ACTIVATED HUMAN MONOCYTES INHIBIT THE INTRACELLULAR MULTIPLICATION OF LEGIONNAIRES' DISEASE BACTERIA*

By MARCUS A. HORWITZ‡ and SAMUEL C. SILVERSTEIN

From the Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021

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

In previous studies, we investigated the role of humoral immunity in Legionnaires' disease (2, 3). We found that virulent in vivo grown *L. pneumophila* bacteria are completely resistant to the bacteriocidal effects of human serum even in the presence of high-titer anti-*L. pneumophila* antibody (2). The bacteria also resist killing by freshly explanted human polymorphonuclear leukocytes and monocytes even in the presence of specific antibody and complement; under optimal conditions, only 0.5 log of an inoculum is killed by these phagocytes (2, 3). Most importantly, *L. pneumophila* bind more avidly to monocytes in the presence of specific antibody and complement, but the bacteria multiply intracellularly as rapidly as when they enter monocytes in the absence of antibody (3). These results indicate that humoral immunity, at least by itself, does not play a decisive role in host defense against Legionnaires' disease.

In this study, we investigate the potential role of cell-mediated immunity in Legionnaires' disease. We demonstrate that (*a*) in vitro activated human monocytes have the capacity to inhibit the multiplication of virulent *L. pneumophila*; (*b*) cytokines produced by mitogen-sensitized human mononuclear cell cultures have the capacity to activate the monocytes such that they can inhibit *L. pneumophila* multiplication; and (*c*) activated monocytes inhibit *L. pneumophila* multiplication both by decreasing phagocytosis and by decreasing the rate of intracellular multiplication.

Materials and Methods

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. Concanavalin A (Con A)[1], three times crystallized and lyophilized, was obtained from Miles-Yeda Ltd., Kankakee, Ill..

Agar. Modified charcoal yeast extract agar was prepared in 100- × 15-mm bacteriologic petri dishes as described (1).

Serum. Venous blood was obtained and clotted, and the serum was separated and stored at

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

−70°C until used as described (4). Normal (nonimmune) human serum (type AB) with an indirect fluorescent antibody (IFA) anti-*L. pneumophila* titer (5) of <1:64 was obtained from an adult donor not known to have ever had Legionnaires' disease. Immune human serum with an IFA anti-*L. pneumophila* titer of 1:4,096 was obtained from an adult donor who had recently recovered from Legionnaires' disease (2).

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 (1).

Human Blood Mononuclear Cells. Mononuclear cells used in experiments with *L. pneumophila* were obtained from the blood of a normal adult donor not known to have ever had Legionnaires' disease and with an IFA anti-*L. pneumophila* titer of <1:64. The blood mononuclear cell fraction was obtained by centrifugation over a Ficoll-sodium diatrizoate solution as previously described (1); the cells were >99% viable by trypan blue exclusion. Examination of a stained cytocentrifuged sample revealed that the mononuclear cell fraction contained ~40% monocytes, 58.5% lymphocytes, and 1.5% polymorphonuclear leukocytes. The adherent subpopulation (containing >90% monocytes) was prepared as described (1). Mononuclear cells used in the production of Con A-induced supernatants were obtained from the buffy coat of blood donated at the New York Blood Center. The buffy coat cells were diluted 1:1 in 0.9% saline and the mononuclear cells were obtained as from blood by centrifugation over a Ficoll-sodium diatrizoate solution (1).

Assay for Activation of Monocytes in Cultures Containing Con A and Lymphocytes. 6×10^6 freshly explanted mononuclear cells (~40% monocytes; ~60% lymphocytes) were incubated in 35-mm plastic petri dishes in 2 ml RPMI 1640 medium containing 25% fresh normal human serum for 1.5 h at 37°C to allow monocytes to adhere to the dishes. At this point in some experiments, control dishes were washed vigorously to remove the nonadherent lymphocyte-enriched fraction of the mononuclear cell population, leaving adherent cells that were >90% monocytes. The monocytes, in the presence or absence of lymphocytes, were then incubated for 24 h at 37°C in 5% CO_2-95% air with Con A at concentrations ranging from 0 to 32 μg/ml as indicated in Results. After 24 h, *L. pneumophila* (2×10^4 colony-forming units [CFU]) were added to the cultures. *L. pneumophila* were not agglutinated by Con A at concentrations up to 1,000 μg/ml. The cultures were incubated at 37°C in 5% CO_2-95% air on a gyratory shaker for 1 h and under stationary conditions thereafter. CFU of *L. pneumophila* in each culture were determined daily as described (1).

Preparation of Supernatants of Con A-sensitized Mononuclear Cell Cultures. 6×10^6 mononuclear cells were incubated in 35-mm plastic petri dishes at 37°C in 5% CO_2-95% air for 0, 24, 48, or 72 h in 2 ml RPMI 1640 medium containing 25% fresh normal human serum and 0-32 μg/ml Con A. At the end of the incubation, the cultures were transferred to conical tubes and the cells were sedimented by centrifugation at 200 g for 10 min at 4°C. The supernatant was removed, filtered through 0.2 μm Millipore filters (Millipore Corp., Bedford, Mass.), and stored at −70°C.

Assay for Activation of Monocytes with Supernatant of Con A-sensitized Mononuclear Cell Cultures. Mononuclear cells (6×10^6) were incubated in 35-mm petri dishes in 2 ml RPMI 1640 medium containing 10% serum for 1.5 h at 37°C in 5% CO_2-95% air to allow monocytes to adhere. The dishes were then vigorously washed to remove the nonadherent lymphocyte-enriched fraction of the mononuclear cell population. The monocyte monolayers were then incubated for 0, 12, 24, or 48 h in 2 ml RPMI 1640 medium containing 15% fresh normal human serum and 0-40% (vol/vol) of Con A-induced supernatant prepared as described above. In some experiments, control monocyte monolayers were incubated with 20% (vol/vol) supernatant of mononuclear cell cultures from which Con A was omitted and/or with 20% (vol/vol) solution of Con A at 16 μg/ml (final concentration 3.2 μg/ml Con A). Monocytes incubated for 48 h with any of these supernatant preparations or control solutions were >99% viable by trypan blue exclusion. 24 or 48 h after explantation of monocytes, *L. pneumophila* (~2 × 10^4 CFU) were added to the cultures. The cultures were incubated at 37°C in 5% CO_2-95% air on a gyratory shaker for 1 h and under stationary conditions thereafter. CFU of *L. pneumophila* in each culture were determined daily, as described (1). In some experiments, leukocytes were lysed by sonication (1) before assay for CFU. In some experiments, 200 μl (10% of the culture

volume) additional fresh Con A-induced supernatant or control medium was added to cultures daily after infection.

Assays for the Binding or Ingestion of L. pneumophila by Supernatant-activated Monocytes. Monocytes in monolayer culture were prepared as in the assay described above. The monolayers were incubated for 24 h at 37°C in 5% CO_2-95% air in 2 ml RPMI 1640 medium containing 15% fresh normal human serum and 20% Con A-induced supernatant or control medium. After 24 h, *L. pneumophila* (5×10^5 CFU) were added to each petri dish and the cultures were incubated for 2 h at 37°C in 5% CO_2-95% air on a gyratory shaker at 100 rpm. At the end of the incubation, the monolayers were vigorously washed four times to remove nonadherent bacteria. Two assays were then performed in parallel. First, to determine the number of viable *L. pneumophila* bound or ingested, the mean CFU/monocyte was calculated from the number of adherent monocytes and CFU in each petri dish. The number of adherent monocytes in each dish was determined by counting monocytes within a calibrated area using an inverted phase-contrast microscope; counts from four different areas of each dish were averaged. CFU were determined after lysing the monocytes by sonication.

Second, to determine the number of *L. pneumophila* bacterial particles bound or ingested, a fluorescent assay was used. Replicate monocyte monolayers were infected and washed as above, and then fixed, stained with fluorescein-conjugated rabbit anti-*L. pneumophila* antiserum, and examined by fluorescence microscopy as described (1). The number of fluorescent bacterial particles bound to or ingested by >1,400 consecutive supernatant-activated and control monocytes and the percentage of these monocytes with ≥1 cell-associated bacterial particle was enumerated microscopically. From these data, the average number of bacterial particles/monocyte was calculated in each experiment.

Electron Microscopy. Monocyte monolayers were prepared, infected, and washed as described in the previous assay except that the monolayers were infected with 7.5×10^7 CFU (3×10^8 bacterial particles) of agar-grown *L. pneumophila* instead of egg yolk-grown bacteria. The agar-grown bacteria were obtained by culturing egg yolk-grown *L. pneumophila* on modified charcoal yeast extract agar for 72 h. These bacteria were 25% viable as determined by counting the number of bacterial particles, using a Petroff-hausser chamber (Arthur H. Thomas Co., Philadelphia, Pa.) and measuring the number of CFU in a suspension of the bacteria. After the monocyte monolayers were washed free of nonadherent bacteria, they were processed for electron microscopy as described (1).

Determination of Doubling Times of L. pneumophila in Supernatant-activated and Control Monocytes. Replicate monocyte monolayers were prepared, infected, and washed free of nonadherent bacteria as in *Assays for the Binding or Ingesting of L. pneumophila by Supernatant-activated Monocytes* described above. In the same way that CFU per petri dish were determined immediately after infection in that assay (after lysing the monocytes by sonication), CFU per petri dish were determined 22 h after infection in this assay. Both data were used to compute the doubling time. The doubling time was determined from the formula

$$\text{doubling time} = \frac{(t - t_0)\log 2}{\log\left(\dfrac{N_t}{N_0}\right)}$$

where t_0 is the time of the first determination of CFU/dish, t is the time of the second determination (22 h later) N_t is the number of CFU/dish at time t, and N_0 is the number of CFU/dish at time t_0.

Assay for Killing of L. pneumophila by Monocytes Activated with Con A-induced Supernatant. Mononuclear cells (6×10^6) suspended in 1.5 ml RPMI 1640 medium containing 20% fresh normal human serum were incubated in plastic test tubes with or without 200 µl Con A-induced supernatant for 24 h at 37°C in 5% CO_2-95% air. After 24 h, 200 µl Con A-induced supernatant was added to cultures to which it had not been added initially. At this point, *L. pneumophila* were incubated for 10 min at 37°C in RPMI 1640 medium containing either 25% normal or 25% immune fresh human serum. Then 10^6 CFU in 100 µl of this medium were added to the mononuclear cell cultures. Normal or immune fresh human serum (200 µl) of the same type in which the *L. pneumophila* were preincubated was also added to cultures, bringing the final volume to 2 ml. The tubes were gassed with 5% CO_2-95% air so that the pH was 7.4,

as judged by the phenol red indicator dye, capped, sealed with Parafilm (American Can Company, Greenwich, Conn.), and incubated for 2 h at 37°C on a gyratory shaker at 300 rpm. At the end of the incubation, the tubes were placed in an ice-water bath to stop the reaction and sonicated for 30 s continuously as described (1, 3). CFU in the medium were determined as described (1).

Sonication of Infected Monocyte and Mononuclear Cell Cultures. This was performed essentially as described (1, 3). The amount of sonic energy used lysed the leukocytes completely but did not reduce bacterial CFU (1, 3).

Results

Monocytes Incubated with Both Con A and Lymphocytes Acquire the Capacity to Inhibit the Intracellular Multiplication of L. pneumophila. We incubated freshly explanted human peripheral blood monocytes in monolayer culture for 24 h in medium containing Con A (16 µg/ml), autologous peripheral blood lymphocytes, both Con A and lymphocytes, or neither Con A nor lymphocytes as described in Materials and Methods. We then added virulent *L. pneumophila* to each culture and determined CFU daily. *L. pneumophila* multiplied several logs in control cultures, but multiplication was inhibited in cultures containing both Con A and lymphocytes (Fig. 1). The inhibition was of intracellular multiplication in monocytes because *L. pneumophila* multiplies only intracellularly under tissue culture conditions (1).
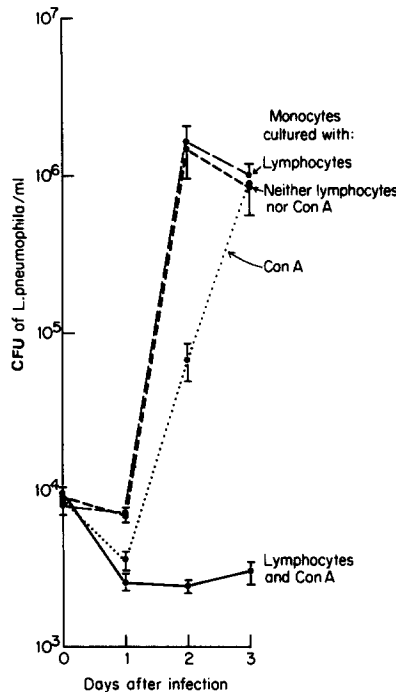

Fig. 1. Monocytes incubated with both Con A and lymphocytes inhibit *L. pneumophila* multiplication. Mononuclear cells (6 × 10⁶) in 2 ml medium containing 25% fresh human serum were incubated in 35-mm plastic petri dishes for 1.5 h at 37°C in 5% CO_2-95% air to allow monocytes to adhere. Some cultures were vigorously washed to remove nonadherent (predominantly lymphoid) cells. These and the other cultures were then further incubated for 24 h with or without Con A (16 µg/ml). *L. pneumophila* (2 × 10⁴ CFU) were then added to each culture and CFU in each culture determined daily. Each point represents the average for three replicate petri dishes ± SE.

To determine the effect of Con A concentration on mononuclear cell inhibition of *L. pneumophila* multiplication, we incubated mononuclear cells with Con A in concentrations ranging from 0 to 16 μg/ml for 24 h. We then infected the cultures with *L. pneumophila* and determined CFU daily as above. Con A exhibited a dose-response pattern in sensitizing mononuclear cells to inhibit *L. pneumophila* multiplication (Fig. 2). In the experiment described in Fig. 2, multiplication was somewhat inhibited in cultures containing 0.5 and 1 μg/ml Con A and maximally inhibited in cultures containing ≥ 2 μg/ml Con A. In several experiments of this type, Con A was consistently maximally effective at ≥ 4 μg/ml.

These studies show that monocytes in Con A-sensitized mononuclear cell cultures acquire the capacity to inhibit the multiplication of *L. pneumophila* and that this phenomenon is lymphocyte dependent. This suggested the possibility that a cytokine produced in the Con A-sensitized mononuclear cell cultures might be involved in functionally altering the monocytes such that they can inhibit *L. penumophila* multiplication. We shall refer to this functional alteration as "activation." We investigated this possibility next.

Monocytes Incubated with Cell-free Supernatant of Con A-sensitized Mononuclear Cell Cultures Acquire the Capacity to Inhibit the Intracellular Multiplication of L. pneumophila. We incubated monocyte monolayers, which had been vigorously washed to remove
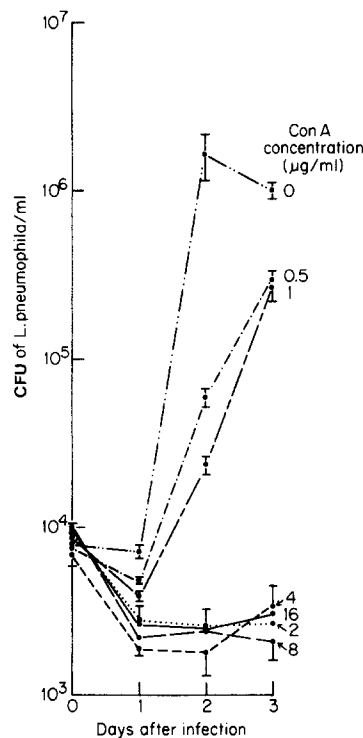

Fig. 2.   Con A exhibits a dose-response pattern in sensitizing mononuclear cells. Mononuclear cells (6 × 10⁶) in 2 ml medium containing 25% serum were incubated with Con A at concentrations ranging from 0 to 16 μg/ml for 24 h at 37°C in 5% CO_2-95% air. *L. pneumophila* (2 × 10⁴ CFU) were then added to each culture and CFU in each culture determined daily as in Fig. 1. Each point represents the average for three replicate petri dishes ± SE.

nonadherent cells (predominantly lymphocytes), in medium containing 0–40% cell-free filtered supernatant of mononuclear cell cultures sensitized for 48 h with 15 μg/ml Con A (see Materials and Methods). After 24 h, we added *L. pneumophila* to the cultures. Monocytes treated with supernatant from Con A-sensitized mononuclear cell cultures inhibited *L. pneumophila* multiplication, and the degree of inhibition was proportional to the concentration of supernatant added (Fig. 3).

We examined activated and nonactivated monocyte monolayers by phase contrast microscopy and noted striking differences in the integrity of the monolayer after infection with *L. pneumophila*. By 48 h after infection, control monocyte monolayers were destroyed but activated monocyte monolayers were intact.

Because supernatant-activated monocytes averaged twice the size of nonactivated monocytes (see below), it was possible that the reduced CFU in the medium of activated monocytes was to some extent due to sequestration of *L. pneumophila* within activated monocytes. We therefore performed control experiments in which we lysed replicate monocyte cultures by sonication to release intracellular bacteria before assaying the culture medium for CFU; sonication at the levels used has no effect on the viability of *L. pneumophila* (1). Sonication did not significantly increase CFU in
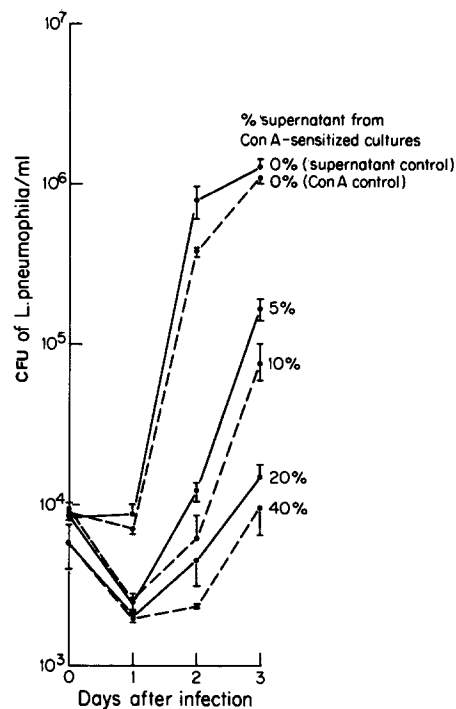

Fig. 3.   Monocytes incubated with the supernatant of Con A-sensitized mononuclear cell cultures inhibit *L. pneumophila* multiplication. Monocytes in monolayer culture were incubated at 37°C in 5% CO_2-95% air in 2 ml RPMI 1640 medium containing 15% fresh normal human serum and 0–40% cell-free supernatant from Con A-sensitized mononuclear cell cultures. Control monolayers were incubated with supernatant from a mononuclear cell culture from which Con A was omitted (supernatant control) or with 15 μg/ml Con A (Con A control). After 24 h, *L. pneumophila* (2×10^4 CFU) were added to the cultures and CFU in each culture determined daily. Each point represents the average for three replicate petri dishes ± SE.

these cultures, whether or not the monocytes were activated. Thus, *L. pneumophila* were not sequestered within the activated monocytes.

We concluded from these experiments that cytokines released into the medium of Con A-sensitized mononuclear cell cultures have the capacity to activate monocytes such that they inhibit *L. pneumophila* multiplication. We next sought to define experimental conditions for generating supernatants with enhanced potency.

Supernatant Potency Is Proportional to the Concentration of Con A Used for Sensitization of the Mononuclear Cell Cultures and Maximal after 48 h of Sensitization. We first studied the effect on supernatant potency of varying the concentration of Con A used to sensitize the mononuclear cell cultures. We prepared five different supernatants by incubating mononuclear cells for 48 h with five concentrations of Con A ranging from 0 to 15 µg/ml. We then tested these supernatants for their capacity to activate monocytes such that they could inhibit *L. pneumophila* multiplication, as described in the legend to Fig. 4.

The capacity of supernatant-activated monocytes to inhibit *L. pneumophila* multi-
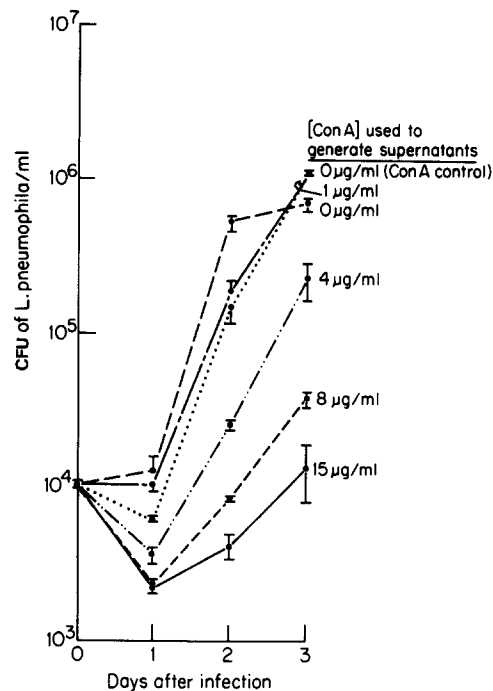

Fig. 4.   Supernatant potency is proportional to the concentration of Con A used for sensitization of the mononuclear cell cultures. Five different supernatants were prepared by incubating mononuclear cells with Con A at concentrations of 0, 1, 4, 8, and 15 µg/ml, as described in Materials and Methods. Then freshly explanted monocytes in monolayer culture were incubated in 2 ml RPMI 1640 medium containing 15% fresh normal human serum and 20% (vol/vol) of the supernatant preparation indicated. An additional set of control monocyte monolayers was similarly incubated in medium containing 20% (vol/vol) solution of Con A at 15 µg/ml (Con A control). After 24 h, *L. pneumophila* (2 × 10⁴ CFU) were added to each culture and CFU in each culture determined daily. In this experiment, 200 µl of fresh supernatant or control Con A-containing solution of the same type as used initially was added to each culture daily after infection. Each point represents the average for three replicate petri dishes ± SE.

plication was proportional to the concentration of Con A used to generate the supernatants up to 15 μg/ml Con A (Fig. 4).

In other experiments, we compared four different supernatants, prepared by incubating mononuclear cells with 15 μg/ml Con A for 0, 24, 48, or 72 h, and found that the 48-h supernatants endowed monocytes with the greatest capacity to inhibit *L. pneumophila* multiplication. Consequently, in all subsequent experiments, we used supernatants generated with 15 μg/ml Con A for 48 h.

Optimal Conditions for Activating Monocytes with Con A-induced Supernatant. We found that the degree of monocyte inhibition of *L. pneumophila* multiplication was proportional to the length of time monocytes were preincubated with Con A-induced supernatant (48 h > 24 h > 12 h) before infection (data not shown). We also found that monocytes treated repeatedly with fresh supernatant had an enhanced capacity to inhibit *L. pneumophila* multiplication (Fig. 5).

Monocytes Progressively Lose Responsiveness to Con A-induced Supernatant and Spontaneous Inhibitory Capacity with Time in Culture. Monocytes kept in culture differentiate and come to resemble tissue macrophages (6). To determine whether monocytes kept in culture retain their capacity to respond to cytokines with inhibition of *L. pneumophila* multiplication, we cultured monocytes for 0, 3, 7, or 10 d before treating them with
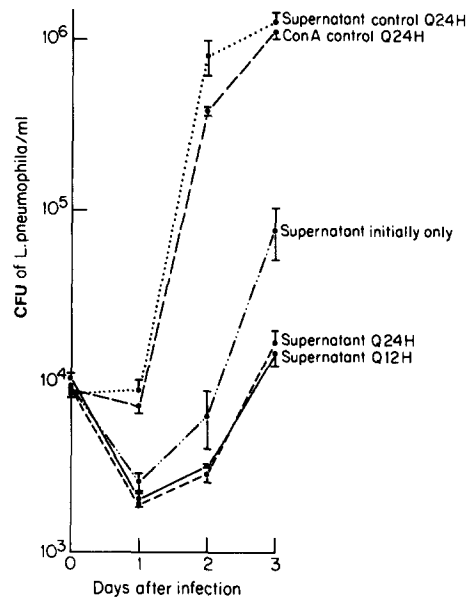

FIG. 5. Monocytes treated repeatedly with Con A-induced supernatant inhibit *L. pneumophila* multiplication more than monocytes treated only before infection. Freshly explanted monocytes in monolayer culture were incubated in RPMI 1640 medium containing 15% fresh normal human serum. Some cultures were treated with Con A-induced supernatant (200 μl or 10% by volume) immediately after explantation and with control supernatant every 24 h thereafter (supernatant initially only). Other cultures were treated with Con A-induced supernatant (200 μl) every 24 h (supernatant Q24H) or every 12 h (supernatant Q12H). Control cultures were treated every 24 h with 200 μl control supernatant (supernatant control Q24H) or 200 μl Con A (15 μg/ml) solution (Con A control Q24H). *L. pneumophila* (2 × 10^4 CFU) were added to all cultures 24 h after explantation, and CFU were determined daily. Each point represents the average for three replicate petri dishes ± SE.

Con A-induced supernatant or control medium. We infected the monocytes 24 h after their first treatment.

With time in culture, monocytes progressively lost their reponsiveness to supernatant, that is, they exhibited a diminished capacity to inhibit *L. pneumophila* multiplication (Fig. 6 A). Interestingly, control monocytes also became less inhibitory to *L. pneumophila* multiplication (Fig. 6 B). Whereas, in freshly explanted control monocytes, *L. pneumophila* did not multiply above the level of the initial inoculum until 24–48 h after infection, in control monocytes cultured ≥3 d, *L. pneumophila* multiplied 0.75–1.5 log above the level of the initial inoculum during the first 24 h after infection (Fig. 6 B).

Morphology of Activated Monocytes. Con A supernatant-treated monocytes were larger, more spread, and more stretched out than nonactivated monocytes (Fig. 7). Activated monocytes covered 1.8 times the surface area of nonactivated monocytes on petri dishes.

Supernatant-activated Monocytes Inhibit L. pneumophila Multiplication Both by Phagocytosing Fewer Bacteria and by Slowing the Rate of Intracellular Multiplication. Activated mononu-
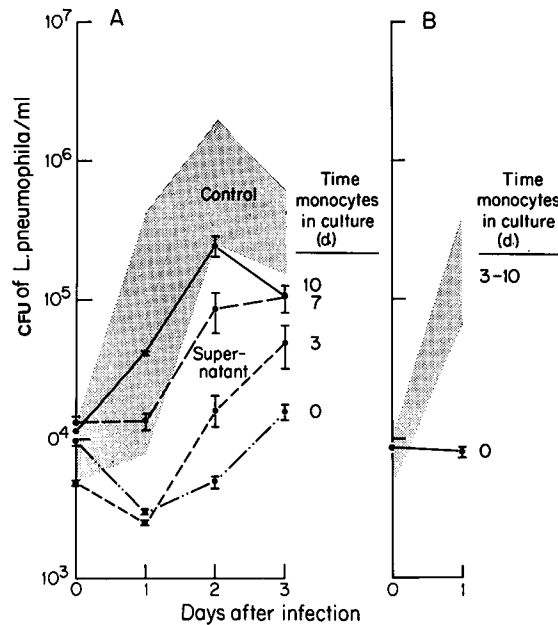

Fig. 6.   A. Monocytes progressively lose their responsiveness to supernatant with time in culture. Freshly explanted monocytes in monolayer culture were maintained in RPMI 1640 medium and 15% fresh normal human serum. Starting 0, 3, 7, or 10 d after explantation, replicate cultures were treated daily with either Con A-induced supernatant (200 µl, i.e., 10% of petri dish volume) or control medium. The monocytes were infected 24 h after their first treatment (i.e., 1, 4, 8, or 11 d after explantation), and CFU determined daily. The stippled area represents the range of *L. pneumophila* CFU in control monocytes, and the lines represent *L. pneumophila* CFU in monocytes treated with Con A-induced supernatant. Each point represents the average for three replicate petri dishes ±SE. B. Nonactivated monocytes lose spontaneous inhibitory capacity with time in culture. Data for CFU in control monocyte cultures during the first 24 h after infection from Fig. 6 A are presented. Monocytes in culture for 0 d (i.e., freshly explanted) and monocytes in culture 3, 7, and 10 d (stippled area) were treated with control medium for 24 h and infected with *L. pneumophila*. The number of CFU per culture was determined at the time of infection and 24 h later.
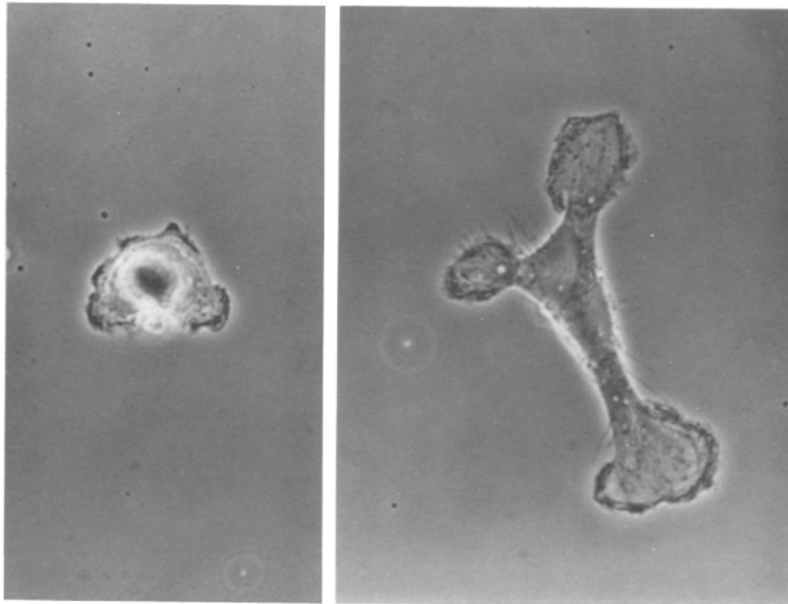
Fig. 7. The morphology of monocytes changes with activation. Freshly explanted monocytes in monolayer culture were incubated in RPMI 1640 medium containing 15% fresh normal human serum and either 10% control medium (left) or Con A-induced supernatant (right). After 48 h, both control and supernatant-activated monocytes were fixed and photographed at the same magnification, using a phase contrast microscope (× 850).

clear phagocytes have been reported to have a diminished phagocytic capacity for a number of particles including several intracellular pathogens (see Discussion). Because *L. pneumophila* must enter monocytes in order to multiply, one way supernatant-activated monocytes might inhibit *L. pneumophila* multiplication is by phagocytosing fewer bacteria. To examine this question, we compared the phagocytic capacities of supernatant-activated and control monocytes by two independent methods within a single experiment. First, we compared monocytes treated with Con A-induced supernatant or control medium for their capacity to bind or ingest viable *L. pneumophila* bacteria, as measured by CFU. We incubated the monocyte monolayers with *L. pneumophila* for 2 h, washed away nonadherent bacteria, counted the number of monocytes in each petri dish, lysed the monocytes by sonication, measured CFU per dish, and calculated CFU per monocyte. It was important to count the number of adherent monocytes because the supernatant-activated monocytes adhered better to petri dishes than the control monocytes when the monolayers were washed to remove nonadherent bacteria (Table I, line A); enhanced adherence of activated human monocytes to a degree comparable to what we observed has been reported by others (7). Control experiments showed that, immediately after washing, >85% of CFU were cell-associated by the criterion that they were released into the medium only when the monocytes were lysed by sonication.

Second, we compared replicate cultures of supernatant-activated and control monocytes for their capacity to bind or ingest *L. pneumophila* bacterial particles, as measured by fluorescence microscopy. We incubated the monocyte monolayers with *L. pneumophila* and washed them as above, fixed them, stained them with fluorescein-

TABLE I

Supernatant-activated Monocytes Inhibit L. pneumophila Multiplication Both by Phagocytosing Fewer Bacteria and Slowing Intracellular Multiplication

	Control monocytes (experiment 1)*	Activated monocytes (experiment 1)	Effect of activation			
			Experiment 1	Experiment 2	Experiment 3	Mean
A. *L. pneumophila* CFU/monocyte						
1. Monocytes/petri dish	344,000 ± 14,000	650,000 ± 37,000	↑1.9×‡	↑1.6×	↑1.6×	↑1.7×
2. CFU/petri dish	1,340 ± 118	1,560 ± 168				
3. CFU/monocyte	3.90×10^{-3}	2.40×10^{-3}	↓38%‡	↓52%	↓67%	↓52.3%
B. *L. pneumophila* particles/monocyte						
1. Monocytes counted	2,243	2,059				
2. Percent monocytes with ≥1 particle	9.6%	6.1%	↓36%§	↓54%	↓50%	↓46.7%
3. Total particles counted	259	147				
4. Particles/monocyte	115×10^{-3}	71×10^{-3}	↓38%§	↓60%	↓63%	↓53.7%
C. Doubling time of *L. pneumophila* during first 22 h after infection						
1. CFU/petri dish (t = 0)	1,340 ± 118	1,560 ± 168				
2. CFU/petri dish (t = 22 h)	192,000 ± 88,000	7,275 ± 4				
3. Doubling time	3.1 h	10.0 h	↑3.2×‡	↑3.2×	↑3.0×	↑3.1×

Freshly explanted monocytes in monolayer culture were treated for 24 h with 400 μl (20% by volume) Con A-induced supernatant or control medium in petri dishes, four of which contained coverslips. The cultures were then incubated with *L. pneumophila* for 2 h and washed to remove nonadherent bacteria. A. Immediately thereafter, supernatant-activated monocytes in three petri dishes and control monocytes in three petri dishes were counted (A1), lysed by sonication, and CFU per petri dish determined (A2). Data are the average for three dishes ±SE. From these data, the average number of CFU per monocyte was calculated (A3). B. Also immediately thereafter, supernatant-activated monocytes in two dishes (each containing three coverslips) and control monocytes in two dishes (also with three coverslips each) were fixed, stained with fluorescein-conjugated rabbit anti-*L. pneumophila* antibody, and examined by fluorescence microscopy. The percentage of >2,000 consecutive supernatant-activated and control monocytes with one or more *L. pneumophila* particle(s) (B1 and B2) and the total number of monocyte-associated particles (B3) were enumerated. From these data, the average number of particles/monocyte was calculated (B4). C. 22 h after infection, supernatant-treated monocytes in three petri dishes and control monocytes in three petri dishes were lysed by sonication and CFU per petri dish were determined (C2). Using data obtained in A (C1), the mean doubling time of *L. pneumophila* in supernatant-activated and control monocytes was calculated as described in Materials and Methods (C3).

* Detailed data are presented for one experiment (experiment 1) under columns headed "Control monocytes" and "Activated monocytes." The data for that experiment and two other experiments (experiment 2 and experiment 3) are summarized and averaged in columns under the heading "Effect of activation."

$$\ddagger \text{ Factor} = \frac{\text{activated monocyte data}}{\text{control monocyte data}}$$

$$\S \text{ Percent} = \frac{\text{control monocyte data} - \text{activated monocyte data}}{\text{control monocyte data}} \times 100$$

conjugated rabbit anti-*L. pneumophila* antibody, examined then by fluorescence microscopy, and counted the number of bacterial particles per monocyte.

Supernatant-activated monocytes bound or ingested fewer *L. pneumophila* than control monocytes as determined by assaying either monocyte-associated CFU or monocyte-associated fluorescent bacterial particles (Table I). In three experiments, supernatant-activated monocytes bound or ingested an average of 52.3% fewer CFU and 53.7% fewer fluorescent bacterial particles (Table I). Within each experiment, the two assays yielded similar results.

To determine whether supernatant-activated and control monocytes phagocytosed these monocyte-associated bacteria, we examined the monocytes by electron micros-

copy. In the same experiment as above, we infected replicate monocyte monolayers with L. pneumophila grown on agar for 72 h. We used once-passed (i.e., egg yolk passed to agar once) agar-grown organisms because we could obtain them in high number and thereby achieve a high particle:monocyte ratio during infection; the high ratio was required to obtain an adequate number of monocyte-associated bacteria in random ultrathin sections of adherent monocytes. Control experiments showed that such once-passed agar-grown bacteria multiply in human monocytes and that their multiplication is inhibited in supernatant-activated monocytes. By electron microscopy, all monocyte-associated bacteria were intracellular in membrane bound vacuoles, whether in supernatant-activated or control monocytes.

These experiments showed that supernatant-activated monocytes phagocytose fewer L. pneumophila bacteria than control monocytes. To determine if supernatant-activated monocytes also slow the rate of intracellular multiplication of L. pneumophila, we determined, in the same experiment, the doubling time of monocyte-associated (i.e., intracellular) bacteria during the first 22 h after infection from the data described in Table I C. During this 22-h period, L. pneumophila multiplication occurred predominantly or exclusively in the initially infected monocytes, which were initially lightly infected; by fluorescence microscopy, >97% of control monocytes and >99% of supernatant-activated monocytes with cell-associated bacterial particles contained two or fewer bacterial particles (not all of which were viable) and none contained more than four. Control experiments showed that at both 0 and 22 h after infection, >85% of the CFU in dishes were monocyte associated by the criterion that they were released into the medium only when the monocytes were lysed by sonication.

The doubling time of L. pneumophila was markedly prolonged in supernatant-activated monocytes in comparison to control monocytes (Table I). In three experiments, the doubling time was prolonged an average of 3.1 times in supernatant-activated cells.

Thus, supernatant-activated monocytes inhibit L. pneumophila multiplication by two mechanisms. They restrict access of bacteria to the intracellular milieu the bacteria require for multiplication, and they slow the multiplication rate of those bacteria that are internalized.

Antibody Does Not Augment the Capacity of Supernatant-activated Monocytes to Inhibit L. pneumophila multiplication. We determined the doubling time of L. pneumophila during the first 22 h after infection in supernatant-activated and control monocytes as above, except that we preincubated the bacteria in 50% immune or normal fresh human serum before infection, and maintained the monocytes in the presence of a 10% concentration of the corresponding immune or control serum throughout the experiment. In supernatant-activated monocytes, the doubling time of antibody-coated L. pneumophila (9.6 h) did not differ significantly from the doubling time of L. pneumophila not treated with specific antibody (9.9 h).

Monocytes Activated with Con A-induced Supernatant Do Not Kill L. pneumophila. To determine whether monocytes treated with Con A-induced supernatant have the capacity to kill L. pneumophila, we incubated mononuclear cells in test tubes with or without Con A-induced supernatant for 24 h. Control experiments showed that monocytes in such cultures resemble monocytes similarly incubated in petri dishes with respect to their capacity to support or inhibit L. pneumophila multiplication (data not shown). After 24 h, we added Con A-induced supernatant to control cultures and

immediately incubated the mononuclear cells with *L. pneumophila* for 2 h in shaking suspension in the presence of normal or immune serum. We then lysed the leukocytes by sonication and determined CFU in the medium.

In the presence of normal seurm, containing complement but not antibody, monocytes did not kill *L. pneumophila* regardless of whether or not the monocytes were activated (Fig. 8). In the presence of immune serum, containing both anti-*L. pneumophila* antibody and complement, both activated and nonactivated monocytes killed *L. pneumophila*, but they killed only a small proportion (0.2 log) of the original inoculum. The activated monocytes did not kill more *L. pneumophila*; in fact, they consistently killed fewer bacteria than nonactivated monocytes, perhaps because they phagocytose less avidly. Thus, Con A-induced supernatant endows the monocytes with the capacity to inhibit the multiplication of *L. pneumophila* but not to kill them.

Discussion

Cell-mediated immunity may be viewed as having an afferent arm—the induction of cytokines, including lymphokines, that activate mononuclear phagocytes, and an
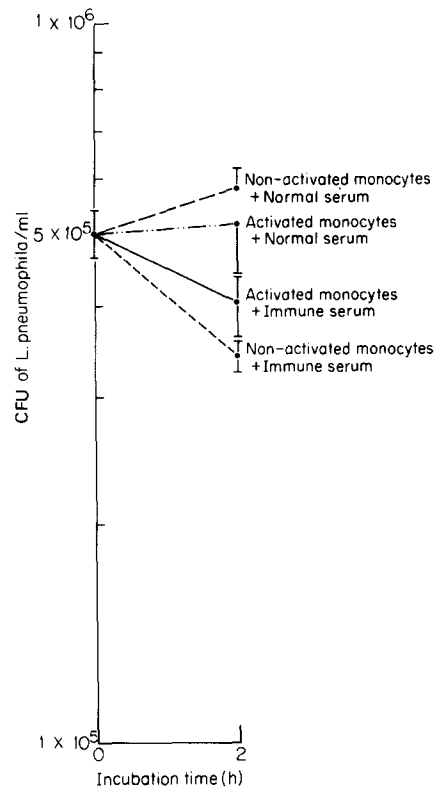

Fig. 8.   Monocytes activated with Con A-induced supernatant do not kill *L. pneumophila*. Mononuclear cells (6 × 10⁶) were incubated in RPMI 1640 medium containing fresh normal human serum with or without Con A-induced supernatant. After 24 h, Con A-induced supernatant was added to cultures not initially treated with it. Then, *L. pneumophila* (10⁶ CFU) that had been preincubated for 10 min at 37°C with either 25% normal or 25% immune fresh human serum were added to each tube along with additional serum of the same type. The cultures were incubated for 2 h at 37°C in 5% CO_2-95% air on a gyratory shaker, after which the leukocytes were lysed by sonication and CFU of *L. pneumophila* in the medium determined. Each point represents the average for two replicate tubes ±SE.

efferent arm—the expression of cytotoxic activity by the activated mononuclear phagocytes. A number of intracellular parasites have been reported to be susceptible to the efferent arm of cell-mediated immunity. Activated mononuclear phagocytes, particularly animal macrophages, have been reported to kill or inhibit the multiplication of *Leishmania enriettii* (8, 9), *Toxoplasma gondii* (10–15), *Trypanosoma cruzi* (16, 17), *Rickettsia tsutsugamushi* (18), *Coxiella burnetti* (19), and the bacterial pathogens *Listeria monocytogenes* (20–23) and *Mycobacterium tuberculosis* (24, 25). Our studies show that *L. pneumophila* is also susceptible to the efferent arm of cell-mediated immunity and that activated human monocytes are capable of inhibiting the intracellular multiplication of this bacterium.

Our studies do not indicate whether *L. pneumophila* antigens induce cell-mediated immunity, i.e., induce sensitized mononuclear cells to produce cytokines capable of activating mononuclear phagocytes to an antimicrobial capacity. However, in guinea pigs, *L. pneumophila* antigens have been reported to induce cutaneous delayed-type hypersensitivity (26), a correlate of cell-mediated immunity. Thus, *L. pneumophila* provokes a specific cell-mediated immune response in animal hosts. It seems likely that it also does so in man.

We have referred in this paper to the activating molecules in Con A-induced supernatant as cytokines because we do not know which cells in the mononuclear cell population produce them. In studies of the mononuclear cells involved in the production of cytokines, the lymphocyte has been shown to be essential. Similarly, in our study the production of Con A-induced cytokine was lymphocyte dependent (Fig. 1). Where it has been examined, these lymphocytes have been reported to be T cells (8, 12, 13, 15, 23).

With time in culture, human monocytes lost both spontaneous and cytokine-dependent capacity to inhibit *L. pneumophila* multiplication; that is, monocytes became progressively less inhibitory to *L. pneumophila* multiplication (Fig. 6 B) and they lost their capacity to respond to Con A-induced supernatant with a slowing of *L. pneumophila* multiplication (Fig. 6 A). Loss of spontaneous and cytokine-dependent inhibitory capacities have been observed in studies of anti-tumor cell activities of mouse macrophages (27, 28). The time course for loss of cytokine-dependent antimicrobial activity in our study is similar to the time course for loss of cytokine-dependent anti-tumor cell activity in one of these studies (27). Interestingly, the time course for loss of spontaneous antimicrobial activity by human monocytes in our study is similar to the time-course for loss of hydrogen peroxide releasing capacity by human monocytes in a study by Nakagawara et al. (29).

Our studies show that activated human monocytes inhibit *L. pneumophila* multiplication in two ways. First, the activated monocytes phagocytose fewer bacteria and thereby restrict access of the bacteria to the intracellular milieu they require for multiplication. Second, these monocytes slow the rate of multiplication of bacteria that are internalized. Both of these phenomena, decreased phagocytosis and decreased rate of multiplication of intracellular parasites, have been observed previously in activated mononuclear phagocytes, howbeit under separate circumstances. Although decreased phagocytosis has not been uniformly observed (30), in vivo activated mouse or guinea pig macrophages have been reported to exhibit decreased phagocytosis of starch granules, latex beads, graphite dust, and formalinized *L. monocytogenes* (30), sheep erythrocytes coated with IgG or with IgM and complement (31), *T. cruzi* (32),

Candida albicans (33), *Cryptococcus neoformans* (34), and *R. tsutsugamushi* (18); in vitro activated human monocytes have been reported to exhibit decreased phagocytosis of latex particles (35). Thus, decreasing phagocytosis and decreasing the rate of intracellular multiplication of pathogens may be general mechanisms by which activated mononuclear phagocytes defend the host against intracellular pathogens.

In this and previous papers (2, 3), we have investigated the potential roles of humoral and cell-mediated immunity in Legionnaires' disease. Humoral immunity appears to play a limited role in host defense against Legionnaires' disease. Antibody and complement promote a modest amount of killing of virulent *L. pneumophila* by human polymorphonuclear leukocytes and monocytes, but these humoral components, which promote entry of *L. pneumophila* into monocytes, do not inhibit the rate of *L. pneumophila* multiplication in monocytes (2, 3). In contrast, cell-mediated immunity appears to play a major role in host defense against Legionnaires' disease. Activated human monocytes inhibit the intracellular multiplication of *L. pneumophila*. Thus, inhibition of *L. pneumophila* multiplication in monocytes is accomplished by activating the monocytes and not by coating the bacteria with antibody and complement.

Legionnaires' disease has a predilection for immunocompromised patients (36–39). Our findings suggest that a defect in cell-mediated immunity may be responsible for this by rendering such patients less capable of limiting the multiplication of Legionnaires' disease bacteria to which they are exposed.

In the normal host, humoral and cell-mediated immunity may work in concert to eliminate *L. pneumophila* bacteria. However, humoral immunity in the absence of cell-mediated immunity theoretically could endanger the host by targeting *L. pneumophila* to monocytes where the bacteria multiply (1, 3). This could occur in both the normal and immunocompromised host. In the normal host, this might occur from immunization with a vaccine that resulted only in antibody production. In an immunocompromised host with intact humoral but impaired cell-mediated immunity, this might occur from immunization (even with a vaccine that in a normal individual would induce both antibody production and cell-mediated immunity), or from prior exposure to Legionnaires' disease.

Summary

We have examined the interaction between virulent egg yolk-grown *L. pneumophila*, Philadelphia 1 strain, and in vitro-activated human monocytes, under antibiotic-free conditions. Freshly explanted human monocytes activated by incubation with concanavalin A (Con A) and human lymphocytes inhibited the intracellular multiplication of *L. pneumophila*. Both Con A and lymphocytes were required for activation. Con A was consistently maximally effective at ≥ 4 µg/ml.

Monocytes activated by incubation with cell-free filtered supernatant from Con A-sensitized mononuclear cell cultures also inhibited the intracellular multiplication of *L. pneumophila*. The most potent supernatant was obtained from mononuclear cell cultures incubated with ≥ 15 µg/ml Con A for 48 h. The degree of monocyte inhibition of *L. pneumophila* multiplication was proportional to the length of time monocytes were preincubated with supernatant ($48 > 24 > 12$ h) and to the concentration of supernatant added ($40\% > 20\% > 10\% > 5\%$). Monocytes treated with supernatant daily were more inhibitory than monocytes treated initially only. With time in

culture, monocytes progressively lost a limited degree of spontaneous inhibitory capacity and also lost their capacity to respond to supernatant with inhibition of *L. pneumophila* multiplication.

Supernatant-activated monocytes inhibited *L. pneumophila* multiplication in two ways. They phagocytosed fewer bacteria, and they slowed the rate of intracellular multiplication of bacteria that were internalized. As was the case with nonactivated monocytes, antibody had no effect on the rate of intracellular multiplication in supernatant-activated monocytes.

Neither supernatant-activated nor nonactivated monocytes killed *L. pneumophila* in the absence of antibody. Both killed a limited proportion of these bacteria in the presence of antibody and complement.

We have previously reported that anti-*L. pneumophila* antibody and complement neither promote effective killing of *L. pneumophila* by human polymorphonuclear leukocytes and monocytes nor inhibit the rate of *L. pneumophila* multiplication in monocytes. These findings and our present report that activated monocytes do inhibit *L. pneumophila* multiplication indicate that cell-mediated immunity plays a major role in host defense against Legionnaires' disease.

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