Legionella pneumophila Inhibits Acidification of Its Phagosome in Human Monocytes

MARCUS A. HORWITZ and FREDERICK R. MAXFIELD
Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York 10021; and Department of Pharmacology, New York University School of Medicine, New York 10016

ABSTRACT We used quantitative fluorescence microscopy to measure the pH of phagosomes in human monocytes that contain virulent *Legionella pneumophila*, a bacterial pathogen that multiplies intracellularly in these phagocytes. The mean pH of phagosomes that contain live *L. pneumophila* was 6.1 in 14 experiments. In the same experiments, the mean pH of phagosomes containing dead *L. pneumophila* averaged 0.8 pH units lower than the mean pH of phagosomes containing live *L. pneumophila*, a difference that was highly significant ($P < 0.01$ in all 14 experiments). In contrast, the mean pH of phagosomes initially containing live *E. coli*, which were then killed by monocytes, was the same as for phagosomes initially containing dead *E. coli*. The mean pH of *L. pneumophila* phagosomes in activated monocytes, which inhibit *L. pneumophila* intracellular multiplication, was the same as in nonactivated monocytes.

To simultaneously measure the pH of different phagosomes within the same monocyte, we digitized and analyzed fluorescence images of monocytes that contained both live *L. pneumophila* and sheep erythrocytes. Within the same monocyte, live *L. pneumophila* phagosomes had a pH of ~6.1 and sheep erythrocyte phagosomes had a pH of ~5.0 or below.

This study demonstrates that *L. pneumophila* is capable of modifying the pH of its phagocytic vacuole. This capability may be critical to the intracellular survival and multiplication of this and other intracellular pathogens.

Legionella pneumophila, the agent of Legionnaires' disease, multiplies intracellularly in human mononuclear phagocytes within a membrane-bound cytoplasmic phagosome (1, 2). Live *L. pneumophila* inhibits fusion of the phagosome with monocyte lysosomes and induces the formation of a novel ribosome-lined phagosome. In contrast, formalin-killed *L. pneumophila* does not inhibit fusion or induce the formation of the specialized phagosome, and the dead bacteria are degraded within the phagolysosome (3, 4).

The intraphagosomal pH has been measured or estimated in several bacteria and other particles that are phagocytized by mammalian phagocytes and later reside in phagolysosomes (5–11). These phagolysosomes become acidified. Since live and formalin-killed *L. pneumophila* have different intracellular destinies, we were interested in comparing the pH of phagosomes containing these two types of bacteria and exploring the possibility that alteration of intraphagosomal pH may play a role in *L. pneumophila* intracellular survival.

In this paper, we used quantitative fluorescence microscopy to measure the pH of monocyte phagosomes that contain *L. pneumophila* and other particles prelabeled with fluorescein-conjugated antibodies. We shall demonstrate (*a*) that phagosomes that contain live *L. pneumophila* have a relatively high pH and one that is 0.8 pH units higher than phagosomes containing formalin-killed *L. pneumophila*; (*b*) that phagosomes initially containing live *E. coli*, which are rapidly killed by monocytes (12) and degraded in phagolysosomes (3), have the same pH as formalin-killed *E. coli*; (*c*) that infection of monocytes with live *L. pneumophila* does not inhibit acidification of phagolysosomes that contain IgG-coated erythrocytes in the same cell; and (*d*) that activation of monocytes does not influence the pH of phagosomes containing live or formalin-killed *L. pneumophila*.

MATERIALS AND METHODS

Media: Dulbecco's phosphate-buffered saline with Ca^{++} and Mg^{++} ions, pH 7.4; PBS without Ca^{++} and Mg^{++}; egg yolk buffer, with or without 1% BSA;

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tryptic soy broth; and RPMI 1640 medium (RPMI)[1] were obtained or prepared as previously described (1, 12). No antibiotics were added to any medium in any of the experiments.

Reagents: Formaldehyde solution, 37% (wt/wt), was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Monensin was obtained from Calbiochem-Behring Corp. (La Jolla, CA).

Antisera: Fluorescein isothiocyanate-conjugated rabbit anti-*L. pneumophila* Knoxville 1 (group 1) antiserum, globulin fraction, was obtained from the Center for Disease Control (Atlanta, GA). Rabbit anti-sheep erythrocyte IgG was obtained from Cordis Laboratories Inc. (Miami, FL). Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate-conjugated goat anti-human IgG were from Cappel Laboratories, Inc. (Cochranville, PA).

Serum: Venous blood was obtained, clotted, serum separated, and stored at −70°C until used, as described (1). Normal (nonimmune) human serum (type AB) with an indirect fluorescent antibody anti-*L. pneumophila* titer of <1:64 was obtained from a single donor (1). Complement was inactivated where indicated by heating the serum at 56°C for 30 min just before use.

Human Blood Mononuclear Cells: Mononuclear cells were obtained from the blood of a normal adult donor as described; the donor was not known to have ever had Legionnaires' disease and the donor's serum had an anti-*L. pneumophila* titer of <1:64 by the indirect fluorescent antibody assay (1).

Activation of Monocytes: In some experiments, mononuclear cells were incubated in Falcon No. 2059 polypropylene test tubes (Falcon Labware, Oxnard, CA) at 37°C for 24 h in 5% CO_2/95% air with 50% concanavalin A (Con A)-stimulated mononuclear cell supernatant to activate the monocytes as described (13). At the end of the incubation, Con A supernatant control was added to control cultures. The Con A supernatant and supernatant control were prepared as described (14).

Bacteria: *L. pneumophila*, Philadelphia 1 strain, was grown in embryonated hens' eggs, then passed one time only on modified charcoal yeast extract agar for 80 h, harvested, suspended in egg yolk buffer, and counted in a Petroff-hausser chamber (Arthur H. Thomas Co., Philadelphia, PA) as described (4). These bacteria were 75–100% viable as determined by measuring the number of colony-forming units in the bacterial suspension. *Escherichia coli*, serotype 09:K29:H$^-$, strain Bi 161-42, an encapsulated serum-resistant strain, was cultured and grown to mid-logarithmic phase in tryptic soy broth, and prepared for experiments as described (12). We obtained formalin-killed *L. pneumophila* and *E. coli* by incubating the bacteria with 2% formalin for 30 min at 4°C in shaking suspension and then washing the bacteria four times by centrifugation as described. The formalin treatment killed 100% of the bacteria as assayed by measuring colony-forming units of the treated suspension.

Preparation of Fluorescein-labeled Bacteria: Fluorescein-labeled *L. pneumophila*, live or formalin-killed, were prepared by incubating *L. pneumophila* at a concentration of 1×10^9 bacterial particles/ml with an equal volume of fluorescein-conjugated rabbit anti-*L. pneumophila* antibody for 20 min at 37°C. Fluorescein-labeled *E. coli*, live or formalin-killed, were prepared by a two-stage procedure. First, the bacteria were labeled with human IgG by incubation at a concentration of 2.5×10^8 bacterial particles/ml at 37°C for 10 min in PBS containing 25% heat-inactivated normal human serum; this serum contains natural antibody (IgG and IgM) against this strain of *E. coli* (12). After this incubation, the bacteria were washed twice by centrifugation at 15,000 *g* for 5 min. Second, the IgG-coated bacteria were labeled with fluorescein by incubation at a concentration of 2.5×10^8 bacterial particles/ml with an equal volume of fluorescein-conjugated goat anti-human IgG at 37°C for 20 min. After the *L. pneumophila* and *E. coli* were labeled, they were washed by centrifugation at 15,000 *g* for 5 min. At these concentrations of bacteria and antibody, the *L. pneumophila* and *E. coli* were saturated with the fluorescein-conjugated antibodies.

Preparation of Fluorescein-labeled Erythrocytes: Sheep erythrocytes were obtained from the Laboratory Animal Research Center (The Rockefeller University) and labeled with fluorescein by a two-stage procedure. First, we coated the erythrocytes with rabbit IgG by incubating them with rabbit anti-sheep erythrocyte IgG as described (15) and washing them by centrifugation in Ca^{++}- and Mg^{++}-free PBS (4°C) for 8 min at 750 *g*. Second, IgG-coated erythrocytes were labeled with fluorescein by incubation at a concentration of 5% (vol/vol) with an equal volume of fluorescein-conjugated goat anti-rabbit IgG at 37°C for 20 min. The erythrocytes were then washed twice by centrifugation in Ca^{++}- and Mg^{++}-free PBS (4°C) for 8 min at 750 *g*.

Phagocytosis of Fluorescein-labeled Particles by Monocytes: Mononuclear cells (2×10^7 cells/ml) were incubated in plastic test tubes with fluorescein-labeled live or formalin-killed *L. pneumophila* (3.75×10^7 cells/ml) or with fluorescein-labeled live or formalin-killed *E. coli* (1×10^7 cells/ml) in 10% fresh normal human serum at 37°C at 5% CO_2/95% air for 30 min on a gyratory shaker at 200 rpm. As a control for autofluorescence (see below), mononuclear cells were also incubated with *L. pneumophila* and *E. coli* that were not labeled with fluorescein, at the same time and under the same conditions. After the incubation, the cells were transferred to 35-mm petri dishes which we prepared especially for fluorescence microscopy by punching 13-mm-diam holes in them and attaching glass coverslips to the bottom surface, as described (16); 100 μl of the cell suspension was placed on the 13-mm-diam glass surface at the bottom of the dishes. The cells were incubated at 37°C in 5% CO_2/95% air for 30 min to allow the monocytes to adhere. Afterwards, the dishes were virgorously washed to remove the nonadherent cells. In experiments not involving erythrocytes, the monocyte monolayers were then incubated at 37°C in 5% CO_2/95% air in 2 ml RPMI containing 5 or 10% fresh human serum until just before examination by fluorescence microscopy. In experiments involving activated monocytes, the monocytes were also incubated with Con A supernatant, Con A supernatant control, or buffer control (RPMI). In experiments involving erythrocytes, the monocyte monolayers were incubated with 0.0125% (vol/vol) fluorescein-labeled or unlabeled IgG-coated erythrocytes in RPMI without serum at 37°C in 5% CO_2/95% air for 30 min, vigorously washed to remove nonadherent erythrocytes, incubated an additional 10 min at 37°C to allow phagocytosis of erythrocytes adhered to the surface of monocytes but not yet ingested, and then cultured in RPMI containing 5% serum until just before examination by fluorescence microscopy.

For examination by fluorescence microscopy, the monocyte monolayers were washed with PBS and cultured in PBS containing 5% serum. At the concentrations of *L. pneumophila*, erythrocytes, and monocytes used, the majority of monocytes with fluorescent particles contained only one or two bacteria and/or only one or two erythrocytes.

Measurement of Phagosomal pH: Since fluorescein has a pH-dependent fluorescence excitation profile, the pH of fluorescein-containing organelles can be determined from the ratio of the fluorescence intensities resulting from 450- and 490-nm excitation (17). The fluorescence intensities of a 40- μm-diam circle containing one to three monocytes that had phagocytized fluorescent bacteria or erythrocytes was measured using a Leitz MPV microscope spectrofluorometer mounted on a Diavert inverted microscope as previously described (16, 18). A similar method has been used with other cells (19, 20). Monocytes containing fluorescent bacteria or erythrocytes were identified by observation under fluorescence illumination using a silicon-intensified target video camera, and the monocytes were centered in the 40-μm-diam measuring and illumination field. We corrected measurements for intrinsic cellular fluorescence (autofluorescence) by making measurements on parallel cultures of monocytes containing bacteria or erythrocytes not labeled with fluorescein. On the average, autofluorescence contributed ~40% of the total fluorescence at 450 nm and 20% at 490 nm. Measurements were made with a 0.1-s illumination time, which produced negligible bleaching. The pH values were obtained from the ratio of the fluorescence intensities by reference to a standard curve (21). Monocytes were incubated with fluorescein-labeled live or formalin-killed *L. pneumophila*, live or formalin-killed *E. coli*, or erythrocytes, and then fixed and equilibrated to pH values between 4.5 and 7.5 in the presence of 10 μM monensin (to collapse pH gradients) to ascertain whether the ratio of fluorescence intensities for these intracellular particles matched those for fluorescein-labeled transferrin used to generate the standard curve (Fig. 1). Over this pH range, the over-all shape of each curve which relates intensity ratios to pH was the same for all particles. Between pH 5.5 and 7.5, all particles yielded the same 450/490 intensity ratios as the labeled proteins (± 0.3 pH units) (Fig. 1).

Digital Image Analysis: Video images were obtained with a modified Dage 65 silicon-intensified target video camera (Dage-MTI Inc., Wabash, MI) and recorded with a Panasonic NV 8030 video tape recorder. Images were digitized using a CAT-800 image digitizer and analyzed using a MINC 11/23 computer. Four frames of all images were averaged to improve the signal-to-noise ratio. Local background fluorescence was determined using previously described programs (18) and was taken as the 25th percentile of intensity in a 32×64 picture element (~6.5×13 μm) region.

Particles with intensities above a threshold value in the 490-nm digitized image were identified as *L. pneumophila* or erythrocytes by visual inspection. The intensities of the same pixels were measured for the 450-nm image, and the 450/490 intensity ratio for each bacterium and erythrocyte was calculated.

Statistics: Data were analyzed by the two-sample Student's *t* test, two-tailed (22) using a subroutine (TTSTT) of the Scientific Subroutines Package supplied by Digital Equipment Corp. (Marlboro, MA).

[1] *Abbreviations used in this paper*: Con A, concanavalin A; RPMI, RPMI 1640 medium.

RESULTS

pH of the *L. pneumophila* Phagosome

To determine the pH of phagosomes containing live *L. pneumophila*, we infected freshly explanted monocytes with fluorescein-labeled *L. pneumophila* in suspension cultures, plated the monocytes on glass coverslips, and determined the pH of the phagosome by quantitative fluorescence microscopy. The average pH in 14 separate experiments was 6.1 (Table I).

We were able to measure the pH of phagosomes containing live *L. pneumophila* because our method of fluorescein labeling the bacteria—coating them with fluorescein-conjugated antibodies—was not bactericidal. This method offered additional advantages. Antibody-coated *L. pneumophila* are completely resistant to the bactericidal effects of serum (23), which allows us to infect monocytes in the presence of complement. Antibody, in the presence of complement, greatly promotes phagocytosis of *L. pneumophila* (24) with minimal influence on subsequent intracellular events; a small proportion of antibody- and complement-coated *L. pneumophila* fuse with lysosomes (3) and, under ideal conditions for phagocyte killing, a small proportion of an inoculum is killed by monocytes (24). However, the other antibody- and complement-coated bacteria multiply in monocytes and at the same rate as *L. pneumophila* that enter monocytes in the absence of serum ligands (24).

To determine if the relatively high pH of the *L. pneumophila* phagosome was dependent upon the bacteria being alive, we measured the pH of phagosomes containing formalin-killed *L. pneumophila* in the same experiments in which we measured the pH of phagosomes containing live bacteria. The average pH of phagosomes containing formalin-killed *L. pneumophila* was 5.4 (Table I). Although there was considerable variation in the average pH of phagosomes in different experiments, the difference in pH between phagosomes containing live and formalin-killed *L. pneumophila* was relatively uniform (0.8 ± 0.2 pH units). This difference was highly significant ($P < 0.01$ for each of the 14 experiments).

We compared the pH of phagosomes containing live *L. pneumophila* with those containing formalin-killed *L. pneumophila* as early as 30 min and as long as 6 h after infection, and we consistently observed the pH difference. In two sets of experiments, we measured the pH of parallel cultures at different times after infection. We performed experiments 3 and 4 (Table I) on the same day and measured parallel cultures 1.5 h (experiment 3) and 6 h (experiment 4) after infection. Similarly, we performed experiments 6 and 7 on the same day and measured parallel cultures 2.5 h (experiment 6) and 3.5 h (experiment 7) after infection (Table I). In both sets of experiments, we observed no dependence of pH on time after infection, and the difference in pH between phagosomes containing live and formalin-killed *L. pneumophila* remained constant.

It was not possible to compare the pH of vacuoles containing live with those containing formalin-killed *L. pneumophila* >6 h after infection because formalin-killed *L. pneumophila* are degraded within monocytes (4). However, in two experiments, we measured the pH of phagosomes containing live *L. pneumophila* 18 h after infection by which time the bacteria had multiplied intracellularly and the monocytes contained large vacuoles with numerous bacteria. In one experiment (No. 10), the pH was 7.0 ± 0.5, and in the other (No. 14), the pH was 6.1 ± 0.7.

In most experiments, we infected and assayed freshly explanted monocytes. However, in three experiments (Nos. 11, 12, and 14) we infected 1-d-old monocytes. As with freshly explanted monocytes, we observed a highly significant pH difference between phagosomes containing live and those containing formalin-killed *L. pneumophila* in 1-d-old monocytes (Table I).

Within each experiment, pH measurements of phagosomes containing live *L. pneumophila* tended to be spread over a

TABLE I

pH of Phagocytic Vacuoles Containing Live or Formalin-killed L. pneumophila

Experiment	pH of Vacuoles containing: Live *L. pneumophila*	Formalin-killed *L. pneumophila*	Δ pH*
1	6.1 ± 0.9‡	5.2 ± 0.2	0.9
	5.9 ± 0.2‡		0.7
2	5.5 ± 0.5	4.6 ± 0.4	0.9
3§	6.2 ± 0.7	5.3 ± 0.1	0.9
4§	6.1 ± 0.5	5.2 ± 0.2	0.9
5	6.0 ± 0.5	5.5 ± 0.3	0.5
6§	6.1 ± 0.9	5.3 ± 0.2	0.8
7§	6.1 ± 1.1	5.3 ± 0.2	0.8
8	5.8 ± 0.6	5.2 ± 0.1	0.6
9	6.0 ± 0.7	5.3 ± 0.2	0.7
10	5.8 ± 0.8‖	5.0 ± 0.3	0.8
	5.7 ± 0.8‖		0.7
11¶	6.9 ± 0.7	6.3 ± 0.7	0.6
12¶	7.4 ± 0.7	6.3 ± 0.7	1.1
13	6.3 ± 0.6‖	5.3 ± 0.2	1.0
	6.0 ± 0.6‖		0.7
14¶	6.3 ± 0.6	5.3 ± 0.2	1.0
Mean	6.1 ± 0.4	5.4 ± 0.4	0.8 ± 0.2

Monocytes were incubated with fluorescein-labeled live or formalin-killed *L. pneumophila* in shaking suspension and allowed to adhere to a glass surface at the bottom of a petri dish. The pH of phagosomes containing fluorescent bacteria was then measured as described in the text. Each pH value is the mean ± SD of 10 to 20 independent pH measurements each from a different measuring and illumination field. The difference between the pH of phagosomes containing live and formalin-killed *L. pneumophila* was statistically significant ($P < 0.01$) in each of the 14 experiments by the two sample Student's *t* test, two-tailed.

*Δ pH = (mean pH of vacuoles containing live *L. pneumophila*) − (mean pH of vacuoles containing formalin-killed *L. pneumophila*).

‡The two values for live *L. pneumophila* are for two different levels of infection. The upper pH value is for monocytes infected with 5×10^7 live *L. pneumophila*/ml and the lower value is for monocytes infected with 1×10^7 live *L. pneumophila*/ ml. The pH value for vacuoles containing formalin-killed *L. pneumophila* was obtained on monocytes incubated with 5×10^7 formalin-killed *L. pneumophila*/ml. In all subsequent experiments, monocytes were infected with 3.75×10^7 live or formalin-killed *L. pneumophila*/ml, as described in the text.

§Experiment 3 was performed on the same day as experiment 4 and experiment 6 was performed on the same day as experiment 7. All other experiments were performed on separate days over a 1-y period.

‖The two values for live *L. pneumophila* in experiments 10 and 13 are for two preparations of live bacteria. The upper value is for live *L. pneumophila* freshly harvested as described in the text. The lower value is for live *L. pneumophila* harvested in the same way and then flash-frozen and maintained at −80°C until use; under these conditions the bacteria maintained their viability (1). In all other experiments, freshly harvested *L. pneumophila* were used.

¶Experiments 11, 12, and 14 were performed with 1-d-old monocytes. In all other experiments, freshly explanted monocytes were used. In experiments 11 and 12, the monocytes were cultured for 1 d in RPMI and serum only. In experiment 14, the monocytes were cultured in addition with Con A supernatant control.

wide range, whereas pH measurements of phagosomes containing formalin-killed *L. pneumophila* were clustered relatively tightly about the mean (see Fig. 4). This is reflected in Table I by a relatively large standard deviation for pH measurements with live *L. pneumophila* and a relatively small standard deviation for pH measurements with formalin-killed *L. pneumophila* in most experiments. We have no obvious explanation for the wide spread of pH measurements obtained with live *L. pneumophila*, although the very low measurements may have been obtained on a small number of dead bacteria in these cultures.

We considered the possibility that the fluorescein label on formalin-killed but not live *L. pneumophila* was altered intracellularly, for example by oxidative metabolites generated by the monocyte, and thus gave a falsely low pH value. However, when we incubated monocytes with live or formalin-killed *L. pneumophila* (or with live or formalin-killed *E. coli*, see below) and then fixed and equilibrated them in the presence of monensin to pH values between 4.5 and 7.5, all these bacterial particles yielded the same 450/490 intensity ratios over the pH range 5.5–7.5 as on the standard curve generated with fluorescein-labeled proteins (± 0.3 pH units) (Fig. 1). At pH 4.5, small differences were observed but the measurements at this pH are less reliable because of the weak fluorescence intensity. When we added methylamine (30 mM) to the medium of living monocytes to collapse intracellular pH gradients, we found that live or formalin-killed *L. pneumophila* both gave the same 450/490 intensity ratio, corresponding to a pH of 7.0.

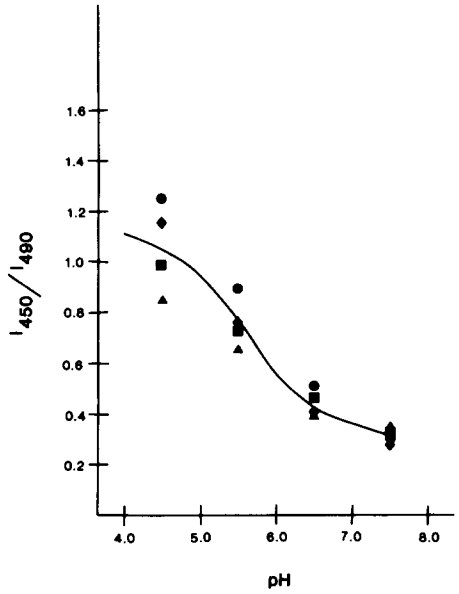

FIGURE 1 Calibration curve for determining pH values from fluorescence measurements. Human mononuclear cells were incubated with various types of fluorescein-labeled bacteria or fluorescein-labeled erythrocytes and plated on coverslip dishes as described in the text. The cells were then fixed in 2% paraformaldehyde in RPMI for 5 min at 23°C and incubated in buffers of different pH values containing monensin (30 μM) to dissipate any residual pH gradients. Fluorescence intensities of groups of one to three monocytes were measured by microspectrofluorometry with 450 and 490 nm excitation. The intensity values were corrected for autofluorescence, and the ratio of intensities, I_{450}/I_{490}, was calculated at each pH. The points show average values from 10 to 20 measurements following phagocytosis of live *L. pneumophila* (◆), formalin-killed *L. pneumophila* (●), live *E. coli* (■), and formalin-killed *E. coli* (▲). The line shows results of a series of 10 measurements made on solutions of a fluorescein-labeled protein (transferrin) as described (21). At pH 4.5, the measurements become relatively inaccurate because of the low fluorescence intensity from fluorescein at acidic pH values. At pH 7.5, the measurements are highly reproducible, but the intensity ratio becomes nearly independent of pH. Between pH 5.5 and 7.5 all of the measurements on phagocytized bacteria agreed with the fluorescein-transferrin curve within ± 0.3 pH units or less.

pH of *E. coli* Phagosomes in Monocytes That Have Ingested Live and Formalin-killed *E. coli*

Unlike *L. pneumophila*, live *E. coli* are rapidly killed by monocytes and reside in phagolysosomes after phagocytosis (3, 12). To determine the pH of the *E. coli* phagosome and the influence on phagosomal pH of killing the *E. coli* with formalin before phagocytosis, we measured the pH of *E. coli* phagosomes in monocytes incubated with live or formalin-killed *E. coli*. By the time we measured the pH of these phagosomes, the live *E. coli* would have been killed by the monocytes (12). The average pH of phagosomes initially containing live *E. coli* was 5.7 ± 0.2 (Table II). This value was not significantly different from the average pH of phagosomes containing formalin-killed *E. coli* (5.6 ± 0.1). In the same experiments, phagosomes containing live and formalin-killed *L. pneumophila* exhibited significant pH differences. Therefore, in contrast to *L. pneumophila*, formalin killing of *E. coli* before phagocytosis did not influence intraphagosomal pH.

Simultaneous pH Measurements of Phagosomes Containing *L. pneumophila* or Erythrocytes in the Same Monocyte

To determine if live *L. pneumophila* induced a general inhibition of phagosome acidification, we simultaneously measured the pH of phagosomes containing live *L. pneumophila* or erythrocytes in the same monocytes. We chose erythrocytes (5 μm diam) for this comparison for two reasons. First, by fluorescence microscopy we could readily distinguish erythrocytes by size and shape from the rod-shaped *L. pneumophila* (~0.5 × 1 μm). Second, we had determined that in

TABLE II
pH of Phagocytic Vacuoles Containing E. coli

Experiment	pH of *E. coli* phagosomes in monocytes that have ingested: Live *E. coli*	Formalin-killed *E. coli*	Δ pH*
3	6.0 ± 0.6	5.7 ± 0.3	0.3
4	5.6 ± 0.3	5.5 ± 0.3	0.1
6	5.7 ± 0.2	5.6 ± 0.2	0.1
8	5.4 ± 0.2	5.7 ± 0.2	−0.3
Mean	5.7 ± 0.2	5.6 ± 0.1	0.05 ± 0.2

Monocytes were incubated with fluorescein-labeled live or formalin-killed *E. coli* and the pH of the phagocytic vacuoles was measured as in Table I. Each pH value is the mean ± SD of 10 to 20 independent measurements. The experiments were carried out in parallel with experiments on *L. pneumophila*, and the experimental numbers correspond to those in Table I. No significant differences were observed between the pH of vacuoles in cells incubated with live or formalin-killed *E. coli* by the two-sample Student's *t* test, two-tailed.

*Δ pH = (mean pH of vacuoles containing live *E. coli*) − (mean pH of vacuoles containing formalin-killed *E. coli*).

monocytes incubated with either erythrocytes or *L. pneumophila* in the same experiment, the average pH of erythrocyte phagosomes (~4.3) was significantly different from that of *L. pneumophila* phagosomes (6.0). To determine if phagosomes within the same monocyte had different pH values, we obtained video images of monocytes containing both fluorescent *L. pneumophila* and erythrocytes (Figs. 2 and 3). Examination of many monocytes containing both particles indicated qualitatively that the vacuoles that contained erythrocytes were more acidic than those that contained *L. pneumophila*. The intensity of the *L. pneumophila* was clearly brighter with 490-nm excitation than with 450-nm excitation; the intensity of the erythrocytes in the same cells was the same at both excitation wavelengths or brighter at 450 nm (Fig. 2, *a* and *b*). This indicates that the vacuoles containing *L. pneumophila* had pH values of ~5.5 or above while the vacuoles containing erythrocytes had pH values of ~5.0 or below.

To make a quantitative measurement of the pH of two vacuoles in the same cell, we digitized some of the video images and determined the 450/490 fluorescence intensity ratio separately for the erythrocyte and *L. pneumophila* vacuoles (Figs. 2, *c* and *d* and 3). The monocyte shown in Fig. 2 contains both an erythrocyte and an *L. pneumophila* bacterium. The pH of the vacuole containing the erythrocyte was 5.0, whereas the pH of the vacuole containing the *L. pneumophila* was 6.1. Similar quantitative results were obtained in two other monocytes (not shown).

We considered the possibility that hemoglobin or some other erythrocyte factor disproportionately quenched or otherwise altered the fluorescence intensities resulting from 450- and 490-nm excitation of the fluorescein label on the erythrocytes and thereby yielded a falsely low pH value. However, when we incubated monocytes with fluorescein-labeled erythrocytes and then fixed and equilibrated them to pH values between 4.5 and 7.5, we obtained the same 450/490 intensity ratios over the pH range 5.5–7.5 as on the standard curve generated with fluorescein-labeled proteins (± 0.3 pH units). Thus, phagosomes of different pH existed within the same monocyte, and the presence of *L. pneumophila* in one phagosome did not apparently influence the pH of other phagosomes in the same monocyte.

Influence of Monocyte Activation on Intraphagosomal pH

Activated monocytes inhibit the intracellular multiplication of *L. pneumophila* (13, 14). To determine if monocyte activation influenced the pH of the *L. pneumophila* phagosome, we activated the monocytes with Con A supernatant before incubating them with *L. pneumophila*. The pH of phagosomes containing live or formalin-killed *L. pneumophila* in activated monocytes was not significantly different from the pH of these phagosomes in parallel cultures of nonactivated monocytes (Table III and Fig. 4).

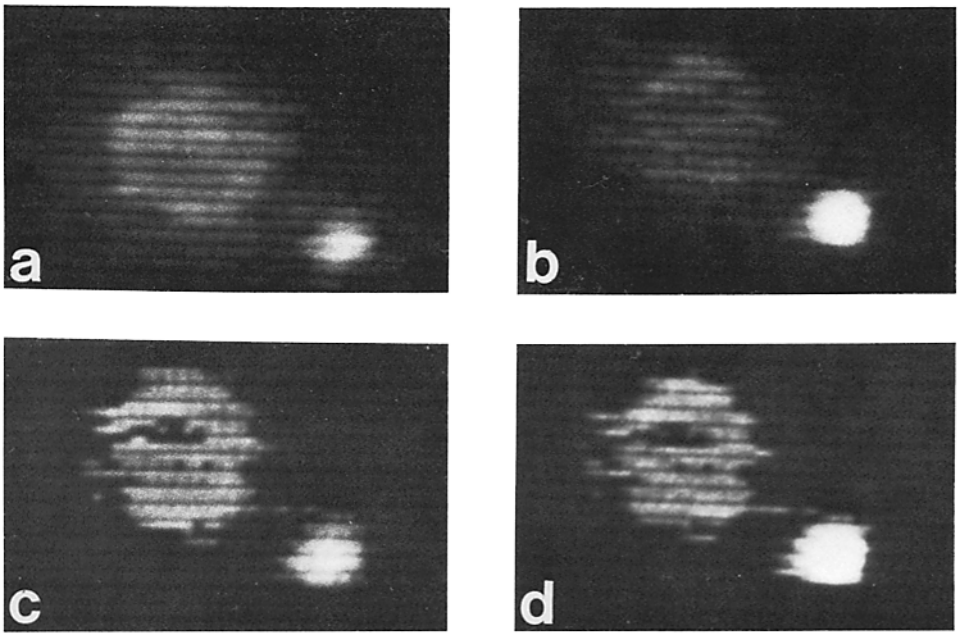

FIGURE 2 Digital image analysis of pH of phagosomes containing *L. pneumophila* or erythrocytes in the same monocyte. Human monocytes were incubated with fluorescein-labeled *L. pneumophila* and fluorescein-labeled sheep erythrocytes as described in the text. Video images of cells under epifluorescent illumination were recorded on videotape using a modified Dage 65 SIT camera as described (18). (*a*) A single monocyte that has phagocytized both *L. pneumophila* and an erythrocyte observed with 450 nm excitation. (*b*) The same cell observed with 490 nm excitation. (*c* and *d*) The images shown in *a* and *b* were digitized and analyzed as described in the text. A locally determined background intensity was subtracted from each picture element intensity. After the background subtraction, a threshold value was applied to the 490 nm image such that the perimeter of the area comprising picture elements with intensity values above this threshold outlined the focused images of the erythrocyte and the *L. pneumophila*. The picture elements with intensity values above this threshold are shown in *d*, and the corresponding picture elements in the 450 nm image are shown in *c*. The intensities for all picture elements corresponding to each particle were summed, and values of $I_{450}/_{490}$ were determined for the erythrocyte and the *L. pneumophila*. Compared with a standard curve, the vacuole that contained the erythrocyte was determined to have a pH of 5.0, whereas the vacuole that contained the *L. pneumophila* had a pH of 6.1. A free erythrocyte in the same video image (not shown in the figure) was processed along with the image shown and had a pH value of 7.4.

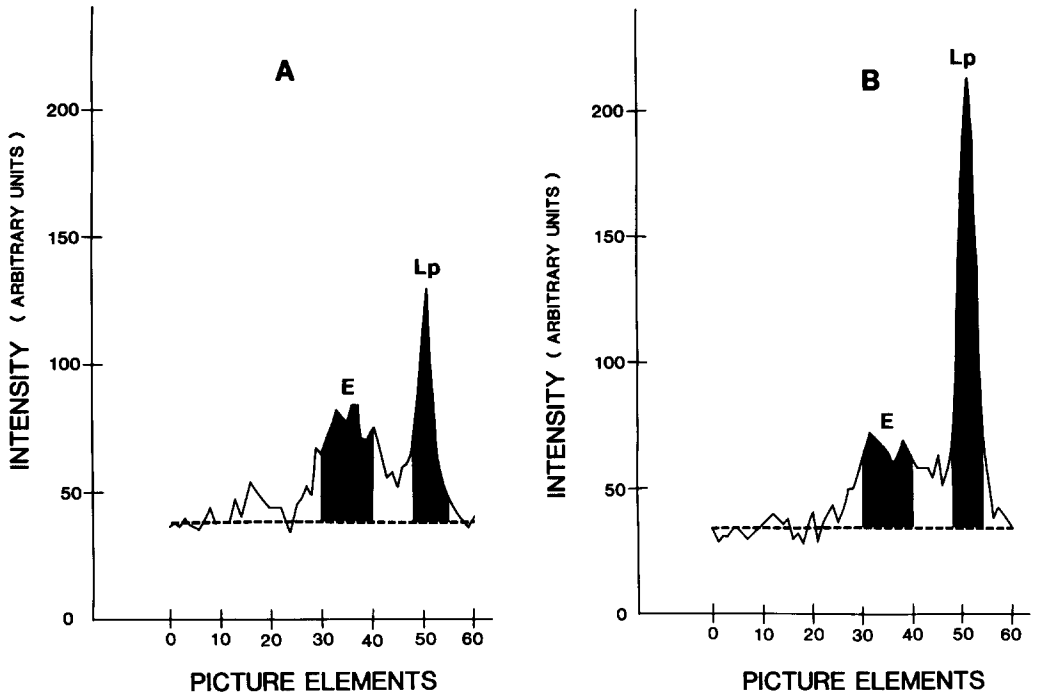

FIGURE 3 Intensity profiles of video images. These curves show the intensities of picture elements along a lane drawn through the center of the erythrocyte and the *L. pneumophila* bacterium pictured in Fig. 2. *A* shows intensities at 450 nm excitation and *B* at 490 nm excitation. The horizontal dashed lines indicate the background values which were subtracted from the intensity measurements before calculating the I_{450}/I_{490} intensity ratio. The shaded areas indicate picture elements along the line with intensity values above the threshold level described in Fig. 2; these are the picture elements along the line that contribute to the images of the erythrocyte (*E*) and the *L. pneumophila* bacterium (*Lp*) and only such picture elements were used in calculating the I_{450}/I_{490} intensity ratio. For picture elements along this line contributing to the erythrocyte image, the intensity at 450 nm excitation was greater than at 490 nm. In contrast, for picture elements along this line contributing to the *L. pneumophila* image, the intensity at 490 nm excitation was greater than at 450 nm.

TABLE III

pH of Phagocytic Vacuoles in Activated and Nonactivated Monocytes

Experiment	State of monocyte activation	pH of vacuoles containing live *L. pneumophila*	pH of vacuoles containing formalin-killed *L. pneumophila*	Δ pH*
11	Activated	6.6 ± 0.9	6.2 ± 0.6	0.4
	Nonactivated	6.9 ± 0.7	6.3 ± 0.7	0.6
14	Activated	6.3 ± 0.7	5.4 ± 0.1	0.9
	Nonactivated	6.3 ± 0.6	5.3 ± 0.2	1.0

Monocytes were cultured for 24 h in the presence of Con A supernatant to activate them (activated monocytes) or in the presence of either RPMI (experiment 11) or Con A supernatant control (experiment 14) (nonactivated monocytes). The monocytes were incubated with fluorescein-labeled live or formalin-killed *L. pneumophila* and the pH of phagosomes was determined as in Tables I and II. Each pH value is the mean ± SD of 10 to 20 independent pH measurements. Data for nonactivated monocytes is transcribed from Table I. The pH values for activated monocytes were not significantly different from those for nonactivated monocytes whether the phagosomes contained live or formalin-killed *L. pneumophila*.

* Δ pH = (mean pH of vacuoles containing live *L. pneumophila*) − (mean pH of vacuoles containing formalin-killed *L. pneumophila*).

DISCUSSION

This study shows that the pH of phagocytic vacuoles containing live *L. pneumophila* is substantially higher (0.8 ± 0.2 pH units) than the pH of vacuoles containing dead *L. pneumophila*. Our study is the first to examine quantitatively the pH of phagocytic vacuoles containing a live intracellular pathogen and to compare this pH with that of vacuoles containing a dead intracellular pathogen. In one previous study, the pH of vacuoles containing a live extracellular pathogen, a yeast, was examined and was reported to be the same for live and dead organisms (10). Similarly, in our study, the pH of vacuoles containing another extracellular pathogen, an encapsulated strain of *E. coli*, was the same for live and dead organisms. It is unlikely that the method of killing *L. pneumophila*, formalin treatment, was responsible for the pH difference between vacuoles containing live and dead *L. pneumophila* since the same method was used to kill *E. coli*, for which no pH difference was found between vacuoles containing live and dead organisms.

With *L. pneumophila*, in contrast to yeast and *E. coli*, live organisms enter a different intracellular compartment than do dead ones. Live *L. pneumophila* enter a phagosome, whereas dead *L. pneumophila* and both live and dead yeast and *E. coli* enter a phagolysosome (3, 10). The pH difference between vacuoles containing live and dead *L. pneumophila*, and the absence of such a difference between vacuoles initially containing live and dead yeast or *E. coli*, may reflect the different intracellular fates of these organisms. Whether pH is a primary determinant of intracellular fate or a consequence of it is unknown. In either case, the capacity of live *L. pneumophila* to modify directly or indirectly the pH of its phagocytic vacuole may be critical to its intracellular survival and multiplication. Consistent with this hypothesis, the capacity of *L. pneumophila* to multiply on artificial medium is strictly pH dependent, with a pH optimum of 6.9 (25).

The technique we used in this study allowed us to measure

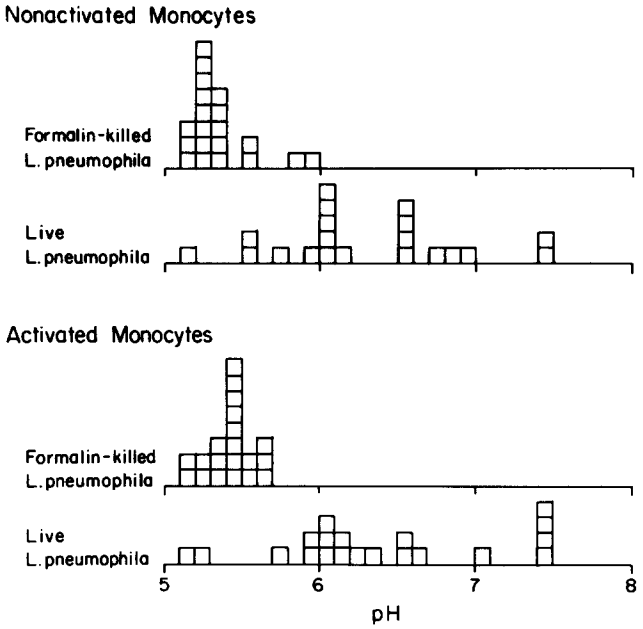

FIGURE 4 pH of *L. pneumophila* phagosomes in activated and nonactivated monocytes. Monocytes were pretreated with Con A supernatant to activate them or with Con A supernatant control. The activated and nonactivated monocytes were incubated with fluorescein-labeled live or formalin-killed *L. pneumophila*, and the pH of phagosomes was determined as in Tables I-III. Experiment 14 (Table III) is shown. Each small box in the figure represents one independent pH measurement. For both live and formalin-killed bacteria, activation of the monocytes did not significantly influence the mean pH values. This experiment was typical of all 14 experiments in that pH measurements on live bacteria were spread over a wide range, whereas pH measurements on formalin-killed *L. pneumophila* were relatively tightly clustered about the mean.

the pH of individual vacuoles in individual cells and different vacuoles in the same cell. In this way, we determined that different phagocytic vacuoles within the same monocyte were at different pH. Vacuoles containing erythrocytes, readily distinguishable by their size and shape from those containing *L. pneumophila*, had a pH of ~5.0 or less in the same monocytes in which live *L. pneumophila* had a pH of ~6.1. The digital image analysis method was used by Yamashiro et al. (16) and Tycko et al. (18) to measure the pH of individual endocytic compartments within the same cell, and by Tanasugarn et al. (26) to measure cytoplasmic and pinosome pH within the same cell.

Activated monocytes inhibit *L. pneumophila* multiplication in two ways (13). First, they phagocytize ~50% fewer bacteria then nonactivated monocytes, thereby restricting access of the bacteria to the intracellular milieu they require for multiplication. Second, they markedly slow the rate of multiplication of those bacteria that are internalized, prolonging the doubling time by threefold or more. The mechanism underlying the inhibition of intracellular multiplication is unknown, which prompted us to study the influence of activation on phagosomal pH. Our study indicates that an alteration in pH is not involved since activation does not influence the pH of phagosomes containing either live or dead *L. pneumophila*. This finding may reflect the fact that activation of monocytes does not markedly influence the capacity of *L. pneumophila* to form a specialized phagosome or inhibit phagosome–lysosome fusion (3).

The mechanism by which *L. pneumophila* inhibits acidification of its phagosome is not known, but three categories of mechanisms may be considered. First, the primary cause may be inhibition of phagosome–lysosome fusion. If so, then the higher pH might be due to differences in pH regulation between phagosomes and phagolysosomes. In some cases, it has been reported that endocytic compartments undergo further acidification as lysosomal fusion occurs (27–29). However, in other cell types, it has been found that the pH of prelysosomal endocytic compartments is about the same as the pH of lysosomes (18, 21, 27). We do not know if phagosomes containing formalin-killed *L. pneumophila* become more acidic after they fuse with lysosomes (see below). One nonlysosomal endocytic compartment, the transferrin-containing *para*-Golgi compartment in Chinese hamster ovary cells, maintains a pH (6.4) that is relatively high compared with that of lysosomes (30). Possibly, the *L. pneumophila* phagosome in human monocytes is in the same class of endocytic compartments as the transferrin-containing compartment in Chinese hamster ovary cells. Second, *L. pneumophila* may release a lipophilic base that facilitates diffusion of protons across the *L. pneumophila* membrane. This would require continuous production of the base by the bacterium at a rate sufficient to match the proton pumping capacity of the phagosome. Third, *L. pneumophila* may modify the phagosome membrane such that the internal pH rises. This could be accomplished by making the membrane leaky to protons (e.g., by insertion of a proton ionophore) or by inhibition of the ATP-dependent acidification mechanism of phagosome membranes (31).

We attempted to determine if acidification of vacuoles containing formalin-killed *L. pneumophila* was inhibited by blocking fusion of these vacuoles with lysosomes. Basing our experiments on Keilian and Cohn's (32) observations that fusion of yeast particles in mouse peritoneal macrophages did not occur within 10 min of phagocytosis at 37°C or within 1 h thereafter at 15°C, we allowed monocytes to phagocytize live or formalin-killed *L. pneumophila* for 5 min at 37°C and to adhere to petri dishes for 5 min at 37°C, and then cooled them to 15°C. Under these conditions, however, we found that acidification of vacuoles containing both live and formalin-killed *L. pneumophila* was blocked (pH of vacuoles containing live or formalin-killed *L. pneumophila* were both ~7.0 for at least 1 h). Evidently, reducing the temperature to 15°C blocks acidification of vacuoles containing live or formalin-killed *L. pneumophila*. Consequently, we were unable to determine if fusion with lysosomes was responsible for the lower pH of phagosomes containing formalin-killed *L. pneumophila* in our study.

Three major differences between phagocytic vacuoles containing live and formalin-killed *L. pneumophila* have now been identified. First, live *L. pneumophila* induces the formation of a specialized phagosome, a process involving interaction between the phagocytic vacuole and monocyte smooth vesicles, mitochondria, and ribosomes (4). In contrast, the phagocytic vacuole containing formalin-killed *L. pneumophila* does not interact with these cellular organelles. Second, the phagocytic vacuole containing live *L. pneumophila* does not fuse with lysosomes, whereas the vacuole containing formalin-killed *L. pneumophila* does fuse (3). Third, as shown in this paper, phagocytic vacuoles containing live *L. pneumophila* maintain a significantly higher pH than phagocytic vacuoles containing formalin-killed *L. pneumophila*. Regard-

ing the first two differences, phagosomes containing live *Toxoplasma gondii* and various *Chlamydia* species interact with at least some of the cytoplasmic organelles that the *L. pneumophila* phagosome interacts with and, like *L. pneumophila*, these intracellular parasites inhibit phagosome–lysosome fusion (3). It is possible that these parasites also share with *L. pneumophila* the capacity to inhibit acidification of their phagosomes and that a common mechanism underlies all these features of phagosomes containing these intracellular parasites. In addition to revealing mechanisms of intracellular survival of parasites, studies of phagosomal pH in infected cells may shed light on mechanisms underlying phagosome–lysosome fusion and other intracellular phenomena.

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