The Crohn’s disease-associated adherent-invasive *Escherichia coli* strain LF82 replicates in mature phagolysosomes within J774 macrophages

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Summary

Adherent-invasive *Escherichia coli* (AIEC) bacteria isolated from Crohn’s disease patients are able to extensively replicate within macrophages in large vacuoles. The mechanism by which AIEC bacteria survive within phagocytic cells is unknown. This report describes the maturation of AIEC LF82-containing phagosomes within J774 macrophages. LF82-containing phagosomes traffic through the endocytic pathway as shown by the sequential acquisition and loss of EEA1 and Rab7 and by accumulation of Lamp-1, Lamp-2 and cathepsin D. We demonstrated that AIEC LF82-containing phagosomes mature into active phagolysosomes where bacteria are exposed to low pH and to the degradative activity of cathepsin D. Finally, we showed that an acidic environment is necessary for replication of AIEC LF82 bacteria within J774 macrophages. Thus, evidence is provided that AIEC LF82 bacteria do not escape from the endocytic pathway but undergo normal interaction with host endomembrane organelles and replicate within acidic and cathepsin D-positive vacuolar phagolysosomes.

Introduction

Crohn’s disease (CD) is an inflammatory bowel disease of unknown aetiology in humans (Duchmann and Zeitz, 1999). CD has features that might be the result of a microbial process in the gut (Sartor et al., 1996; Elson, 2000; Podolsky, 2002; Shanahan, 2002). Some characteristic pathological elements of CD, including aphthous ulcers of the mucosa, mural abscesses, and macrophage and epithelioid cell granulomas also occur in well-recognized infectious disease such as shigellosis, salmonellosis and *Yersinia* enterocolitis, in which invasive-ness is an essential virulence factor of the bacteria involved (Zumla and James, 1996). However, these pathogenic bacteria have not been found associated with CD. *Escherichia coli* DNA was detected in 80% of microdissected granulomas of CD patients, which suggests a possible role for *E. coli* in CD lesions (Ryan et al., 2004). Besides, we recently reported that the ileal mucosa of 36.7% of CD patients is abnormally colonized by pathogenic *E. coli* strains termed AIEC for adherent-invasive *E. coli*, which are able to adhere to and to invade intestinal epithelial cells (Darfeuille-Michaud et al., 1998; 2004; Boudeau et al., 1999). They are also able to replicate extensively in large vacuoles within macrophages without triggering host cell death (Glasser et al., 2001; Darfeuille-Michaud et al., 2004).

Macrophages serve as the first line of defence by eliminating indesirable microorganisms. These professional phagocytic cells engulf bacteria within phagosomes that rapidly evolve into bactericidal organelles termed phagolysosomes. As they mature into digestive organelles, phagosomes progressively acidify and interact with the endosomal network and/or the biosynthetic pathway (Desjardins et al., 1994; Desjardins, 1995; Claus et al., 1998; Ullrich et al., 1999; Deretic et al., 2004; Becker et al., 2005). Two predominant models exist to explain the biogenesis of phagolysosomes: the ‘vesicle shuttle’ model, which supports that transport intermediates deriving from endocytic organelles are targeted to phagosomes, and the ‘kiss and run’ model, which proposes that phagosomes undergo transient and partial fusion with endocytic organelles (Desjardins et al., 1994; Desjardins, 1995; Gu and Gruenberg, 1999; Gruenberg, 2001; Harrison et al., 2003). This dynamic process is modulated by the sequential appearance and disappearance of proteins on the membrane. First, plasma membrane proteins, including the transferrin receptor (TfR), which initially compose the phagosome, disappear and are replaced sequentially by proteins present in early endosomes (e.g. early endosomes antigen 1 or EEA1, and Rab5 GTPase). The fol-
allowing event leads to the late endosome stage of maturation, characterized by the acquisition of specific markers such as the Rab7 GTPase and peripheral transmembrane glycoproteins Lamps. Finally, as the phagosomes continue to acidify and accumulate different Rabs, more Lamps, cathepsin D and other acid hydrolases, they develop lysosomal traits. In addition to this well-characterized endocytic pathway, cells possess another defensive mechanism against invading pathogens, the autophagic pathway (Mizushima et al., 2002; Reggiori and Klionsky, 2002; Yoshimori, 2004).

Inside host cells, certain intracellular pathogens control the fate of their membrane-bound compartments, and escape host degradation by interfering with the endocytic pathway or by infiltrating the autophagic route (Meresse et al., 1999; Dorn et al., 2002). Intravacular pathogens have evolved several different strategies of finding a successful intracellular replication niche. One strategy developed by Mycobacteria and Salmonella enterica serovar Typhimurium within phagocytic cells is the establishment of vacuoles specifically retaining or excluding proteins that govern phagosome maturation. Indeed, aberrant distribution of the EEA1/Rab5 and Rab7 GTPases has been correlated with virulence for these pathogens (Clemens and Horwitz, 1995; Via et al., 1997; Hashim et al., 2000; Knodler and Steele-Mortimer, 2003). A second strategy used by a subgroup of vacuolar pathogens such as Brucella abortus (Pizarro-Cerda et al., 1998a,b), Coxiella burnetii (Beron et al., 2002) and Porphyromonas gingivalis (Dorn et al., 2001) is to infiltrate the autophagic pathway of host cells. The transit through autophagic pathway allows B. abortus and Legionella pneumophila to gain access to the endoplasmic reticulum (ER) (Pizarro-Cerda et al., 1998a,b; Amer and Swanson, 2005).

The strategy used by AIEC bacteria to resist macrophage degradation is unknown. The aim of the present study was to define the biogenesis of the large phagosomes containing numerous live AIEC LF82 bacteria within J774 macrophages. Our results demonstrate that AIEC LF82-containing phagosomes transit along the classical endocytic pathway, and do not infiltrate the autophagic route. AIEC LF82 bacteria are able to survive and to replicate within a compartment harbouring hostile features of mature phagolysosomes including acid pH and active cathepsin D. Moreover, the acidic vacuolar environment is necessary for replication of AIEC LF82 bacteria within J774 macrophages.

Results

AIEC LF82-containing phagosomes rapidly acquire early endosomal markers

Experiments using an antibody directed against the early endocytic antigen 1, EEA1, were performed to investigate the interactions of AIEC LF82-containing phagosomes with early endocytic compartments within J774 macrophages. EEA1 was found on 52.8 ± 2.9% of LF82-containing phagosomes after 10 min of infection, and at this time point the protein appeared to be evenly distributed around bacteria-containing phagosomes (Table 1 and Fig. 1). After 20 min of infection, 30.0 ± 3.0% of the LF82-containing phagosomes were still EEA1-positive but this immunostaining decreased rapidly as at 60 min post-infection EEA1 was detected only on 17.1 ± 1.9% of LF82-containing phagosomes and less than 1.4 ± 0.5% of them were EEA1-positive at 24 h post-infection. These experiments show that EEA1 was delivered to and rapidly removed from LF82-containing phagosomes. The localization of EEA1 on AIEC LF82-containing phagosomes may represent a prerequisite for the subsequent acquisition of late endocytic characteristics.

AIEC LF82-containing phagosomes acquire features of late endosomes

In order to further determine the nature of the AIEC LF82-replicating compartment within J774 macrophages, immunofluorescence experiments were performed using antibodies directed against late endosomal specific markers, the Rab7 GTPase and the membrane glycoprotein Lamp-1. After 20 min of infection, 54.4 ± 2.9% of LF82-containing phagosomes were Rab7-positive (Table 1 and Fig. 2). The interaction between LF82-containing phagosomes and Rab7 was transient because at 60 min post-infection only 8.5 ± 0.9% of LF82-containing phagosomes were Rab7-positive. Lamp-1 was detected only on

| % of colocalization on AIEC LF82-containing phagosomes ± SEM |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| 10 min | 20 min | 60 min | 24 h |
| EEA1 | 52.8 ± 2.9 | 30.0 ± 3.0 | 17.1 ± 1.9 | 1.4 ± 0.5 |
| Rab7 | 7.5 ± 1.6 | 54.4 ± 2.9 | 8.5 ± 0.9 | 1.1 ± 0.8 |
| Lamp-1 | 21.7 ± 3.9 | 27.7 ± 5.2 | 67.1 ± 4.0 | 56.7 ± 2.4 |
| Cathepsin D | 2.8 ± 0.3 | 20.3 ± 4.2 | 34.6 ± 2.0 | 66.1 ± 2.2 |

a. For each point, at least 100 AIEC LF82-containing vacuoles were counted and scored for the presence or absence of markers. The results shown are the mean percentage of marker colocalization on AIEC LF82-containing phagosomes ± standard error of the mean (SEM). At least three independent experiments were performed.

b. J774 macrophages grown on coverslips were infected with GFP-expressing AIEC strain LF82 as described in Experimental procedures.

c. The endosomal/lysosomal markers were localized using goat antibodies against EEA1, chicken antibodies against Rab7, rat antibodies against Lamp-1 and rabbit antibodies against cathepsin D, followed by corresponding secondary antibodies and visualized by confocal microscopy.

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Fig. 1. The early endosome-associated antigen 1, EEA1, localizes on AIEC LF82-containing phagosomes immediately after infection. J774 macrophages were infected with GFP-expressing AIEC strain LF82 for 10 min and monolayers were then fixed immediately. Labelling of EEA1 was performed using goat antibodies to EEA1 and Alexa 594-conjugated donkey anti-goat IgG secondary antibodies.

Fig. 2. The Rab7 GTPase transiently localizes on AIEC LF82-containing phagosomes after 20 min of infection and is then removed at later times. J774 macrophages were infected with GFP-expressing AIEC strain LF82 for 20 min (20 min post-infection, p.i.) and then fixed immediately, or fixed after a further 40 min incubation period in fresh cell culture medium containing gentamicin (60 min p.i.). Labelling of Rab7 was performed using rabbit antibodies against Rab7 and secondary antibodies Alexa 594-conjugated donkey anti-rabbit IgG.
27.7% ± 5.2% of LF82-containing phagosomes after 20 min of infection (Table 1 and Fig. 3). At 60 min post-infection, 67.1% ± 4.0% of the bacteria-containing phagosomes were Lamp-1-positive. Taken together, these results demonstrate that during early time post-infection, LF82-containing phagosomes progressed from EEA1-positive compartments to Lamp-1-positive compartments, with a rapid loss of EEA1 and a transient acquisition of Rab7.

AIEC LF82-containing phagosomes harbour markers of phagolysosomes

At the end of the normal maturation, phagosomes possess lysosomal traits. They accumulate Lamps, continue to acidify, and acquire hydrolytic enzymes, such as cathepsin D, which are required for the efficient degradation of the phagosomal contents. Analysis of infected macrophages at 8 h and 24 h post-infection demonstrated that
AIEC LF82-containing phagosomes were positive for both Lamp-1 and Lamp-2 (Table 1, Figs 3 and 4B). AIEC LF82-containing phagosomes were further analysed by monitoring the presence of cathepsin D using an antibody directed against this luminal hydrolase (Fig. 4A). After 20 min of infection, 20.3% ± 4.2% of AIEC LF82-containing phagosomes were positive for cathepsin D. The percentage of LF82-containing phagosomes that

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**Fig. 4.** AIEC LF82 bacteria replicate within acid phagosomes harbouring characteristic markers of phagolysosomes. 
A. J774 macrophages were infected with GFP-expressing AIEC strain LF82 for 20 min and then incubated for 40 min (60 min post-infection, p.i.) or for 24 h (24 h p.i.) in fresh cell culture medium containing gentamicin. Labelling of cathepsin D was performed using rabbit anti-cathepsin D antibodies and Alexa 594-conjugated donkey anti-rabbit secondary antibodies.

B. J774 macrophages were infected with GFP-expressing AIEC strain LF82 for 20 min and then incubated for 24 h (24 h p.i.) in fresh cell culture medium containing gentamicin. Labelling of Lamp-2 was performed using rat antibodies to the murine Lamp-2 and Alexa 594-conjugated goat anti-murine secondary antibodies. Colocalization of LysoTracker Red DND-99 with GFP expressing AIEC LF82 bacteria was performed as described in Experimental procedures.

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are cathepsin D-positive increased with time and 66.1% ± 2.2% of them were positively labelled at 24 h post-infection (Table 1 and Fig. 4A). Thus, the progressive acquisition in time of Lamps on the membrane of the AIEC LF82-containing phagosomes was accompanied by an accumulation of the intraluminal cathepsin D, suggesting that these phagosomes mature into phagolysosomes.

**Maturation of AIEC LF82-containing phagosomes does not involve interaction with the autophagic pathway**

To investigate the possible involvement of the autophagic pathway in the maturation of AIEC LF82-containing phagosomes, the localization of LC3, an autophagosome-specific membrane marker, was analysed. For this purpose, J774 macrophages were transiently transfected with the plasmid pEGFP-LC3. As a positive control, macrophages were subjected to starvation, a conventional inducer of autophagy (Fig. 5A). Numerous GFP-LC3-labelled vacuoles were observed in starved cells. As shown in Fig. 5B and C, GFP-LC3 was not recruited on AIEC LF82-containing phagosomes neither at 60 min post-infection nor at 24 h post-infection. No colocalization between GFP-LC3 and LF82 bacteria-containing phagosomes was observed at very early (10 min and 20 min) or later (4 h and 8 h) post-infection times (data not shown). These results suggest that AIEC LF82-containing phagosomes do not interact with the autophagic pathway. However, a transient association of LC3 with autophagosomes cannot be ruled out.

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**Fig. 5.** AIEC LF82-containing phagosomes do not colocalize with LC3, the specific marker of the autophagic pathway. J774 macrophages were transiently transfected with the plasmid pEGFP-LC3.

A. Transfected J774 cells were incubated in complete medium (control, left panel) or in starvation medium for 4 h (starvation, right panel).

B. Transfected J774 cells were infected with AIEC strain LF82 for 20 min and then incubated for 40 min in fresh cell culture medium containing gentamicin.

C. Transfected J774 cells were infected with AIEC strain for 20 min and then incubated for 24 h in fresh cell culture medium containing gentamicin. Bacteria were revealed using rabbit antiserum directed against lipopolysaccharide O83 and Alexa 594-conjugated donkey anti-rabbit secondary antibodies.
AIEC LF82 bacteria replicate within mature phagolysosomes with low pH and active cathepsin D

As AIEC LF82-containing phagosomes were cathepsin D-positive, we performed experiments to define if this protease was in its immature or active form. An acid pH is necessary for a functional and optimal activity of cathepsin D, thus the pH of bacteria-containing phagosomes was sensed using LysoTracker Red DND-99, a fluorescent acidotropic component used to probe acid organelles. At 24 h post-infection, the large phagosomes within which AIEC LF82 bacteria had extensively replicated were positively marked with the LysoTracker Red (Fig. 4B), indicating that the LF82-containing phagosomes were acidic compartments.

To determine the activity of cathepsin D, which localized within AIEC LF82 bacteria-containing phagosomes, experiments using NIRF probe were performed (Fig. 6). NIRF probe is a dual fluorochrome (Cy5.5 and FITC) cathepsin D-activable probe. If cathepsin D is immature, the close spatial proximity of the multiple fluorochromes results in quenching of Cy5.5 fluorochromes and only a FITC signal is detected, which indicates a well internalization of the probe into the cell. Besides, in the presence of active cathepsin D, peptidic substrates present on the NIRF probe are cleaved resulting in a release of free Cy5.5 and thus by detection of a Cy5.5 signal (Tung et al., 1999). As a positive control, Texas Red dextran conjugates were used (Fig. 6A). The distribution of the Cy5.5 fluorescence of the NIRF probe revealed the presence of an active proteolytic form of cathepsin D within LF82-containing phagosomes at 24 h post-infection, suggesting that AIEC bacteria have the ability to replicate in an environment with active protease (Fig. 6B).

Replication ability of AIEC LF82 bacteria is impaired when the acid pH of the vacuolar compartment is neutralized

We studied the effect of vacuolar pH-neutralizing reagents on the replication ability of AIEC LF82 bacteria within J774 macrophages. Two reagents raising the pH of acid compartments were used, ammonium chloride (NH₄Cl) and chloroquine, which are lysosomotropic components that are membrane permeative in their non-protonated forms but impermeative when protonated in acid phagosomes. None of the reagents tested was directly lethal to either the bacteria or the host cells at the concentrations used. Growth curves of AIEC LF82 in the presence of chloroquine and NH₄Cl are presented in Fig. 7A. As chloroquine and NH₄Cl are lysosomotropic drugs, they presumably reach higher concentrations in the phagocytic vacuoles than those added originally. To exclude the possibility that higher concentrations of these drugs could be bactericidal, LF82 bacteria were also grown in RPMI medium supplemented with drugs at 100-fold higher concentrations (chloroquine 200 µM and NH₄Cl 200 mM) than those used during experiments with macrophages. In the pres-
ence of chloroquine at 2 μM or NH₄Cl at 2 mM, growth curves were identical to those of bacteria grown in the absence of these compounds. At concentrations 100-fold of those used in experiments with macrophages, only slight decreases in bacterial growth were observed.

Treatments with NH₄Cl and chloroquine decreased the replication of intracellular AIEC LF82 bacteria in a dose-dependent manner (Fig. 7B). When macrophages were treated with chloroquine at 2 μM or NH₄Cl at 2 mM, inhibitions of LF82 intracellular bacteria replication were observed. Percentages of intracellular bacteria at 24 h post-infection relative to those at 1 h post-infection, taken as 100%, were 69.2% ± 16.8% in the presence of chloroquine and 109.9% ± 18.8% in the presence of NH₄Cl compared with 361.9% ± 33.0% in absence of treatment. The decrease in bacterial replication of intramacrophagial LF82 bacteria after 24 h of cell treatment with chloroquine or NH₄Cl was confirmed by confocal microscopic examination. At 24 h post-infection chloroquine or NH₄Cl-treated and -infected macrophages showed phagosomes containing only a few bacteria compared with untreated macrophages, which exhibited large phagosomes containing numerous bacteria (Fig. 7C). These data demonstrated that an acidic environment is required for...
intracellular replication of AIEC strain LF82 within J774 macrophages.

**Exogenous iron supplementation does not restore replication of AIEC LF82 within chloroquine or NH4Cl treated-macrophages**

The intracellular transport and mobilization of iron are dependent upon an acidic environment in endocytic vesicles. As vacuolar pH-neutralizing reagents such as chloroquine and NH4Cl have been reported to interfere with iron availability for intracellular bacteria (Baynes et al., 1987), we investigated if the decrease in intracellular LF82 replication could be restored following supplementation with exogenous iron. The addition of ferric citrate at 10 µM did not restore the replication level of the AIEC LF82 bacteria within macrophages (Fig. 7B). A very slight increase in bacterial replication was observed with chloroquine-treated macrophages but higher concentrations of ferric citrate did not induce any increase in bacterial replication (data not shown). These results indicated that the decrease in the intracellular replication of LF82 bacteria observed after intraphagosomal pH neutralization was not attributed to impaired iron acquisition.

**Discussion**

The ileal mucosa of patients with CD is abnormally colonized by pathogenic AIEC strains, which have the ability to extensively replicate within J774 macrophages in spacious phagosomes, without inducing host cell death (Glasser et al., 2001; Darfeuille-Michaud et al., 2004). The aim of the study was to investigate whether AIEC reference strain LF82 avoid the bactericidal degradative activities of macrophages, by creating a specific replicative niche or by adapting to the hostile phagolysosomal environment. As biogenesis of AIEC-containing phagosomes may play a crucial role in creating a replicative niche, interactions of these phagosomes with endocytic and autophagic pathways were followed from early to late events within J774 cells.

After infection, AIEC LF82-containing phagosomes rapidly acquire EEA1, as previously observed with S. enterica serovar Typhimurium (Steele-Mortimer et al., 1999; Knodler and Steele-Mortimer, 2003). As EEA1 is a major effector of the Rab5 GTPase, and as these proteins are associated with vesicle tethering and SNARE docking, which are involved in the process of membrane fusion, transient acquisition of EEA1 can confer to AIEC-phagosomes the ability to reach the late endosomal stage of maturation (Desjardins, 1995; Simonsen et al., 1998; Dulos et al., 2003). This event involves in particular the acquisition of the Rab7 GTPase (Desjardins et al., 1994; Desjardins, 1995). Pathogenic bacteria such as *Mycobacterium tuberculosis* and *Mycobacterium avium* inhibit the phagosome–lysosome fusion and replicate within an immature phagosome by blocking vesicle maturation between the stages controlled by Rab5 and Rab7 (Clements and Horwitz, 1996; Via et al., 1997). In the case of AIEC strain LF82, the association of Rab7 with LF82-containing phagosomes occurred but little to no colocalization of Rab7 was detected on AIEC LF82-containing phagosomes at 60 min post-infection. This indicates that in contrast to what observed with Mycobacteria, the Rab7 GTPase was not retained on LF82-containing phagosomes, suggesting that they could evolved into phagolysosomes.

Salmonella-containing vacuoles also acquire Rab7 and further Lamp-1, but by bypassing the mannose-6-phosphate receptor (M6PR) compartment, they do not acquire lysosomal hydrolytic enzymes including cathepsin D (Buchmeier and Heffron, 1991; Rathman et al., 1997; Hashim et al., 2000; Garvis et al., 2001; Knodler and Steele-Mortimer, 2003). A gradual increased amount of Lamp-1 on LF82-containing phagosomes was observed at 60 min post-infection, and this Lamp-1 level was maintained until 24 h post-infection. At this time point, LF82-containing phagosomes were also Lamp-2-positive. In contrast to *Salmonella*-containing vacuoles, the progressive acquisition of Lamps on LF82-containing phagosomes was accompanied by an increased accumulation of the intraluminal cathepsin D. However, the mechanism by which cathepsin D is delivered to the LF82-containing phagosomes remains to be investigated. Indeed, this hydrolase can be delivered either by the endocytic pathway or via the biosynthetic pathway by involving the trans-Golgi network (Claus et al., 1998; Deretic et al., 2004).

As AIEC LF82-containing phagosomes display lysosomal features, LF82 bacteria could reside in an autophagolysosome-like compartment. Indeed, autophagy participates to the establishment of replicative compartments for pathogens such as B. abortus, C. burnetii, L. pneumophila and P. gingivalis (Pizarro-Cerda et al., 1998b; Dorn et al., 2001; Beron et al., 2002; Amer and Swanson, 2005). No translocation of the highly specific autophagosomal marker LC3 from the cytosol to the membrane of AIEC LF82-containing phagosomes was observed within J774 macrophages expressing GFP-LC3 at times post-infection studied. This suggests that AIEC LF82-containing phagosomes do not divert to a compartment with autophagic characteristics. However, LC3 association with autophagosomes can be transient and the involvement of autophagy in the process of phagosomal maturation, though unlikely, cannot be definitively excluded.

As most of the AIEC LF82-containing phagosomes were cathepsin D-positive at 24 h post-infection, and as immunocytochemical techniques do not address the biolog-
rical activity of cathepsin D, experiments were performed to investigate whether this protease was active. Cathepsin D matures by an internal cleavage induced by the low luminal pH of late endocytic compartments (Rijnboutt et al., 1992). At 24 h post-infection, spacious cathepsin D-positive vacuoles containing LF82 bacteria were positive for LysoTracker, a fluorescent acidotropic component used to probe acid organelles, which indicates that these compartments had low pH. Moreover, the dual fluorescent NIRF probe was used to image cathepsin D intraphagosomal activity. Confocal microscopy analysis revealed the presence of an active proteolytic form of cathepsin D within LF82-containing phagosomes. Thus, AIEC LF82-containing phagosomes mature into active phagolysosomes where bacteria are exposed to acid pH and also to degradative proteases. The way in which AIEC bacteria resist the attacks of protease activities is under current investigation. Bacterial cytoplasmic membrane is one of the targets for most antimicrobial peptides (Epand and Vogel, 1999; Hancock and Rozek, 2002). However, recent studies suggest that lipopolysaccharide (LPS) constitutes the first protective layer that controls the binding of peptides and their insertion into the cytoplasmic membrane (Papo and Shai, 2003; 2005). Thus, it can be speculated that the LPS by itself or the expression of specific proteins on the cell surface could protect AIEC bacteria from antimicrobial peptides. Nor can it be excluded that AIEC bacteria may exploit these proteases to generate amino acids that contribute to their high replication within the phagolysosomes.

Acid vacuolar pH could play a key role in the AIEC LF82 replication within macrophages. Treatment of J774 macrophages with vacuolar pH-neutralizing agents such as chloroquine and NH₄Cl inhibited the replication of intracellular LF82 bacteria. However, as these vacuolar pH-neutralizing agents are known to interfere with iron transport and mobilization, we checked that the defect in intracellular AIEC LF82 bacteria replication was not attributed to impaired iron availability. Iron supplementation has been reported to restore intracellular replication of Francisella tularensis and L. pneumophila in chloroquine and/or NH₄Cl-treated cells (Byrd and Horwitz, 1991; Fortier et al., 1995), but we observed no increase in AIEC LF82 intracellular replication. Thus, although it is clear that neutralizing the endosomal pH interferes with AIEC LF82 bacteria replication, the mechanism of this inhibition needs to be investigated. As already observed with C. burnetii, LF82 bacteria may take advantage of the proton gradient to drive biochemical reactions or nutrient/metabolite transports that are essential for growth (Hackett and Williams, 1981; 1983; Chen et al., 1990). Interestingly, we have already reported that low pH represents a key signal for AIEC LF82 bacteria to induce the expression and/or regulation of genes required for intraphagosomal replication (Bringer et al., 2005). Indeed, the stress protein HtrA plays an essential role in the adaptation of intracellular AIEC LF82 bacteria to low pH conditions. Moreover, the LF82-ΔhtrA isogenic mutant has a reduced rate of growth in an acid medium that partly reproduces the microenvironment of the phagosome, and htrA gene expression was highly upregulated in bacteria grown under these in vitro conditions and also in intramacrophagic bacteria. The importance of phagosomal acidification for intramacrophagic survival and/or replication has already been reported. An acidic environment constitutes the physiological signal mediating the virB operon expression in B. abortus and the gene expression of the SPI-2 type III secretion system and of the two-component system PhoP/PhoS in Salmonella Typhimurium (Alpuche Aranda et al., 1992; Beuzon et al., 1999; Steele-Mortimer et al., 2000; Boschiroli et al., 2002).

In summary, we have provided evidence that in contrast to many pathogens that escape from the normal endocytic pathway or infiltrate autophagy, AIEC LF82 bacteria are taken up by macrophages within phagosomes, which mature without diverting from the classical endocytic pathway, and which share features with phagolysosomes. To survive and replicate in the harsh environment encountered inside these compartments, including acid pH and proteolytic activity of cathepsin D, AIEC LF82 bacteria may have evolved from non-pathogenic bacteria by elaborating adaptation mechanisms for which acid pH constitutes a key signal to activate the expression of virulence genes.

Experimental procedures

Bacterial strain, plasmids and culture condition

AIEC strain LF82 was isolated from a chronic ileal lesion of a patient with CD and belongs to E. coli serotype O83:H1. The plasmid pFPV25.1, which harbours the green fluorescent protein (GFP), was used to visualize bacteria for confocal microscopy analysis (Valdivia and Falkow, 1997). The plasmid pEGFP-LC3 was kindly provided by Tamotsu Toshimori, National Institute of Genetics, Mishima-Shizuoka, Japan (Kabeya et al., 2000). Luria–Bertani (LB) broth or LB agar plates were used for standard cultivation (Institut Pasteur Production).

Culture and infection of J774 macrophages

J774 is a mouse macrophage cell line that was derived from a tumour in a female BALB/c mouse and has been shown to possess characteristics of macrophages. The murine macrophage-like cell line J774 (American Type Culture Collection No. TIB67) was maintained in an atmosphere containing 5% CO₂ at 37°C in RPMI 1640 medium (Biowhittaker Cambrex Company, Verviers, Belgium) supplemented with 10% (v/v) fetal calf serum (FCS; Biowhittaker) and 1% L-Glutamine (Invitrogen, Cergy-Pontoise, France). Cells were seeded in 24-well tissue culture plates at a density of 1 × 10⁵ cells cm⁻² and were grown for 18 h
in an atmosphere containing 5% CO₂ at 37°C. Before infection, cell monolayers were washed twice with PBS and the medium was replaced with 1 ml of RPMI 1640 supplemented with 10% heat inactivated FCS. J774 monolayers were infected at a multiplicity of infection (moi) of 100 bacteria per macrophage. After 10 min of centrifugation at 1000 g, corresponding to 10 min of infection and after supplementary 10 min incubation at 37°C with 5% CO₂, corresponding to 20 min of infection, infected macrophages were washed twice with PBS, and fresh cell culture medium containing 20 μg ml⁻¹ of gentamicin (Gm) was added for 40 min, corresponding to the 60 min post-infection time point, or was added for 8 h or for 24 h, corresponding to the 8 h and 24 h post-infection time point.

**Macrophage survival assay**

Bacterial uptake, survival and replication were measured by Gm protection assay. Cells were infected as described above. After 10 min of centrifugation at 1000 g and a 10 min incubation period at 37°C with 5% CO₂, infected macrophages were washed twice with PBS, and fresh cell culture medium containing 20 μg ml⁻¹ of Gm was added for a 1 h or a 24 h period. To determine the number of intracellular bacteria, cell monolayers were washed once with PBS, and 0.5 ml of 1% Triton X-100 (Sigma Chemical, St Louis, MO) in deionized water was added to each well for 5 min to lyse eukaryotic cells. This concentration of Triton X-100 had no effect on bacterial viability for at least 30 min. Samples were mixed, diluted, and plated onto LB agar plates to determine the number of colony-forming units (cfu) recovered from the lysed monolayers. The number of intracellular bacteria was determined after 1 h and 24 h of Gm treatment. Bacterial replication was expressed as the mean percentage of bacteria recovered after 24 h post-infection relative to the number of bacteria recovered after 1 h of Gm treatment, defined as 100%.

**Antibodies**

Goat antibodies against EEA1 (sc-6415) were obtained from Santa Cruz biotechnology, CA, USA. Rat antibodies against Lamp-1 (No. 1D4B) and rat antibodies against Lamp-2 (No. ABL-93) were obtained from the Developmental Studies Hybridoma Bank, Baltimore, MD, USA. Chicken antibodies against Rab7 were kindly provided by Angela Wandinger-Ness (University of New Mexico, Albuquerque, USA) and rabbit antibodies against Rab7 have been described elsewhere (Meresse et al., 1997). Rabbit antibodies against cathepsin D were purchased from DAKO Corporation (Carpinteria, CA, USA). Rabbit antiserum against E. coli LPS O83 was generously provided by Lothar Beutin (Department of Biological Safety, Robert Koch Institut, Berlin, Germany). Secondary antibodies used were: Alexa 594-conjugated goat anti-murine IgG, Alexa 594-conjugated donkey anti-goat IgG, Alexa 594-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and Fluo Probes 546-conjugated goat anti-chicken IgG (Molecular Probes, Interchim, Montluçon, France).

**LysoTracker probe**

The fluorescent basic amine probe LysoTracker Red DND-99 was obtained from Molecular Probes. J774 macrophages were infected with GFP expressing-AIEC strain LF82 as described above. LysoTracker was diluted at a final concentration of 50 nM in complete culture medium containing 20 μg ml⁻¹ of Gm. One hour before the end of the experiment, the medium was replaced by a medium containing LysoTracker. Cells were washed five times with PBS and then fixed with 3% paraformaldehyde (PFA), pH 7.4, in PBS for 10 min at room temperature. Colocalization of LysoTracker with bacteria was determined using confocal microscopy.

**Cathepsin D activity measurement**

J774 macrophages were infected as described above. The cathepsin D-sensitive near-infrared fluorescence (NIRF) probe was prepared as previously described (Tung et al., 1999; 2000). It was conjugated with FITC to monitor probe internalization and with Cy5.5 marker that became fluorescent in the near-infrared spectrum after cathepsin D activation. NIRF at 0.1 μM was added to infected cells 1 h before the end of the experiment. J774 cells were then washed to remove the free NIRF probe and were fixed with 3% PFA, pH 7.4, in PBS for 10 min at room temperature. As a positive control, Texas Red dextran conjugates, 400 000 MW (Molecular Probes) were used. The colocalization of bacteria and dextran conjugates with the NIRF probe was examined by confocal microscopy.

**Transient transfection with pEGFP-LC3**

Adherent cells at 90% confluence were transfected during 5 h with the plasmid pEGFP-LC3 (3 μg DNA) using lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. After transfection, cells were washed twice with PBS, and complete RPMI medium was added until infection. Transfected-cells were infected with AIEC strain LF82 as described above and processed for confocal microscopy.

**Induction of autophagy**

Autophagy was induced by starvation (Mortimore and Schworer, 1977; Munafo and Colombo, 2001). LC3-GFP transfected cells were washed three times with PBS at 37°C and incubated in 1 ml of Opti-MEM I reduced Medium (Invitrogen) for 4 h.

**Addition of vacuolar-pH-neutralizing reagents and ferric citrate to infected cells**

Reagents used to neutralize the vacuolar pH were: ammonium chloride (NH₄Cl; Sigma) used at 1 or 2 mM and chloroquine (Sigma) used at 1 or 2 μM. Ferric citrate (Sigma) was used at 10, 50 and 100 μM. Macrophages were infected as described below. The reagents were added to cells at 1 h post-infection. Infected and untreated cells were analysed in parallel. All experiments were performed in duplicate. Results are expressed as the number of viable intracellular bacteria at 24 h post-infection relative to that obtained after 1 h post-infection, taken as 100%. Macrophage viability was checked by trypan blue dye exclusion assays and by measurement of lactate dehydrogenase (LDH) release. To test that these reagents were not bactericidal, growth curves were performed in RPMI.
medium supplemented with chloroquine at 2 or 200 µM or NH₄Cl at 2 or 200 mM.

**Immunofluorescence and confocal microscopy**

Fixation was performed with 3% PFA, pH 7.4, in PBS for 10 min at room temperature. Fixed cells were washed three times in PBS and permeabilized by incubation in PBS containing 0.1% saponin (Sigma). Primary and secondary antibodies were diluted in PBS containing 0.1% saponin and 5% horse serum (Sigma). Coverslides were incubated with primary antibodies for 30 min at room temperature, washed in PBS containing 0.1% saponin and then incubated for 30 min in obscurity with secondary antibodies. Coverslides were mounted onto glass slides using Mowiol (Aldrich, Steinheim, Germany). When necessary, the actin cytoskeleton was stained for 15 min with TRITC-labelled-phalloidin at 1 µg ml⁻¹ (Sigma). Cells were observed with Leica TCS 4DA confocal microscope (CNRS Marseille Luminy, France) and with Olympus Fluoview (IFR Santé Université d’Auvergne, Clermont-Ferrand, France). To determine the percentage of positive AIEC LF82-containing phagosomes for a specific marker, at least 100 bacteria-containing phagosomes were counted and scored for the presence or absence of the marker protein. Each confocal microscopy image is representative of three independent experiments.

**Statistical analysis**

Student’s t-test was used for comparison of the two groups of data. All experiments were repeated at least three times. A P-value less than or equal to 0.05 was considered statistically significant.

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