Phagosomal Acidification Is Not a Prerequisite for Intracellular Multiplication of *Legionella pneumophila* in Human Monocytes

Hagen Wieland,¹ Friedrich Goetz,² and Birgid Neumeister ¹

¹Department of Transfusion Medicine, University Hospitals Tübingen, and ²Microbial Genetics, University of Tübingen, Tübingen, Germany

*Legionella pneumophila* is able to multiply in a variety of eukaryotic cells. Contradictory results have been published on the significance of phagosomal acidification in the intracellular multiplication of *Legionella* species in monocytes. Therefore, we analyzed the phagosomal pH values in 2 different types of human monocytes throughout the intracellular-replication cycles of 2 *Legionella* species that have different rates of intracellular multiplication. Our results show that phagosomal acidification is not a prerequisite for intracellular replication of *Legionella pneumophila* in human monocytes.

*Legionella pneumophila*, an intracellular pathogen and the causative agent of Legionnaire’s disease, is able to persist and to multiply in a variety of eukaryotic host cells, including protozoa and human monocytes [1–4]. In contrast, many other members of the genus *Legionella*, such as *L. steigerwaltii*, are not able to multiply in human monocytes [3, 4]. After being ingested by human peripheral-blood monocytes (PBMs), *L. pneumophila* resides and multiplies in a special compartment that evades phagosome-lysosome fusion and subsequent acidification during the first 6 h after infection [5, 6]. It has also been shown that, in macrophages of *A/J* mice, *L. pneumophila* blocks the maturation and acidification of its own phagosome only during the first hours after being ingested. The multiplication of *L. pneumophila* was maximal in macrophages whose endosomal compartment became acidic, and it was therefore assumed that phagosomal acidification may be a necessary prerequisite for intracellular replication [7]. It remained to be determined whether phagosomal acidification also is required for intracellular multiplication of *L. pneumophila* in human monocytes. Therefore, using a newly developed, cell-permeable, low-toxic, pH-sensitive fluorescent dye, we studied the kinetics of phagosomal acidification throughout the entire intracellular-replication cycle and correlated the phagosomal pH values with the intracellular-replication rates of 2 different *Legionella* species that exhibit different replication capabilities in human monocytes. To control for intracellular fate and for the determination of pH values, we used *Escherichia coli*, because it is an organism that is killed via the classic endolysosomal pathway.

**Materials and methods.** A fresh human isolate of *L. pneumophila* (serogroup 1; strain Pontiac), obtained from a patient with severe *Legionella* pneumonia, was provided by Prof. G. Ruckdeschel (Munich, Germany). *L. steigerwaltii* ATCC 35303 was obtained from the American Type Culture Collection. Both strains were grown on buffered charcoal yeast extract–α agar (BioTest) for 3 days at 37°C in 5% CO₂. *E. coli* DH5α was grown on Luria broth (LB) agar (Oxoid) overnight at 37°C in 5% CO₂.

To determine growth curves, bacteria obtained from a fresh liquid culture produced overnight in buffered yeast extract (BYE) broth were suspended in 30 mL of BYE broth at an OD₅₇₈nm of 0.02. Growth was assessed by the determination of the optical density at OD₅₇₈nm.

For the labeling of bacteria, *L. pneumophila*, *L. steigerwaltii*, and *E. coli* were transformed using a plasmid coding for enhanced green-fluorescent protein (EGFP), as described elsewhere [8]. The plasmid was provided by Dr. M. Steinert (Institute for Molecular Infection Biology, University of Würzburg, Germany).

PBMs were prepared from buffy coats that had been made from donated blood, by use of a lymphocyte-separation medium (PAA Laboratories) and a Monocyte Isolation Kit (Miltenyi Biotech), both according to their manufacturers’ instructions. Informed consent was obtained from blood donors for the use ofuffy coats after blood processing, for in vitro infection with *Legionella* species. Mono Mac 6 (MM6) cells were donated by Dr. H. W. L. Ziegler-Heitbrock (University of Leicester, United Kingdom). The cells were cultured as described elsewhere [4].

Both infection of human monocytes with EGF-labeled bacteria and assays for intracellular multiplication were performed as described elsewhere [4]. For infection of MM6 cells, 2 × 10⁶ cells were coincubated with 2 × 10⁴ bacteria, providing a bacterium:cell ratio of 100:1; PBMs were infected with 2 ×
10^7 bacteria, providing a bacterium:cell ratio of 10:1. All experiments were performed at least 3 times.

Except for some minor changes described below, the intracellular-killing assays using *E. coli* DH5α were performed in the same manner as has been described for the infection with legionellae. MM6 cells and freshly isolated PBMs were infected with *E. coli* DH5α at a bacterium:cell ratio of 10:1. After 20 min, extracellular bacteria were removed by 2 washings with RPMI 1640; the cells were then incubated in culture medium containing 10 μg of gentamicin/mL, to prevent extracellular multiplication of uningested bacteria. Every hour for 5 h after infection, disrupted samples were plated out on LB agar, in appropriate dilutions. After incubation, colonies were counted for 24 h at 37°C in 5% CO2.

Infected human monocytic cells (1 × 10^7) were stained for 10 min at 37°C in 5% CO2, with gentle shaking, by use of a 10 μmol/L final concentration of SNARF-5F-AM-ester (Molecular Probes) in PBS. The cells were then washed 3 times with culture medium, to remove any traces of free dye, and were incubated for 1 h in culture medium at 37°C in 5% CO2, to allow recovery from the staining process; afterward, phagosomal pH values were determined immediately.

To determine phagosomal pH values, infected and stained cells were centrifuged for 10 min at 400 g and then were resuspended in PBS. Five microliters of PBS were dropped into a microwell of an immunology slide (Bioworld) and were covered with a coverslip; for 3 min the cells were allowed to attach to the glass surface of the slide. *Legionella*-filled phagosomes were located by EGFP fluorescence microscopy (λex = 488 nm), and an optical alignment was performed by focusing inside the phagosome. SNARF-5F fluorescence emissions, in response to an excitation wavelength of 543 nm, at nm 580 nm, and nm were simultaneously collected in 2 channels640 nm), and an optical alignment was performed by focusing in-internal-density filters and to reduce photobleaching and cell damage caused by laser illumination, neutral-density filters and ~11% of maximum laser power were used for imaging. Images were collected using a C-Apo ×63 oil-immersion objective.

SNARF-5F dual-emission ratios of phagosomes were evaluated using Leica Confocal Evaluation Software, and the pH value for each individual cell was calculated. To establish calibration curves, the absolute intracellular pH values were calibrated in situ, by treatment with 10 μmol of nigericin/L (Molecular Probes) in high-K+ solutions of varying pH (5.6–8.2) [9].

For endosomal tracer experiments, lysosomes were fluorescently prelabeled with the soluble endosomal tracer Texas red–ovalbumin (Molecular Probes), as described elsewhere [7]. Afterward, MM6 cells were infected with EGFP-tagged *L. pneumophila*, as described elsewhere [4]. Seventeen to eighteen hours after infection, the infected cells were analyzed by microscopy using a Zeiss Axioskop equipped with a Plan-Neofluar ×100/1.30 oil-immersion objective. In each of the 2 experiments, highly replicative vacuoles (n = 30) were evaluated by visual identification of the EGFP-tagged intracellular bacteria and subsequent quantification of phagosome-lysosome fusion events, by means of detectable Texas red fluorescence inside the vacuoles.

**Results.** The number of intracellular *L. pneumophila* increased ~2 logarithms, in both PBMs (figure 1A) and MM6 cells (figure 1B), after phagocytosis, whereas the number of intracellular *L. steigerwaltii* slightly declined after phagocytosis (figure 1A and 1B). Thus, in terms of their ability to support or inhibit the intracellular multiplication of *Legionella* species, we did not find any difference between MM6 cells and PBMs.

However, MM6 cells and PBMs differed in their ability to kill ingested *E. coli*. Although, under the same experimental conditions, PBMs phagocytized ~10 times more bacteria than did MM6 cells, after 5 h of incubation the number of intracellular *E. coli* was lower, and killing was more rapid, in PBMs than in MM6 cells (figure 1C).

To determine acidification of *E. coli* phagosomes, human monocytes were allowed to phagocytize *E. coli*, and phagosomal pH values were gaged 90 min and 3 h after ingestion, for MM6 cells, and 90 min after ingestion, for PBMs. In contrast to PBMs (figure 2A), the immortal monocyctic cell line MM6 did not acidify phagosomes containing *E. coli* (figure 2B). The pH of *E. coli* phagosomes in MM6 cells was nearly neutral (~7.3) 90 min after infection, whereas the pH of *E. coli* phagosomes in PBMs was acidic (5.6–6.2; mean, 5.88) 90 min after infection (figure 2A and 2B).

When infected with the 2 *Legionella* species, MM6 cells again did not acidify their phagosomes. Despite efficient intracellular multiplication in MM6 cells (figure 1B), phagosomes of *L. pneumophila* in these host cells showed, 2 h after infection, pH values of ~6.6, which increased, 25–48 h after infection, up to ~7.3. The phagosomes of the nonreplicative *L. steigerwaltii* showed, 2 h after infection, pH values of ~6.9; 24 h after infection, ~7.2; and 48 h after infection, ~7.9 (figure 2B). Thus, although the pH values of the *L. pneumophila* phagosomes in MM6 cells were slightly lower than those of the *L. steigerwaltii* phagosomes in MM6 cells, the pH retained neutral values during intracellular *Legionella* multiplication.

After uptake of bacteria by PBMs, the phagosomal pH was ~6.7 both for *L. pneumophila* 3 h after infection and for *L. steigerwaltii* 4 h after infection. The pH of *L. steigerwaltii* phagosomes then rose, 21 h after infection, to ~6.9 and remained at this value for up to 48 h after infection. The pH of *L. pneumophila* phagosomes slightly decreased, 20 h after infection, to ~6.4 (figure 2A). Thus, in contrast to *L. steigerwaltii* phagosomes, *L. pneumophila* phagosomes slightly acidified during intracellular replication in PBMs. The phagosomes of both *Legionella* species showed lower pH values in PBMs, compared
Intracellular fate of *Legionella pneumophila*, *L. steigerwaltii*, and *Escherichia coli* in human peripheral-blood monocytes (PBM) and Mono Mac 6 (MM6) cells. PBM (A) were infected with either of the 2 *Legionella* species at a bacterium:cell ratio of 10:1; MM6 cells (B) were infected with either of the 2 *Legionella* species at a bacterium:cell ratio of 100:1; and both PBM and MM6 cells were infected with *E. coli* (C) at a bacterium:cell ratio of 10:1. After phagocytosis and removal of extracellular bacteria, cells were incubated for the periods of time indicated, and the number of viable bacteria (after multiplication in the host cells) was determined, by hypotonic disruption of the host cells and by culture of the fluid on agar. Growth curves show a representative of 6 independent experiments.

with those in MM6 cells, whereas rates of intracellular multiplication were nearly identical in the 2 host-cell types.

In situ pH calibration with SNARF-5F was performed in the presence of 10 μmol of nigericin/L (figure 2C). To show that SNARF-5F has no direct toxic effect on the viability of *L. pneumophila*, SNARF-5F was added to *L. pneumophila* liquid cultures in the concentration used for staining. The dye did not affect bacterial viability (data not shown) and was, therefore, suitable for use in the measurement of the pH values of bacterial phagosomes. To investigate the endosomal accessibility of *L. pneumophila* phagosomes, lysosomes of MM6 cells were labeled with Texas red–ovalbumin prior to infection. Fluorescence microscopy 17–18 h after infection revealed that 46.5% ± 4.9% (mean ± SD; n = 60) of the vacuoles had acquired Texas red fluorescence, indicative of their merging with the endosomal pathway.

Discussion. It has been demonstrated that, within the first hours of the infection cycle, *L. pneumophila* phagosomes in PBM evade phagosome-lysosome fusion and subsequent acidification [5, 6]. Moreover, evidence has been reported that *L. pneumophila* phagosomes in human monocytes remain neutral up to 18 h after infection [6]. However, a recent study has demonstrated that phagosomal maturation in *Legionella*-infected bone marrow–derived macrophages in A/J mice is delayed, not abolished, and had partially occurred 16 h after infection [7]. The associated vacuolar pH declined from ∼7.4 (at 3–6 h after infection) to ∼5.6 (at 16–22 h after infection). Acquisition of lysosomal proteins and phagosomal acidification correlated with the bacterial-replication phase of *L. pneumophila*. Nonreplicative *Legionella* species were not tested [7]. To clarify these obvious discrepancies, we used a newly developed, low-toxic, cell-permeable, pH-sensitive fluorescent dye to analyze phagosomal acidification in 2 different types of human monocytes, throughout the entire intracellular-replication cycles of 2 *Legionella* species that have different rates of intracellular multiplication.

In the present study, we have shown that neither the replicative *L. pneumophila* nor the nonreplicative *L. steigerwaltii*
Figure 2. Phagosomal acidification in human peripheral-blood monocytes (PBM) (A) and Mono Mac 6 (MM6) cells (B), after ingestion of either Legionella pneumophila, L. steigerwaltii, or Escherichia coli. PBM were infected with either of the 2 Legionella species at a bacterium:cell ratio of 10:1; MM6 cells were infected with either of the 2 Legionella species at a bacterium:cell ratio of 100:1; and both PBM and MM6 cells were infected with E. coli (C) at a bacterium:cell ratio of 10:1. After phagocytosis and removal of extracellular bacteria, cells were incubated for the periods of time indicated. Mean ± SD pH values of 6 independent experiments are shown. In situ pH calibration (C) was performed in the presence of 10 μM of nigericin; each point represents the mean ± SD of 3 independent measurements. The dual-emission ratio was calculated as λ_{640} ± 3 nm/ λ_{580} ± 3 nm.

causes sustainable phagosomal acidification in MM6 cells, despite the considerable difference between the multiplication rates of the 2 species. A new finding was that these host cells were also unable to acidify E. coli phagosomes, which remained at a neutral pH of ~7.3, corresponding to common cytoplasmic pH values [9]. We therefore presume that the immortal cell line MM6 is constitutively unable to acidify bacterial phagosomes at all. Intracellular-killing assays revealed that MM6 cells are not able to kill phagocytized E. coli as efficiently as are PBM. The lack of phagosomal acidification in MM6 cells presumably contributes to this impaired killing. Because vacuolar acidification (which has been demonstrated to be unnecessary for phagosomal maturation in human fibroblasts [10]) constitutes only a part of the endocytic pathway, MM6 cells seem to retain, to some extent, the ability to kill E. coli.

In contrast to MM6 cells, PBM were able to acidify E. coli phagosomes (mean pH 5.88) as soon as 90 min after infection. The pH of L. pneumophila phagosomes in PBM decreased only slightly, 24 h after infection (~6.4), whereas the pH of L. steigerwaltii phagosomes rose to ~6.9 during the infection cycle. The pH values of L. pneumophila phagosomes in PBM were considerably higher than those obtained in an investigation of infected bone marrow–derived murine macrophages, which revealed a phagosomal pH, 16–22 h after infection with L. pneumo-
Mycobacterium tuberculosis are capable of preventing the acidification of their own phagosomes. Legionella steigerwaltii may indicate that intracellular multiplication of L. pneumophila requires a more acidic pH in PBM. However, this inference does not seem to be true, because we obtained identical rates of multiplication in a human monocytic cell line, MM6, that is not able to acidify bacterial phagosomes.

In addition to L. pneumophila, other intracellular bacteria are capable of preventing the acidification of their own phagosomes. Mycobacterium tuberculosis and M. avium reside in a neutral phagosomal environment in human macrophages; moreover, M. tuberculosis partly inhibits or retards the maturation of its phagosome. M. avium phagosomes in murine J774 macrophages remain at a pH of ~6.2 during the entire infection cycle and exhibit partial inhibition of maturation.

In contrast, Coxiella burnetii resides in an acidic phagosome in THP-1 monocytes, regardless of the virulence of the strain (although, in terms of their capacity to inhibit phagosomal maturation, virulent strains differ from avirulent strains).

The results of the endosomal tracer experiments using L. pneumophila–infected MM6 cells show that, 17–18 h after infection, approximately half of the vacuoles containing replicating legionellae had already fused with lysosomes. As has been recently demonstrated for phagosomes of human fibroblasts after ingestion of collagen-coated beads, maturation of L. pneumophila phagosomes in MM6 cells occurs despite the absence of vacuolar acidification. In combination with the consistency of phagosomal pH values, the results of the present study provide additional evidence that vacuolar acidification is not necessarily linked to the phagosomal-maturation process.

Our results, obtained using a new, pH-sensitive fluorescent dye, confirm the previous results reported by Horwitz and Maxfield, who found that L. pneumophila phagosomes in PBM acidify only very slightly. In addition, the total absence of phagosomal acidification in L. pneumophila–infected MM6 cells and the associated strong intracellular multiplication of the bacteria demonstrate that, whereas an acidic environment may be an attendant phenomenon of intracellular multiplication in human monocytes, it is not necessary for it.

References