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Activation of Rho and Rab GTPases dissociates *Brucella abortus* internalization from intracellular trafficking

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Summary

***Brucella abortus* is an intracellular pathogen that relies on unconventional virulence factors to infect hosts. In non-professional phagocytes, Rho GTPases-activation by the *Escherichia coli* cytotoxic necrotizing factor (CNF) promoted massive *Brucella* entrance by membrane ruffling, a mechanism that differs from the common mode of entrance used by this bacterium in non-treated cells. Cytotoxic necrotizing factor treatment, however, did not alter the intracellular route followed by the wild type or non-virulent defined mutants. In contrast, expression of a constitutively active Rab5Q79L GTPase did not alter cell-invasion by *Brucella* but hampered its ability to reach the endoplasmic reticulum. The CNF-induced *Brucella* super-infection did not reduce the ability of host cells to synthesize DNA and progress through the cell cycle. Furthermore, CNF-treatment increased the isolation of *Brucella*-containing compartments by a factor of 15. These results demonstrate that in non-professional phagocytic cells, *Brucella* manipulates two different sets of GTPases during its biogenesis, being internalization and intracellular trafficking two consecutive but independent processes. Besides, CNF-induced super-infection demonstrates that *Brucella* does not interfere with crucial cellular processes and has shown its potential as tool to characterize the intracellular compartments occupied by this bacterium.**

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Introduction

Brucella organisms are intracellular parasites of mammals, causing severe syndromes generally known as brucellosis (Moreno and Moriyón, 2001). During its infection cycle in humans and animals, *Brucella* uses professional and non-professional phagocytes as host cells. The first kind of cells, mainly macrophages, sustain limited replication and constitute efficient vehicles for spreading the bacteria to different tissues. In the second category, trophoblasts of the gravid uterus of animals constitute the main replicating niche of virulent *Brucella*, simultaneously being the disseminating site for infecting the fetus (Anderson and Cheville, 1986; Anderson *et al*., 1986). In contrast to other pathogenic bacteria, no classical virulence factors such as exotoxins, cytolysins, capsules, fimbria, flagella, plasmids, lysogenic phages, resistant forms, antigenic variation, endotoxic lipopolysaccharide (LPS) or apoptotic inducers have been described in *Brucella* organisms (Moreno and Moriyón, 2001). Instead, the virulence factors in *Brucella* are those molecular determinants that allow the bacteria to invade (Guzmán-Verri *et al*., 2001), resist intracellular killing (Moreno and Moriyón, 2001) and reach their replicating niche in professional and non-professional phagocytes (Detilleux *et al*., 1990a,b; Pizarro-Cerdá, 1998; Pizarro-Cerdá *et al*., 1998).

In *Mycobacterium*, opsonization favours invasion as well as the intracellular localization of the bacterium in compartments that diverge from the degradative pathway (Schorey *et al*., 1997). In *Samonella*, the intracellular trafficking pattern of bacteria-containing phagosomes is independent of the mechanism of bacterial entry (Rathman *et al*., 1997). In *Brucella* the mode by which the bacterium is internalized by professional phagocytes seems to be linked to its fate within vacuolar compartments. For instance, phagocytosis of opsonized *Brucella* through Fc and complement receptors favours bacterial intracellular destruction (Harmon *et al*., 1988, 1989; Gross *et al*., 1998), indicating that phagosomes are more prone to fuse with lysosomes when *Brucella* is internalized by these means. In the case of non-professional phagocytes, it is

not known if the intracellular trafficking and fate of *Brucella* are linked to its mode of entry. Proper understanding of the relationship between these two phenomena is important to dissect virulence mechanisms and to comprehend the biogenesis of *Brucella* inside cells.

It has been demonstrated that different sets of small GTPases are needed for bacterial penetration and intracellular trafficking. GTPases of the Rho subfamily such as Rho, Rac and Cdc42, known to be linked to cytoskeletal functions, have been demonstrated to play a crucial role during penetration of intracellular pathogens to cells (Dramsi and Cossart, 1998). Similarly, Rab small GTPases, such as Rab5 and Rab7, frequently associated with early and late intracellular endosomes, respectively, play a primary function in the intracellular trafficking associated with intracellular pathogens (Alvarez-Domínguez *et al*., 1996; Méresse *et al*., 1999). Invasion of virulent *Brucella abortus* in non-professional phagocytes requires small GTPases of the Rho subfamily and direct activation of Cdc42 (Guzmán-Verri *et al*., 2001). Once inside cells, *Brucella* localizes in early phagosomes, where it avoids fusion with late endosomes and lysosomes. Then, the bacterium redirects its trafficking to autophagosomes to reach its final destination at the endoplasmic reticulum (ER), where it replicates extensively (Pizarro-Cerdá *et al*., 1998). Therefore, it seems plausible that by manipulating these two different types of small GTPases, we could discern whether or not the molecular mechanisms leading to *Brucella* internalization determine its intracellular trafficking. Indeed, the experiments designed in this work demonstrate that *Brucella* internalization and intracellular trafficking are two independent molecular events in non-professional phagocytes. In addition we show that replicating *B. abortus* does not interfere with crucial cellular processes and that this bacterium is capable of replicating in alternative compartments different from the ER.

Results

CNF-treatment overcomes low permissibility of non-professional phagocytes to B. abortus

In vitro experiments indicate that the number of virulent *Brucella* organisms associated to cells and the proportion of infected cells is low in comparison to other intracellular bacteria. In order to quantify this observation, monolayers of HeLa and BHK cells were infected with *B. abortus* at different multiplicities of infection (MOI) for 30 min and the percentage of cells associated with bacteria determined. At a MOI as high as 50 000 only approximately 25% of the HeLa cells were associated with bacteria whereas at a MOI of 500 only 5% of the cells were associated with bacteria (Fig. 1A). It has been observed that BHK cells are significantly more permissive to *B. abortus* invasion than other non-professional phagocytic cell lines (Pizarro-Cerdá *et al*., 1998). When the percentage of BHK cells associated with bacteria at a MOI as high as 50 000 was determined, no more than 60% of the cells were associated with brucellae (Fig. 1A). These experiments confirmed previous reports (Detilleux *et al*., 1990a; Pizarro-Cerdá *et al*., 1998) indicating that non-professional phagocytic cell lines posses low permissiveness to *B. abortus* infections.

Small GTPases of the Rho subfamily are involved in the mechanism of entrance of *B. abortus* to non-professional phagocytes (Guzmán-Verri *et al*., 2001). Because the *Escherichia coli* cytotoxic necrotizing factor (CNF) has the ability to permanently activate several of these GTPases (Flatau *et al*., 1997), we evaluated the ability of *B. abortus* to invade and replicate in CNF-treated cells. Consistent with previous reports (Pizarro-Cerdá *et al*., 1998), after an initial stationary phase of 18 h, virulent *B. abortus* 2308 replicates successfully in non-intoxicated HeLa cells, reaching their highest intracellular concentration at 48 h (Fig. 1B). Under the same MOI conditions (500 bacteria per cell), *B. abortus* invades and replicates more efficiently in CNF-treated cells than in non-treated cells (Fig. 1C). The number of infected cells with *B. abortus* was 85%, whereas the number of bacteria per cell was doubled. The number of bacteria replicating within the monolayer was 10 times higher in CNF-treated cells when compared to non-treated cells (Fig. 1D), indicating that this bacterium is fully able to invade and replicate within these intoxicated cells. Although the poorly invasive *B. abortus bvr*S mutant was able to invade CNF treated cells in higher numbers as compared to its invasion to non-intoxicated cells, the replication of this mutant strain was still very limited (Fig. 1B and C) and intracellular destruction was evident afterwards (see below).

B. abortus *enters CNF-treated cells by membrane ruffling*

Because CNF intoxication could be used to internalize a greater number of *B. abortus* into non-professional phagocytes, it was important to determine the mechanism of entrance. When visualized by electron microscopy, no particular eukaryotic host cell membrane rearrangement was observed upon contact with virulent 2308 or non-virulent *B. abortus* (Fig. 2A and B). In contrast, both virulent and non-virulent *B. abortus* strains were ingested by the dynamic membrane ruffling induced by the toxin in CNF-treated cells (Fig. 2C and D). Similarly, after *Brucella* infection of non-treated cells, only minor cytoskeletal rearrangements were observed by fluorescence microscopy using fluorescein isothiocyanate (FITC)-phalloidin labelled actin filaments (Fig. 2E). On the contrary, bacteria not only associated with host cells to a greater extent in CNF-treated cells but they also closely localized to massive

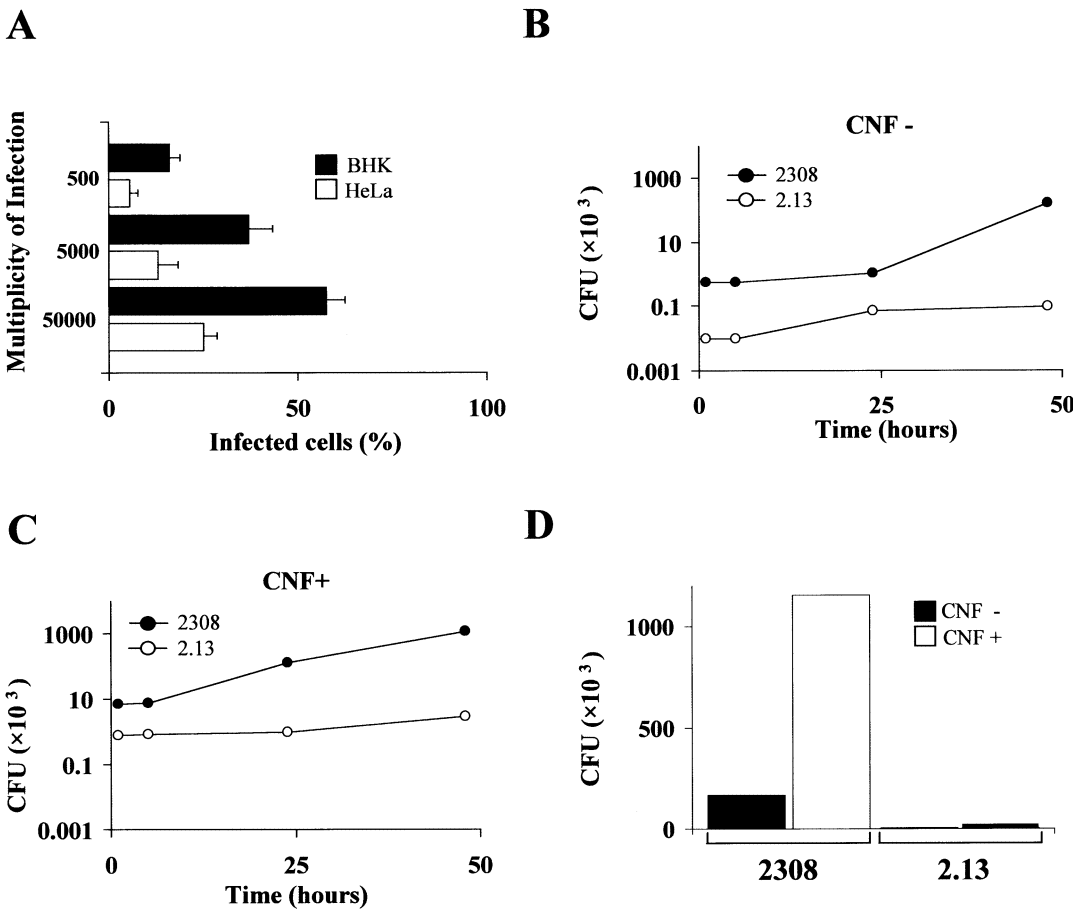


Fig. 1. Effect of CNF intoxication on *B. abortus* association to cells and intracellular replication.
A. Non-intoxicated cells were infected with virulent *B. abortus* 2308 at the indicated MOI for 30 min. Then cells were washed, fixed and bacteria visualized with FITC-anti-LPS antibodies. The percentage of cells associated with bacteria was calculated.
B. Non-intoxicated HeLa cells were infected with the virulent *B. abortus* 2308 or the non-virulent *bvr*S mutant *B. abortus* 2.13 for 30 min at a MOI of 500. Extracellular bacteria were killed by addition of 100 μg ml^{-1} gentamicin for 30 min. After the indicated incubation times, cells were lysed with 0.1% Triton in PBS and intracellularly replicating bacteria determined by CFU counting.
C. HeLa cells were intoxicated with CNF for 2 h. After intoxication, cells were infected with either the virulent *B. abortus* 2308 or the *bvr*S mutant *B. abortus* 2.13 for 30 min at a MOI of 500 and processed as described in B.
D. Number of CFUs obtained after 48 h infection reflecting replication of *B. abortus* 2308 or *B. abortus* 2.13 in untreated or CNF-intoxicated HeLa cells. The standard deviation in B–D was less that 5% at all points.

actin rearrangements induced by the CNF (Fig. 2F). Thus, *B. abortus* penetrates CNF-treated cells through membrane ruffles elicited in response to the activated GTPases of the Rho subfamily, a reminiscence of the strategy used by some enteric bacteria, such as *Salmonella*, to invade non-professional phagocytic cells (Scherer and Miller, 2001).

Rho GTPases activation promotes B. abortus *internalization without altering intracellular trafficking*

Consistent with previous results obtained in non-phagocytic cell lines (Pizarro-Cerdá *et al*., 1998), virulent *B. abortus* 2308 was found to avoid fusion with lysosomes. After transient localization in early phagosomes, *Brucella* transits through autophagosomes, which are lysosome-associated membrane glycoprotein (LAMP)-2 positive/calnexin positive/cathepsin D negative compartments. Then, virulent *Brucella* finally reaches the ER co-localizing with the marker calnexin but not with LAMP-2. In contrast, the non-virulent *B. abortus bvrS* mutant never reaches the ER as it is unable to avoid lysosome fusion, as revealed by its co-localization with the lysosomal marker cathepsin D, followed by its destruction within these digestive compartments (Sola-Landa *et al*., 1998). As the mechanism of entry of *B. abortus* into CNF intoxicated cells differs from standard conditions, it was important to determine if the intracellular trafficking was also altered.

At 4 h post infection, virulent *B. abortus* 2308 did not co-localize with cathepsin D although it showed a clear

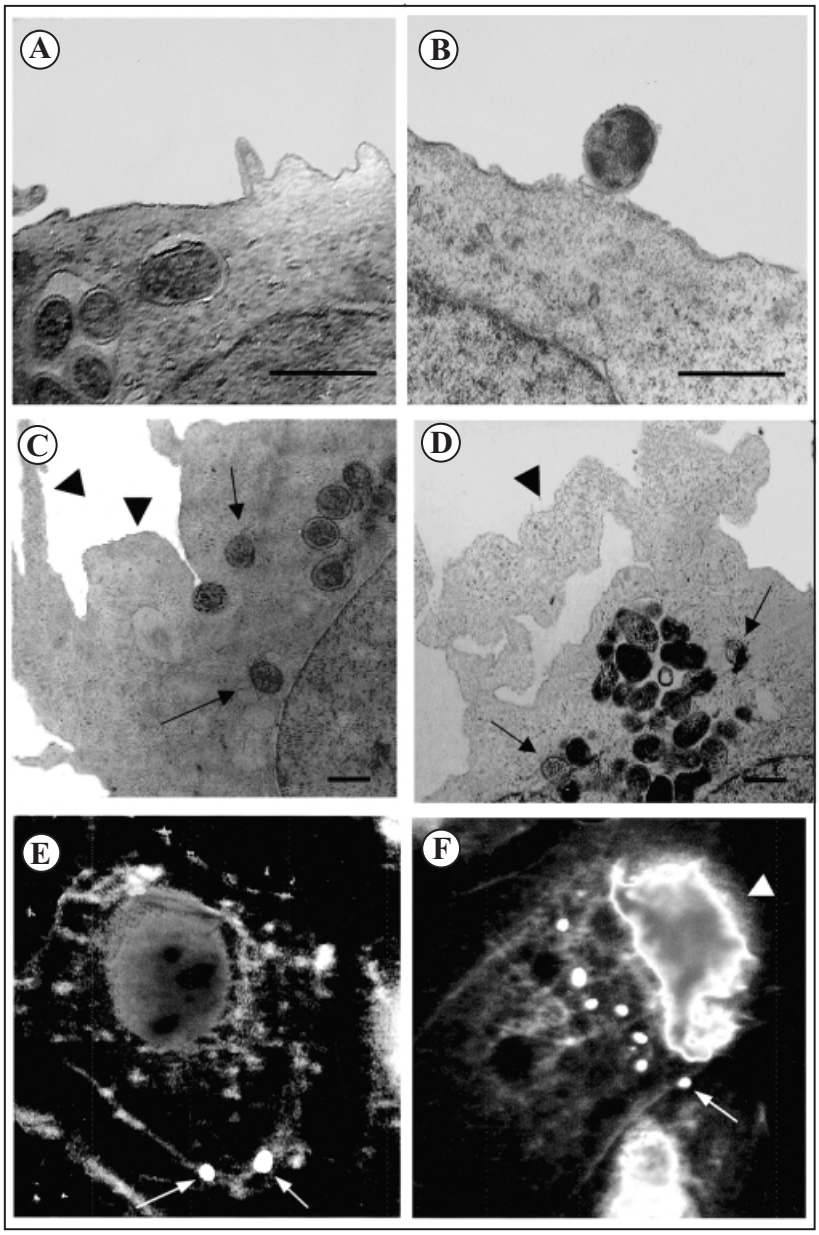


Fig. 2. Microscopic analysis of the entrance of *B. abortus* on CNF-intoxicated HeLa cells. Untreated (A, B and E) and 2 h CNF-intoxicated (C, D and F) HeLa cells were infected with virulent *B. abortus* 2308 (A, C, E and F) or non-virulent *B. abortus* 2.13 *bvr*S mutant (B and D) for 30 min at a MOI of 5000. After infection monolayers were washed, fixed and processed for transmission electron microscopy (A–D) or fluorescence microscopy (E and F) using anti-*Brucella* antibodies to detect cell-associated bacteria and FITC-labelled phalloidin to detect polymerized actin. Arrows indicate the intracellular brucellae, while arrowheads point to membrane ruffles. Bar: 1 μm.

co-localization with LAMP-2. After 8 h, there was still some co-localization with LAMP-2 and calnexin. After 24 h *B. abortus* 2308 displayed an extensive co-localization with calnexin but not with LAMP-2 or cathepsin D, indicating its presence in the ER (Fig. 3A). Importantly, the intracellular virulent bacteria displayed an intact structure reflecting their viability. In contrast, non-virulent *B. abortus bvr*S mutant extensively co-localized with cathepsin D and LAMP-2 as early as 4 h. After 24 h the bacteria remained within cathepsin D positive/calnexin negative compartments indicating fusion of their vacuoles with lysosomes (Fig. 3B). The *bvr*S mutant, in contrast to the virulent *Brucella*, lost its intact appearance and only bacterial debris were seen after 24 h. Thus, even if CNF induces the entrance of *B. abortus* through membrane ruffling, the intracellular routes of both the virulent and non-virulent *Brucella* are conserved in these intoxicated cells.

Expression of constitutively active Rab5 interferes with B. abortus *trafficking but not with internalization*

We studied the influence of another set of GTPases, Rab proteins, in the entrance and trafficking of virulent *B. abortus* to non-professional phagocytic cells. It is known that Rab5 controls early endocytosis whereas Rab7 plays a role in late endocytic events (Gorvel *et al.*, 1991; Méresse

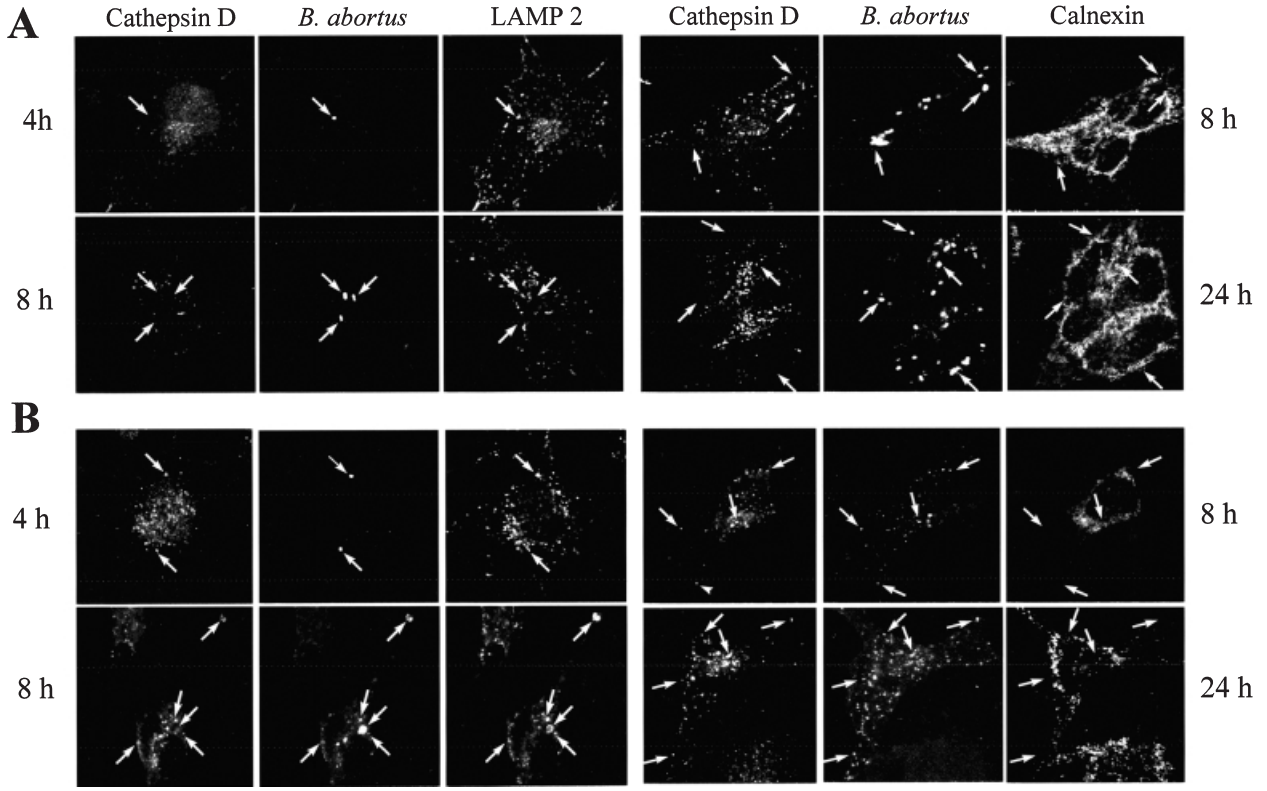


Fig. 3. Intracellular trafficking of *B. abortus* on CNF-intoxicated HeLa cells. Monolayers were intoxicated for 2 h with CNF and then infected with *B. abortus* for 30 min at a MOI of 500. Extracellular bacteria were killed by addition of 100 μg ml^{-1} gentamicin for 30 min. After the indicated incubation times in the presence of 5 μg ml^{-1} gentamicin cells were fixed and immunostained using antibodies against cathepsin D, *B. abortus* LPS, LAMP-2 and calnexin.
A. Virulent *B. abortus* 2308.
B. Non-virulent *B. abortus* 2.13 *bvr*S.

et al., 1995). NIH3T3 cells were selected for these experiments due to the high efficiency in obtaining stable Rab5 and Rab7 transfectants (Fig. 4) as described in *Experimental procedures*. The internalization mode, the intracellular route and the replication profile of *B. abortus* in infected NIH3T3 cells were indistinguishable from those observed in HeLa cells (data not shown).

Because Rab5 stimulates fluid phase-dependent phagocytosis but not receptor-dependent phagocytosis (Duclos *et al*., 2000), we determined whether *B. abortus* uptake was altered in Myc-Rab5Q79L-transfected cells. *Brucella* entered Myc-Rab5Q79L-expressing cells at the same rate than control or Myc-Rab7Q67L-transfected cells (Fig. 5B). The intracellular trafficking of *B. abortus* in Myc-Rab7Q67L-expressing cells was the same as in controls, efficiently reaching its replicating niche at the ER, without acquiring markers of late endosomes or lysosomes (data not shown). In contrast, a significant proportion of the internalized *B. abortus* was retained and capable of multiplying within Myc-Rab5Q79L-positive giant early endosomes (Fig. 5A). Altogether these results

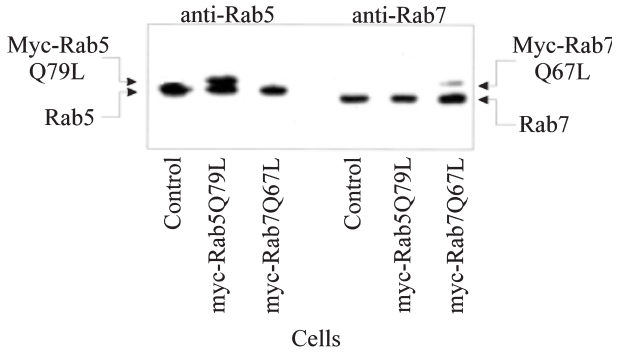
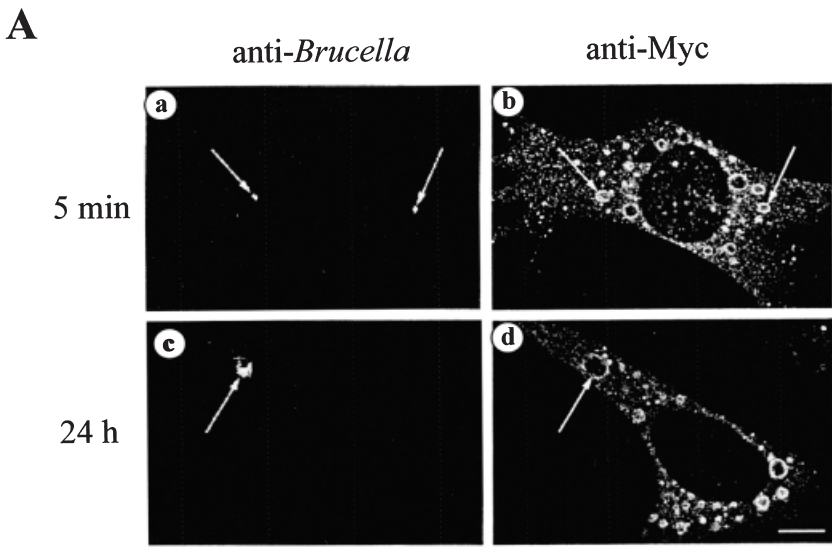


Fig. 4. Expression of constitutively active Rab5 and Rab7 GTPases in stable transfectants. Twenty micrograms of total lysate proteins from Myc-Rab5Q79L- and Myc-Rab7Q67L-NIH3T3 cells were separated in 12% SDS-PAGE, transferred to immobilon-P and analysed by Western blot using affinity purified anti-Rab5 or anti-Rab7 antibodies in combination with a peroxidase conjugate. Bands corresponding to endogenous and Rab mutants are shown.



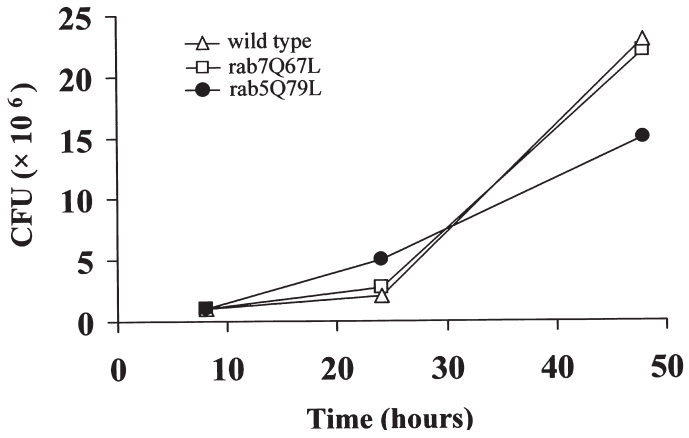


Fig. 5. *B. abortus* trafficking and replication in Rab-overexpressing cells.
A. NIH3T3 stably transfected with Myc-tagged Rab5Q79 L cells grown on 13 mm glass slides were infected with *B. abortus* 2308 and processed after 5 min (a and b) or 24 h (c and d). Slides were processed for immunofluorescence with anti-*Brucella* antibodies (a and c) or anti-Myc antibodies (b and d). Bar: 20 μm. Arrows in c and d show a cluster of replicating bacteria within a giant early endosome.
B. Monolayers of wild-type NIH3T3 cells (white triangles) or NIH3T3 cells overexpressing Rab5Q79 L (black circles) or Rab7Q67 L (white squares) were infected 1 h with *B. abortus* 2308. After infection, cells were washed and extracellular bacteria killed by gentamicin addition. At the indicated times, monolayers were lysed and CFU corresponding to intracellular bacteria were determined. The values represent the media of three independent experiments with standard deviations less than 5% at each point.

indicate that *B. abortus* internalization does not depend on Rab proteins and that intracellular trafficking to the ER includes passing through Rab5-containing but not Rab7-containing-compartments. Furthermore, despite being retained in early compartments, *B. abortus* is still capable of avoiding fusion with lysosomes and achieving replication in compartments other than the ER.

B. abortus *does not interfere with host-DNA synthesis, cell mitosis and cytokinesis*

It has been repeatedly observed that the low number of cells infected by *B. abortus* eventually sustain a massive replication of the bacterium (Detilleux *et al*., 1990a). Thus, we took advantage of certain side-effects produced by CNF intoxication to study how the massive replication of *B. abortus* affects basic cellular processes. CNF treatment inhibits the cytokinesis of intoxicated cells while not affecting karyokinesis resulting in the generation of multinucleated cells (Flatau *et al*., 1997). The presence of multinucleated cells is then an indicator of successful division of the nucleus without cell division. When *B. abortus*-infected CNF-intoxicated HeLa cells were further incubated for 24 and 48 h, it was evident that the massive bacterial growth, commonly observed in control cells, also took place in intoxicated cells, indicating that CNF treatment does not interfere with bacterial replication. Besides, it was observed that a large proportion of the massively infected cells after 48 h were multinucleated as well, suggesting that *B. abortus* infection was not interfering with the mitotic process (Fig. 6A). The proportion of infected and non-infected CNF-treated cells showing more than one nuclei was almost identical, demonstrating that, in fact, the presence *B. abortus* inside host cells does not

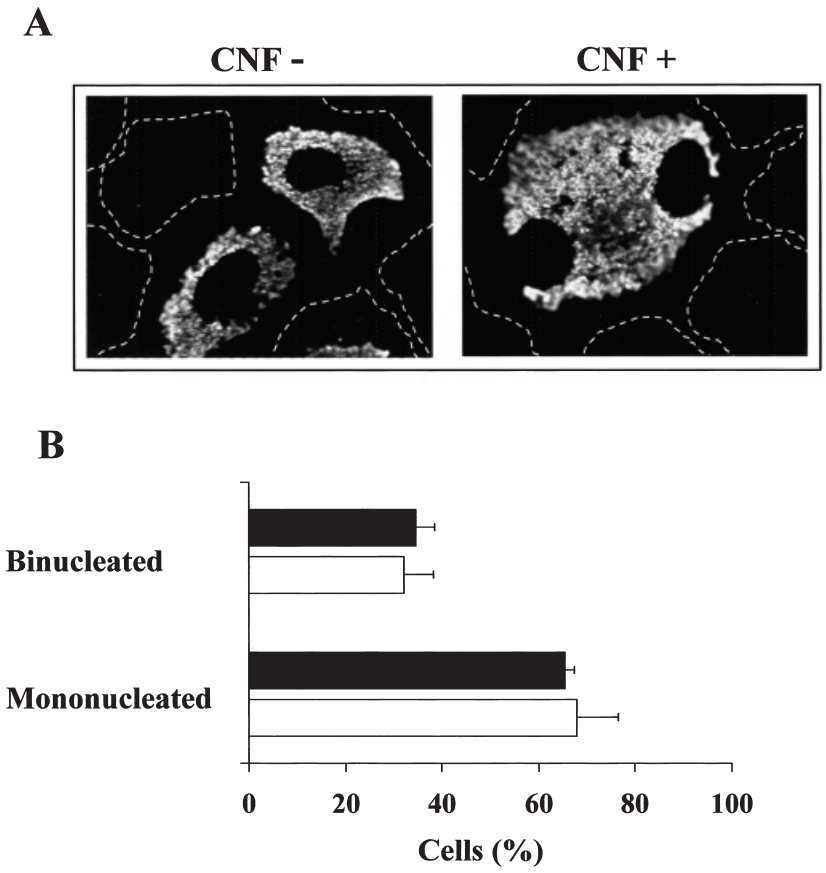


Fig. 6. Effect of *B. abortus* infection in host cell cytokinesis.
A. HeLa cells grown on 13 mm glass slides were treated 2 h with CNF or left untreated. Cells were then infected with *B. abortus* 2308 for 30 min at a MOI of 500. Extracellular bacteria were killed by addition of 100 μg ml^{-1} gentamicin for 30 min, washed and cells further incubated for 48 h in the presence of 5 μg ml^{-1} of gentamicin. Cells were then fixed in 3.7% paraformaldehyde and processed for immunofluorescence using anti-*B. abortus* LPS antibodies. Cells were photographed at 1000× magnification. Dashed lines represent neighbouring non-infected cells.
B. Three hundred infected cells (black bars) and 300 non-infected cells (white bars) were counted and the percentage of mononucleated and binucleated cells was calculated. The mean and standard deviation of three separated experiments is shown.

interfere with nuclear division (Fig. 6B). Though in non-CNF-treated cells is not possible to determine by microscopic examination which cells had gone through mitosis, the presence of pairs of massively infected cells surrounded by uninfected cells suggested that these have recently gone through cytokinesis (Fig. 6A). In this respect it is also interesting to note that intercellular spread of *Brucella* organisms does not occur, as is the case of other intracellular pathogens such as *Listeria* and *Shigella* (Dramsi and Cossart, 1998). Moreover, new infections from released *Brucella* are precluded due to the presence of bactericidal antibiotic in the media (Pizarro-Cerdá *et al.*, 1998).

DNA synthesis was examined by bromodeoxyuridine (BrdU) incorporation into S phase cells followed by fluorescent detection using monoclonal antibodies against the nucleotide analogue. When *B. abortus* infected CNF-treated and non-treated HeLa cells were processed in this manner it was evident from microscopic examination the presence of massively infected cells with fluorescent nuclei indicating that the infection does not alter the normal DNA synthesis machinery of the host cell (Fig. 7A). Quantification of the percentages of infected and non-infected cells in S phase gave almost identical results confirming this hypothesis (Fig. 7B). Thus, intracellular replication of *B. abortus* does not interfere with DNA synthesis, nuclear and cellular division of host cells.

B. abortus*-containing phagosomes are more abundant in CNF-treated cells*

We took advantage of the augmented entrance of *B. abortus* into CNF-intoxicated cells to isolate early *Brucella*-containing phagosomes. Post nuclear supernatants (PNS) prepared from non-CNF-treated HeLa cells infected with virulent *B. abortus* 2308 for 90 min were separated on a discontinuous sucrose gradient. Colony forming units (CFU)-counting after plating of the different fractions indicated the presence of the *Brucella*-containing compartments in the 20–40% interface (Fig. 8A). When the same experiment was repeated using CNF-treated cells the *Brucella*-containing compartments were recovered from the same fraction as the non-intoxicated cells suggesting that the density of the compartments was not altered by the intoxication. When compared in terms of number, up to 15 times more *Brucella*-containing compartments were recovered from CNF-treated cells than from non-treated counterparts (Fig. 8A) mirroring the

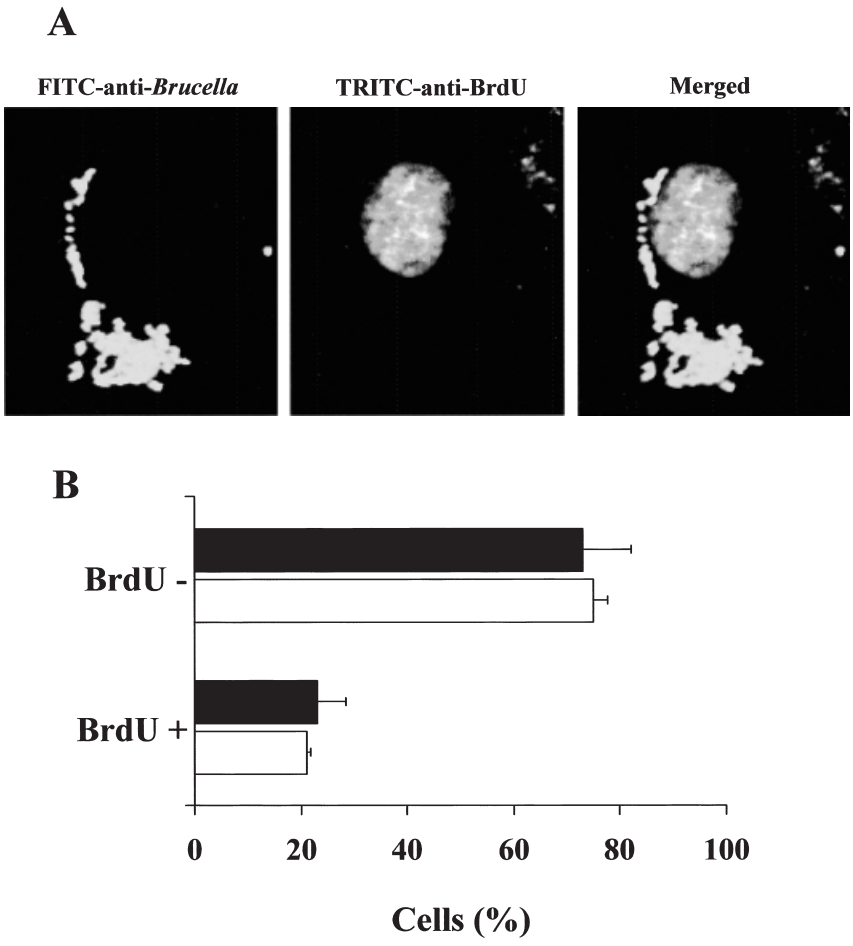


Fig. 7. Effect of *B. abortus* infection in host DNA synthesis.
A. HeLa cells were infected with *B. abortus* 2308 for 30 min at a MOI of 500. Extracellular bacteria were killed by addition of 100 μg ml^{-1} gentamicin for 30 min and cells further incubated for 44 h in the presence of 5 μg ml^{-1} of gentamicin. MEM supplemented with 10 μg ml^{-1} of BrdU was added to the cells for additional 4 h. Cells were then fixed with 3.7% paraformaldehyde and doubled-immunostained using antibodies against *B. abortus* LPS (FITC-labelled) and BrdU epitope (TRITC-labelled). *B. abortus* infected cells were photographed at 1000× magnification.
B. Three hundred infected cells (black bars) and 300 non-infected cells (white bars) were counted and the percentage of DNA-synthesizing cells was calculated for each population. The mean and standard deviation for three separated experiments is shown.

increase in *B. abortus* entrance induced by the activation of Rho GTPases (Fig. 1). Even though *Brucella*-containing compartments were purified in the presence of gentamicin to avoid contamination with bacteria without eukaryotic membranes, the integrity of the isolated compartments was corroborated by immunofluorescence. Freshly prepared *Brucella*-containing compartments were incubated with FITC-labelled anti-*Brucella* antibodies. After six washings and fixation steps, *Brucella*-containing compartments were permeabilized and further incubated with tetrarhodamine isothiocyanate (TRITC)-labelled anti-*Brucella* antibodies. With this protocol, bacteria contained in compartments are stained red whereas free bacteria are stained green. The majority (more than 90%) of the *Brucella*-containing compartments isolated either from non-intoxicated or from CNF-treated cells showed a red fluorescence, indicating the presence of a host-derived lipid bilayer surrounding the bacteria (Fig. 8B). Similar results were observed with the vaccine strain *B. abortus* S19, however, lower quantities on *Brucella* containing vesicles were recovered (data not shown). Thus, CNF treatment increases the yield of *Brucella*-containing compartments isolated from infected cells without apparent alteration neither to the integrity nor to the biological and physical properties of the compartments.

Discussion

Virulent *Brucella* organisms penetrate non-professional phagocytes by modest actin recruitment through molecular mechanisms that involve small GTPases of the Rho subfamily and direct activation of Cdc42 (Guzmán-Verri *et al.*, 2001). However, in contrast to other intracellular bacterial pathogens that also recruit small GTPases of the Rho subfamily and actively penetrate non-professional phagocytes (Dramsi and Cossart, 1998), *Brucella* is not capable of attaching to every single cell in a monolayer. This low permissibility of *Brucella* uptake is not only an *in vitro* artifact observed in non-professional phagocytic cell lines grown in the laboratory, but also a general phenomenon displayed by non-professional phagocytes. For instance, exposure of chorioallantoic trophoblasts (the key target cell in pregnant females) as long as 10 h to *B. abortus* 2308 at a rate of 200 bacteria per cell resulted in

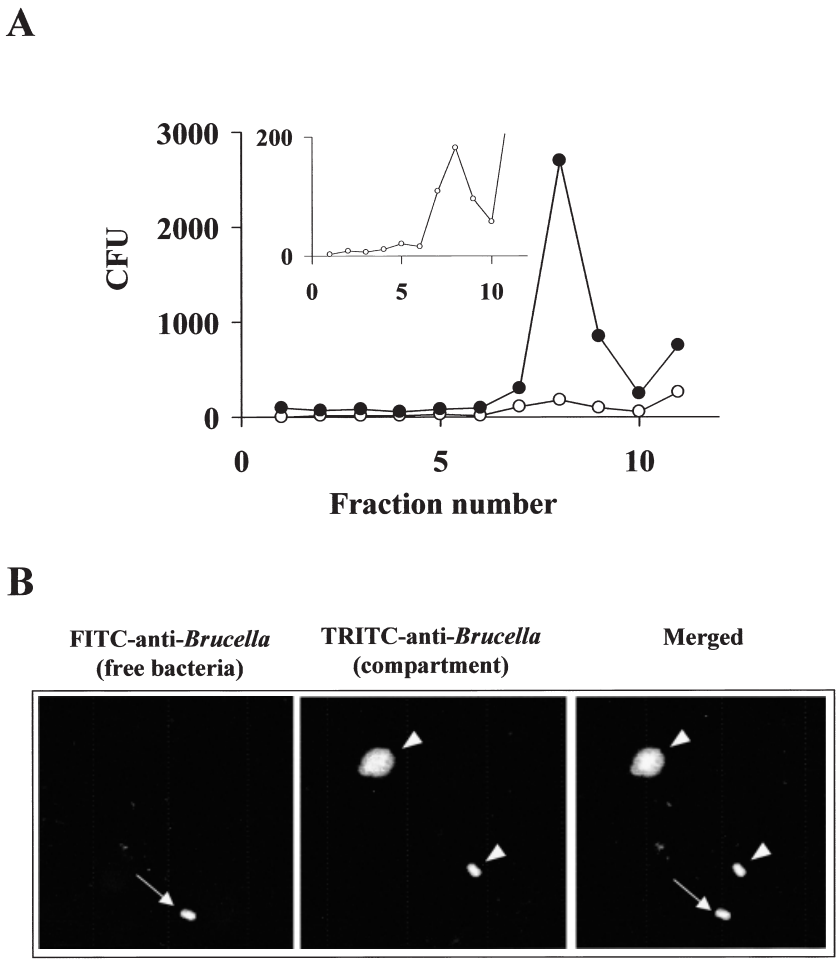


Fig. 8. Isolation of *B. abortus*-containing compartments from CNF-intoxicated cells.
A. HeLa cells were intoxicated with CNF for 2 h (black circles) or left untreated (white circles). Insert corresponds to the same untreated infected cells (white circles), but at different scale in order to appreciate the profile of the peak. Cells were infected with *B. abortus* 2308 at a MOI of 5000 for 15 min and extracellular bacteria killed by addition of 100 μg ml^{-1} of gentamicin. Infected cells were further incubated for 60 min. After incubation cells were disrupted, PNS prepared as described in *Experimental procedures* and loaded on a discontinuous sucrose gradient containing 5 μg ml^{-1} gentamicin. After 2 h centrifugation at 100 000 × *g*, 12 fractions were collected, plated on TSA and CFU for each fraction determined.
B. Fraction 8 from A was incubated with FITC-conjugated anti-*Brucella* LPS antibodies for 30 min. After incubation, *Brucella* containing compartments were pelleted and fixed onto a 13 mm glass slide. After permeabilization with 0.5% Triton, the slides were incubated with TRITC-conjugated anti-*Brucella* LPS antibodies for 30 min. Bacteria within compartments are stained red (TRITC-labelled) whereas free bacteria are stained green (FITC-labelled).

only 40% of infection (Samartino *et al*., 1994). Furthermore, ultrastructural analysis of placentas from intravenously *Brucella*-injected goats revealed the presence of both infected and non-infected trophoblasts; whereas the former group of cells suffered a great distension of the ER where the bacterium was visualized multiplying, the latter group exhibited identical morphometric characteristics as trophoblasts extracted from non-inoculated animals (Anderson *et al*., 1986). These results resemble the observations made by us and others in HeLa cells where the few parasited cells sustain a heavy replication in the ER while neighbouring cells remain essentially uninfected. Thus, the use of HeLa cells is clearly justified as a model to understand the *in vivo* events occurring in non-professional phagocytic cells during the development of brucellosis.

It is known that independent activation of Rho, Rac and Cdc42 favours binding and penetration of *Brucella* to cells, while inactivation of these GTPases inhibits bacterial invasion (Guzmán-Verri *et al*., 2001). Accordingly, pool activation of these small GTPases by CNF greatly promotes the internalization of *Brucella* to non-professional phagocytes, overcoming their low infectivity. The high internalization rate of *B. abortus* in CNF-treated cells could be due to an increase in the number of 'physiological receptors' or to the acquisition of a 'phagocytic' phenotype by these cells as previously reported (Fiorentini *et al*., 1997). The latter possibility is, however, more plausible considering the ultrastructural and light microscopy analysis, demonstrating the entrance of *B. abortus* through membrane ruffles. This hypothesis is also sustained by the observation that the poorly invasive *B. abortus bvr*S mutant is also ingested by the same route and in larger numbers by CNF-treated cells. Alternatively, both possibilities may work in conjunction, favouring the binding and penetration of *Brucella* to CNF-intoxicated cells.

Considering that in CNF-treated cells *Brucella* enters through a different mechanism to the one used under normal conditions, might lead to the assumption that the intracellular trafficking of this bacterium would be also altered, as it seems to be the case of professional phagocytes (Harmon *et al*., 1988, 1989; Gross *et al*., 1998).

However, when the intracellular trafficking of virulent and non-virulent *B. abortus* strains was studied in CNF-treated cells, no differences were observed in relation to control cells. Virulent *Brucella* strain was able to reach its replicating niche at the ER, passing through compartments compatible with autophagosomes, whereas the non-virulent *bvr*S mutant was directed to lysosomes and destroyed. This means that regardless of the mechanism of entrance, virulent *Brucella* is able to sense its intracellular status and respond to it by unknown molecular mechanisms in order to direct its trafficking to the ER in non-professional phagocytes. Additionally, it is important to stress that the 'phagocytic behaviour' induced by CNF in epithelial cells applies only for the internalization step of *Brucella* biogenesis as the intracellular route followed by the bacterium does not reflect the heterogeneous pattern observed in activated professional phagocytes, but rather a characteristic pattern determined in non-professional phagocytic cells.

Constitutive expression of active forms of Rab5 or Rab7 did not influence the internalization of *Brucella* in non-professional phagocytes, suggesting that this bacterium is physiologically internalized by receptor-mediated phagocytosis. Indeed, phagocytosis with the involvement of receptors seems to be mediated by GTPases of the Rho subfamily (Caron and Hall, 1998) rather than Rab proteins (Duclos *et al*., 2000). On the other hand, expression of constitutively active Rab5, but not Rab7, did alter *Brucella* intracellular trafficking. Therefore, while *Brucella* specifically activates Cdc42 during its penetration to cells (Guzmán-Verri *et al*., 2001), normal expression of Rab5 but not Rab7 GTPases in phagosomes is necessary to guide its intracellular trafficking. This property differentiates *Brucella* from other intracellular pathogens such as *Salmonella* where an adequate Rab7 function is required in order to create the final intracellular niche (Méresse *et al*., 1999). Furthermore, the fact that Rab7 overexpression does not alter the intracellular trafficking of *B. abortus*, is consistent with previous results indicating that *Brucella* does not transit through late endosomes, but rather actively deviates its route to alternative compartments (Pizarro-Cerdá *et al*., 1998).

The above observations indicate that *B. abortus* has developed different strategies in order to manipulate two sets of small GTPases to control two separate steps in its biogenesis: internalization and intracellular trafficking. The hypothesis of two separate stepwise independent molecular mechanisms of penetration and intracellular trafficking is also supported by the recent discovery that *Brucella* mutants deficient in the type IV secretion system *vir*B are defective in intracellular trafficking but not in the entrance to HeLa cells (Comerci *et al*., 2001; Delrue *et al*., 2001). Similarly, the entrance of the poorly invasive *B. abortus bvr*S mutant is greatly enhanced in CNF-treated cells. However, this strain is finally directed to lysosomes and destroyed indicating that the two component BvrS-BvrR system does not only control the entrance of the bacterium to the cell (Sola-Landa *et al*., 1998), but that is also involved in the control of *B. abortus* intracellular trafficking.

Infection of cell types where two different sets of small GTPases were manipulated, also resolved additional questions related to *Brucella* parasitism. One relevant phenomenon was the demonstration that normal DNA replication, mitotic and cytokinetic processes of massively *Brucella*-infected cells are not altered. These results are consistent with previous reports where *Brucella*-infected Vero cells were observed in different phases of the mitotic cell cycle (Detilleux *et al*., 1990a). It has been recently reported that infection with *B. suis* prevents apoptosis in human THP-1 monocytes (Gross *et al*., 2000), presumably as a strategy to sustain extensive proliferation. Our results imply that a similar phenomenon could occur in non-professional phagocytic cells.

The ability of *Brucella* to survive and achieve limited replication in giant Rab5 compartments different from the ER was also a relevant observation indicating that although captured within giant early phagosomes, the bacterium could adapt and achieve some replication level. This result is also consistent with the observation that non-polar *vir*B *Brucella* mutants are capable of surviving and establishing limited replication outside the ER (Comerci *et al*., 2001). Therefore, the limiting step in *Brucella* intracellular survival within non-professional phagocytic cells lines is not only the ability to redirect its trafficking to the ER, but also to render its early phagosome non-fusogenic with late endosomes and subsequently with lysosomes, as indicated in previous investigations (Pizarro-Cerdá *et al*., 1998).

Efforts are currently in progress to characterize the proteomic composition of phagosomes containing inert particles and intracellular pathogens (Ferrari *et al*., 1999; Pizarro-Cerdá *et al*., 2002). A significant fraction of these efforts is carried on phagocytic cell lines. However, in macrophages the characterization of *Brucella* phagosomes would be hampered by the heterogeneity of the compartments where the bacteria are found (Arenas *et al*., 2000; Moreno and Moriyón, 2001). In contrast, in non-professional phagocytes *Brucella* follows a more uniform intracellular trafficking, acquiring specific markers stepwise and departing from the phagolysosomal route (Pizarro-Cerdá *et al*., 1998). Thus, the use of CNF to isolate *Brucella*-containing compartments from superinfected cells will be a valuable tool to study the intracellular lifestyle of this bacterium. Additionally, this could lead to the generation of chronically infected cell lines, a model which would allow the detection of the intracellular antigenic repertoire produced under brucellosis infections

with the subsequent implications for treatment and prophylaxis.

Experimental procedures

Bacterial strains, plasmids and cell cultures

Bacterial strains were routinely grown in tryptic soy medium (Gibco). Virulent *B. abortus* 2308 is a wild-type, virulent smooth-LPS strain described elsewhere (Sangari and Agüero, 1991). *Brucella abortus* 2.13 is a smooth LPS, poorly invasive 2308 derivative with a Tn*5* insertion in *bvrS* (Sola-Landa *et al*., 1998). *Brucella abortus* S19 is an attenuated vaccine strain (Moreno and Moriyón, 2001). Human cervix carcinoma cells (HeLa; American type culture collection no. CCL-2), baby hamster kidney cells (BHK-21; American type culture collection no. CCL-10) and mouse 3T3 fibroblast (NIH3T3; National Institute of Health, USA) were grown at 37°C under 5% CO_2 in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, 2.5% sodium bicarbonate and 1% glutamine (all from Gibco). Penicillin (100 units ml^{-1}) and streptomycin (100 μg ml^{-1}) routinely added, were excluded from cell cultures during *Brucella* infections.

Plasmids encoding for Myc epitope-tagged constitutively active Rab5 (Myc-Rab5Q79L) and Myc epitope-tagged constitutively active Rab7 (Myc-Rab7Q67L) were constructed as previously described (Méresse *et al*., 1995; Duclos *et al*., 2000). NIH3T3 cells were transfected with Myc-Rab5Q79L- and Myc-Rab7Q67L-containing plasmids by electroporation (Duclos *et al*. 2000). Selection of stable NIH3T3/Myc-Rab5Q79L and NIH3T3/Myc-Rab7Q67L transformants has been described in previous works (Méresse *et al*., 1995). The correct expression of Myc-tagged Rab5 and Rab7 mutants in transfectants was checked before infection by Western blotting. Transfected cells expressed endogenous Rab5 and Rab7 along with the Myc-tagged Rab5 or Rab7 mutant GTPases respectively (Fig. 4). The constitutive expression of Myc-Rab5Q79L and Myc-Rab767L in giant early and late endosomes, respectively, was further confirmed by the action of bafilomycin A1 on these transfected cells. This drug inhibits the vacuolar H ± ATPase and induces rapid vacuolation disruption and redistribution of early (Rab5-containing) but not late (Rab7-containing) endosomes (D'Arrigo *et al*., 1997; Duclos *et al*., 2000), confirming the location of these recombinant GTPases in their respective compartments. As LAMP proteins are found in compartments intersecting with endocytosis and lysosomal fusion, the moderate decrease observed of LAMP-1 in giant endosomes was commensurate with the disrupting action of bafilomycine A-1 on early endosomes (data not shown).

Binding and invasion assays

HeLa, BHK-21, NIH3T3 and NIH3T3/Myc-Rab transformants cells were grown to subconfluency in 24-well tissue culture plates. Cytotoxic necrotizing factor was purified according to Falzano *et al*. (1993). Cytotoxic necrotizing factor-intoxication of HeLa cells was always carried out 2 h before *B. abortus* infection at a concentration of 3 ng ml^{-1}. After intoxication, the monolayer was washed once with cold phosphate-buffered saline, 0.01 M, pH 7.4 (PBS) and kept at 4°C until infection. Infections were carried out using an overnight culture of *B. abortus* diluted in Eagle's minimal essential medium diluted to reach the desired MOI. Plates were centrifuged at 300 *g* at 4°C, incubated for 30 min at 37°C under 5% CO_2 and washed three times with PBS. Extracellular bacteria were killed by adding Eagle's minimal essential medium supplemented with 100 μg ml^{-1} gentamicin for 30 min and cells incubated for the indicated times in the presence of 5 μg ml^{-1} gentamicin. Plates were then washed with PBS. Cells were lysed by adding 0.1% Triton X-100 for 10 min. Aliquots were plated in tryptic soy agar (Gibco) and incubated at 37°C for 3 days for determination of CFU.

Fluorescence and transmission electron microscopy

Cells (5×10^5) were grown on 12 mm glass slides and inoculated with *B. abortus* as described above. Samples were processed for double immunofluorescence as previously described (Pizarro-Cerdá *et al*., 1998). FITC-phalloidin (Sigma) (0.5 μg ml^{-1}) was added to stain polymerized actin cytoskeleton. Quantification of cells associated to bacteria was performed in at least 500 cells. For transmission electron microscopy, cell monolayers infected with an overnight culture of *B. abortus* 2308 were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde (Merck) in 0.1 M phosphate buffer. Samples were placed in 1% O_SO_4 solution for 1 h for fixation, dehydrated in graded concentration of ethanol and infiltrated with Spurr resin. Thin sections on 300-mesh colloidon-coated grids were stained with uranyl acetate and lead Sato's solution (Robinson *et al*., 1987). Preparations were examined with a Hitachi H-7100 electron microscope operating at 75 kV.

For confocal microscopy, cells were grown, infected and processed as described above and processed for double and triple immunofluorescence analysis as described previously (Pizarro-Cerdá *et al*., 1998). The following antibodies were used to localize different intracellular compartments: rabbit polyclonal anti-human LAMP-1 and LAMP-2 (Dr M. Fukuda, The Burnham Institute, La Jolla, CA, USA), rabbit polyclonal anti-cathepsin D (Dr S. Kornfeld, Washington University School of Medicine, St Louis, MO, USA), rabbit polyclonal anti-calnexin (Dr A. Helenius, Institute of Biochemistry, Zurich, Switzerland), rabbit anti-Rab5 (Duclos *et al*., 2000), rabbit anti-Rab7 (Méresse *et al*., 1999) and monoclonal antibody against Myc epitope (Santa Cruz Biotechnology). Cow polyclonal antibodies against *B. abortus* 2308 were used to detect internalized *Brucella* (Pizarro-Cerdá *et al*., 1998). TRITC-conjugated anti-mouse antibody (Sigma), FITC-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories) and TRITC-conjugated rabbit anti-cow antibodies (Sigma) were used as developing antibodies. Indirect immunofluorescence and confocal analysis were performed using a Leica TCS 4D microscope (Leica Lasrtechnik Gmbh, Heidelberg, Germany) under oil immersion.

Assay of DNA synthesis

Cells were infected with *B. abortus* 2308 for 20, 44 and 68 h. After incubation at 37°C the medium was changed for fresh medium containing 10 μg ml^{-1} BrdU (Sigma) and further incubated for 4 h. Cells were then fixed and permeabilized and incubated with monoclonal anti-BrdU (Clone BU-33; Sigma). Cells were further incubated with TRITC-rabbit anti-mouse conjugate and BrdU-incorporating cells were visualized by fluorescence microscopy.

Phagosome isolation

HeLa cells grown on six-well plates were intoxicated with CNF for 2 h or left untreated. Cells were then infected with *B. abortus* 2308 or vaccine strain S19 at a MOI of 5000 for 15 min. After washing with PBS, extracellular bacteria were killed by addition of 100 μg ml^{-1} gentamicin for 30 min. Cells were further incubated for 30 min in the presence of 5 μg ml^{-1} gentamicin. Cells were scrapped off the plates in lysis buffer (250 mM sucrose, 0.5 mM EGTA, 0.1 mM PMSF and 10 μg ml^{-1} leupeptin, 10 mM HEPES, pH 7.4) and *Brucella*-containing compartments released by passing the cell suspension 30 times through a 27-needle coupled to a 1 ml syringe. After 10 s centrifugation at 13000 r.p.m., the compartment-containing fraction was loaded on the top of a discontinuous sucrose gradient (40, 20 and 8.5%) and centrifuged at 30000 r.p.m. on a SW-41 rotor (Beckman) for 2 h after which 12 × 1 ml fractions were collected from the top of the tube. A fixed volume of each fraction was plated on ATS to determine CFUs. Alternatively, free *Brucella* organisms in fraction number 8 (containing the 20%–40% sucrose interface) were visualized by incubation with cow FITC-labelled anti-*Brucella* antibodies for 30 min at 4°C. Compartments were then pelleted by diluting in lysis buffer and centrifuging at 13000 r.p.m. for 10 min. Pelleted compartments were fixed on a 13 mm glass slides with 3.7% paraformaldehyde and permeabilized with 0.5% Triton. *Brucella* contained within compartments were visualized by incubation with rabbit polyclonal anti-*Brucella* antibodies and revealed with TRITC-labelled anti-rabbit conjugate.

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