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Introduction

Legionella pneumophila is the causative agent of a severe pneumonia known as Legionnaires’ disease. The Gram-negative, facultative intracellular pathogen replicates within and ultimately kills human monocytes, alveolar macrophages and free-living amoebae (Horwitz and Silverstein, 1980; Rowbotham, 1980; Nash et al., 1984). Within host cells, L. pneumophila creates a specialized phagosome that is initially pH neutral and does not fuse with lysosomes (Horwitz, 1983a; Horwitz and Maxfield, 1984; Bozue and Johnson, 1996). Phagosomes containing L. pneumophila successively associate with smooth vesicles, mitochondria and endoplasmic reticulum (Horwitz, 1983b; Swanson and Isberg, 1995; Abu Kwaik, 1996) and, at late stages, may acidify and communicate with the endosomal/lysosomal network (Sturgill-Koszycki and Swanson, 2000).

The L. pneumophila icm (intracellular multiplication)/dot (defective organelle trafficking) genes, located in two chromosomal regions, are required for intracellular multiplication and host cell killing (reviewed by Kirby and Isberg, 1998; Segal and Shuman, 1998a). Chromosomal region I harbours the genes icmV, -W, -X and dotA, -B, -E, -J, -D (Marra et al., 1992; Berger et al., 1994; Brand et al., 1994; Vogel et al., 1998), and region II contains the genes icmT, -S, -R, -Q, -P, -O, -N, -M, -L, -K, -E, -G, -C, -D, -J, -B, tphA and icmF (Segal and Shuman, 1997; Andrews et al., 1998; Purcell and Shuman, 1998; Segal et al., 1997; Vogel et al., 1998). The icm/dot genes are also indispensable for growth within the amoeba Acanthamoeba castellanii (Segal and Shuman, 1999a) and Dictyostelium discoideum (Solomon et al., 2000), indicating that L. pneumophila’s mechanism of intracellular growth is evolutionarily conserved. Biochemical analysis of soluble Icm proteins indicated that IcmQ and -R, as well as IcmS and -W, bind directly to each other (Coers et al., 2000), and that IcmX is secreted (Matthews and Roy, 2000). Mutant strains lacking these genes showed discernable phenotypes, as the icmR, -Q and -W but not the icmS and -W mutants were defective for immediate cytotoxicity caused by contact-dependent pore formation in the macrophage membrane (Zuckman et al., 1999; Coers et al., 2000).

Some of the icm/dot genes are required for conjugation of the mobilizable plasmid RSF1010 (Segal et al., 1998; Segal and Shuman, 1998b; Vogel et al., 1998). This result corresponds with the finding that a majority of the Icm/Dot dependent upregulation of phagocytosis by Legionella pneumophila

Summary

Legionella pneumophila is the causative agent of Legionnaires’ disease, a severe pneumonia. Dependent on the icm/dot loci, L. pneumophila survives and replicates in macrophages and amoebae within a specialized phagosome that does not fuse with lysosomes. Here, we report that phagocytosis of wild-type L. pneumophila is more efficient than uptake of icm/dot mutants. Compared with the wild-type strain JR32, about 10 times fewer icm/dot mutant bacteria were recovered from HL-60 macrophages in a gentamicin protection assay. The defect in phagocytosis of the mutants could be complemented by supplying the corresponding genes on a plasmid. Using fluorescence microscopy and green fluorescent protein (GFP)-expressing strains, 10–20 times fewer icm/dot mutant bacteria were found to be internalized by HL-60 cells and human monocyte-derived macrophages (HMMΦ). Compared with icm/dot mutants, wild-type L. pneumophila infected two to three times more macrophages and yielded a population of highly infected host cells (15–70 bacteria per macrophage) that was not observed with icm/dot mutant strains. Wild-type and icmT mutant bacteria were found to adhere similarly and compete for binding to HMMΦ. In addition, wild-type L. pneumophila was also phagocytosed more efficiently by Acanthamoeba castellanii, indicating that the process is independent of adherence receptor(s). Wild-type L. pneumophila enhanced phagocytosis of an icmT mutant strain in a synchronous co-infection, suggesting that increased phagocytosis results from (a) secreted effector(s) acting in trans.

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proteins share significant sequence similarity with plasmid-encoded conjugation systems (Segal and Shuman, 1998b; 1999b). Furthermore, IcmE and DotB are homologous to proteins of the Agrobacterium tumefaciens Vir type IV conjugation system (VirB10, VirB11) (Vogel et al., 1998). L. pneumophila also harbours the lvh genes, which encode a type IV secretion system distinct from Icm/Dot (Segal et al., 1999). The Lvh system is dispensable for multiplication within macrophages and A. castellanii but partially required for conjugation. Interestingly, lvh--dotB or lvh--icmE double mutants are completely defective for conjugation.

Legionella pneumophila is phagocytosed by macrophages and A. castellanii by coiling or conventional phagocytosis (Horwitz, 1984; Bozue and Johnson, 1996). Phagocytosis of the bacteria by human monocytes involves complement receptor (CR) 1 and CR3 and is enhanced under different conditions, such as opsonization with fresh serum (Payne and Horwitz, 1987; Marra et al., 1990) or immunoglobulins (Horwitz and Silverstein, 1981; Nash et al., 1984) or previous growth in A. castellanii (Cirillo et al., 1999). However, the mechanism and receptors mediating phagocytosis do not seem to influence the intracellular fate of L. pneumophila fundamentally.

Legionella pneumophila wild-type but not icm/dot mutant bacteria block maturation of the phagosome within minutes after internalization (Roy et al., 1998; Wiater et al., 1998). This finding prompted us to investigate whether phagocytosis was also affected by the pathogen. In this study, we compared the uptake of L. pneumophila wild-type with icm/dot mutant strains using a gentamicin protection assay and fluorescence microscopy. We found that the icm/dot mutants are defective for efficient phagocytosis by macrophages and A. castellanii. The defect in efficient phagocytosis of the icm/dot mutants was not the result of a defect in adherence. However, efficient uptake of an icmT mutant could be partially restored in a co-infection with wild-type bacteria, indicating that secreted effector(s) are involved. These results suggest that the L. pneumophila icm/dot genes function to stimulate uptake of the bacteria upon initial contact of the pathogen with its host cell.

Results

Legionella pneumophila icm/dot mutants are defective for phagocytosis by HL-60 cells

Within minutes after internalization, wild-type L. pneumophila but not icm/dot mutant bacteria are found in a compartment that neither fuses with lysosomes nor acidifies (Roy et al., 1998; Wiater et al., 1998). In addition, expression of dotA before macrophage uptake but not continuous signalling by the Icm/Dot transporter is required to establish a replication-permissive phagosome (Roy et al., 1998; Coers et al., 1999). The rapid kinetics of the endocytic block prompted us to analyse phagocytosis of L. pneumophila in detail. As a host cell, we initially chose HL-60 macrophages, a well-established model of L. pneumophila uptake, intracellular multiplication and host cell killing (Marra et al., 1990).

Differentiated human HL-60 cells were infected with L. pneumophila wild-type strain JR32 or icm/dot mutants and, after killing extracellular bacteria with gentamicin, the cells were lysed, and the number of viable intracellular bacteria was determined. Using this assay, we recovered about 10 times more viable wild-type L. pneumophila than icm/dot mutants from the HL-60 macrophages (Fig. 1A). This phenotype was statistically significant (P < 0.04, Student’s t-test) and was observed for icm/dot mutants in chromosomal region II (icmT, -S, -R, -Q, -P, -O, -N, -M, -L, -K, -E, -G, -C, -O, -J, -B, -F) as well as region I (dotB, -A, icmV, -X). The L. pneumophila strains carried the mobilizable plasmid pMMB207αab (pMMB), the non-mobilizable derivative pMMB207αb-Km-14 (pMMB-MobA) or plasmids encoding icmM, -K, -E (pMKEx) or icmM, -L, -E (pMLE). The latter two plasmids were introduced into the icmL or icmK mutants, respectively, to rule out polar effects on downstream genes in the putative operon formed by icmMLKE (Segal et al., 1998). Disruption of tphA, a gene in region II, did not impair phagocytosis. This result corresponds with the finding that tphA is also not required for intracellular multiplication within macrophages (Purcell and Shuman, 1998). In addition, the phenotype of the icmF mutant was somewhat weaker than that of other icm/dot mutants, correlating with its only partial requirement for intracellular multiplication (Purcell and Shuman, 1998).

Treatment of the HL-60 macrophages with cytochalasin B, an inhibitor of actin polymerization, led to the recovery of about 10–20 times less wild-type bacteria (P < 0.01), indicating that, under these conditions, phagocytosis was largely abolished, and gentamicin eradicated extracellular L. pneumophila. Cytochalasin might reduce the adherence of differentiated HL-60 cells (data not shown), thus decreasing the number of gentamicin-resistant intracellular cfu observed. However, cytochalasin-dependent decrease in phagocytosis was confirmed in independent experiments using green fluorescent protein (GFP)-expressing L. pneumophila and immunofluorescence microscopy as a more direct assay to enumerate intracellular bacteria (see below).

The defect in efficient phagocytosis of the icm/dot mutant strains was complemented by supplying the corresponding icm/dot genes on plasmids (Fig. 1B; P < 0.05). In general, complementation was more efficient if the complementing genes were encoded by
the non-mobilizable plasmid pMMB207ab-Km-14 rather than by the mobilizable plasmids pMMB207 or pMMB207ab. Moreover, the wild-type strain JR32 transformed with pMMB207ab was phagocytosed less efficiently than JR32 transformed with pMMB207ab-Km-14 ($P = 0.02$). These findings are consistent with the idea that conjugal nucleoprotein complexes compete with effector proteins as substrates of the Icm/Dot transport

machinery during phagocytosis as well as in later steps of intracellular multiplication (Segal and Shuman, 1998b).

Differentiated HL-60 cells are not very efficient phagocytes (see below; data not shown) and, therefore, the cells were routinely infected with L. pneumophila at a high multiplicity of infection (MOI) of 100. However, wild-type L. pneumophila were also more efficiently phagocytosed than icmT mutant bacteria at lower MOIs of 5–50 bacteria per macrophage (Fig. 1C).

The gentamicin sensitivity of L. pneumophila wild-type strain JR32 and the icmT mutant GS3011 was compared by incubating the strains with different concentrations of gentamicin for 1 h. No significant differences in the susceptibility towards gentamicin were found, ruling out the possibility that the apparent difference in phagocytic efficiency between wild-type and icm/dot mutant strains was caused by increased drug sensitivity of the mutant strains (data not shown).

We considered the possibility that the decreased number of viable icm/dot mutant bacteria resulted from more rapid killing of the latter by the HL-60 cells within the standard 1 h infection time. To test this idea, gentamicin-treated infected cells were lysed as early as 15 min after infection. At this time point, about five times fewer icmT mutant bacteria were recovered from the cells compared with wild-type strain JR32 \((P=0.001)\), and the defect for phagocytosis was complemented by supplying plasmid-encoded icmT (data not shown). If phagocytosis was blocked with cytochalasin B, about 15 times more bacteria were killed within 15 min \((P=0.002)\), thus validating the short incubation time with gentamicin. From these results, it seems unlikely that the differences in viable wild-type and icm/dot mutant bacteria result from more efficient killing of the mutants.

The apparent efficiency of phagocytosis varied to some degree between independent experiments (Fig. 1). After differentiation, the HL-60 cells did not always adhere similarly to the wells and, consequently, different numbers of infected cells were left after the washing steps (see Experimental procedures). Importantly, however, in 10 independent experiments performed in triplicate, about 10 times more intracellular viable wild-type L. pneumophila were recovered compared with icm/dot mutant bacteria (data not shown).

In an independent approach, we used fluorescence microscopy to quantify phagocytosis of GFP-expressing wild-type or icm/dot mutant L. pneumophila directly. Infected HL-60 cells were washed and fixed 20 min after infection, and extracellular bacteria were immunolabelled with a rhodamine-conjugated, serotype-specific anti-L. pneumophila antibody. Thus, intracellular (green) bacteria could easily be distinguished from extracellular (yellow; superimposition of green and red) bacteria (Fig. 2A). Wild-type and icm/dot mutant strains grown to stationary phase were examined by fluorescence microscopy for GFP expression before infections. Although \(>95\%\) of wild-type bacteria and icm/dot mutants expressed GFP, individual bacteria showed some differences in fluorescence intensity. Thus, more faintly green fluorescent bacteria might appear red in the overlay fluorescence image. In addition, the green and red fluorescence of some unattached bacteria did not co-localize in the overlay image, as these bacteria moved during the time required to acquire two image series of 40 sections with different fluorescence filters.

Compared with wild-type strain JR32, 10–20 times fewer icm/dot mutants \(\text{icmT}^-\) and \(\text{dotA}^-\) were scored within differentiated HL-60 cells \((P<0.03)\). The wild-type strain was phagocytosed about 10 times more efficiently than an icmT mutant strain, regardless of whether the bacteria had been grown to stationary phase on agar plates or in liquid culture (data not shown). The icmN mutant was phagocytosed about two times less efficiently than wild-type bacteria. As expected, the tphA mutant was phagocytosed as efficiently as wild type, and cytochalasin D severely diminished the uptake of wild-type bacteria.

Interestingly, individual macrophages infected with JR32 phagocytosed large numbers of bacteria (up to 60 bacteria per macrophage), resulting in a population of highly infected cells that was not observed in infections with the icmT mutant (Fig. 2C) or the icmS, -R, -Q, -P, -O, -W and dotB and -A mutants (data not shown). Furthermore, JR32 infected more HL-60 macrophages (31 \(\pm\) 6\%) than the icmT mutant (10 \(\pm\) 7\%) or the other icm/dot mutants.

Similar results were obtained using flow cytometry to analyse HL-60 cells infected with GFP-expressing L. pneumophila strains. Compared with HL-60 cells infected with the wild-type strain JR32, two to three times fewer cells infected with icm/dot mutant strains \(\text{icmT}^-\) and \(\text{dotB}^-\) showed a fluorescence intensity higher than the intrinsic fluorescence of uninfected cells (data not shown).

We also compared phagocytosis of GFP-expressing JR32 or icmT mutant bacteria by murine J774A.1 macrophages. Using fluorescence microscopy, these cells were found to phagocytose approximately three times more wild-type L. pneumophila than icmT mutants (data not shown).

In summary, the above experiments demonstrate that L. pneumophila icm/dot mutants are taken up less efficiently by human and mouse macrophages, indicating that the secretion system encoded by these genes governs the initial interaction of the bacterium with its host cell.

**Human monocyte-derived macrophages phagocytose icm/dot mutants less efficiently than wild-type L. pneumophila**

We next addressed the question whether L. pneumophila
icm/dot mutants are defective for uptake not only by macrophage cell lines but also by primary macrophages. Human monocytes were isolated from buffy coats and matured into macrophages to yield human monocyte-derived macrophages (HMMΦ). Within these host cells, the wild-type strain JR32 replicated, and icm mutants (icmT, -S) were killed (data not shown). To test whether the phagocytic efficiency of HMMΦ is affected by the L. pneumophila icm/dot genes, the macrophages were infected with either the GFP-expressing wild-type strain JR32 or icm/dot mutants, and intracellular bacteria were counted by fluorescence microscopy (Fig. 3A) as described above. At 20 min after infection, approximately eight times (icmT, -S, -R), four times (icmQ), 15 times (icmP, -O) or 35 times (icmW) fewer intracellular icm mutants were phagocytosed compared with wild-type bacteria (Fig. 3B; P < 0.013). The dotB and icmN mutant strains were phagocytosed by HMMΦ about two times less efficiently than wild-type, and the tphA mutant was not impaired for phagocytic uptake. Cytochalasin D-treated

![Image of fluorescence microscopy](image_url)

**Fig. 2.** Fluorescence microscopy of HL-60 macrophages infected with wild-type and icm/dot mutant L. pneumophila. 
A. Fluorescence micrographs of differentiated HL-60 cells infected for 20 min at an MOI of 150 with GFP-expressing wild-type bacteria (JR32) in the absence and presence of cytochalasin D or with an icmT mutant (LELA4086). Extracellular bacteria were labelled with a rhodamine-conjugated antibody. Z-section images of infected cells were merged into one layer, and either red and green fluorescence images (bottom) or fluorescence images and a brightfield picture (top) were overlain. Thus, intracellular bacteria are labelled green, extracellular bacteria are labelled yellow (red and green superimposed). Bar represents 10 μm. 
B. icm/dot mutants are defective for efficient phagocytosis by HL-60 cells. Intracellular wild-type bacteria (JR32), icm/dot mutants (LELA4086, GS3001, GS3012, LELEA3463, GS3002, GS3003, GS3007, LELE2883, LELE3118, LM1445) or a tphA mutant strain (MW627) were counted within 100 randomly selected macrophages. Mean and standard deviation of three independent experiments are shown. 
C. Wild-type and icmT mutant bacteria are distributed differently within HL-60 cells, yielding a population of highly infected cells (12–60 bacteria per macrophage) specific for the wild-type strain. Individual wild-type and icmT mutant bacteria were counted within 100 HL-60 cells and plotted against the number of macrophages containing the same number of intracellular bacteria. The sum of three independent experiments is shown. 

HMM\(\Phi\) phagocytosed < 1% of wild-type \(L.\) pneumophila compared with untreated cells \((P = 0.008)\).

Strikingly, the distribution of wild-type and \(icmT\) mutant bacteria within HMM\(\Phi\) was different (Fig. 3C). Most HMM\(\Phi\) infected with the \(icmT\) mutant strain contained only one or a few bacteria per macrophage. In contrast, many HMM\(\Phi\) infected with wild-type \(L.\) pneumophila contained several bacteria per macrophage, and a population of heavily infected macrophages (16–70 bacteria per macrophage) was only observed for HMM\(\Phi\) infected with wild-type bacteria. The population of highly infected macrophages was also absent from infections with the \(icmS\), -\(R\), -\(Q\), -\(P\), -\(O\) and -\(W\) mutant bacteria (data not shown). Significantly more HMM\(\Phi\) were infected by wild-type \(L.\) pneumophila than by the \(icmT\) mutant (64 ± 12% or 35 ± 3% respectively; \(P = 0.03\)) or other \(icm\)
mutants. As expected, HMMΦ were more efficient phagocytes than HL-60 cells and phagocytosed approximately three to four times more wild-type as well as icm/dot mutant bacteria (Figs 2B and 3B).

HMMΦ were infected with GFP-expressing wild-type or icmT mutant bacteria at different MOIs (Fig. 3D). At an MOI of 5 or 10, wild-type as well as icmT mutant bacteria were phagocytosed with similar efficiency, and the overall uptake of bacteria was very low (10–15 bacteria per 50 macrophages). At an MOI of 25, however, a threshold was observed, at which the difference in phagocytosis between wild type and the icm mutant became significant (P = 0.02). At MOIs from 25 to 100, the number of intracellular bacteria increased (up to 600 bacteria per 50 macrophages), whereas the ratio of wild-type to icmT mutant bacteria remained about 6–15.

The lvh genes complement a dotB mutant for efficient phagocytosis

The dotB and icmE genes show sequence homology to two genes within the L. pneumophila lvh locus, encoding a type IV secretion system distinct from the icm/dot-encoded apparatus (Segal et al., 1999). The Lvh system is dispensable for intracellular growth of L. pneumophila in HL-60 cells and A. castellanii. Furthermore, deletion of lvh, dotB or icmE individually has only a minor effect on conjugation. However, the lvh–dotB or lvh–icmE double mutants conjugate five orders of magnitude less efficiently (Segal et al., 1999). In order to test whether the lvh locus is involved in phagocytosis, we infected HMMΦ with GFP-expressing lvh, lvh–dotB or lvh–icmE mutant strains (Fig. 4). Whereas the lvh mutant was phagocytosed similarly to the wild-type and dotB mutant strains, phagocytosis of the lvh–dotB mutant was severely diminished (P = 0.01). The icmE mutant and, consequently, the lvh–icmE mutant was also impaired for phagocytosis. As both the lvhB11 and dotB genes share homology with the large family of virB11 ATPases, these results suggest that lvhB11 can substitute for the dotB gene not only for conjugation but also for a virulence-related trait such as phagocytic efficiency.

Wild-type and icmT mutant L. pneumophila adhere similarly and compete for binding to HMMΦ, and uptake is triggered in trans

The differences in phagocytic efficiency between L. pneumophila wild type and icm/dot mutants might result from either decreased adherence of the mutants to the host cell or a defect in bacterial signalling. We chose HMMΦ to analyse binding of L. pneumophila to macrophages, as L. pneumophila was found to adhere well to these cells (Fig. 3A). The HMMΦ were treated with cytochalasin D to block phagocytosis and infected with either GFP-expressing wild-type or icmT mutant strains. Extracellular bacteria were labelled with a rhodamine-conjugated anti-L. pneumophila antibody. Similar numbers of extracellular bacteria were counted on HMMΦ infected with either wild-type or icmT mutant bacteria (Fig. 5A, ‘single strains’). Therefore, the differences in phagocytic efficiency between wild-type and icmT mutant bacteria cannot be accounted for by differences in adherence. Equal numbers of bacteria were also counted in co-infection experiments with unlabelled wild-type and GFP-expressing icmT bacteria (Fig. 5A, ‘competition’). Furthermore, compared with infections with one bacterial strain, 50% fewer wild-type and icmT mutant bacteria adhered to the macrophages in the co-infections (P = 0.04). These results indicate that wild-type and icmT mutant bacteria adhering to macrophages compete for the same receptor(s) on HMMΦ.

In additional experiments, adherence of wild-type or icm/dot mutant strains to HL-60 cells or HMMΦ was compared by determining colony-forming units (cfu). Cytochalasin D-treated macrophages were infected with bacteria and, after removing non-adherent bacteria by washing, the cells were lysed, and cfu were determined. Similar numbers of icm/dot mutants (icmT, -S, -R, -Q, -P, -O, -N, -M, -E, -G, -C, -D, -J, -B, tphA, -F, dotB, -A, icmV, -W, -X) and wild-type L. pneumophila were recovered in this assay, indicating that similar numbers of wild-type and icm/dot mutant bacteria adhered to cytochalasin D-treated macrophages (data not shown).

To test whether stimulation of phagocytosis by wild-type L. pneumophila would promote phagocytosis of an icmT mutant strain in a co-infection, GFP-expressing icmT mutant bacteria were used to infect HMMΦ simultaneously with either unlabelled wild-type or unlabelled icmT mutant bacteria. The MOI of the unlabelled strains was kept constant at 100, and GFP-expressing icmT was used at two different MOIs (Fig. 5B). At an MOI of 50 (P = 0.007) or 125 (P = 0.032), about two times more GFP-expressing icmT bacteria were phagocytosed in the presence of unlabelled JR32 compared with co-infections with the unlabelled icmT mutant. Therefore, wild-type L. pneumophila promotes the phagocytosis of icmT mutant bacteria, suggesting that secreted effector molecules are involved. Given that wild-type and icmT mutant bacteria adhere similarly to macrophages and compete for the same receptor(s), this result argues that, independently of adherence, wild-type L. pneumophila promotes phagocytosis by secreting (an) effector(s).

Acanthamoeba castellanii phagocytoses icm/dot mutants less efficiently than wild-type L. pneumophila

Growth of L. pneumophila in its natural host A. castellanii
requires the icm/dot genes (Segal and Shuman, 1999a). To analyse whether these genes are also involved in phagocytosis of L. pneumophila by A. castellanii, the amoebaæ were infected with either the GFP-expressing wild-type strain JR32 or icm/dot mutants. Extracellular bacteria were labelled with a rhodamine-conjugated anti-L. pneumophila antibody, and intracellular bacteria were counted by fluorescence microscopy as described above for macrophages. At 20 min after infection, about three to four times fewer icm mutants (icmT, -S, -P, -O) were phagocytosed compared with wild-type bacteria (Fig. 6A, P < 0.05). Wild-type L. pneumophila was more efficiently phagocytosed than icmT mutant bacteria at MOIs as low as 1 or 5 (Fig. 6B). These results indicate that the icm/dot genes are involved in the initial steps of the interaction between L. pneumophila and amoebaæ. Moreover, phagocytosis of L. pneumophila by amoebaæ or macrophages seems to be mechanistically similar.

Discussion

Intracellular multiplication of L. pneumophila depends on several properties of the organism. The ability of the bacteria to survive the antimicrobial defences of phagocytic host cells is the first challenge that must be met. Phagocytosis of most other organisms results in the formation of a phagosome that rapidly fuses with lysosomes, and the internalized organism is either killed or can tolerate the inhospitable environment of the phagolysosome. Legionella and some other intracellular pathogens have the ability to prevent phagosome—lysosome fusion (Sinai and Joiner, 1997; Garcia-del Portillo, 1999). Genetic analysis has revealed that the icm/dot gene products are required for preventing phagosome—lysosome fusion, intracellular multiplication and organelle trafficking (Kirby and Isberg, 1998; Segal and Shuman, 1998a; Vogel and Isberg, 1999). Very rapidly after internalization, it is possible to distinguish whether Legionella-containing phagosomes have either fused or not fused with lysosomes. Phagosomes that have not fused provide a hospitable environment in which the bacteria multiply within a vacuole and eventually kill the host cell. Legionella contained in fused phagolysosomes, however, are unable to grow and are destroyed. Approximately 85% of the phagosomes containing viable wild-type bacteria do not fuse and are called ‘specialized’ or ‘Legionella-specific’ phagosomes. In contrast, > 90% of the phagosomes containing either dead bacteria or viable icm/dot mutant bacteria fuse and form phagolysosomes (Horwitz, 1987; Roy et al., 1998; Wiater et al., 1998). Thus, the ability of the bacteria to influence the fusion properties of the phagosome is key to the ultimate fate of the bacteria.

It has been proposed that the icm/dot complex functions to deliver effectors to the host cell (Kirby and Isberg, 1998; Segal and Shuman, 1998a). One presumed function of the effectors is to modify the process of phagocytosis. The experiments described here tested the idea that L. pneumophila influences the process of phagocytosis itself. We found that wild-type L. pneumophila are taken up by phagocytic host cells more efficiently than icm/dot mutants (Figs 1A, 2B, 3B and 6A). Not only was the net number of bacteria taken up higher, but wild-type bacteria were found in very high numbers inside individual phagocytes (Figs 2C and 3C). These heavily infected cells were not detected in infections with icm/dot mutants. The defect in phagocytosis of an icmT mutant was not the result of a defect in adherence (Fig. 5A) and, because similar results were obtained using mammalian or protozoan host cells, these effects appear to be independent of the receptor(s) used by the bacteria to adhere to host cells. The adherence receptor used by L. pneumophila does not seem to affect its intracellular survival profoundly, as the bacterium multiplies within phagocytes after being taken up under different opsonizing or non-opsonizing conditions (Horwitz and Silverstein, 1981; Nash et al., 1984; Payne and Horwitz, 1987; Marra et al., 1990; Cirillo et al., 1999). Interestingly, in co-infection experiments, the presence of wild-type L. pneumophila could promote increased uptake of an icmT mutant in trans (Fig. 5B). This finding provides evidence that the Icm/Dot transport system indeed

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**Fig. 4.** The lvh genes complement a dotB mutant for efficient phagocytosis. HMMB were infected at an MOI of 100 with GFP-expressing wild-type bacteria (JR32) or icm/dot (LELA2883, LELA4432), lvh (GS28G) or lvh–icm/dot (LELA2883-28, LELA4432-28) mutant strains. Extracellular bacteria were labelled with a rhodamine-conjugated antibody, and intracellular bacteria were counted within 50 randomly selected macrophages. The lvh genes, encoding a type IV secretion system distinct from the icm/dot-encoded apparatus, functionally complemented a dotB mutant for efficient phagocytosis. Mean and standard deviation of triplicates performed in two independent experiments are shown.
secretes (an) effector(s). In accordance with this result, wild-type \textit{L. pneumophila} was recently found to rescue a \textit{dotA} mutant if the two strains were fed to the macrophages simultaneously but not sequentially, and the wild-type strain supported intracellular multiplication of \textit{dotA} mutant bacteria residing in the same phagosome but not in distinct phagosomes (Coers \textit{et al.}, 1999).

Upregulation of phagocytosis by Icm/Dot-secreted effectors might immediately lead to either a \textit{Legionella}-specific or a non-specific phagosome that is subsequently modified by wild-type but not \textit{icm/dot} mutant \textit{Legionella}. In a ‘concerted’-type model, a \textit{Legionella} effector triggers increased uptake into a specialized type of phagosome that does not fuse with lysosomes (Fig. 7, pathway A). In the absence of the effector, normal phagocytosis results in the formation of a phagosome that does fuse with lysosomes, eventually leading to an acidic phagolysosome, in which \textit{icm/dot} mutants are degraded (Sadosky \textit{et al.}, 1993; Purcell and Shuman, 1998; Solomon \textit{et al.}, 2000). Alternatively, in a ‘sequential’-type model, \textit{L. pneumophila} could upregulate phagocytosis by altering aspects of cell physiology related to phagocytosis (pathway B). This might occur by increasing the rate of membrane recycling or stimulating the turnover of actin filaments. In this case, although the quantity of phagosome formation is altered, the quality of wild-type and mutant phagosomes is the same. The effector(s) would then modify the newly formed phagosome. Although the concerted model makes an attractive functional connection between phagosome formation and phagosome fate, we cannot distinguish these models at the present time.

Many of the experiments reported here were carried out by infecting host cells with MOIs of up to 100 bacteria per cell. The fact that the differences in uptake between wild-type and mutant \textit{Legionella} were more apparent at the higher MOI raised several interesting issues. First, do the high MOIs used kill the host cells by the immediate cytotoxicity phenomenon (Husmann and Johnson, 1994; Kirby \textit{et al.}, 1998)? Our results indicate that, not only are the cells alive enough to carry out phagocytosis, but also the permeability barrier of the infected cells seems to be intact, as the anti-\textit{Legionella} antibodies used to distinguish intracellular from extracellular bacteria are excluded from the cells (Figs 2A and 3A). Secondly, is the high MOI infection ‘physiologically relevant’? There are several possible answers because the exact conditions of infection of protozoa in the wild and alveolar macrophages in human lungs are not known. After a successful infection, large numbers of newly replicated bacteria are released from lysed host cells. If new host cells are in the same vicinity, a high MOI would indeed occur. In the case of Legionnaires’ disease, whether or not the bacteria alone or bacteria within infected protozoa (e.g. \textit{Hartmannella vermiformis}) are the infectious agent has been debated.

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\textbf{Fig. 5.} Wild-type and \textit{icmT} mutant bacteria adhere similarly and compete for binding to \textit{HMM\textsuperscript{F}}, and uptake is triggered \textit{in trans}. A. \textit{HMM\textsuperscript{F}} were treated with cytochalasin D and infected at an MOI of 100 with either a GFP-expressing wild-type strain (JR32, closed bars) or a GFP-expressing \textit{icmT} mutant strain (GS3011, open bars) alone (‘single strains’) or synchronously with unlabelled wild-type and GFP-expressing \textit{icmT} mutants at an MOI of 100 each (‘competition’). Extracellular bacteria were labelled with a rhodamine-conjugated antibody and counted on 50 randomly selected macrophages. Similar numbers of wild-type and \textit{icmT} mutant bacteria adhered to \textit{HMM\textsuperscript{F}} and, in co-infections, the strains competed for the same adherence receptor(s). Mean and standard deviation of quintuplets (single strains) or quadruplets (competition) performed in three or two independent experiments, respectively, are shown. B. \textit{HMM\textsuperscript{F}} were co-infected with a GFP-expressing \textit{icmT} mutant strain (GS3011, MOI 50 or 125) and either unlabelled wild-type (JR32, closed bars) or unlabelled \textit{icmT} mutant bacteria (GS3011, open bars) at an MOI of 100 each. After labelling extracellular bacteria with a rhodamine-conjugated antibody, intracellular bacteria were scored within 50 randomly selected macrophages. Twice as many GFP-expressing \textit{icmT} bacteria were phagocytosed in the presence of JR32 compared with co-infections with the \textit{icmT} mutant. Results are the mean and standard deviation of three independent experiments.
Infected protozoa harbour many hundreds of organisms and could release them inside alveoli. During experimental aerosol infection of animals, suspensions of $10^8$ to $10^9$ organisms ml$^{-1}$ are used to nebulize the air in a closed compartment (Marra et al., 1992), and the exact MOI with respect to alveolar macrophages in the animal lungs is difficult to quantify. Finally, are the experiments described here comparable with the data presented here. Our results presented here represent the first report of a bacterial secretion system (Icm/Dot) promoting phagocytosis by professional phagocytes. In contrast, inhibition of phagocytosis by pathogenic bacteria using a type III secretion system is a well-known phenomenon. Pathogens avoiding phagocytosis by this means include *Yersinia* spp. (Lian et al., 1987), enteropathogenic *Escherichia coli* (Goosney et al., 1999) and *Pseudomonas aeruginosa* (reviewed by Ernst, 2000). Only recently, *Helicobacter pylori* has been reported to resist phagocytosis by macrophages and polymorphonuclear neutrophils using a type IV secretion system (Allen et al., 2000; Ramarao et al., 2000). Interestingly, within 2 h after internalization, virulent *H. pylori* type I (harbouring the *cag* pathogenicity island), but not type II strains, induced the formation of homotypic fusions of phagosomes, yielding ‘megasomes’ with multiple viable bacteria (Allen et al., 2000). The *H. pylori* effector(s) affect phagocytosis in *trans* as, after preinfection of macrophages, the pathogen prevented the internalization of latex beads as well as opsonin-independent phagocytosis of *Neisseria gonorrhoeae* (Ramarao et al., 2000). Similarly, extracellular wild-type *L. pneumophila* triggered the uptake of *icmT* mutant bacteria in a co-infection experiment in *trans* (Fig. 5B). However, once internalized, the *L. pneumophila* effector(s) act only in cis (within a discrete phagosome) rather than perturbing endocytosis in general (Coers et al., 1999). The different effects of *L. pneumophila* and *H. pylori* on phagocytosis represents yet another example of the notion that the effectors of conserved secretion systems may perform opposite functions and are tailored for the unique needs of specific pathogens. Other plant and animal pathogens harbouring type IV secretion systems include *A. tumefaciens*, *Bordetella pertussis*, *Rickettsia prowazekii* and *Brucella* spp. (Christie and Vogel, 2000). However, for *L. pneumophila* and most other bacteria, the effector(s) exerting virulence functions have not yet been identified.

### Experimental procedures

#### Reagents

Bacto agar, proteose peptone and yeast extract were purchased from Difco Laboratories. Phosphate-buffered saline (PBS) was from BioWhittaker. RPMI-1640 medium (RPMI), L-glutamine (Gln) and Dulbecco’s phosphate-buffered salt solution (DPBS) were from Mediatech. Fetal calf serum (FCS) was purchased from Sigma-Aldrich, and normal human AB serum (NHS) was from Gemini Bio-Products. Ficoll-Paque Plus and Percoll were obtained from Amersham Pharmacia.
The rabbit anti-*L. pneumophila* Philadelphia 1 rhodamine-conjugated antibody was purchased from m-Tech, and the mounting media Citifluor no. 1 was from Ted Pella. All other reagents were from Sigma-Aldrich.

**Bacteria and plasmids**

The bacterial strains used in this study are listed in Table 1. *L. pneumophila* was grown on CYE agar plates (Feeley et al., 1979) or AYE broth (Horwitz and Silverstein, 1983). Antibiotics were used at the following concentrations: chloramphenicol (5 µg ml⁻¹); gentamicin (10 µg ml⁻¹); and kanamycin (50 µg ml⁻¹).

Strain GS3012 was constructed by replacing the *icmR* gene with a kanamycin resistance cassette by allelic exchange with plasmid pGS-Le-38-Km-1 as described before (Segal and Shuman, 1997). Plasmid pGS-Lp-32 (Segal and Shuman, 1997) was digested with *Eco*RV, and a 1721 bp fragment (236–1957 in the sequence) containing the *icmTSR* genes was cloned into *pUC-18* digested with *Hin*clII to generate pGS-Lp-38. Subsequently, the kanamycin resistance cassette was cloned into the *Dra*III and *Sac*I sites (located in the *icmR* gene) of pGS-Lp-38 to generate pGS-Lp-38-Km-1. This plasmid was digested with *Pvu*II, and the fragment containing the disrupted *icmR* gene was cloned into the *Eco*RV site of the allelic exchange vector pLAW-344 (Wiater et al., 1994) to generate pGS-Le-38-Km-1.

Strain LM1445 was constructed by replacing the *icmW* gene with a gentamicin resistance cassette by allelic exchange with plasmid pLM804 as described above for strain GS3012. Plasmids were introduced into the *L. pneumophila* strains by electroporation or conjugation as described previously (Segal and Shuman, 1998b). The plasmids used in this study are listed in Table 2. Plasmid pGS-GFP-04 was constructed by digesting plasmid pGFP-mut-2 (Cormack et al., 1996) with *Eco*RI and *Hin*dIII and cloning the released fragment into the same sites of pMMB207. In this vector, the *gfp* gene is under the control of the *P*<sub>tac</sub> promoter. Plasmid pGS-BCD-05 was constructed by screening the *L. pneumophila* pLAFR1 library (Szeto and Shuman, 1990) using a fragment from the plasmid pAB-11 as a probe. Plasmid pAB-11 harbours the group V *Eco*RI fragment from strain LELA2883 that contains an insertion in the *dotB* gene (Sadosky et al., 1993). Several positive...
cosmids were identified. One of them (pGS-cos-4) was digested with BamHI, and a 12 kb fragment containing the dotBCD genes was subcloned into pUC-18 to generate pGS-BCD-01. The insert from this plasmid was cloned using BamHI sites into pMMB207α·Km-14 to generate pGS-BCD-05.

**Cell culture of macrophages and A. castellanii**

The human leukaemia cell line HL-60 (ATCC CCL-240), murine J774A.1 macrophages (ATCC TIB-67) and human monocyte-derived macrophages (HMMF) were grown in a humidified atmosphere of 5% CO$_2$ at 37°C in RPMI supplemented with 2 mM Gln and 10% (v/v) heat-inactivated (56°C, 30 min) FCS (HL-60, J774A.1) or 10% (v/v) heat-inactivated NHS (HMMF) respectively.

Peripheral blood monocytes (PBMs) were isolated from buffy coats obtained through the New York Blood Center. The PBMs were isolated by Ficoll-Paque Plus and Percoll gradient centrifugations as described previously (Cozzi et al., 1984; Hilbi et al., 1997). Briefly, 38 ml of buffy coat diluted 1:5 with PBS was placed on 11.4 ml of Ficoll-Paque Plus and centrifuged at 900 g for 30 min at room temperature. To remove platelets, 5 ml of washed leucocytes was placed on 15 ml of heat-inactivated FCS and centrifuged at 200 g for 15 min. The leucocytes were resuspended in 14 ml of RPMI containing 2 mM Gln and 10% (v/v) heat-inactivated FCS, placed on the same volume of 46% Percoll and centrifuged at 600 g for 30 min. Washed PBMs were resuspended at a density of $10^6$ cells ml$^{-1}$ in RPMI containing 2 mM Gln and 10% (v/v) heat-inactivated NHS and matured in Teflon beakers for at least 5 days to yield HMMF. In some experiments, the PBMs were matured directly on sterile coverslips in 24-well plates.

*Acanthamoeba castellanii* (ATCC 30234) was grown in 30 ml of proteose peptone–yeast extract–glucose medium (PYG) in a 75 cm$^2$ tissue culture flask at 28°C (Moffat and Tompkins, 1992). The amoebeae were split once a week, fed with PYG 24 h before use and prepared for infections as described previously (Segal and Shuman, 1999a). Briefly, non-adherent amoebeae were removed by gently shaking the flask and replacing the medium with fresh PYG. Adherent amoebeae were detached by tapping the flask vigorously. The resulting suspension was centrifuged for 10 min at 220 g, resuspended at a concentration of $4 \times 10^5$ amoebeae ml$^{-1}$ and used to plate on sterile coverslips (see below) or to inoculate 30 ml of fresh PYG (50–100 μl 30 ml$^{-1}$).

**Gentamicin protection assay**

HL-60 cells (1 $\times 10^5$ per well, 96-well plate) were differentiated into macrophages by incubation for 48 h with 100 ng ml$^{-1}$ phorbol 12-myristate 13-acetate (PMA) in RPMI supplemented with 2 mM Gln and 10% (v/v) fresh NHS (RGN medium). Before the infection, the HL-60 cells were placed for 1 h in RGN medium containing cytochalasin D (10 μM) where indicated. The bacterial strains used for the infections were grown on CYE agar plates for 2–3 days and harboured the plasmid pMMB207 (icmD, -J, -B), pMMB207α·Km-14 (icmR, -Q, -M, -L, -K, -E, -X, dotA), pMMB207α·Km-14 (icmT, -S, -P, -O, -N, -G, -C, -F, tphA, dotB) or the corresponding complementing plasmids (Table 2). Bacteria suspended in RGN medium (OD$_{600}$ of 0.3 $\times 5 \times 10^8$ cells ml$^{-1}$) were added to the plates with amoebeae to a final concentration of $10^8$ cells ml$^{-1}$ and incubated for 24 h at 28°C. After incubation, the plates were washed 3 times with RGN medium and 1 ml of 10% (v/v) heat-inactivated NHS was added to each well. The plates were incubated for 1 h and then washed 3 times with RGN medium. The amoebeae were then removed by 5 min of centrifugation at 1,000 g, and the amoebeae-free supernatant was harvested and plated on CYE agar plates. The number of amoebeae recovered was determined by colony-forming unit (CFU) count. The results were expressed as the number of CFU recovered per amoebeae added to the plates. The experiment was performed in triplicate and the results were expressed as the mean ± standard deviation.

**Table 1. *L. pneumophila* strains.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and features</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS286</td>
<td>Jr32 lhr::Gm</td>
<td>Segal et al. (1999)</td>
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<td>GS3001</td>
<td>Jr32 icmS3001::Km</td>
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<td>Jr32 icmO3002::Km</td>
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<td>Jr32 icmN3007::Km</td>
<td>Segal et al. (1998)</td>
</tr>
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<td>GS3008</td>
<td>Jr32 icmM3008::Km</td>
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</tr>
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<td>GS3009</td>
<td>Jr32 icmL3008::Km</td>
<td>Segal et al. (1998)</td>
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<td>GS3010</td>
<td>Jr32 icmK3010::Km</td>
<td>Segal et al. (1998)</td>
</tr>
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<td>GS3011</td>
<td>Jr32 icmT3011::Km</td>
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<td>GS3012</td>
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<td>This study</td>
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<td>LM1445</td>
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<td>MW656</td>
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<td>Purcell and Shuman (1998)</td>
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bacteria ml$^{-1}$) were centrifuged onto the HL-60 cells (700 g, 10 min) at an MOI of 100, and the infected cells were incubated for another 10 min at 37°C. To kill extracellular bacteria, the infected macrophages were washed twice with PBS containing 0.1 mg ml$^{-1}$ gentamicin and incubated for another 40 min in RGN containing 0.1 mg ml$^{-1}$ gentamicin. At 60 min after infection, the infected macrophages were lysed with 0.2 ml H$_2$O for 10 min, and aliquots of the lysates were plated on CYE agar plates. Intracellular viable bacteria were counted as cfu. As an input control, dilutions of the bacterial suspensions in RGN containing 0.1 mg ml$^{-1}$ gentamicin for 1 h, or in buffer, respectively, and incubated for 1 h with a rhodamine-conjugated rabbit anti-\textit{L. pneumophila} Philadelphia 1 antibody, diluted 1:100 in blocking buffer. After washing five times, the coverslips were mounted onto microscopy slides using the mounting medium Citifluor no. 1.

\textbf{Immunofluorescence staining and fluorescence microscopy}

For immunofluorescence, the phagocytes were seeded on sterile coverslips in 24-well plates at the following densities: HL-60 cells (1.5 x 10$^6$ per well, differentiated for 48 h with PMA), HMM$^\Phi$ (0.25 x 10$^6$ per well, overnight) or \textit{A. castellanii} (0.25 x 10$^6$ per well, overnight). Before infection, the phagocytes were washed two or three times with RPMI containing 2 mM Gln (HL-60, HMM$^\Phi$) or Ac buffer (\textit{A. castellanii}) (Moffat and Tompkins, 1992), respectively, and placed in 0.5 ml of RGN (HL-60, HMM$^\Phi$) or Ac buffer (\textit{A. castellanii}), containing cytochalasin D (10 $\mu$M, 1 h) where indicated.

The \textit{L. pneumophila} strains used for the infections were grown to stationary phase either on CYE plates for 2–3 days (HL-60 cells) or in AYE broth for about 24 h (HMM$^\Phi$, \textit{A. castellanii}). Before infection, the bacteria were suspended or diluted, respectively, in RGN (HL-60, HMM$^\Phi$) or Ac buffer (\textit{A. castellanii}), and the phagocytes were infected with bacteria at the MOIs indicated. In all experiments, the bacteria were centrifuged onto the phagocytes (700 g, 10 min) to synchronize infection and incubated for another 10 min at 37°C (macrophages) or 28°C (\textit{A. castellanii}). Infected macrophages were washed five times with DPBS and fixed for 15 min with DPBS containing 3.7% formaldehyde. Infected \textit{A. castellanii} were washed five times with 20% DPBS (Kaiser \textit{et al.}, 1999) and fixed for 15 min with 20% DPBS containing 3.7% formaldehyde. The fixed cells were washed five times, and incubated for 30 min with 5% non-fat milk in DPBS or 20% DPBS (blocking buffer), respectively, and incubated for 1 h with a rhodamine-conjugated rabbit anti-\textit{L. pneumophila} Philadelphia 1 antibody, diluted 1:100 in blocking buffer. After washing five times, the coverslips were mounted onto microscopy slides using the mounting medium Citifluor no. 1.

\textbf{Statistical analysis}

Statistical analysis was performed by two-tailed Student’s \textit{t}-test for paired samples, taking \(P < 0.05\) as significant.

\textbf{Acknowledgements}

We wish to thank Steven Greenberg for critical reading of the manuscript, Dianne Cox and Jens Husemann for stimulating discussions, and Richard Friedman for help with the statistical analysis. We are also grateful to Laura M. Hales for constructing the \textit{icm}W mutant strain, and Carmen Rodriguez for superb technical assistance. Brendan Cormack and Stanley Falkow kindly provided plasmid pGFP-mut-2. This

\textbf{Table 2. Plasmids used in this study.}

<table>
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<th>Reference</th>
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<td>gfp in pMMB207</td>
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<td>Segal and Shuman (1998b)</td>
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</table>

\textit{a}. Used for complementation of the icm\textit{M} mutant strain GS3008.

\textit{b}. Used for complementation of the icm\textit{D} mutant strain LEELA 1205.
work was supported by a fellowship from the Swiss National Science Foundation to H.H. and NIH grant AI 23549 to H.A.S.

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