Essential role of the VirB machinery in the maturation of the Brucella abortus-containing vacuole

Diego J. Comerci,1,2† Maria José Martínez-Lorenzo,3† Rodrigo Sieira,1 Jean-Pierre Gorvel3 and Rodolfo A. Ugalde1
1Instituto de Investigaciones Bioteclénológicas, Universidad Nacional de General San Martín, CONICET, Avenida General Paz entre Constituyentes y Albarrelos, San Martín 1650, Buenos Aires, Argentina.
2Comisión Nacional de Energía Atómica, División Agropecuaria, Centro Atómico Ezeiza 1804, Buenos Aires, Argentina.
3Centre d’Immunologie INSERM-CNRS de Marseille-Luminy, Case 906-13288, Marseille Cedex 9, France.

Introduction

Brucella spp. are Gram-negative facultative intracellular pathogens that cause brucellosis, a worldwide-distributed zoonosis affecting a broad range of mammals, ranging from dolphins and domestic animals to humans (Enright, 1990). Brucellosis remains endemic in many developing countries, where it causes important economic losses (Corbel, 1997). In humans, brucellosis is a serious debilitating disease characterized by diverse pathological manifestations such as undulant fever, osteoarthritic complications, endocarditis and several neurological disorders. In domestic animals such as cattle, goats and sheep, the outstanding manifestation of the pathology is the abortion of pregnant females and the sterility of males as a result of the colonization of placenta, fetal tissues and sexual organs (Nicoletti, 1989; Corbel, 1997).

The mechanisms by which Brucella bind and penetrate cells are not fully characterized, but several observations at the electron microscopic level suggest that they are actively internalized in non-professional phagocytes by local membrane extensions that resemble the zipper-phagocytosis mechanism observed during Listeria infection (J. Pizarro-Cerdá, P. Montcourrier and J.-P. Gorvel, unpublished). However, the reduced penetration in HeLa cells and murine macrophages displayed by Brucella mutants in the two-component regulatory system BvrS–BvrR (Sola-Landa et al., 1998) suggests that Brucella actively promote their internalization in both cell types. During initial steps of internalization into professional and non-professional phagocytes, Brucella abortus is transiently found in a vacuole characterized by the presence of early endosomal antigens such as EEA1 and the transferrin receptor. Between 50 min to 1 h post-infection (p.i.), B. abortus bypasses late endosomal compartments, localizing in a multimerembranous, ribosome-associated vacuole that resembles an autophagosome, as revealed by electron microscopy analysis, co-localization with autophagy modulation studies (Pizarro-Cerda et al., 1998a,b).

However, the autophagosome is not the replication niche of the bacteria. At later times p.i., virulent bacteria are found in an endoplasmic reticulum (ER)-like compartment in which massive replication occurs. Electron microscopy analyses of trophoblastic and Vero-infected cells also revealed that multiplication of virulent B. abortus occurs in an ER-like compartment (Anderson and Cheville, 1986; Meador and

Summary

In epithelial cells, the intracellular pathogen Brucella abortus escapes from the endocytic pathway, exploits the autophagic machinery of the host cell and establishes a unique replication niche in the endoplasmic reticulum. The molecular mechanisms underlying these processes are still poorly understood. Recently, a B. abortus type IV-related secretion system encoded by the virB operon has been described as being involved in the intracellular trafficking of the bacteria. In this study, we have analysed the intracellular pathway of B. abortus virB10 mutant strains by confocal microscopy. We demonstrate that a functional virB operon is essential for the biogenesis of the Brucella-containing vacuole. Polar mutation preventing the transcription of virB10 and downstream sequences did not allow Brucella to bypass the endocytic pathway. Consequently, polar mutant-containing vacuoles fused with lysosomes in which bacteria underwent a degradation process. In contrast, virB10 non-polar mutants were capable of avoiding interactions with the endocytic pathway but, diverging to wild-type Brucella, were unable to reach the endoplasmic reticulum to establish their intracellular replication niche and seemed to be recycled to the cell surface. Based on the two particular phenotypes described in this work, a model of maturation of the Brucella-containing vacuole is proposed.
Deyoe, 1989; Detilleux et al., 1990; Pizarro-Cerda et al., 1998a). Taken together, these data indicate that pathogenic B. abortus escapes from the endocytic pathway, exploits the autophagic machinery of the host cell and establishes its replication niche in an ER-like organelle.

Several intracellular pathogens have evolved different ways of controlling the maturation of their membrane-bound compartments, circumventing host defences and further degradation, transforming them into nutrient-rich environments in which bacteria replicate. Members of the genera Shigella, Listeria and Rickettsia evade the degradative endocytic pathway, escaping from their nascent vacuoles and replicating in the cytoplasm of the host cell. Others, such as Chlamydia and Legionella, rapidly avoid interactions with the endocytic pathway to replicate within a specialized vacuole located in the biosynthetic pathway (Meresse et al., 1999a). Infection of human macrophages by Legionella pneumophila occurs by a sequential multistep process. Once the vacuole has been formed, it undergoes several recruitment events that include association with smooth vesicles, mitochondria and rough ER. The bacteria multiply within this specialized vacuoles (Horwitz, 1983a,b). The intracellular fate of L. pneumophila is determined by a type IV-related secretion system encoded by 24 dot/icm virulence genes (Segal et al., 1998; Vogel et al., 1998). Vacuoles containing L. pneumophila dot and icm mutants fuse with lysosomes, resulting in a severe intracellular growth defect. dotA mutants rapidly accumulate LAMP1 and Rab7, indicating that DotA plays a fundamental role in the inhibition of vacuole–lysosome fusion (Roy et al., 1998). This avirulent mutant has the ability to multiply in a vacuole formed by wild-type L. pneumophila, indicating that factors secreted by the Dot/ICM systems are necessary and sufficient to establish a replicative niche (Coers et al., 1999).

The molecular mechanisms that allow Brucella to evade the endocytic pathway and to establish its unique replication niche are still poorly understood. Sola-Landa et al. (1998) have demonstrated that the two-component regulatory system bvrS–bvrR is essential for Brucella to invade the host cell, effectively reaching the autophagosome and escaping the lysosome fusion. These results suggest that genes under the control of this system may be important for the establishment of the intracellular replication niche. Recently, the virB operon of Brucella has been identified (O’Callaghan et al., 1999; Ugalde, 1999; Sieira et al., 2000). This operon is composed of 13 open reading frames (ORFs) that share homology with other bacterial type IV secretion systems known to be involved in the intracellular trafficking of pathogens, as is the case for the dot and icm genes of L. pneumophila. Polar mutations introduced in the first gene of the operon, virB1, abolish the ability of Brucella to replicate intracellularly, indicating that this system is essential for the intracellular lifestyle of this pathogen. Mice infection performed with polar and non-polar mutations in virB10 demonstrated that the virB operon is a major determinant of Brucella virulence (Sieira et al., 2000).

In the present work, we analyse the intracellular traffic of B. abortus virB10 polar and non-polar mutants in HeLa cells. Our results show that the intracellular fate of the Brucella-containing vacuole is determined by the type IV secretion system encoded by the virB operon.

Results

Both B. abortus virB10 polar and non-polar mutants interact with early endosomes

In a recent study, we showed that virulent B. abortus S2308 was able to multiply efficiently within HeLa cells (Pizarro-Cerda et al., 1999). During S2308 infection, a lag period of 10 h was observed, during which bacteria were found within cells but did not replicate, followed by an exponential growth period with massive bacterial replication. In contrast, polar and non-polar mutations introduced in virB10 affected the ability of bacteria to replicate intracellularly, producing an avirulent phenotype observed previously in HeLa cells and infected mice (Sieira et al., 2000).

We then focused on the intracellular fate of both B. abortus virB10 polar and non-polar mutant-containing vacuoles in HeLa cells.

As for virulent B. abortus S2308, both B. abortus virB10 polar and non-polar mutants were found, between 5 and 10 min after inoculation, in early vacuoles characterized by the presence of the early endosome-specific peripheral membrane protein EEA1 (Mu et al., 1995) (Fig. 1A). At 10 min p.i., between 10% and 15% of wild-type or mutants strains co-localized with the early endosomal marker to the same extent as phagosomes containing latex beads (Fig. 1B). At 40 min p.i., <2% of either wild-type, mutant strains or latex beads co-localize with EEA1 (Fig. 1B). The low levels of co-localization of both bacteria or inert particles with EEA1 (~15%) suggest that the entry into HeLa cells was not synchronous and that, once inside the cell, the interaction with early endosomal compartments was rapid and transient. No difference was observed in bacterial adherence or internalization among the mutants and parental strain (not shown). These results indicate that early events of biogenesis in Brucella-containing vacuoles were not affected by mutations interrupting the transcription of virB10 and downstream sequences.

B. abortus S2308 and virB10 non-polar compartments are devoid of the lysosomal enzyme cathepsin D, whereas the virB10 polar mutant undergoes degradation

It has been proposed that pathogenic Brucella inhibits the
fusion between bacterial vacuoles and lysosomes (Frenchick et al., 1985; Pizarro-Cerda et al., 1999). Therefore, we compared the acquisition of lysosomal-associated proteins by Brucella-containing vacuoles. Both virB10 polar and non-polar mutant-containing vacuoles gradually accumulated the late endosomal–lysosomal marker LAMP1 (Fig. 2). At 4 h p.i., 80–90% of vacuoles were labelled for LAMP1 (Fig. 5), as already observed for the wild-type S2308 (Pizarro-Cerda et al., 1998a). Four hours after inoculation, wild-type, virB10 polar and non-polar mutants seemed to follow the same kinetics of acquisition of LAMP1. However, from 4 h p.i. up to 12 h p.i., LAMP1 started to be excluded from the S2308-containing strains in contrast to both virB10 polar and non-polar mutant strains, which retained the lysosomal membrane protein on their vacuole (Figs 2 and 5).

We then analysed the distribution of the lysosomal acid hydrolase cathepsin D, known to accumulate inside lysosomes. Cathepsin D was gradually recruited in virB10 polar mutant-containing compartments (94% of positive vacuoles at 4 h p.i.) with the same kinetics as LAMP1 (Fig. 5). Both intact bacteria and bacterial degradation products detected by anti-Brucella lipopolysaccharide (LPS) antibodies co-localize with cathepsin D at 12 h and 24 h p.i. (Fig. 3, middle), suggesting that the attenuated polar mutant had interacted with lysosomes. In contrast, most of the vacuoles containing wild-type or virB10 non-polar mutant excluded cathepsin D. Indeed, at 12 h p.i., the lysosomal enzyme was detected in <10% of the vacuoles containing either S2308 or virB10 non-polar strains (Fig. 5).

Taken together, these results demonstrate that the virB10 polar mutant failed to evade the endocytic pathway and was targeted to lysosomes for further degradation. These results are consistent with the rapid clearance of the polar mutant that we have described previously (Sieira et al., 2000). This implies that a complete virB secretion system is essential for Brucella to avoid degradation by lysosomes. In contrast, the vacuoles containing the virB10 non-polar mutant remained LAMP1 positive and never acquired the lysosomal marker cathepsin D, suggesting that the VirB machinery devoid of VirB10 only is still competent to avoid interactions with lysosomes.

B. abortus virB10 polar and non-polar mutant compartments are devoid of ER markers

We then looked for the presence of ER markers such as EEA1 is detected in Brucella-containing vacuoles. HeLa cells were incubated with a suspension of latex beads or infected with S2308, virB10 polar or non-polar mutant strains for 5 min at 37°C, washed and incubated further for 10 min and 40 min in cell culture medium, washed and immediately processed for double indirect immunofluorescence as described.

A. Distribution of EEA1 (right) and bacteria (left) 10 min after internalization. Vacuoles containing internalized bacteria are labelled with EEA1 (arrows in A). Bar represents 10 μm.

B. Distribution of EEA1 in vacuoles containing latex beads, S2308, virB10 polar or non-polar mutant strains at 5, 10 and 40 min p.i. The percentages of vacuoles containing EEA1 were calculated as described in Experimental procedures. Data represent mean ± SD from two independent experiments.
sec61β, calnexin and calreticulin in the Brucella-containing vacuoles. The ER translocator sec61β was recruited in the S2308-containing vacuoles from 4 h onwards (Fig. 5), as described previously (Pizarro-Cerda et al., 1998a). At 24 h p.i., calreticulin was found to decorate the vacuoles containing the S2308 replicating bacteria (Fig. 4, top). The same results were observed with calnexin (not shown). In contrast, virB10 polar and non-polar mutant strains were unable to recruit sec61β (Fig. 5) and calreticulin (Fig. 4, middle and bottom). As a control, the virB10 non-polar mutant complemented with the pBBRvirB10 plasmid (Sieira et al., 2000), which allows the expression of virB10 under a lac promoter, displayed the same behaviour as that of the wild type, reaching and replicating in an ER-like compartment (Fig. 6). Similarly, the virB10 polar mutant harbouring a cosmid containing the complete virB operon also recovered the virulent phenotype of S2308 and access to a calreticulin-positive ER compartment (not shown).

These results indicate that the virB10 non-polar mutant bypassed the late endosomal–lysosomal compartment in a similar manner to that of the wild-type strain. However, the mutant failed to complete the vacuole maturation normally characterized by the exclusion of LAMP1 and the acquisition of ER markers and, consequently, was unable to establish its intracellular replication niche. This indicates that the virB secretion system devoid only of VirB10 retains the ability to prevent fusion of the vacuoles with lysosomes, but is still incompetent to establish a replicative niche.

The virB10 non-polar mutant is recycled to the host cell surface

As we did not detect the virB10 non-polar mutant either in lysosomes or in an ER-like compartment, we looked for its cellular location. Surprisingly, from 12 h p.i. onwards, a growing number of virB10 non-polar mutant bacteria were detected in the proximity of the cell surface (Figs 2 and 3), a phenomenon that was never observed at early time points after inoculation. To determine whether bacteria were intracellular or cell-associated extracellular bacteria, we fluorescein isothiocyanate (FITC)-coupled donkey anti-rabbit IgG before cell permeabilization with saponin. Samples were then permeabilized and incubated further with bovine anti-LPS, followed by TxR-coupled donkey anti-bovine IgG. The number of intracellular and extracellular bacteria per infected cell at 24 h p.i. was estimated. Figure 7 shows that, at 24 h p.i., a low number of virulent wild-type bacteria was found extracellular (mean number of seven bacteria/infected cell), whereas a significantly higher...
containing vacuoles. HeLa cells were infected with S2308, virB10

Fig. 3. Cathepsin D is concentrated in virB10 polar mutant-containing vacuoles. HeLa cells were infected with S2308, virB10 polar or non-polar mutant strains for 1 h. After 24 h of incubation, cells were processed for double indirect immunofluorescence as described. Distribution of cathepsin D (right) and bacteria (left) at 24 h p.i. B. abortus virB10 polar mutant undergoes a degradation process seen at 24 h p.i. (middle), in which both intact bacteria (arrows) and degradation products (arrowheads) co-localize with the lysosomal marker. S2308 has established its intracellular replication niche (top). B. abortus virB10 non-polar mutants do not co-localize with cathepsin D (arrows at the bottom). Note that numerous bacteria are localized at the cell surface delimited by a dotted line (lower left). Bar represents 10 μm.

number of virB10 non-polar bacteria was found on the cell surface (mean number of 20 bacteria infected cell⁻¹), contrasting with the number of intracellular wild-type bacteria that was much higher than that of the virB10 non-polar mutant (44 bacteria cell⁻¹ versus 0.4 bacteria cell⁻¹ respectively). In contrast, the mean numbers of intracellular and extracellular virB10 polar mutants cell⁻¹ were 1 and 0 at 24 h p.i., respectively, owing to the fact that most of the polar mutants were degraded after interaction with lysosomes.

Discussion
Brucella is a facultative intracellular pathogen that has evolved the capacity to evade the host defence mechanisms and to establish an appropriate replication niche. After host cell invasion, Brucella has been found to survive and replicate within membrane-bound compartments of professional and non-professional phagocytes (Liautard et al., 1996). Brucella rapidly interacts first with the early endosomes and bypasses late endosomal–lysosomal compartments. Then, the pathogen exploits the autophagic machinery and establishes a unique replication niche in the ER (PizarroCerda et al., 1999). Up to now, the bacterial genes involved in the maturation process of Brucella-containing vacuoles still remains to be characterized. Recently, our group and others have described the virB operon of Brucella (O’Callaghan et al., 1999; Ugalde, 1999; Sieira et al., 2000). The B. abortus virB operon is a collinear arrangement of 13 ORFs highly homologous to other bacterial type IV secretion systems. Mice infection studies and intracellular replication assays have demonstrated that the virB operon constitutes a major determinant of Brucella virulence (Sieira et al., 2000).

In this study, we have analysed the intracellular pathway followed by virB10 mutant strains of B. abortus after infection. We demonstrate that a functional type IV related secretion system, encoded by the virB operon, is essential for the biogenesis of the Brucella-containing vacuole. We have examined the distribution of parental and mutant strains by double immunofluorescence in HeLa cells. We show that, during the first ≈10 min after inoculation, bacteria acquire early endosomal markers such as EEA1, demonstrating that Brucella-containing vacuoles are able to interact with early endosomes. Interaction of intracellular parasites with early endosomal compartments has already been demonstrated for Mycobacterium tuberculosis and Mycobacterium avium in human monocyte-derived macrophages (de Chastelier et al., 1995; Clemens and Horwitz, 1996). Recent data have also shown that Salmonella typhimurium acquires EEA1 before being targeted to lysosomal glycoprotein-containing vesicles (Meresse et al., 1999b; Steele-Mortimer et al., 1999). No difference in bacterial adherence and internalization was observed between wild-type and virB10 mutant strains. Moreover, co-localization studies of Brucella virB mutant-containing vacuoles with early endocytic markers at short times p.i. demonstrate that virB10 and downstream sequences are not involved in either the invasion process or the first steps of internalization within non-professional phagocytes.

We show that, although vacuoles containing the pathogenic parental Brucella do not fuse with cathepsin D-positive compartments, the virB10 polar mutant rapidly acquired LAMP1 and cathepsin D, thus indicating that vacuoles containing the avirulent B. abortus virB10 polar mutant interacted with lysosomes.
Polar mutation preventing the transcription of \textit{virB10} and downstream sequences (\textit{virB11}, \textit{ORF12} and \textit{ORF13}) affects the process by which \textit{Brucella} effectively bypasses the endocytic pathway. Vacuoles containing the polar mutant fuse with lysosomes and undergo a degradation process. This is in agreement with our previous observations showing that polar mutations of \textit{virB10} are responsible for an avirulent phenotype (Sieira et al., 2000). \textit{virB10} encodes a predicted 388-amino-acid transmembrane protein with unknown function that has homologues in all bacterial type IV secretion systems and in the mating pair formation complex of IncP RK2 plasmid. The \textit{Agrobacterium tumefaciens} VirB10 is essential for tumorigenesis (Berger and Christie, 1994). VirB11 is a predicted 362-amino-acid cytoplasmic or inner membrane protein that has a conserved Walker A NTP-binding motif that has been shown to be essential in \textit{A. tumefaciens} and \textit{Bordetella pertussis} (Stephens et al., 1995; Kotob and Burns, 1997). This protein has homologues in both type IV and type II secretion systems, the mating pair formation of IncP RK2 plasmid and pilin biosynthesis genes of \textit{Pseudomonas aeruginosa} and \textit{Neisseria gonorrhoeae}.

\begin{figure}[h]
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\caption{\textit{B. abortus virB10}-containing vacuoles do not express the ER marker calreticulin. HeLa cells were inoculated with S2308, \textit{virB10} polar or non-polar mutant strains for 1 h. After 24 h of incubation, cells were processed for double indirect immunofluorescence as described. Distribution of calreticulin (middle), bacteria (left) and three-colour images obtained by merging the two images (right) at 24 h p.i. Arrowheads indicate bacteria that not co-localize with the ER marker. Calreticulin does not co-localize with intracellular \textit{B. abortus virB10} polar or non-polar mutant strains (arrowheads in middle and bottom), whereas a large number of wild-type S2308 bacteria has established its intracellular replication niche and co-localizes with the ER marker (dotted line at the top). Bar represents 10 \textmu m.}
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\end{figure}
ORF12 and ORF13 are exclusively found in the Brucella virB operon (O’Callaghan et al., 1999; Sieira et al., 2000). Gene disruption experiments demonstrated that ORF12 is not essential for intracellular replication of Brucella suis (O’Callaghan et al., 1999). ORF13 encodes a predicted protein of 83 amino acids that has no homology with any protein deposited in the data banks. The question whether both ORF12 and ORF13 are really functional remains to be elucidated.

As the kanamycin resistance cassette inserted in virB10 has strong terminator sequences that affect the transcription of not only virB10 but also virB11 and downstream sequences, it is conceivable that the Br. abortus virB10 polar mutant has a non-functional VirB complex. We do not have formal proof of how the Brucella VirB complex acts, but comparison of this system with the well-characterized A. tumefaciens VirB operon (Christie, 1997) and the B. pertussis Ptl operon (Weiss et al., 1993) allows us to suppose that it is a multimolecular export machinery. Whatever the actual function of the Brucella VirB system, the intracellular trafficking of the virB10 polar mutant described here implies that the VirB complex per se or putative effector molecules secreted by this system are involved in avoiding fusion of the Brucella-containing vacuole with lysosomes.

The studies performed with a virB10 non-polar mutant show that this strain is capable of evading the endocytic pathway but is unable to reach the ER to establish the intracellular replication niche. We hypothesize that the B. abortus virB10 polar mutant may have an impaired but partially functional type IV secretion machinery. This incomplete transport system might be competent to deliver effectors that avoid interactions between the Brucella-containing vacuole and lysosomes, but is unable to complete the maturation pathway of the vacuole to the establishment of the replication niche. However, from 12 h p.i. onwards, the virB10 non-polar mutant seemed to be recycled to the host cell surface. More experiments are needed to analyse the mechanism of this recycling pathway further.

Previous studies have established that, in HeLa cells, intracellular Brucella follows a two-step coupled process:
another intracellular pathogen, *Legionella pneumophila*, has evolved a similar way to modulate its nascent vacuole. Upon entry, vacuoles containing *L. pneumophila* sequentially associate with smooth vesicles, mitochondria and the ER (Horwitz, 1983a,b). The replicative vacuole fails to acquire late endosomal–lysosomal markers and the ER (Horwitz, 1983a,b). The replicative vacuole sequentially associate with smooth vesicles, mitochondria and the ER (Horwitz, 1983a,b). The replicative vacuole fails to acquire late endosomal–lysosomal markers and the ER (Horwitz, 1983a,b). The replicative vacuole sequentially associate with smooth vesicles, mitochondria and the ER (Horwitz, 1983a,b). The replicative vacuole sequentially associate with smooth vesicles, mitochondria and the ER (Horwitz, 1983a,b). The replicative vacuole sequentially associate with smooth vesicles, mitochondria and the ER (Horwitz, 1983a,b). The replicative vacuole sequentially associate with smooth vesicles, mitochondria and the ER (Horwitz, 1983a,b). The replicative vacuole sequentially associate with smooth vesicles, mitochondria and the ER (Horwitz, 1983a,b). The replicative vacuole sequentially associate with smooth vesicles, mitochondria and the ER (Horwitz, 1983a,b).

![Fig. 7. Numbers of extracellular and intracellular B. abortus per infected HeLa cell at 24 h p.i. HeLa cells were infected with virulent wild-type S2308, virB10 non-polar or virB10 polar mutant strains for 1 h. At 24 h p.i., cells were washed, fixed and processed for double indirect immunofluorescence as described. Extracellular bacteria were fluorescently labelled by incubating with rabbit anti-LPS followed by FITC-coupled donkey anti-rabbit IgG before cell permeabilization with saponin. Then, the samples were permeabilized and incubated further with bovine anti-LPS, followed by TRF-coupled donkey anti-bovine IgG. The number of extracellular (white bars) and intracellular (black bars) bacteria per host cell were recorded by confocal laser scanning microscopy. Data represent means ± SD of two determinations made in duplicate.](image-url)

Based on the two particular phenotypes described in this work, we propose a model of maturation in the Brucella-containing vacuole in which at least two distinct putative effector molecules are secreted by the VirB machinery: one acting to avoid the degradative pathway (evidenced by the *virB10* polar mutant) and the second needed to establish the intracellular replication niche (evidenced by the *virB10* non-polar mutant). Future experiments are required to solve this matter.

**Experimental procedures**

**Bacterial strains**

*B. abortus* S2308 is a CO2-independent virulent smooth strain. The *B. abortus* virB10 polar mutant is a derivative of S2308 with an insertion of a Kan R polar cassette at the *Nru* site of *virB10*. The isogenic strain *B. abortus* virB10 non-polar mutant has an insertion of a Gm R cassette devoid of transcriptional terminator inserted at the same *Nru* site (Sieira et al., 2000). The complemented *B. abortus* virB10 polar mutant harbours a pVK102 cosmid containing a complete *virB* operon (Sieira et al., 2000). The *B. abortus* virB10 non-polar mutant was complemented with pBBR-virB10, a replicative vector for Brucellae containing the entire *virB10* under the lac promoter. Bacteria were grown at 37°C on a rotary shaker in tryptic soy broth (TSB) (Difco) to stationary phase, and aliquots were frozen at −70°C in TSB–30% glycerol. For each experiment, a log-phase culture of bacteria was prepared in 5 ml of TSB for 15–17 h at 37°C with agitation to allow bacterial growth. When necessary, antibiotics were added to a final concentration of: kanamycin (50 μg ml⁻¹), gentamicin (2.5 μg ml⁻¹), ampicillin (50 μg ml⁻¹) and tetracycline (3 μg ml⁻¹). Bacterial numbers were estimated by comparing the optical density at 600 nm with a standard curve and determined by retrospective counts.

**Antibodies and fluorescent probes**

Rabbit polyclonal anti-early endosomal antigen 1 (EEA1) was obtained from Dr H. Stemmark, The Norwegian Radium Hospital, Oslo, Norway; rabbit polyclonal anti-human LAMP1 was obtained from Dr M. Fukuda, The Burnham Institute, La Jolla, CA, USA; rabbit polyclonal anti-cathepsin D was a gift from Dr S. Kornfeld, Washington University School of Medicine, St Louis, MO, USA; rabbit polyclonal anti-calreticulin and anti-calnexin were purchased from Affinity Bio reagents. Rabbit polyclonal anti-secl615 was a gift from Bernhard Dobberstein, ZMBH, Heidelberg, Germany. Cow and rabbit polyclonal anti-*B. abortus* 2308 antibodies were prepared using standard procedures (Pizarro-Cerda et al., 1998a). The secondary antibodies used were: FITC-conjugated donkey anti-rabbit IgG; FITC-conjugated donkey anti-goat IgG; FITC-conjugated donkey anti-mouse IgG; Texas
red (TxR)-conjugated goat anti-cow IgG and Cy5 conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories).

**Cell culture**

HeLa cells were grown in 75 cm² flasks (Falcon; Becton-Dickinson) at 37°C in a 5% CO₂ atmosphere in minimal Dulbecco’s essential medium (MDEM; Gibco BRL) containing 10% fetal calf serum (FCS) and 2 mM glutamine without antibiotics (cell culture medium). Cells were used between passages 1 and 15 and were split 1:10 or 1:4 twice per week. For monolayer inoculations, 24-well tissue culture plates (Falcon; Becton-Dickinson) were seeded with 50 μl of 1 × 10⁶ or 1 × 10⁷ cells well⁻¹ (for confocal microscopy analysis, cells were deposited in 24-well tissue culture plates containing 12-mm-diameter glass coverslips).

**Bacterial inoculation and latex bead incubation**

We established previously a protocol for both the incubation of HeLa cells with a suspension of latex beads and the infection of HeLa cells by various strains of B. abortus (Pizarro-Cerda *et al.*, 1998a; Sola-Landa *et al.*, 1998). Log-phase growing cultures of B. abortus were prepared as described previously (Sieira *et al.*, 2000). After HeLa cells were seeded in 24-well plates (10⁵ cells well⁻¹), medium was removed and cells were inoculated with 1 ml of minimal essential medium (MEM; Gibco) supplemented with 5% FCS and 2 mM glutamine (cell culture medium) containing 5 × 10⁷ cfu of bacteria, without antibiotics or a 1:20 000 dilution of a 10% solution of dyed latex beads. In order to ensure a close contact between cells and bacteria, culture plates were centrifuged for 10 min at 1000 r.p.m. at room temperature and placed in an incubator under a 5% CO₂ atmosphere at 37°C. After 1 h, wells were washed five times with PBS (pH 7.4) and incubated further with cell culture medium containing 100 μg ml⁻¹ gentamicin and 50 μg ml⁻¹ streptomycin to eliminate remaining extracellular *Brucellae*.

**Analytic and quantitative immunofluorescence**

At different times after inoculation, coverslips were washed to remove non-adherent bacteria (five times with PBS) and fixed for 15 min in 3% paraformaldehyde, pH 7.4, at room temperature. Cells were then washed three times with PBS, incubated for 10 min with PBS–50 mM NH₄Cl in order to quench free aldehyde groups. Coverslips were incubated with primary antibodies directed against different host intracellular markers for 20 min at room temperature, washed in PBS containing 0.1% saponin and then incubated with FITC- or TxR-coupled donkey anti-rabbit or anti-mouse antibodies in a PBS–5% horse serum–0.1% saponin solution. The coverslips were mounted onto glass slides using Mowiol (Aldrich). Cells were observed on a Leica TCS 4DA microscope using 100× oil immersion objective. The laser lines used were 488 nm (FITC), 568 nm (TxR) and 647 nm (Cy5). Projections were saved in TIFF format and imported to ADOBE PHOTOSHOP where images were merged using RGB format. To determine the percentages of bacteria or latex beads that co-localized with the studied intracellular markers, we counted a minimum of 100 intracellular bacteria (revealed by indirect immunofluorescence) or latex beads (red auto-fluorescence emission) in the red channel, and then the samples were observed further through the FITC channel. The assays were performed in duplicate. The limits of the cells can be seen on highly contrasted confocal images enabling the drawing of a dotted line using the appropriate tool from the PHOTOSHOP software.

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