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Brucella intracellular life: from invasion to intracellular replication

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Abstract

Brucella organisms are pathogens that ultimate goal is to propagate in their preferred niche, the cell. Upon cell contact the bacteria is internalized via receptor molecules by activating small GTPases of the Rho subfamily and by a moderate recruitment of actin filaments. Once inside cells, *Brucella* localizes in early phagosomes, where it avoids fusion with late endosomes and lysosomes. These early events require the control of Rab small GTPases, and cytokines such as the G-CSF. Then, the bacterium redirects its trafficking to autophagosomes and finally reaches the endoplasmic reticulum, where it extensively replicates. Some of the bacterial molecular determinants involved in the internalization and early events after ingestion are controlled by the BvrS/BvrR two component regulatory system, whereas the intracellular trafficking beyond this early compartments are controlled by the VirB type IV secretion system. Once inside the endoplasmic reticulum, *Brucella* extensively replicates without restricting basic cellular functions or inducing obvious damage to cells. The integrity of *Brucella* LPS on the bacterial surface is one of the required factors for *Brucella* intracellular survival, and therefore for virulence.

Keywords: *Brucella*; BvrR/BvrS; *vir*B; Bacterial invasion; Intracellular trafficking

1. Introduction

Members of the genus *Brucella* are pathogenic bacteria of humans and animals exceedingly well adapted to its host and not surviving for extended periods of time in open conditions. Although the classical definition of *Brucella* species describe these bacteria as facultative intracellular parasites, this definition does not honor their true nature which is better understood as a facultative extracellular intracellular parasites. That means

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that the *Brucella* preferred niche is the intracellular environment of host cells. This environment sustains extensive replication, allowing bacterial expansion and the subsequent transmission to new host cells, frequently achieved through the heavily infected aborted fetus. 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 true virulence elements of *Brucella* are those molecular determinants that allow them 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 (Pizarro-Cerdá et al., 1998, 1999; Detilleux et al., 1990a,b). The events attained by *Brucella* parasites during its biogenesis in cells, marked by its binding to the host membrane and culminating in its intracellular replication within vacuolar compartments, are resumed in Fig. 1. This review mainly deals with these phenomena, in the understanding that other important mechanism influencing these events, mainly those involved with the immune response, will be covered by other works in this issue.

2. *Brucella* invasion to cells

Once *Brucella* invades mucosa, professional phagocytes lying underneath the submucosa ingest the bacterium. It has been shown that M cells, macrophages and neutrophils ingest *Brucella* by zipper-like phagocytosis (Ackermann et al., 1988). Opsonized *Brucella* are internalized via complement and Fc receptors in macrophages and monocytes, whereas non-opsonized *Brucella* seems to penetrate via lectin or fibronectin receptors, in addition to other unknown receptors (Campbell et al., 1994; Harmon et al., 1988). It has been reported, however, that opsonized *Brucella*, preferentially phagocytized by activated macrophages via immunological receptors, are more prone to be destroyed than bacteria invading macrophages by other means (Gross et al., 2000; Harmon et al., 1988, 1989). In this sense, the mode by which *Brucella* is internalized by professional phagocytes seems to be linked to its fate within intracellular compartments. On the contrary, in non-professional phagocytes this seems not to be the case. In these cells, the mode of entrance does not interfere with the intracellular trafficking of the ingested *Brucella* (Chaves-Olarte et al., unpublished results).

Despite the fact that no cellular receptor or bacterial ligands have been identified in non professional-phagocytes and *Brucella* organisms, respectively, there is indirect evidence of specific molecules participating in the binding of *Brucella* to cells. For instance, it has been demonstrated that not all epithelial cells in a monolayer are permissive to *Brucella* infection, restricting the binding just to a few cells. Once *Brucella* organisms bind, minor host cellular projections upon contact are generated and the bacteria are mostly located between cell–cell boundaries rather than in the cell body (Chaves-Olarte et al., unpublished results). In cells treated with *Clostridium difficile* TcdB toxin, in which retraction of the cell body is evident, *Brucella* binds to cellular spikes attached to the substrate, suggesting the participation of adhesion plaques (Guzmán-Verri et al., 2001). Similarly, in polarized epithelial cells *Brucella* preferentially binds to the basolateral plane rather than to the

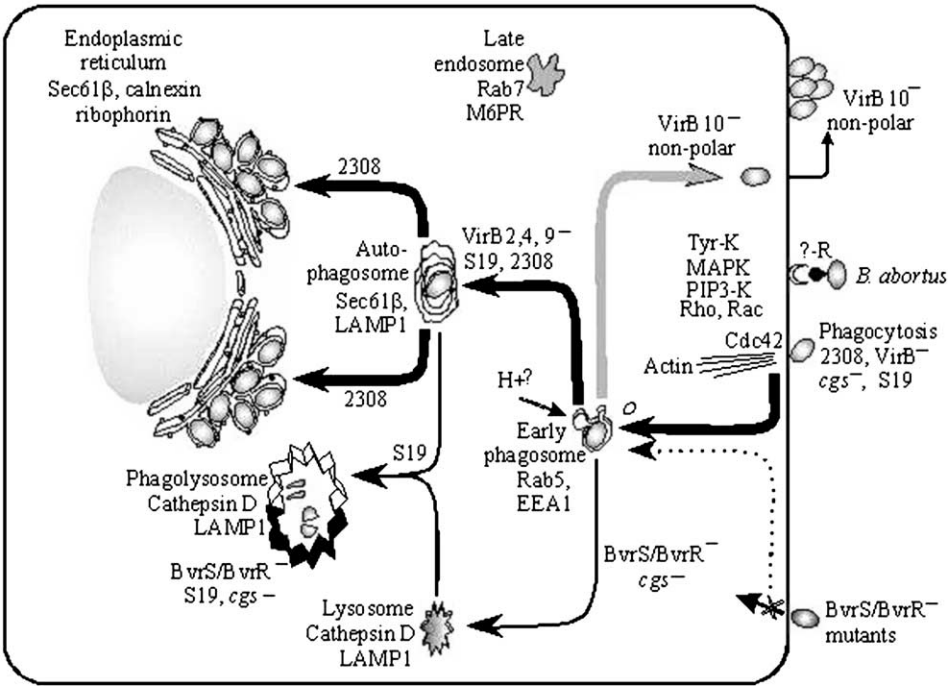

Fig. 1. Schematic model of *B. abortus* invasion and intracellular trafficking in epithelial HeLa cells. *Brucella* organisms bind to discrete sites of cells via unknown receptor molecules (?-R). Then the bacteria penetrate by a discrete phagocytosis with moderate recruitment of actin filaments, activation of small GTPases (Cdc42, Rac and Rho) but direct recruitment of Cdc42 and signals mediated by second messengers (Tyr-K, MAP-K, and PIP3-K). The ingested bacterium, is initially routed to early phagocytic compartments (marked by Rab5 and EEA1) which may be acidified (H^+) by acquisition of specific proton pumps (?). In HeLa cells most of the ingested virulent brucellae are routed (thick black arrows) to the endoplasmic reticulum (sec1β, calnexin, ribophorin) by the autophagocytic route (marked by LAMP1 and sec61β), whereas only a few bacteria are directed (thin black arrows) to phagolysosomes. Some null mutants (BvrS/BvrR$^-$) are defective in penetration (crossed arrow) and when ingested (doted arrow) are incapable of avoiding fusion with lysosomes (marked cathepsin D, LAMP1). Other mutants are not defective in penetration but cannot avoid fusion with lysosomes (cgs$^-$), whereas others transit from autophagosomes to phagolysosomes (S19, VirB 2, 4 and 9 null mutants) or from early phagosomes to the cell membrane (nonpolar VirB10; gray thick arrow). Signals for apoptosis inhibition may be released from the *Brucella* replicating niche. Notice that virulent *Brucella* does not transit through late endosomes (marked withRab7, M6PR).

apical surface (Chaves-Olarte et al., unpublished observations). In HeLa cells, it seems that *Brucella* is ingested through a mechanism which involves a discrete recruitment of actin filaments which are evident at the binding site. Although zipper-like phagocytosis has been observed in these cells (Pizarro-Cerdá et al., 1999), it seems to be an exceptional event (Detilleux et al., 1990a,b; Kuzumawati et al., 2000).

The participation of actin cytoskeleton at the site of attachment observed by fluorescent and electron microscopy has been confirmed by the use of actin-depolimerizing agents such as cytochalasin D or with myosin inhibitors such as 2,3-butanedione monoxime. As it has been demonstrated for other bacteria, it seems also that a modest participation of

microtubules is necessary for *Brucella* uptake (Guzmán-Verri et al., 2001). Given the growing evidence for potential interactions between microtubule and actin networks, it is feasible that bacterial pathogens exploiting one network would also depend on the other. Concomitantly to the recruitment of cytoskeletal structures, activation of small GTPases of the Rho subfamily such as Rho, Rac and Cdc42 are required for *Brucella* internalization in non-professional phagocytes (Guzmán-Verri et al., 2001). This group of GTPases, known to be cytoskeletal regulators, participates in the internalization of several intracellular parasitic bacteria. By interacting with one or more of these molecular switches, pathogenic bacteria are capable to modulate their invasion to cells. In the case of virulent *B. abortus*, Cdc42 is the only GTPase directly activated upon contact with host cells. However, specific activation or inhibition of other GTPases also promote or hamper *Brucella* invasion to epithelial cells. This indicates that although *Brucella* does not directly activate Rho or Rac, the bacterium is capable of taking advantage of the GTPase pools kept in cells. Indeed, specific inhibition of Rac by clostridial toxins totally abrogates the entrance of *Brucella* to cells, reflecting the importance of this GTPase. The role of this protein in *Brucella* internalization is further stressed by the fact that Rac has been described as potential link between the microtubule and actin networks since microtubule growth induces Rac activation and therefore lamellopodium formation (Waterman-Storer et al., 1999).

In addition to the molecular mediators described above, the uptake of killed or alive *Brucella* by non-professional-phagocytes is also suppressed by inhibitors of energy metabolism (iodoacetate and dinitrophenol), inhibitors of receptor-mediated endocytosis (amantadine and methylamine) and by repressors of endosomal acidification (chloroquine, ammonium chloride and monensin). These drugs are capable of inhibiting penetration when added at the same time as the bacterial inoculum (8 h), but not when added after the inoculation period (Detilleux et al., 1991). This suggests that the infection process not only occurs via receptor molecules but that it also requires energy input from the host cell.

Invasion is also inhibited by chemicals and toxins that increase the levels of cyclic-AMP (dibutyl-cyclic-AMP and *Vibrio cholerae* enterotoxin), but is stimulated by toxins and chemicals that increase the levels of cyclic-GMP (*Escherichia coli* enterotoxin A and dibutyl-cyclic-GMP). This suggests an inverse relationship between these two second-messengers during *Brucella* infection. Similarly, wortmanin (which inhibits the PIP3 kinase) considerably reduces the internalization of *Brucella* by epithelial cells, suggesting involvement of PIP3-kinase phosphorylation during this process (Guzmán-Verri et al., 2001). Since the level of cyclic GMP usually increases when the inositol phospholipid pathway is activated, it is likely that binding of *Brucella* to cells also stimulates the generation of IP3 via phospholipase C activation. Other cellular kinases, such as tyrosine kinases and MAP kinases, seem to be required for internalization into non-professional phagocytes, since an inhibition of these enzymes hampers bacterial penetration.

3. *Brucella* molecular determinants for cell invasion

It has been demonstrated that rough *Brucella* attaches in higher numbers to cells than smooth *Brucella*, although the former bacteria is less efficient in invading cells than the

latter (Sola-Landa et al., 1998; Detilleux et al., 1990a,b). This phenomenon suggests that *O*-polysaccharide and the structurally related native hapten (NH) have a role in the invasion of *Brucella* to cells. At least in the case of immunoglobulin opsonised *Brucella*, these two molecules are known to be relevant, since they are the principal determinants for antibody binding. In spite of this, it is also known that these polysaccharide molecules are not prone to be directly opsonised by complement generated by activation of the alternative pathway, suggesting a role of the *O*-polysaccharide in virulence (Moreno et al., 1981). Chromosomal analysis has revealed various putative genes potently coding for adhesins, invasins and *vir*G-like genes for attachment and actin recruitment in cells (DelVecchio et al., 2002). Up to know these molecules have not been yet identified in *Brucella* cell envelope, calling for the generation of mutants in order to determine if they are indeed functional genes coding for these proteins.

It has been determined that the BvrS–BvrR two component system coding for a histidine kinase sensor and a regulator protein is involved in the internalization of *Brucella* in both professional and non-professional phagocytic cells (Sola-Landa et al., 1998). The molecular properties, the phylogenetic relationship and the functional details of this system are described by López-Goñi et al. (2002). In general terms, this system seems to be involved in the structural and functional homeostasis of the outer membrane. *Brucella bvr*S and *bvr*R null mutants, although not exactly equivalent, are defective in several outer membrane proteins, mainly Omp3a (former Omp25) and Omp3b as well as in the structure of the LPS molecule, but the O chain seems to be intact. The BvrS/BvrR null mutants are incapable of directly activating the cdc42 small GTPase upon cell contact and in consequence incompetent for invading cells, remaining bound to the host cell surface, thus, indicating that this two-component system controls the expression of molecular determinants necessary for invasion (Guzmán-Verri et al., 2001). Interestingly, an Omp3a (Omp25) deletion mutant displaying low attenuation (Edmonds et al., 2001) is not equivalent to the *bvr* null mutants, in the sense that the dynamics of infection and the replication profile in host cells is different between both mutants. This suggests that the overall defect in the *bvr* null mutants is not restricted to the sole absence of this protein, but rather implicates a more complex interaction of molecular events that needed to be dissected.

4. *Brucella* intracellular trafficking

Once virulent *Brucella* is ingested, the bacterium redirects its intracellular trafficking through a unique pathway until reaching its replicating niche. However, this trafficking is not homogeneous in all cells. Indeed, professional phagocytes are more prone to kill the ingested brucellae within phagolysomes than non-professional phagocytes. In certain phagocytes, such as in neutrophils, *Brucella* merely seems to withstand the intracellular microbicidal actions during a certain period of time, without bacterial replication. In other professional phagocytes, such as monocytes and macrophages, the majority of ingested bacteria seem to be destroyed within phagolysosomes, after resisting bactericidal killing mechanisms for relatively short period of times. In infected cell cultures, following bacteria engulfment only 15–30% of the initially infected macrophages sustain replication after a

period of 48 h, as shown by microscopy techniques. This indicates that only a few of the internalized bacteria are capable of avoiding lysosome fusion and redirect their trafficking to their final replicating niche. These concomitant events (bacterial destruction versus replication), more evident in activated macrophages than in naive phagocytes, cause a complex mosaic of *Brucella*-containing compartments, mainly during the first hour after infection. Therefore, it is not surprising that controversy regarding the intracellular trafficking of *Brucella* in macrophages arose. In general terms, our previous and recent experiences indicate that the intracellular trafficking of those *Brucella* organisms which finally reach their replication niche in macrophages, is not essentially different from that observed in non-professional phagocytes (Pizarro-Cerdá et al., 1999; Pizarro-Cerdá et al., in preparation). Indeed, from the bulk of ingested *Brucella*, only a few are capable of redirecting their intracellular trafficking, while most of them are destroyed within the phagolysosomes of macrophages. Those that successfully evade lysosomal fusion are capable of extensively replicating within membrane-bound compartments resembling the endoplasmic reticulum (ER) just as it is the case in epithelial cells. Following these arguments, the intracellular trafficking events described in non-professional phagocytes such as HeLa, Vero, trophoblast and 3T3 fibroblasts, are also valid for *Brucella* intracellular trafficking in macrophages.

During the first minutes after invasion of non-professional phagocytes, both the virulent *B. abortus* as well as the attenuated vaccine S19 interacts with an intracellular compartment related to the early endosomal network. This is confirmed by the presence of markers, such as the transferrin receptor, the small GTP-binding protein rab5 or the early endosomal antigen 1 (EEA1) in the *Brucella*-containing compartments (Pizarro-Cerdá et al., 1998). This association with the early endocytic network is transient, since after 10 min of internalization, the number of *Brucella*-containing compartments labeled either with rab5 or EEA1 decreases significantly and no labeling is detected with these markers after 30 min post-inoculation (Pizarro-Cerdá et al., 1998; Chaves-Olarte et al., unpublished results). The integrity of the early endosomal system is relevant to the subsequent normal trafficking of *B. abortus* in host cells. For instance, an important fraction of the internalized parasites are unable to escape from the giant early *Brucella*-containing compartments in cells expressing the activated membrane-bound form of the Rab5 GTPase. Although these giant early phagosomes labeled with Rab5 allow limited *Brucella* multiplication, after 48 h proliferation *Brucella* replication decreases (Chaves-Olarte et al., unpublished results). On the contrary, *Brucella* intracellular trafficking and replication in cells transfected with the active form of the late endosomal Rab7 GTPase, are not affected. These data suggest that while Rab5 is transiently necessary for the first steps of *Brucella* intracellular trafficking, Rab7 is not required for subsequent *Brucella*-containing vacuole biogenesis. Indeed, internalized *Brucella* intersects with early endosomes, but not with late endosomes (Pizarro-Cerdá et al., 1998). Interestingly, activation of GTPases of the Rho subfamily do not affect the intracellular trafficking of *Brucella*. This indicates that, at least in non-professional phagocytes, *Brucella* is capable of modulating two different sets of GTPases in a compartmentalized manner: GTPases required for invasion and those required for intracellular trafficking (Chaves-Olarte et al., unpublished results). This molecular dissociation demonstrates that internalization and intracellular trafficking are two independent events during *B. abortus* infection in non-professional phagocytes.

In contrast to dead bacteria-containing phagosomes which transiently interact with late endocytic compartments, characterized by the presence of the small GTP-binding protein Rab7 and the mannose 6-phosphate receptors, virulent *Brucella* or the vaccine S19 do not localize within these late compartments. Despite the fact that *Brucella* does not transit through the late endosome network, vacuole acidification seems to be required, since chloroquine, ammonium chloride and monensin (all substances that inhibit endosomal acidification) are capable of reducing the number of intracellular bacteria at early but not at later times after infection (Detilleux et al., 1991). This requirement of acidification in early times during intracellular trafficking has also been observed in macrophages (Porte et al., 1999). Acidification step without the acquisition of lysosomal markers may be necessary for the activation of virulence genes as it occurs with other parasites (Buchmeier and Heffron, 1990).

Following these initial steps, the *Brucella*-containing vacuole is transformed gradually. One hour after internalization, both virulent strain 2308 and attenuated strain 19 are present in an intracellular multimembranous compartment decorated with both the lysosomal-associated membrane protein (LAMP) 1 and the endoplasmic reticulum markers calreticulin or sec61β, but devoid of the luminal lysosomal hydrolase cathepsin D. Several criteria permitted the identification of this late *Brucella*-containing compartment as an autophagosome. First, the multimembranous nature of the LAMP1-positive cathepsin D-negative vacuole is highly reminiscent of autophagosomes. Second, this compartment is labeled by monodansylcadaverine, a marker known to accumulate in autophagosomal bodies. Third, several endoplasmic reticulum markers are present in this *Brucella*-containing vacuole, attesting to an endoplasmic reticulum-related origin of this compartment. Fourth, modulation of the autophagocytic process regulates the intracellular fate of the internalized brucellae (Pizarro-Cerdá et al., 1998).

The presence of LAMP1 in the late *Brucella*-containing compartment could be explained by a direct delivery of this molecule from the Golgi complex to the maturing autophagosomes. This molecule could be present in these compartments accidentally as a bystander, only as an outcome of the different trafficking pathways followed by this molecule, transported in certain cases to the plasma membrane before being delivered to the lysosomes. However, the actual function of the LAMP family of glycoproteins has not been clearly defined, and it would be interesting to determine if this molecule actually plays an active role that could be relevant to the intracellular survival intracellular pathogens. The association of an intracellular pathogen with the autophagic pathway is not unique to *B. abortus*, and it has also been observed in the case of *Legionella* (Swanson and Isberg, 1995) and *Porphyromonas gingivalis* (Dorn et al., 2001). It is not known how these bacteria are able to interact with the autophagic cascade. An interaction between early endocytic compartments and autophagic vacuoles has already been detected (Liou et al., 1997), indicating that a physical connection could exist between early *Brucella*-containing compartments and autophagosomes. Among several scenarios that may be conceived to explain the transfer of these pathogens from one intracellular compartment to the other, we favor the possibility of autophagosomal vacuoles formed by selective invagination of the endoplasmic reticulum around *Brucella*-containing compartments. However, these event should be selective since these *Brucella*-containing compartment resembling autophagosomes do not possess some other endoplasmic reticulum markers,

such as BiP or ribophorin in (Pizarro-Cerdá et al., 1998). Therefore, it seems that only a specialized regions of the endoplasmic reticulum are involved in autophagosome formation or selective fusion events in the manner of the ''kiss and run'' hypothesis (Storrie and Desjardins, 1996) where only a certain markers are deposited from the endoplasmic reticulum to autophagic vacuole.

In contrast to what is observed with certain mutants or killed bacteria, most of the intracellular virulent *Brucella*-containing phagosomes lose the LAMP1 and never acquire lysosomal proteins in nonprofessional phagocytes. However, this final *Brucella*-containing compartment retains the sec61β labeling and acquires other markers of the endoplasmic reticulum, such as the protein disulfide isomerase and calnexin (Pizarro-Cerdá et al., 1998). The morphology of the final *Brucella*-containing compartment also differs from autophagosomes in that only a single membrane is detected around the replicating brucellae, and their intracellular location correspond to the perinuclear area of the infected cells (Detilleux et al., 1990a; Pizarro-Cerdá et al., 1998). Treatment of target cells with proaerolysin before *Brucella* inoculation impairs the bacterial replication process and induces the degradation of virulent strain 2308 (Pizarro-Cerdá et al., 1998), suggesting that the integrity of the structure of the endoplasmic reticulum is indispensable for the appropriate multiplication of *B. abortus*. All these data indicates that the virulent *B. abortus* transits from autophagosomes to the endoplasmic reticulum of host cells, where actual bacterial multiplication occurs, confirming previous ultrastructural studies in trophoblasts and other mammalian cell lines (Anderson and Cheville, 1986; Detilleux et al., 1990a).

The benefits involved in the association of intracellular pathogens with the host-cell endoplasmic reticulum have not been characterized yet. In addition of being a strategy for avoiding lysosomal fusion during the final steps of intracellular invasion, association of *B. abortus* with the host endoplasmic reticulum could be a means of obtaining metabolites synthesized or translocated to this compartment (Sinai and Joiner, 1997a; Swanson and Isberg, 1995). The strategy would be to take advantage of the biosynthetic enzymes, protein-conducting channels or peptide pores to increase the local nutrient supply, fulfilling the complex nutritional requirements for the bacterial growth. A possible prediction of this model would be that the cellular stock of short-lived molecules would decrease in *B. abortus*-infected cells due to the shortage of amino acids and peptides or to the blocking of the biosynthetic process of new proteins in the endoplasmic reticulum. However, heavily infected cells show no decrease in stock levels of short-lived molecules, such as LAMP1 and LAMP2 (J. Pizarro-Cerdá, personal communication). As with other intracellular parasites, it is still not clear why the endoplasmic reticulum is preferred over others locations. Although *Brucella* is capable of achieving limited replication in giant early phagosomes of cells constitutively expressing activated forms of Rab5 small GTPase (Chaves-Olarte et al., 2002), and certain *Brucella* virB null mutants achieve limited replication in the cell membrane (Comerci et al., 2001), none of these alternative niches sustain replication as the endoplasmic reticulum.

In the pregnant animal, *Brucella* organisms preferentially replicate in placental trophoblasts during the middle and late stages of gestation, only after these cells actively secret steroids. The reason for this affinity and the process leading to abortion after midgestation

are not known. Infected trophoblasts produce cortisol, a steroidal hormone not normally generated by the placenta (Enright and Samartino, 1994). Increased levels of prostaglandin F2α and decreased production of progesterone, coupled with increased synthesis of estrogens and cortisol in the *B. abortus*-infected trophoblast at mid- and late-stages of gestation, are identical to the hormonal changes occurring at term in normal cattle with the initiation of parturition. Intracellular *Brucella* probably induces the synthesis of steroids and modifies the metabolism of prostaglandin precursors, such as arachidonic acid, because these hormones may be used as growth factors by the bacteria. The increased hydrophobicity of the *Brucella* outer-membrane together with the preference of the bacteria for replicating within the ER (Anderson et al., 1986a,b), may represent an evolutionary adaptation for using hydrophobic substances available within this compartment in trophoblasts.

5. *Brucella* molecular determinants for intracellular trafficking

As previously stated, *Brucella* invasion and intracellular trafficking in non-professional phagocytes are two independent phenomena which could be discerned by the differential activation of Rho and Rab small GTPases, devoted to the recruitment of cytoskeletal structures and vesicles, respectively. Recent discovery of a type IV secretion system equivalent to that of plant pathogens, has been described to control the intracellular trafficking of *Brucella* but not the invasion to cells (Comerci et al., 2001; Delrue et al., 2001), supporting the concept that invasion and intracellular trafficking are two separated events in these cells. Mutations in *vir*B12 and *vir*B13 do not demonstrate obvious defects. On the other hand, polar mutations in the *vir*B1 and *vir*B10 abolish the ability of *Brucella* to replicate in mice. In cell culture, these mutants are destroyed within phagolysosomes. Attenuated non-polar *vir*B2, *vir*B4, *vir*B8, v*ir*B9 and *vir*B10 *Brucella* mutants are capable of penetrating cells as the same rate as the virulent wild-type *Brucella*, transit through EEA1-positive early compartments and then localize in LAMP1-positive compartments at early times of infection. Following this period of time, *Brucella vir*B mutants are retained within compartments devoid of lysosomal cathepsin hydrolases but retain LAMP1 and sec61β markers. After 12 h, a large proportion of intracellular non-polar *B. abortus* *vir*B10 mutants are recycled to the cell surface. Once outside, this mutant seems to replicate adhered to the cell plasma membrane. Altogether, these results indicate that the *vir*B genes are not necessary for controlling the first events of *Brucella* biogenesis in cells such as attachment, internalization or avoiding fusion with lysosomal compartments. It rather seems that the *vir*B operon coding for *Brucella* type IV secretion system is required for regulating the intracellular trafficking from autophagosome-like compartments to the ER.

How this secretion system controls these important steps during intracellular trafficking is still unknown. It is tempting to speculate that type IV secretion system may export bacterial effectors, able to modulate the biogenesis pathway of the *Brucella*-containing compartment, allowing the new-formed ‘‘organelle’’ to mature in the replication niche. The putative *vir*B10 and *vir*B11 genes seem to code for an internal transmembrane protein of unknown function (structural?) and for a cytoplasmic or inner membrane

protein that has a conserved Walker A NTP-binding motif, respectively. Gene reporter analysis has revealed that the expression of the *Brucella* VirB system is activated during the first 12 h after internalization. Then, the expression of the system diminishes, corresponding to the replicating time in the ER (Comerci D. J., personal communication). Therefore, it seems that the type IV secretion machinery is required for controlling the trafficking of *Brucella* from autophagosomes to its replicating niche within the ER. Once the bacterium has reached this organelle, the secretion apparatus may be turned off.

6. Modulation of the host cell cycle

One of the interesting features about *Brucella* parasitism is that the bacterium is capable of extensively replicating intracellularly without restricting basic cellular functions or inducing obvious damage to cells. For instance, high numbers of bacteria within the ER cause constriction of the cell nucleus, however, invasion to this organelle is never attained. In the same direction, crucial processes of the cell cycle such as DNA synthesis chromosome condensation, mitosis, karyokinesis and cytokinesis are not inhibited, despite the fact of the high number of bacteria replicating within the ER (Chaves-Olarte et al., unpublished results). In addition, treatment of cells with cycloheximide does not inhibit *Brucella* replication, suggesting that the de novo host protein synthesis is not required during intracellular parasitism (Detilleux et al., 1991). All these observations are in agreement with investigations showing that *Brucella* organisms are able to inhibit programmed cell death in both professional (Gross et al., 2000) and non-professional (Rojas et al., unpublished results) phagocytes. This phenomenon is not restricted to the infected cells, since non-infected neighbor cells are also protected suggesting that is caused by a substance released by the cells and may be promoted by replicating *Brucella*. It is difficult to imagine how such a massive replication of intracellular bacteria seems not to interfere with these important physiological pathways. In this sense, it is tempting to speculate that *Brucella* produces a collection of molecular determinants promoting host cell survival and cell proliferation for its own benefit.

The question still remains on to which molecular mechanisms *Brucella* depend to be released from cells once it has achieved extensive intracellular replication. The recent discovery of *Brucella* putative genes potentially coding for hemolysins and their corresponding proteins for secretion may bring some light (DelVecchio et al., 2002). Indeed, it has been shown that *Legionella* hemolysins form pores and disintegrate the vacuolar and cell membranes soon after intracellular bacterial replication ceased (Abu-Kwaik, 2001), suggesting that a similar phenomenon could take place with intravacuolar *Brucella*. In the case of infected erythrophagocytic and chorioallantoic trophoblasts, the extensive bacterial replication eventually generates necrosis after bacterial release of the cells, with the concomitant inflammatory process. A similar phenomenon is observed in massively infected fetal tissues, suggesting that necrosis is the final outcome of the infected cell. After the bacteria are released, the infection to other cells proceeds. In contrast to pathogens such as *Listeria* or *Shigella*, *Brucella* does not infect cells by intracellular spreading from cell to cell.

7. Control of trafficking and intracellular replication by cytokines

During *Brucella*-infection several cytokines such as IFNγ, TNFα, IL-2, IL-10, and IL-12 control the intracellular growth of *Brucella* strains within macrophages, whereas IL-1α, IL-4, IL-6 and GM-CSF do not have clear effects (Golding et al., 2001). Among the various cytokines, IFNγ is the most relevant for generating macrophages with strong activity for killing intracellular *Brucella* (Murphy et al., 2001). Furthermore, IL-2, IL-10 and IL-12, all cytokines that influence the acquired cellular resistance and specifically contribute to control the *Brucella* multiplication, seem to work via the IFNγ-dependent pathway.

The role of TNFα in the control of intracellular *Brucella* replication is elusive. In contrast to what has been observed in murine macrophages, *Brucella* strains do not induce TNFα in human macrophages and live *Brucella* is capable of inhibiting the production of this cytokine (Caron et al., 1994, 1996). However, pretreatment of human macrophages with exogenous TNFα, significantly inhibits the rate of *Brucella* intracellular replication. TNFα may not be essential for the induction of acquired cellular resistance but it is likely to directly activate effector cells by limiting the multiplication of intracellular *Brucella* (Ottones et al., 2000a,b; Zhan et al., 1996).

It has been demonstrated that bactericidal action of activated macrophages and expression of specific cytokines depend upon the expression of the transcriptional factor NF-IL6 (Tanaka et al., 1995). Upon activation of NF-IL6 knockout macrophages by IFNγ, induction of the transcription of TNFα, IL-6, IL-1β, GM-CSF, M-CSF, IL-10, and IL-12 is comparable to that observed in normal mice (Tanaka et al., 1995). Strikingly, no induction of G-CSF expression is observed in NF-IL6 knockout mice, being this defect restricted to macrophages and fibroblasts (Tanaka et al., 1995). In addition, the production of reactive oxygen intermediates is lower in NF-IL6 than that in control macrophages (Tanaka et al., 1995), suggesting that NF-IL6 may control the expression of other elements of the respiratory burst. Indeed, it is known that G-CSF enhances the respiratory burst in phagocytes (Yuan et al., 1993). Despite this, NO synthetase is not impaired in NF-IL6 knockout macrophages (Tanaka et al., 1995). The NO synthetase is associated to intracellular membrane vesicles different from lysosomes and peroxisomes in murine macrophages (Vodovotz et al., 1995). The general outcome is that NF-IL6 knockout mice are more susceptible to bacterial intracellular infections than normal mice, indicating that this transcriptional factor plays a role in controlling intracellular parasites (Tanaka et al., 1995). Certainly, attenuated *B. abortus*S19 vaccine is capable of replicating in NF-IL6 knockout but not in wild type murine macrophages. The role of NF-IL6 in the inhibition of intracellular bacterial replication is related to its control of endocytosis and membrane fusion between endosomes and *Brucella*-containing phagosomes. Strikingly, addition of exogenous G-CSF to NF-IL6 knockout macrophages restores not only the morphology of endosomes but also the fusion between endosomes with phagosomes and the corresponding bactericidal activity against S19.

During *Brucella* infection, it has been observed that endocytosis but not recycling is affected in NF-IL6 knockout macrophages, suggesting that G-CSF promotes fusion events in early phagosomes (Pizarro-Cerdá et al., 1999). Since in NF-IL6 knockout macrophages, NO synthetase is not impaired, vesicles containing this oxidative mediators could be

translocated to *Brucella*-containing phagosomes after treatment with G-CSF. By restoring endosome-phagosome fusion, this cytokine eventually would allow other elements of the respiratory burst present in endocytic compartments to reach the *Brucella*-containing phagosomes and thus to partially re-establish the bactericidal activity of NF-IL6 knockout macrophages (Pizarro-Cerdá et al., 1999). Under these conditions, attenuated *Brucella* could be targeted to lysosomes and killed, whereas virulent bacteria could still replicate but to a lesser extent than in resting macrophages.

8. LPS integrity is essential for modulating *Bucella* intracellular survival

The most conspicuous structural defect that renders *Brucella* organisms avirulent, is the absence of the *O*-polysaccharide and the concomitant absence of the related NH polysaccharide molecules. In other words, a defect that results from the dissociation from smooth to rough phenotype (Allen et al., 1998; Detilleux et al., 1990a,b; Freer et al., 1996; Kreutzer et al., 1979; Martínez-de-Tejada et al., 1995; Riley and Robertson, 1984b; Stevens et al., 1994b). In general, smooth *Brucella* are more resistant than rough strains to the killing action of professional phagocytes and of intracellular bactericidal substances of phagocytes. In addition mutant *B. abortus* strains possessing a defect in the lipid A of LPS are also non-virulent, readily killed by macrophages and also more susceptible to bactericidal action of cationic substances such as defensives and lysozyme (see accompanying article, López-Goñi et al., 2002). Altogether these results clearly indicate that the integrity of LPS molecule on the bacterial surface is one of the required factors for *Brucella* for intracellular survival, and then for virulence. It is precisely in the structure of *Brucella* LPS, in relation with other outer membrane molecules that lays the explanation for the observed lower virulence of LPS defective mutants.

For many years it has been known that the biological activities of *Brucella* LPS diverge from conventional endotoxins and that this characteristic is linked to its particular chemical structure (Leong et al., 1970; Moreno et al., 1981; Rasool et al., 1992), a fact that has been corroborated by several works (Goldstein et al., 1992; López-Urrutia et al., 2000). Indeed, it has become evident the existence of a different family of LPSs which diverge form classical endotoxins not only in their molecular structures but also in their biological activities. For instance, other LPSs molecules derived from intracellular bacteria such as *Legionella*, *Rickettsia*, *Porphyromonas*, *Coxiella*, *Chlamydia* and even from plant cell associated pathogens such *Agrobacterium* display low endotoxicity (Amano et al., 1987; Brade, 1999; Helander et al., 1982; Hollingdale et al., 1980; Neumeister et al., 1998; Pulendran et al., 2001). This characteristic is necessarily linked to their non-classical chemical structure diverging from the ‘‘typical’’ structure of LPS largely presented in text books. Moreover, several of the LPSs isolated from these intracellular bacteria, including *Brucella*, are biological active in the endotoxic resistant C3H/HeJ mice, suggesting that these molecules do not use the classical Toll-like receptors, mainly type 2 and 4. In addition these LPSs induce a lower and different profile of cytokines and do not use the CD14 receptor commonly employed by the ‘‘classical’’ LPSs. It is tempting to speculate that the low biological activity of these LPSs, which eventually fail or induce low activation of microbicidal mechanisms through cytokine networks (e.g. respiratory burst), constitute a

favorable evolutionary trait for a group of intracellular bacteria that must survive for protracted periods within host cells.

The role of LPS in the permeability properties and in resistance to bactericidal substances has been definitively established in a series of experiments involving *Brucella* strains and the construction of LPS chimeras (Freer et al., 1996; Martínez-de-Tejada et al., 1995). When the heterologous LPS inserted in the outer membrane of susceptible bacteria corresponds to the less sensitive smooth *B. abortus*, the chimeras are more resistant to bactericidal cationic molecules. In contrast, when LPS is from the more sensitive bacteria, the chimeras are more susceptible to the action of bactericidal peptides. There is a direct correlation between the amount of heterologous smooth LPS on the surface of chimeric cells and sensitivity to bactericidal substances (Freer et al., 1996). Although this particular resistance to bactericidal molecules is related to the core and lipid A structures, there is a contribution of the *O*-polysaccharide and the associated NH, as suggested by the difference in susceptibility between the rough and smooth *Brucella* strains (Freer et al., 1996; Martínez-de-Tejada et al., 1995). It is worth noting that the resistance of *Brucella* LPS to cationic molecules is more conspicuous when it is integrated in its native outer-membrane. This is demonstrated by the fact that *Brucella* smooth LPS micelles are partially permeabilized by the action of bactericidal peptides, whereas *Brucella* cells are not (Freer et al., 1996).

Brucella LPS is actively released by the replicating bacterium inside cells (Moreno et al., unpublished results). It has been observed that in macrophages, *Brucella* LPS transits through and enriched MHC class II compartment strongly associating to these MHC proteins but not MHC-I molecules (Forestier et al., 1999). LPS-MHC class II complexes form stable clusters, similar to those detected by peptide loaded histocompatibility molecules. In contrast to protein antigens, however, the integrity of the *B. abortus* LPS located within lysosomal like compartments remains unaltered for prolonged periods of time. The absence of the adequate cellular enzymatic machinery to degrade *Brucella* LPS seems to be the obvious explanation for this phenomenon. Interestingly, a significant proportion of *Brucella* LPS recycles to the cell membrane in association with MHC class II molecules forming large macrodomains. One of the consequences of these association is that the *B. abortus* LPS acts as a broad APCs down-regulator for protein antigen presentation to T cells (Forestier et al., 2000). In agreement to the biogenesis of *Brucella* LPS within APCs, this impairment is confined to the MHC class II but not to MHC-I restricted antigen presentation pathway. This dawn-regulating effect is neither related to the decrease of MHC class II surface expression nor to a deficient uptake or processing of protein antigens. Moreover, *Brucella* LPS does not prevent the formation of compact forms of MHC class II in association with the nominal peptide antigen, suggesting that loading of peptides on MHC class II is not inhibited by this bacterial glycolipid molecule. In addition to this down regulation of protein antigens, we have demonstrated that the low endotoxic *B. abortus* LPS not only associates to MHC class II molecules in the cell surface of APCs, but that this association leads to an specific activation of CD4+ T cells. These primed lymphocytes recognize in a restricted manner the *Brucella* LPS molecule in the context syngenic MHC class II proteins (Fourquet et al., unpublished results). In conclusion, it seems that *Brucella* LPS released inside phagocytic cells, acts not only as immunomodulator for protein antigen presentation, but that is also capable of trigger LPS-specific Th cells in addition to B lymphocytes.

The overall higher hydrophobicity of *Brucella* cell envelopes and the close association among the outer membrane macromolecules, are factors implicated in the selective penetration of nutrients inside the bacterial cell. It has been demonstrated that the absence of a barrier to hydrophobic substances is linked to the structure of the *Brucella* LPS core and lipid A (Freer et al., 1996; Martínez-de-Tejada et al., 1995; Velasco et al., 2000). The possible advantages of a hydrophobic envelope for intracellular α-2 subclass *Proteobacteria* has been suggested by the finding that *Rhizobium* LPS becomes highly hydrophobic during bacteroid development (Kannenberg and Carlson, 2001). The net result of this structural change in the LPS is that intracellular bacteroids have a more hydrophobic outer membrane than the free-living rhizobiae. This adaptive condition could promote the exchange of nutrients and favor intracellular life of the bacteroids. Obvious comparisons between the intracellular lifestyle of *Brucella* and *Rhizobium* emerge, since these two bacteria are phylogenetically close relatives (Moreno et al., 1990). The permeability of the *Brucella* outer membrane to sexual hormones and siderophores may be linked the hydrophobic properties of the outer membrane and in consequence to integrity of the *Brucella* LPS.S.

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