Localization of *Legionella* bacteria within ribosome-studded phagosomes is not restricted to *Legionella pneumophila*

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**Abstract**

In this report, we investigate the intracellular fate of selected members of the genus *Legionella* within the monocytic cell line Mono Mac 6 cells. By means of electron microscopy and immunocytochemistry, we could show that *Legionella pneumophila* as well as *Legionella longbeachae* are able to induce ribosome-studded phagosomes which associate with the rough endoplasmic reticulum (RER), whereas *Legionella micdadei* remains to be located within smooth phagosomes but also shows signs of RER association. In addition, we could demonstrate a remarkable correlation between the phagosome type and the morphological phenotype of intracellular bacteria: within ribosome-studded phagosomes, bacteria generally lacked the outer coat of low electron density whereas bacteria within the smooth phagosomes still possessed this outer coat. The virulence factors responsible for inhibition of phagosome maturation and their distribution within the genus *Legionella* as well as the biological significance of the morphological difference of bacteria within smooth and ER-associated phagosomes remain to be investigated. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Members of the genus *Legionella* are Gram-negative, rod-shaped bacteria which survive and multiply efficiently in human monocytes, alveolar macrophages [1] and macrophage-like cell lines such as U937, HL-60 and Mono Mac 6 (MM6) cells [2–4]. *Legionella (L.) pneumophila* is the cause of severe community-acquired and nosocomial pneumonia [5], whereas other species are able to induce pneumonia only in immunocompromised patients [6–8]. Infection results when legionellae are inhaled as an aerosol. After uptake in coils of plasma membrane of alveolar macrophages, *L. pneumophila* is located in a membrane-bound phagosome that evades fusion with lysosomes [9,10]. Within the first few hours of infection, mitochondria and smooth vesicles are recruited around the phagosome and the phagosome is routed to a perinuclear position in the cell, where it appears as a ribosome-studded vacuole [11]. This appearance results from an intimate association between the phagosome and the rough endoplasmic reticulum (RER) [11,12]. Since mutants of *L. pneumophila* that fail to establish ribosome-studded vacuoles also fail to replicate intracellularly, the formation of this specialized phagosome is believed to be essential for intracellular survival and replication of *L. pneumophila* within mammalian host cells [12–14]. Recent data have shown that, during formation of ribosome-studded phagosome, *L. pneumophila* is able to alter the maturation of its phagosome along the endosomal/lysosomal pathway thus preventing the acquisition of late endosomal and lysosomal markers such as CD63, LAMP-1, LAMP-2 and cathepsin D [15–17] and maintaining markers of the endoplasmic reticulum such as BiP [12].

In contrast to *L. pneumophila*, little is known about the intracellular fate of other *Legionella* species. Examination of *L. micdadei*-infected macrophages revealed that replication of these bacteria occurred within ribosome-free phagosomes.
osomes [18,19]. Other Legionella species were investigated only with respect to intracellular multiplication [3,20,21], however, the ultrastructural changes in such infections are still to be characterized.

In this report, we investigate the intracellular fate of selected members of the genus Legionella within MM6 cells. Species were chosen which, in accordance to their human prevalence, show different multiplication rates within these host cells [3].

2. Materials and methods

2.1. Legionella bacterium

L. pneumophila serogroup 1 (Philadelphia-1) ATCC 33152 (isolated from human lung), L. longbeachae serogroup 1 ATCC 33462 (isolated from human lung), and L. micdadei ATCC 33218 (isolated from human blood via yolk sac), were obtained from the American Type Culture Collection (ATCC). L. pneumophila serogroup 1 strain Pontiac (isolated from a patient with severe Legionella pneumonia and passaged less than three times on BCYEα-agar) was kindly provided by Prof. Dr. Ruckdeschel (Munich, Germany).

All strains were grown on BCYEα-agar (Oxoid, Wesel, Germany) at 35°C in 5% CO₂ for 5 days.

2.2. MM6 cells and intracellular multiplication

MM6 cells were kindly donated by Professor H.W.L. Ziegler-Heitbrock (University of Munich, Germany) and cultured as replicative non-adherent monocytes as previously described [3,22]. Infection with legionellae and statistical analysis were performed as recently reported [3]. Briefly, 2 × 10⁶ MM6 cells were infected with 2 × 10⁶ legionellae. After an incubation of 2 h at 37°C, remaining extracellular bacteria were killed by gentamicin (75 μg ml⁻¹) treatment. After 1 h of incubation, the medium was replaced with antibiotic-free medium. This time was defined as the 0 h value. The cells were then incubated for an additional 72 h at 35°C in 5% CO₂. To determine the number of viable legionellae after multiplication in MM6 cells, CFU was determined every 24 h by plating 100 μl of serial 10-fold dilutions from a pool of supernatant and lysis fluid onto BCYEα-agar. Colonies were counted on day 5 after incubation at 35°C in 5% CO₂. Growth rates and 95% confidence intervals of Legionella multiplication in MM6 cells were calculated using the statistics package JMP, version 3.1.5, as recently described [3].

2.3. Preparation of MM6 cells for electron microscopy

All chemicals were purchased from Merck, Darmstadt, Germany if not specially indicated. After 5 and 27 h of incubation (2 and 24 h after removing gentamicin from the coculture), MM6 cells were harvested by centrifugation at 400 × g for 10 min. The pellet was fixed for 2 h in 2.5% glutaraldehyde in HMSS (0.1 M HEPES, Sigma; 0.15 M NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl₂ and 1 mM MgCl₂), pH 7.4 at 4°C. Next, the cells were washed in TBS–glycine (50 mM Tris–HCl, 150 mM NaCl, 1 mM CaCl₂, pH 7.6, containing 50 mM glycine), embedded in 10% gelatin in TBS and centrifuged. The pellet in gelatin was fixed overnight at 4°C in the fixative mentioned above. Small cubes of cell-containing gelatin were prepared and transferred to 0.1 M cacodylate buffer, pH 7.4. The specimens were washed twice and postfixed for 1 h in 1% osmiumtetroxide (Paesel, Frankfurt, Germany) in cacodylate buffer, washed twice and dehydrated in a graded ethanol series. The 70% ethanol step was saturated with uranylaceetate for contrast enhancement and carried out at 4°C overnight. Dehydration was completed with propyleneoxide, followed by a mixture of 1:1 propyleneoxide and Araldite (Serva, Heidelberg, Germany). Specimens were embedded in Araldite and polymerized for 48 h at 60°C. Ultrathin sections were cut using an Ultracut R ultramicrotome (Leica, Bensheim, Germany) and mounted on pioloform coated copper grids. They were stained with lead citrate and observed and documented with a Zeiss CEM 902 electron microscope (Zeiss, Jena, Germany).

2.4. Antibodies

L. pneumophila was localized by using a mouse monoclonal antibody to L. pneumophila serogroup 1 (Lp46, Bio- trend, Cologne, Germany). As primary antibody for localization of L. micdadei and L. longbeachae, a mouse monoclonal anti-Legionella heat shock protein 60 antibody (anti-HSP-60, kindly provided by Dr. Steinmetz and Prof. Bitter-Suermann, Institute for Medical Microbiology, University of Hannover) was used. Secondary antibody was Cy3-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany). For controls, the primary antibody was omitted. Detection of colocalization with RER was performed by using a polyclonal antibody to calnexin (SPA-860, StressGen, Victoria, Canada) [23]. As secondary antibody, a Cy2-conjugated goat anti-rabbit antibody (Dianova, Hamburg, Germany) was used.

2.5. Immunocytochemical procedures

After 5 and 27 h of incubation (2 and 24 h after removing gentamicin from the coculture), MM6 cells were harvested by centrifugation at 400 × g for 10 min. The pellet was fixed for 1 h in 4% freshly depolymerized formaldehyde in HMSS, pH 7.4 at 4°C. Cells were washed in TBS–glycine, embedded in 10% gelatin in TBS and centrifuged. The pellet in gelatin was fixed overnight at 4°C in the fixative mentioned above. Small cubes of cell-containing gelatin were transferred to 1.6 M sucrose/2% polyvinylpyrrolidone (MW 10 000; Sigma) overnight, mounted on...
specimen-pins (Leica) and rapidly frozen by injection into liquid propane in a cryo-preparation chamber (Leica). Semithin cryosections (200 nm) were cut at −70°C using a Leica Ultracut R ultramicrotome equipped with a Leica FCR cryo-chamber (Leica). Sections were mounted on poly-L-lysine (Sigma, Munich, Germany) coated cover-slips, postfixed in 4% formaldehyde/HMSS on ice and washed in TBS containing 1 mM CaCl₂. Antibodies were diluted in 5% (w/v) skimmed milk, 0.3% (v/v) Triton X-100 (Serva), and 0.04% (w/v) NaN₃ in TBS. After several washes in TBS, nuclear staining was achieved by incubation in 0.5 μg ml⁻¹ 4,6-diamidino-2-phenylindole (DAPI; Boehringer, Mannheim, Germany) in TBS for 5 min. Sections were mounted in 90% glycerol/10% TBS containing 1 mg ml⁻¹ p-phenylene diamine (Sigma) and analyzed using a Zeiss Axiophot microscope equipped with triple band pass fluorescence filters for simultaneous visualization and documentation (Zeiss).

3. Results

3.1. Intracellular growth of Legionella bacteria

The two strains of L. pneumophila serogroup 1 (Pontiac and Philadelphia) were able to multiply within MM6 cells, the fresh clinical isolate (Pontiac) showed a higher intracellular multiplication rate than the agar-adapted strain Philadelphia-1 ATCC 33152. L. micdadei showed a moderate multiplication rate, whereas L. longbeachae was unable to multiply within MM6 cells (Fig. 1).

3.2. Electron microscopy

By using conventional transmission electron microscopy, we investigated the morphological characteristics of intracellular Legionella bacteria and their association with cellular compartments of infected monocytes over a time course of 27 h of incubation (24 h after removing gentamicin from the culture) (Fig. 2).

After 2 h (5 h of incubation), L. pneumophila serogroup 1 (Pontiac) was found in phagosomes which appeared decorated with vesicles and mitochondria. Ribosome-studded phagosomes could not be detected at this time point. Bacteria were surrounded by a coat of low electron density. These outer coats were frequently found without internal bacteria, sometimes leaving them collapsed and coiled within the phagosomes (Fig. 2a).

After 24 h (27 h of incubation), bacteria of this strain were numerous within the infected monocytes, reflecting their high rate of intracellular replication. Bacteria were now exclusively located in RER-associated ribosome-studded phagosomes. In contrast to the phagosomes formed after 2 h, legionellae within RER-associated phagosomes after 24 h exhibited an uncoated and vacuolated appearance (Fig. 2b).

The agar-adapted strain of L. pneumophila serogroup 1 (Philadelphia-1) ATCC 33152 only scarcely formed RER-associated phagosomes and the bacteria mostly exhibited the coated phenotype after 24 h (Fig. 2c). Coats without internal bacteria were present. Occasionally, both coated and uncoated legionellae as well as ‘empty coats’ were found within a single phagosome (Fig. 2d).

![Fig. 1. Intracellular growth of Legionella bacteria used for infection of MM6 cells in a bacterium to cell ratio of 100:1. Data are expressed as 95% confidence intervals of exponential growth rates/reduction rates (per h).](https://academic.oup.com/femsle/article-abstract/192/1/145/633572)
Fig. 2. Electron micrograph of a MM6 cell after infection with different *Legionella* strains. (a) MM6 cell, 5 h after infection with *L. pneumophila* serogroup 1 (Pontiac) ATCC 33152. Bacteria are found in phagosomes which appear decorated with vesicles and mitochondria. Ribosome-studded phagosomes could not be detected at this time point. Bacteria seem to be surrounded by a coat of low electron density. These outer coats are frequently found without internal bacteria, sometimes collapsed and coiled within the phagosomes. (b) MM6 cell, 24 h after infection with *L. pneumophila* serogroup 1 (Pontiac) ATCC 33152. Bacteria are now exclusively located in RER-associated ribosome-studded phagosomes (arrows) and exhibit an uncoated and vacuolated appearance. (c) MM6 cell, 24 h after infection with the agar-adapted strain of *L. pneumophila* serogroup 1 (Philadelphia-1) ATCC 33152. Bacteria are engulfed in smooth phagosomes and exhibit the coated phenotype. Coats without internal bacteria are present. (d) MM6 cell, 24 h after infection with the agar-adapted strain of *L. pneumophila* serogroup 1 (Philadelphia-1) ATCC 33152. Both coated and uncoated bacteria as well as 'empty coats' are found within a single phagosome. (e, f) MM6 cell, 24 h after infection with *L. micdadei* ATCC 33218. Bacteria lack the characteristic coat of low electron density, the outer bacterial membrane is distorted and ruffled. Although the phagosome associates with mitochondria and nucleus, ribosome-studded phagosomes could never be observed. (g, h) MM6 cell, 24 h after infection with *L. longbeachae* serogroup 1 ATCC 33462. Two morphologically distinct phagosome types within the infected monocyte can be demonstrated: One type is intimately associated with the RER (arrow) and is found in the vicinity of the nuclear envelope. Bacteria lack the outer coat of low electron density (black star). The other type represents a smooth phagosome that does not associate with the RER. It is located in the cell periphery. Bacteria seem to have an external coat (white star).
Infections with *L. micdadei* were fundamentally different in one way: the characteristic coat of low electron density was never observed to surround bacteria of this species. Instead, the outer bacterial membrane was regularly distorted and ruffled. Although the phagosome was found to be associated with mitochondria and nucleus, ribosome-studded phagosomes could never be observed (Fig. 2e and f).

After 24 h, infections with *L. longbeachae* exhibited two morphologically distinct phagosome types within the infected monocytes. One type was intimately associated with the RER and was found in the vicinity of the nuclear envelope, whereas the other type did not show association with the RER and was usually located in the cell periphery. Interestingly, the morphology of the engulfed bacteria was again different depending on the type of phagosome. Within the RER-associated phagosome, bacteria generally lacked the outer coat of low electron density which was present in the other phagosome type (Fig. 2g and h). Additionally, bacteria in ribosome-studded phagosomes contained typical vacuoles which were otherwise absent. Thus, the appearances of bacteria and phagosomes were found to be variable but highly correlated.

### 3.3. Immunocytochemistry

To investigate the relationships between phagosomes and the host ER, the colocalization of calnexin with the *Legionella*-specific components Hsp60 and *L. pneumophila* LPS, respectively, were examined (Fig. 3).

Double labelling of calnexin and LPS revealed a high degree of colocalization in infections with *L. pneumophila* serogroup 1 (Pontiac), both after 2 and 24 h. In contrast, infections with *L. pneumophila* serogroup 1 (Philadelphia-1) ATCC 33152 only scarcely showed signs of colocalization. Although formation of ribosome-studded phagosomes could never be observed in infections with *L. micdadei*, immunocytochemistry revealed signs of colocalization between *Legionella* Hsp60 and calnexin. Infections with *L. longbeachae* showed little colocalization after 24 h of incubation.

### 4. Discussion

Morphological investigations which identified an association between intracellular *L. pneumophila* and the RER of infected macrophages have led to the speculation that localization of the pathogen in specialized phagosomes is essential for its ability to multiply efficiently and, therefore, is linked to virulence [11,12,16]. In accordance to previous reports [11,12], in the present investigation we could demonstrate that *L. micdadei* serogroup 1 (Pontiac), a wild-type strain isolated from a patient with severe *Legionella* pneumonia and passaged less than three times on BCYE-agar, is located within smooth phagosomes early during infection of MM6 cells, and is engulfed in ribosome-studded phagosomes 24 h after infection. Colocalization of phagosomes with the ER as revealed by association of intracellular bacteria with the resident ER marker calnexin could be detected 2 h as well as 24 h after infection. This strain has shown the highest rate of intracellular replication within MM6 cells. An agar-adapted ATCC strain of *L. pneumophila* revealed a lesser degree of multiplication. RER-associated phagosomes and colocalization were only scarcely found. The cause of this attenuation of intracellular multiplication and phagosome formation is not known. Several replication-associated loci of the *L. pneumophila* genome have been shown to play a fundamental role in regulating phagosome trafficking decisions after uptake into macrophages, such as *dotA* [24], *dotH, dotI* and *dotO* [25], *icmW* [26] and *icmX* [27]. Whether one or more of these factors are mutated in *L. pneumophila* serogroup 1 (Philadelphia-1) ATCC 33152, due to prolonged passage on BCYE-agar, remains to be determined.

In addition, we examined two *Legionella* species which induce severe pulmonary disease only in the immunocompromised host [6–8] and which did not show intracellular multiplication within MM6 cells [3]. In infections with *L. micdadei*, bacteria never became surrounded by a membrane derived from the RER but, nevertheless, showed colocalization with calnexin. Previous reports have already shown the lack of ribosome-studded phagosomes in monocytes infected with *L. micdadei*, but these studies only used electron microscopy without confirmation by immunocytochemistry [18,19]. Thus, *L. micdadei* seems to exhibit an intracellular pathway different from *L. pneumophila*. The morphological appearance of the *L. micdadei* envelope can be explained by a thick peptidoglycan-like layer between the inner and the outer membranes and an extra layer of low electron density attached to the cell envelope which are unique to this species [28–30].

Infections with *L. longbeachae* revealed smooth as well as ER-derived phagosomes within one monocyte. The ultrastructural characteristics of this species within host cells has never been investigated before. Here we show that not only *L. pneumophila* but also *L. longbeachae* is able to induce RER-derived phagosomes. The cause of the appearance of two different phagosomes within one monocyte is not clear. We can not exclude the concomitant existence of more and less virulent bacteria among the population of *L. longbeachae* serogroup 1 ATCC 33462 and that during phagocytosis, using the multiplicity of infection of 100:1, one and the same monocyte was able to phagocytose a virulent bacterium which was then engulfed in a ribosome-studded phagosome as well as an attenuated bacterium which was included into a smooth phagosome [31]. On the other hand, it is possible that the location of bacteria in different phagosomes at this time point represents an intermediate stage of progression of phagosome formation which seems to be delayed in case...
of infections with \textit{L. longbeachae} in comparison to \textit{L. pneumophila}. This would implicate that the formation of ribosome-studded phagosomes may be a common step in the course of infections with several \textit{Legionella} species and that the speed of progression of phagosome formation is triggered by the virulence of a given species.

The mechanisms utilized by legionellae to inhibit the maturation of its phagosome into a phagolysosome are not known. Coers et al. recently proposed a model in which factors transported by the \textit{L. pneumophila} Dot/Icm transporter act to remodel the phagosome into a specialized organelle, and that, once this compartment has been established, nutrients and additional membrane that support bacterial growth inside the vacuole are delivered by the host cell [31].

In this study, we could also observe a close correlation between phagosome type and morphological phenotype of bacteria. As shown for \textit{L. pneumophila} as well as for \textit{L. longbeachae}, bacteria engulfed in smooth phagosomes were surrounded by an external layer of low electron density adherent to their outer membrane. In addition, empty outer coats, in part collapsed and coiled, were frequently found in these phagosomes. In contrast, legionellae located in ribosome-studded phagosomes consistently showed a lack of the external layer. Empty coats could never be demonstrated. This indicates that legionellae may possess this capsular structure to survive in an extracellular environment and loose it during the progression of intracellular replication. This change of morphological phenotype may be associated with the frequently described phenotypical modulation of \textit{Legionella} in response to the intracellular environment leading to enhancement of virulence after intracellular multiplication [32–36]. The composition of the empty layers as well as their intracellular propagation remain to be elucidated.

In conclusion, we could show that not only \textit{L. pneumophila} but also \textit{L. longbeachae} are able to induce ribosome-studded phagosomes and associate with RER whereas \textit{L. micdadei} remains to be located within smooth phagosomes but, nevertheless, also shows signs of ER association. In addition, we could demonstrate a remarkable correlation between phagosome type and morphological phenotype of bacteria. The virulence factors responsible for inhibition of phagosome maturation and their distribution within the genus \textit{Legionella} as well as the biological significance of the morphological difference of bacteria within smooth and RER-associated phagosomes remain to be investigated.

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