and complement	64%	2.6	166†	75
	Complement	52%	1.7	88	
2	Antibody and complement	73%	2.6	190†	74
	Complement	31%	1.3	40	

5×10^6 *L. pneumophila* were preincubated with anti-*L. pneumophila* antibody and complement (fresh human immune serum) or complement alone (fresh normal human serum). The bacteria were then incubated with alveolar macrophages (5×10^5/ml) in 1.0 ml RPMI and incubated on a rotary shaker for 1 h at 37°C in 5% CO_2-95% air. Afterwards, the alveolar macrophages were prepared for fluorescence microscopy and electron microscopy as described in the Methods. By fluorescence microscopy, ≥180 consecutive alveolar macrophages were counted and macrophage-associated fluorescent bacterial particles enumerated. By electron microscopy, ≥50 consecutive macrophage-associated bacteria were counted and determined to be either intracellular or attached to the outside of the macrophage. * The index of macrophage-*L. pneumophila* association is the percentage of alveolar macrophages with bound or ingested *L. pneumophila* multiplied by the average number of *L. pneumophila*/macrophage with ≥1 associated bacteria. † The ratio of the index for antibody and complement to the index for complement alone is 1.9 in experiment 1 and 4.8 in experiment 2 (Mean±SEM = 3.3±1.0).

were viable by trypan blue exclusion, compared with only 80% of macrophages treated with 50–100 μg/ml hydrocortisone. At lower concentrations of hydrocortisone (≤25 μg/ml), macrophage viability remained high (≥92%) and the monolayers intact even after 96 h of incubation with hydrocortisone.

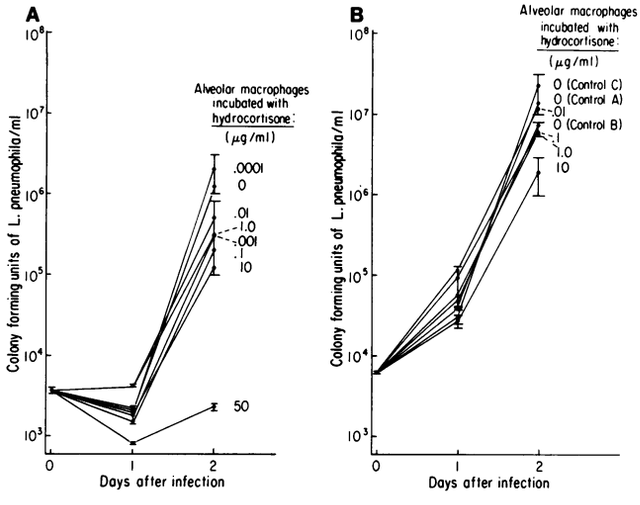

Figure 5. Hydrocortisone does not accelerate *L. pneumophila* multiplication in alveolar macrophages. Alveolar macrophages in monolayer culture were incubated with various concentrations of hydrocortisone for 48 h (*A*) or 96 h (*B*) and then infected with *L. pneumophila*. In *A*, macrophages were incubated with 0–50 μg/ml hydrocortisone. In *B*, macrophages were incubated with 0–10 μg/ml hydrocortisone or with hydrocortisone control solutions (A, B, and C) that had the same ethanol content as solutions that contained 10, 1, and 0.1 μg/ml hydrocortisone, respectively. CFU of *L. pneumophila* per milliliter were determined daily. Data points represents the mean of three replicate wells±SEM.

In one experiment, we collected the supernatants from hydrocortisone-treated macrophage monolayers at 0, 24, 72, and 96 h after the onset of incubation for chromatographic assay of hydrocortisone concentration. There was no diminution in hydrocortisone concentration during the 96 h of incubation. We next examined the effect of hydrocortisone on supernatant-mediated activation of alveolar macrophages. We incubated alveolar macrophages in monolayer culture first with hydrocortisone (0.001–50 μg/ml) for 20 min, 24 h, or 72 h, and then with Con A supernatant or supernatant control for 24 h, and infected the macrophages with *L. pneumophila*. Hydrocortisone had no significant influence on *L. pneumophila* multiplication in activated alveolar macrophages, i.e., in comparison with nonactivated macrophages, activated macrophages inhibited *L. pneumophila* multiplication to about the same extent whether or not the macrophages were pretreated with corticosteroids (Fig. 6 *B*).

In similar experiments, we investigated the influence of hydrocortisone on *L. pneumophila* multiplication in activated and nonactivated human peripheral blood monocytes. As with alveolar macrophages, hydrocortisone did not affect *L. pneumophila* multiplication in activated or nonactivated monocytes (Fig. 6 *A*). Monocyte viability was not reduced by incubation with hydrocortisone in concentrations up to 100 μg/ml.

Hydrocortisone did influence the morphology of alveolar macrophage and monocyte cultures. By phase-contrast microscopy, alveolar macrophages (or monocytes) that were treated with hydrocortisone (1–25 μg/ml) exhibited less spreading than cells treated with hydrocortisone control solution. Also, activated alveolar macrophages and monocytes treated with hydrocortisone (1–25 μg/ml) exhibited less aggregation

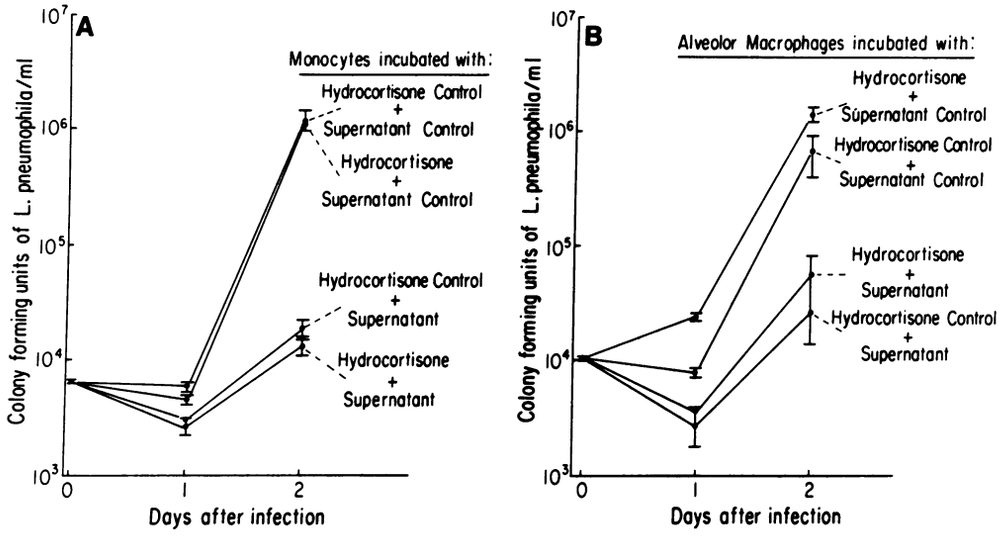

Figure 6. Hydrocortisone does not influence activation of monocytes or alveolar macrophages. (*A*) Monocytes in monolayer culture were incubated with hydrocortisone (50 μg/ml) or hydrocortisone control (containing an equivalent concentration of ethanol) and with Con A supernatant or supernatant control (prepared as described in Methods) for 24 h at 37°C in 5% CO_2-95% air and then infected with *L. pneumophila*. CFU of *L. pneumophila* per milliliter were determined daily. Data points represents the mean of three replicate wells±SEM. (*B*) Alveolar macrophages in monolayer culture were incubated first with hydrocortisone (10 μg/ml) or hydrocortisone control for 24 h and then with supernatant or supernatant control for 24 h at 37°C at 5% CO_2-95% air. The cultures were then infected with *L. pneumophila*. CFU of *L. pneumophila* per milliliter were determined daily. Data points represents the mean of three replicate wells±SEM.

than activated macrophages and monocytes treated with hydrocortisone control solution.

Discussion

These experiments show that *L. pneumophila* multiplies within human alveolar macrophages, the mononuclear phagocyte that is located at the site of infection in Legionnaires' disease. These findings may have important implications for the pathogenesis of Legionnaires' disease. Multiplication within alveolar macrophages is the likely means by which *L. pneumophila* proliferates in the human lung. Indeed, this may be the exclusive means by which the bacterium multiplies in the lung if *L. pneumophila,* which does not multiply extracellularly in vitro under tissue culture conditions (3), also does not multiply extracellularly in vivo. Although alveolar macrophages provide a satisfactory cytoplasmic milieu for *L. pneumophila* multiplication, these macrophages do possess some spontaneous inhibitory capacity against *L. pneumophila* that is lost with time in culture; *L. pneumophila* multiplies more rapidly in 4-d-old than in freshly explanted alveolar macrophages. Human monocytes have also been found to exhibit a time-dependent loss of spontaneous inhibitory capacity against *L. pneumophila* in vitro (7).

Inside alveolar macrophages, *L. pneumophila* resides in a membrane-bound cytoplasmic vacuole that is lined on the cytoplasmic side by ribosomes. The ribosomes are separated from the vacuolar membrane by a gap of ~100 Å. This morphologic feature, which is evidently unique to this bacterium, also has been observed in human monocytes that were infected with *L. pneumophila* in vitro (3, 18) and in alveolar macrophages in human lung tissue specimens from patients with Legionnaires' disease (2). In human monocytes, the ribosome-lined vacuole is formed 4–8 h after phagocytosis of *L. pneumophila* (18). The role of the ribosomes in *L. pneumophila* intracellular survival and multiplication is not known (2).

Previous studies from this laboratory have shown that patients with Legionnaires' disease develop cell-mediated immunity to *L. pneumophila* (8). Circulating mononuclear cells from these patients, in response to *L. pneumophila* antigens, generate cytokines that activate monocytes such that they inhibit *L. pneumophila* intracellular replication. In the current study, we found that human alveolar macrophages are also activated by cytokines (derived from Con A-stimulated human blood mononuclear cells), and that these activated alveolar macrophages inhibit intracellular *L. pneumophila* replication. These findings strongly support the hypotheses that cell-mediated immunity plays a major role in host defense against *L. pneumophila* and that human alveolar macrophages can serve as the effector cell in this defense.

Activation of alveolar macrophages significantly inhibited (85–99% inhibition of growth) but did not completely suppress *L. pneumophila* multiplication. However, in these experiments, alveolar macrophages were activated by a single 24-h treatment

with cytokines (Con A supernatant) before infection with *L. pneumophila*. With monocytes, inhibition of *L. pneumophila* multiplication can be increased by prolonging the duration of cytokine treatment before infection with *L. pneumophila* or by adding daily doses of cytokine during the assay (7).

To our knowledge, this is the first demonstration that a resident human macrophage, in this case an alveolar macrophage, can be activated in vitro by mononuclear cell products (cytokines or lymphokines) to a higher state of antimicrobial capacity. Previous investigations have examined the response to lymphokines of animal macrophages. Lymphokine (or cytokine)-treated animal alveolar macrophages have been shown to have altered adherence, migration, or aggregation, and increased anti-microbial activity against *Nocardia asteroides* and *Listeria monocytogenes* (19–23). Studies with human macrophages may be more relevant to human immunity than studies with animal macrophages because of interspecies differences in macrophage effector functions (24).

Humoral immunity also develops in response to *L. pneumophila* infection, but in vivo observations and in vitro studies indicate that its contribution to host resistance against this organism is minor. The presence of circulating anti-*L. pneumophila* antibody does not appear to protect patients against relapse or reinfection with Legionnaires' disease (25). In vitro studies from this laboratory have shown that anti-*L. pneumophila* antibody does not promote complement killing of *L. pneumophila*. Moreover, while antibody and complement promote efficient ingestion of *L. pneumophila* by polymorphonuclear leukocytes and monocytes, only a modest proportion (0.5 log) of the ingested bacteria are killed by these phagocytes (9, 10). Most importantly, antibody and complement fail to inhibit the intracellular multiplication of *L. pneumophila* in monocytes (10). The present study provides further in vitro evidence that humoral immunity has a limited role in host defense against *L. pneumophila*. Antibody and complement facilitate phagocytosis of *L. pneumophila* by alveolar macrophages but not appreciable killing (0–10% of the original inoculum). The resistance of *L. pneumophila* to killing is perhaps in part due to a low in vitro antibacterial capacity of alveolar macrophages relative to other phagocytes. Although alveolar macrophages in vitro ingest and kill a variety of potential pulmonary pathogens (*S. aureus, E. coli, T. gondii, S. pneumoniae, L. monocytogenes, N. asteroides,* and *K. pneumoniae*), their bactericidal capacity against a given target organism is low compared with other phagocytes (17, 19, 24, 26–29). Similarly, in our study, alveolar macrophages were unable to reduce CFU of *E. coli* below the level of the original inoculum, whereas monocytes were able to reduce CFU of *E. coli* by two logs (99%) in the same assay.

This poor in vitro performance of alveolar macrophages is perplexing in view of reports that, in animals, alveolar macrophages appear to readily kill certain bacteria (4–6, 30). One potential factor in their poor performance in killing assays might be this transfer from the in vivo environment (air-fluid interface of the alveolus) to a radically different in vitro environment (submergence in fluid) in which they may not function optimally. Another factor may be the absence of alveolar-lining material, which appears to have antibacterial activity and can augment alveolar macrophage anti-bacterial capacity (31–34).

The risk of infection and severity of illness with *L. pneumophila* appear to be increased in patients who receive prolonged therapy with corticosteroids (11–13). These observations are not surprising, as corticosteroids are generally believed to predispose patients to pneumonia as well as to infections with various intracellular pathogens (35). In animal studies, the influence of steroids on pulmonary defenses has varied according to the organism studied. Guinea pigs treated with corticosteroids exhibit unimpaired pulmonary clearance of *P. aeruginosa* (36), but diminished intrapulmonary killing of *L. monocytogenes* and increased extrapulmonary dissemination of this organism (37). Mice that are treated with corticosteroids exhibit impaired pulmonary clearance of *S. aureus* (38).

The mechanism(s) underlying the deleterious influence of steroids on pulmonary defenses have not been clearly identified. Animal studies on the influence of steroids on the number of T lymphocytes and macrophages in the lung have yielded conflicting results—sometimes the number of these cells in bronchoalveolar lavage fluid has been reduced (37, 39) and sometimes not (40–42). Studies on the influence of steroids on macrophage effector function have shown variable effects. Peritoneal macrophages from mice treated with a low dose of hydrocortisone for 3 d did not demonstrate a significant decrease in phagocytosis or killing of several different bacteria (43). However, alveolar macrophages from guinea pigs that were treated with a higher steroid dose for 7 d displayed decreased antibody-dependent cellular cytotoxicity and anti-bacterial activity and chemotaxis (37, 44, 45). In vitro treatment of macrophages with steroids had no effect on alveolar macrophage antibody-dependent cellular cytotoxicity (44); however, it did reduce the intrinsic anti-trypanocidal activity of human monocyte-derived macrophages (46). A major way in which steroids could influence host defense against intracellular pathogens would be to impair macrophage responsiveness to lymphokines. Steroid-treated macrophages have been reported to exhibit impaired lymphokine-mediated aggregation (22, 47), migration (48), and release of oxygen intermediates (49, 50). However the steroid effect on lymphokine-boosted macrophage antimicrobial activity has been inconsistent; anti-toxoplasma (49) but not anti-trypanosoma activity (46) was impaired.

In our experiments, treatment of alveolar macrophages with pharmacologic but not cytotoxic doses of hydrocortisone for up to 48 h in vitro had no influence on the rate or extent of intracellular *L. pneumophila* multiplication. Thus, hydrocortisone did not reduce the spontaneous anti-*L. pneumophila* activity of alveolar macrophages. Moreover, hydrocortisone treatment of alveolar macrophages for up to 3 d and monocytes for up to 4 d did not reduce the cytokine-induced anti-*L.*

pneumophila activity of these mononuclear phagocytes. Hydrocortisone did impair macrophage aggregation in response to cytokines, which indicated that steroids can exert independent effects on lymphokine-mediated responses.

Why different studies have yielded divergent findings on the influence of steroids on lymphokine-mediated macrophage antimicrobial activity is not clear. Methodological differences may account for the disparities. The studies involved different macrophages (human alveolar, human monocyte derived, mouse peritoneal), different target organisms (*L. pneumophila, T. cruzi, T. gondii*), and different lymphokine preparations (46, 49).

In conclusion, our experiments indicate that the increased susceptibility to *L. pnemophila* infection in patients treated with steroids is not due to suppression of alveolar macrophage spontaneous or cytokine-induced anti-*L. pneumophila* activity. The mechanism by which steroids impair host resistance against *L. pneumophila* remains unknown. Possibly, steroids suppress other immune effector cells such as lymphocytes. Animal studies may clarify this issue.

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