Legionella pneumophila Inhibits Acidification of Its Phagosome in Human Monocytes

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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;
tryptic soy broth; and RPMI 1640 medium (RPMI) 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 strain (group 1) antiserum, globulin fraction, was obtained from the Center for Disease Control (Atlanta, GA). Rabbit anti-sheep erythrocyte IgG was obtained from Cappel 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% CO2/95% 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 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 O9:K29:H1, strain B16-42, 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 x 10^8 bacterial particles/ml with an equal volume of fluorescein-conjugated rabbit anti-L. pneumophila antibody for 20 min at 4°C. The fluorescein-conjugated L. pneumophila labeled E. coli, but not formalin-killed, E. coli was produced by a two-stage procedure. First, the bacteria were labeled with human IgG by incubation at a concentration of 2.5 x 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 or 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).

Phagocytosis of Fluorescein-labeled Particles by Monocytes: Mononuclear cells (2 x 10^6 cells/ml) were incubated in plastic test tubes with fluorescein-labeled live or formalin-killed L. pneumophila (3.75 x 10^9 cells/ml) or live or formalin-killed E. coli (1 x 10^9 cells/ml) in 10% fresh normal human serum at 37°C at 5% CO2/95% 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 plastic dishes with 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% CO2/95% air for 30 min to allow the monocytes to adhere. Afterwards, the dishes were vigorously washed to remove the nonadherent cells. In experiments not involving erythrocytes, the monocyte monolayers were then incubated at 37°C in 5% CO2/95% 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 then incubated at 37°C in 5% CO2/95% air in 2 ml RPMI containing 5 or 10% fresh human 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. pneumophilia, 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 phagocytosed 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 heating. The value for 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 63 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-400 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 x 64 pixel element (~6.5 x 13 μm) region.

Particles with intensities above a threshold value in the 490-nm digitized image were identified as L. pneumophila or erythrocytes. 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).
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. The two values for live L. pneumophila containing a small proportion of an inoculum is killed by monocytes (24).

Although we consistently observed the pH difference, 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
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.

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 |
|---------------------------------------------|
| pH of E. coli phagosomes in monocytes that have ingested |
| Experiment | Live E. coli | Formalin-killed E. coli | ΔpH* |
| 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.

ΔpH* = (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 ΔI<sub>490</sub> 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.
This study shows that the pH of phagocytic vacuoles containing a live intracellular pathogen 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...
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-

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 than 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).
Legionella Inhibits Phagosome Acidification

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