Brucella alters endocytic pathway in J774 macrophages

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Brucella is a facultative intracellular bacterium which causes chronic infections in mammals by surviving and replicating within host cells. The putative role of the endoplasmic reticulum (ER) in the formation of the phagosome in non-professional phagocytes is supported by several research groups, but still leaves open the question of the fate of Brucella inside professional phagocytes and its resistance mechanisms therein. Macrophages are particularly important for the survival and spreading of Brucella during infection. The intracellular transport of Brucella in these cells has not been thoroughly characterized. To study the maturation process of Brucella-containing phagosomes in phagocytes, we comparatively monitored the intracellular transport of a virulent strain (2308) with two vaccine strains (S19 and RB51) in J774 macrophages. Then, we compared the behavior of all three strains studied through transmission electron microscopy. The results indicate that the virulent strain not only occupies two different kinds of compartments but also alters the endocytic pathway of the cell it parasitizes, unlike what has been reported for non-professional phagocytes, like HeLa cell. Besides, differences are observed in the behavior of both Brucella abortus vaccine strains.

Introduction

Brucella is a facultative intracellular bacterium which causes chronic infections in mammals by surviving and replicating within host cells. Previous studies suggest that Brucella intracellular survival might be related to chemical factors, extracellularly or intracellularly released by the bacterium, which allow it to avoid destruction by phagocytic cells.

Generally, the above mentioned investigations have focused on demonstrating inhibition by Brucella of one or more of the bactericidal mechanisms of phagocytes, namely: respiratory burst, phagosome-lysosome fusion, endocytic and autophagic pathways.

**Brucella abortus** vaccine strain S19 is the only strain that does not metabolize erythritol. Since it lacks D-erythrulose 1-phosphate dehydrogenase, its growth is inhibited in the presence of erythritol because of the toxic accumulation of D-erythrulose 1-phosphate.1

Studies on the pathogenic mechanisms of **Brucella abortus** in animals have been conducted on trophoblast cell lines.2 Intracellular localization in the trophoblast was detected for phagosomes and for the endoplasmic reticulum (ER). This suggests that Brucella replicates in the ER lumen using translocation proteins for its metabolism.3,4 Although **Brucella abortus** infection of fetal tissues and its multiplication therein happen early in pregnancy, miscarriage frequently occurs during the second half of pregnancy. The reasons determining this course of events have not yet been clearly elucidated. It has been suggested that increased susceptibility to infection at late stages of pregnancy could be explained by the presence of erythritol in these tissues. However, the importance of this alcohol has been disputed, because there is no evident difference in erythritol levels among different stages of pregnancy in cattle. Moreover, comparative studies of the strain of Brucella which is inhibited by erythritol (vaccine strain S19) and the virulent strain show a similar rate of growth. Strain S19 of attenuated virulence is found in the smooth phase with its complete lipopolysaccharide (LPS). Samartino and Enright1 suggested the presence of stimulating and inhibiting factors in trophoblast tissues, which could determine substantial changes in Brucella metabolic and secretory products during pregnancy. These differences, related to tissue growth and differentiation, might explain a different development of *B. abortus* at early and late stages of pregnancy.1,2,5,7

A study by Pizarro-Cerdá et al.8 suggested that, in non-professional phagocytic (HeLa) cells, Brucella localizes in compartments of the ER to protect itself from destruction. To reach that protected niche, it takes advantage of the phagocytic pathway while avoiding fusion with lysosomes.8,9

From the foregoing we can regard Brucella as a pathogenic bacterium able to persist in tissues with important components of the mononuclear phagocytic system as well as in reproductive organs and epithelial cells. The mechanisms involved in this cell tropism, the pathways used to enter cells and the survival strategies within these cells and in different hosts are not yet clear.

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Brucella abortus S19, universally used as a vaccine, has some disadvantages, among them its failure to induce immunity in 25–35% of cases, its ability to cause chronic infections in livestock and its being pathogenic to human beings.

*Brucella abortus* RB51, derived from virulent strain 2308, is a stable, rough, attenuated mutant used as a vaccine in the USA. RB51 does not express a significant amount of the O polysaccharide of LPS due to a gene disruption. The putative role of the ER in the formation of the phagosome in non-professional phagocytes is supported by several research groups, but still leaves open the question of the fate of Brucella inside professional phagocytes and its resistance mechanisms therein.10

In previous studies, we compared the behavior of the virulent strain with heat-killed *Brucella abortus* in professional phagocytes, as J 774 macrophages. Results showed that Brucella significantly delays fusion with preformed lysosomes and prevents interaction with newly formed endosomes. We observed a very limited co-localization of *Brucella abortus* with markers of the autophagic pathway and the ER. Moreover, there was no preferential co-localization of live versus heat-killed bacteria with these markers.11-13

Macrophages are particularly important for the survival and spreading of Brucella during infection.14 The intracellular transport of Brucella in these cells has not been thoroughly characterized. To study the maturation process of Brucella-containing phagosomes in phagocytes, we comparatively monitored the intracellular transport of a virulent strain (2308) with two vaccine strains (S19 and RB51), in J 774 macrophages, a well-characterized professional phagocyte as a murine macrophage. Then we compared its behavior with rough *Brucella abortus* vaccine strain RB51, through transmission electron microscopy.

### Results

**Acidification of phagosomes containing *Brucella abortus* of attenuated virulence.** Previous results from our laboratory in macrophages J 774 indicate that when virulent bacteria are compared with dead bacteria, the former are found in acidic compartments in a high percentage (50%) 2 h after entry. This percentage is maintained after 20 h of incubation with few changes.11 We were interested in assessing whether the kinetics of acidification of the vaccine strain was different from that of virulent strain 2308. To this end, we monitored the pH of phagosomes that contained both strains of living bacteria through the co-localization with LysoSensor, which is a weak base that accumulates in acidic compartments and fluoresces at acidic pH (pKa = 5.2).

As shown in Table 1, approximately 40% of the bacteria of attenuated virulence (S19) arrived at acidic compartments quickly after 2 h of incubation. This result is similar to those obtained with bacteria killed by heat (44%) and live virulent bacteria (44%).11 After a long incubation period (20 h), 66% of strain S19 bacteria are found inside acidic compartments. Most of these bacteria were digested after 20 hours of incubation, counting them turned out to be quite difficult, (Table 1 and Fig. 1, co-localization). On the other hand, 2308 *Brucella abortus* had a similar percentage (46%) after the 20 h incubation compared with the 2 h incubation and with previous experiments (Table 1 and Fig. 1).

These results indicate that, for a short incubation period, strain S19 behaves much more like virulent strain 2308 than phagocytosed dead bacteria.11 The higher fraction of the vaccine strain in acidic compartments after the long incubation period and its higher degree of digestion suggests that its arrival in phagosomes is more efficient than for the virulent strain.

**Relationship of phagosomes containing *Brucella abortus* S19 with the autophagic pathway and with the endoplasmic reticulum.** According to other authors, in non-professional phagocytes (HeLa cells) *B. abortus* transits through the autophagic pathway to go to the ER, where it survives. However, our results for macrophages indicate that virulent *B. abortus* would travel transitorily, in a small percentage, through autophagosome compartments, because most of them survive and endure the adverse environment inside modified or normal phagosome compartments, which would not be related to either autophagosomes or the ER.

*Brucella abortus* S19 has high genetic homology with the virulent strain and its LPS is complete, but its virulence is attenuated, although it can still cause disease in humans and animals. All of these characteristics seem to imply a different behavior in intracellular pathogenicity for this kind of bacteria. We decided to verify whether strain S19 displays a different behavior than strain 2308 in terms of interaction with the autophagic pathway and the ER, by comparing in macrophages the fate of these two strains differing in virulence.

The transport of *Brucella abortus* S19 and 2308, marked with tetramethylrhodamine (TAMRA), was monitored in J 774 cells incubated with monodansylcadaverine (MDC), a fluorescent marker for autophagosomes. The results indicated that most of the Brucella-containing phagosomes did not co-localize with MDC (Table 2 and Fig. 2). During the first hours of internalization, the percentage of co-localization was similar for both strains. No significant changes with longer periods of incubation were observed for any of the bacterial strains used.

To determine whether *Brucella abortus* S19 traverses the ER, we fixed and incubated the cells with a fluorescent probe, 3,3′-dioctyloxacarbocyanine iodide (DiOC6). Co-localization with the ER was almost never observed for either virulent or attenuated strain. Even after 20 h of incubation it was not possible to observe any virulent bacterium that would co-localize with compartments compatible with the ER (Table 2 and Fig. 3).

| Table 1. Percentages of vaccine (S19) and virulent (2308) Brucella—containing phagosomes that colocalize with markers of acidic compartments |
| --- | --- | --- | --- |
| **Time** | **Brucella S19** | **Brucella 2308** | **Acidic compartment marker** |
| 2 h | 36%(70) | 44%(41) | TAMRA LysoSensor |
| 20 h | 66%(53) | 46%(67) | TAMRA LysoSensor |
strains were preferentially present in phagolysosomes (Figs. 6 and 7). Generally, strains S19 and RB51 reside in large phagosomes, all of them containing gold, with abundant membranous structures within them. In Figures 6 and 7 it can be observed that, in macrophages, B. abortus neither transits through the autophagic pathway nor goes to the ER.

**Relationship between Brucella-containing phagosomes and the endocytic pathway.**

*Brucella abortus* strain S19 shows no important differences from virulent strain 2308. Both of them go through acidic compartments and are localized neither in structures of the ER nor in macrophage autophagosomes. However, the fact remains that most of strain S19 does not survive within macrophages, being degraded after 24 hours, whereas strain 2308 is able to survive and divide in these cells.

An important characteristic of phagosomes containing the virulent strain is that they do not interact with recently formed endosomes. To determine whether the vaccine strains (S19 and RB51) show the same behavior, an experiment was designed to simultaneously compare the interaction of phagosomes containing *B. abortus* S19, RB51 or 2308 with endosomes and lysosomes, in J774 macrophages.

We marked the lysosomes with 60 nm colloidal gold particles, which were incorporated into the macrophages for 60 min. After repeated rinses, their access to lysosomes was allowed by incubation during 3 h at 37°C. Both strains of bacteria were internalized during 15 min at 37°C. After rinsing, the macrophages were incubated at 37°C for 20 h. Early endosomes were marked with 20-nm colloidal gold particles, through their internalization during 15 min before fixing the cells. Presence of endosome and lysosome markers was monitored by transmission electron microscopy. Phagosomes containing lysosome marker (60 nm gold particles) or endosome marker (20 nm gold particles) and those with no markers were counted.

After 2 h of incubation we observed that a large fraction of *B. abortus* strains were localized in lysosome compartments, namely 60% of the virulent strain and 95% of attenuated strains S19 and RB51. On the other hand, a low number of virulent bacteria were in endosome compartments (10%), whereas the bacteria corresponding to the vaccine strains were there in a five-fold larger proportion (50–60%). After 2–20 h of incubation we observed similar results for the three strains (Fig. 4).

The results obtained with electron microscopy showed that, after 2–20 h of incubation in macrophages, the virulent bacterium (2308) generally occupied two kinds of compartments: modified phagosomes and phagolysosomes (Fig. 5, upper and lower parts, respectively). Conversely, the vaccine strains were preferentially present in phagolysosomes (Figs. 6 and 7). Generally, strains S19 and RB51 reside in large phagosomes, all of them containing gold, with abundant membranous structures within them. In Figures 6 and 7 it can be observed...
that the phagosome containing bacteria of attenuated virulence appears to be very fusogenic, since vesicles in a process of fusion are discernible. In contrast, virulent strain 2308 clearly occupies two different kinds of compartments. In the two upper parts of Figure 5, Brucella is seen in a small phagosome with its membranous borders near the bacterial wall and mostly with no colloidal gold of any size inside it \((\text{modified phagosomes})\). In the lower parts, there are very spacious vesicles containing the bacterium and membranous debris, with 60-nm gold particles indicating that it is a lysosomal structure \((\text{phagolysosomes})\). In neither case \((\text{modified phagosome} \text{ or } \text{phagolysosome})\) does the bacterial morphology show any sign of digestion. These results support those presented by Arenas et al.\(^\text{11}\)

These data suggested that there are differences in the transport of strains S19, RB51 and 2308. The vaccine strains localize only in phagolysosomes that interact with de novo formed endosomes and do not appear within modified phagosomes.

Morphological alterations of \textit{Brucella abortus} strains. The results previously presented enabled us to suggest that the transport of \textit{Brucella abortus} S19 and RB51 through the endocytic pathway has different characteristics than that of virulent strain 2308. Next study we evaluated through electron microscopy, the morphological alteration that the vaccine strain displays after a long period of incubation and determined whether there were significant differences compared to live or dead strain 2308.

In order to perform said comparisons, we quantified the morphological alterations observed in the bacteria (heat-killed and live \textit{B. abortus} 2308 and S19), after 20 h of internalization, using the previous experiments in which early and late endosomes were marked with colloidal gold particles of different size.

In Figure 8 it may be noted that bacteria which are incorporated dead are efficiently digested compared to live virulent strain ones \((p < 0.001)\), and the same is true for strain S19 when compared to live strain 2308 bacteria \((p < 0.001)\). In comparing digested fractions of heat-killed and strain S19 bacteria, it becomes evident that, although both are efficiently digested, the percentage of digestion is lower for the vaccine strain than for heat-killed bacteria.

| Time  | Brucella S19 | Brucella 2308 |
|-------|--------------|---------------|
|       |               |               |
| MDC   | 2 h           | 6\% (33)      | 4\% (45)      |
|       | 20 h          | 3\% (56)      | 6\% (74)      |
| ER (DiOC6) | 2 h           | 3\% (33)      | 3\% (30)      |
|       | 20 h          | 4\% (26)      | 0\% (38)      |
These results confirm that *Brucella abortus* 2308, in contrast to vaccine strains, resists digestion within the macrophage. Despite efficient digestion of *B. abortus* S19 after 20 h, the fraction of digested bacteria is lower than for heat-killed *B. abortus* 2308, showing that the bacterium of attenuated virulence does not behave as an inert particle (bacterium killed by heat) within the endocytic pathway. Similar results were observed for RB51 (data not shown).

**Fusion between endosomes and lysosomes in macrophages infected with *Brucella abortus***. The work reported up to this point allowed us to establish that, in macrophages, the virulent strain of *B. abortus* delays phagosome fusion with lysosomes, prevents fusion with early endosomes, and survives inside two compartments, one of them exhibiting phagolysosome features and the other one having different characteristics.

The last issue put forth in the present study was determining whether transport of endocytic material to lysosomes was altered in the Brucella-infected macrophage. We performed a count of the fusions of lysosome compartments (60 nm gold particles) with endosomes (20 nm gold particles). The percentage of vesicles with 60 nm gold particles that also contained 20 nm gold particles was taken as an indicator of transport to lysosomes. This count was performed in macrophages which contained at least one phagosome with *Brucella abortus* (infected macrophage) and in macrophages containing no bacteria (non-infected macrophage), using always the same sample. Macrophages were incubated with *B. abortus* 2308 and S19 (as a live vaccine strain).

In **Figure 9**, endosome-lysosome fusion percentages for macrophages containing *B. abortus* (virulent and vaccine strain) are presented as a bar graph. In non-infected cells of both samples, fusion percentage was about 20%. A similar percentage of fusion was observed in strain S19-infected macrophages. However, the count performed in macrophages infected with strain 2308 showed a significantly lower percentage of fusion.

Since differences between lysosome-endosome fusions in macrophages infected with strain 2308 and in non-infected macrophages are significant (*p < 0.01*), it may be asserted not only that Brucella has the capacity to alter the characteristics of the phagosome containing it, but also that its presence alters the endocytic pathway of the infected macrophage. Additionally, these results show that, in contrast to strain 2308, strain S19 affects neither endosome-lysosome fusion nor endocytic transport.

**Discussion**

Professional phagocytes are the major effectors of the immune cell response, being capable of eliminating a great variety of microorganisms through their ingestion into phagosomes, where they find a hostile environment that determines their destruction.
The immune innate response begins with the phagocytosis of pathogenic bacteria, which will unleash the adaptive response. To fulfill this function, professional phagocytes possess a variety of receptors that recognize sequences preserved on the surface of pathogenic bacteria.

These sequences (motives) play an essential role in the invasion mechanisms of pathogens; they include mannan, peptides, LPS, and teichoic acids. The bactericidal environment of the phagocytic vacuole is related to the presence of reactive oxygen species, hydrolytic enzymes, and antimicrobial peptides. Low pH and lack of nutrient availability further augment the toxicity of the phagosome environment. The precise path that the microorganism will follow depends on the type of receptor involved in the process of phagocytosis.

In many cases, the microorganism uses binding to receptors only to enter the cell, starting a series of strategies to be kept within the endocytic pathway or to escape towards other compartments.15,16

Thus, phagocytosis is a highly complex phenomenon, in which many cell components are involved, that requires simultaneous activation of several signaling routes. Besides, pathogens respond adaptively to these phenomena, altering the normal phagocytic process at different stages of its course.

Intracellular parasites use different strategies, of which alteration of the normal process of phagosome maturation has been described for many of them, like Mycobacterium, Legionella, Chlamydia, and Listeria.17,18 Within the genus Brucella, different species use diverse strategies to survive inside professional and non-professional phagocytes. Many authors have reported that inhibition of the phagosome-lysosome fusion by Brucella is one of its strategies for survival,19-21 for which the O-chain of the LPS would be important because it would govern the intracellular behavior of the bacterium at an early stage.21

Our observations are based specifically on the study of the maturation process of phagosomes containing Brucella abortus in J 774 macrophages. Through electronic microscopy we have studied the merging of de novo formed phagosomes with preexisting late compartments marked with colloidal gold particles, and additionally the arrival of colloidal gold particles recently incorporated into Brucella-containing phagosomes.

This follow-up of Brucella through electronic microscopy allowed us to determine that this bacterium delays lysosome fusion and prevents interaction of recently formed endosomes with phagosomes containing the bacterium.

Differences in the compartments that the bacterium occupies according to its viability and virulence status (virulent and attenuated vaccine strain and heat-killed bacteria), indicate that Brucella actively modulates the maturation process of the compartments containing it. This observation would be in disagreement with the report by Rittig et al.22 who assume that formation of the special phagosome that Brucella occupies would not depend on the viability or virulence of the bacterium but on some unknown components of its membrane. They worked with virulent bacteria, avirulent mutants (virB9 of the B. suis strain 1330) and heat- or formaldehyde-killed bacteria, all of which induce special phagosomes or small phagosomes with
similar morphology and relative frequency. We believe that viability and virulence status would be related to membrane components; therefore the intracellular behavior of bacteria could depend on all these factors or on other factors not yet elucidated.

The kinetics of intracellular replication and viability of the bacterium reflected in the number of Colony Forming Units (CFU) is similar to that observed by other authors in epithelial cells. Active bacterial reproduction begins after 5 h of incubation. The peak of the logarithmic phase of intracellular bacterial growth is reached 30 h after internalization.

Opsonization of the bacterium does not affect its ability to reproduce inside the macrophage. Besides, the transport alteration caused by Brucella is independent of opsonization of the bacterium with specific antibodies though, certainly, the number of entering bacteria increases when entry is through the Fc receptor of the macrophage.

When the entering bacterium is opsonized, it should unleash the respiratory burst in the macrophage, with the consistent generation of oxidized intermediary reagents. Nevertheless, studies suggest that in Brucella-containing phagosomes this mechanism is uncommon or inhibited. When Brucella arrives at lysosome compartments it does not prevent phagosome acidification but is capable of tolerating the pH of the phagosomal vesicle. Some authors have reported that early acidification of the phagosome would favor the survival of B. suis, perhaps allowing the expression of proteins necessary to support its viability.

Our results agree with observations with R. sive in view that, in comparing vaccine S19 with the highly virulent 2308 and dead bacteria, the percentage of bacteria arriving at acidic compartments in the first hours of intracellular trafficking (2 h) is similar for all three types of bacteria (about 40%).

After long periods of incubation, the bacterium of attenuated virulence is present in acidic compartments in a larger percentage than the virulent strain, which suggests that the former is more efficiently transported to phagolysosomes, where it is finally digested. Later studies performed by other authors confirm a low pH for Brucella-containing compartments; therefore the intracellular behavior of bacteria with specific antibodies though, certainly, the number of entering bacteria increases when entry is through the Fc receptor of the macrophage.

In non-professional phagocytes, Brucella used the autophagic pathway to go to the ER, where it reproduced. However, in macrophages the process would not be similar. Our results for macrophages show that a very low percentage of bacteria (independently of their condition of viability) would travel this route. In phagocytes, the endocytic pathway is more active than in cell types like HeLa or CHO. It is very likely that, in cellular lines like HeLa, the lower activity of the mechanisms of membrane fusion-fission allows interaction of phagosomes with early autophagic vesicles instead of with endosomes or other structures.

The presence of the bacterium in this type of early autophagic vesicle may explain its lower fusogenicity. The relationship between Brucella-containing autophagosomes and some regions of the ER may be associated with the origin of these autophagosomes. The mechanisms used by Brucella to delay fusion with late endosome compartments in macrophages may not be sufficient to drive the phagosome along the autophagic pathway and, even if they do, phagosomes may quickly evolve into autophagolysosomes instead of into ER-related vesicles.

Our results are supported by the counting of a large number of bacteria (including strains S19 and RB51) through fluorescence microscopy, and corroborated by studies on professional phagocytes carried out by other authors.

Besides, trafficking of B. abortus in the ER has been studied for other cell lines such as trophoblasts derived from bovine placentae and for epithelial cells. In macrophages, participation of the ER as membrane source of supply for the formation of the phagosome in early stages has been documented in recent work.

Nevertheless, in studies conducted with monocytes-macrophages of human origin, Rittig et al. observed no presence of Brucella in related structures within autophagosomes or ER, which is in keeping with our finding suggesting that the compartments used by Brucella are different for professional and

Figure 6. Relationship between Brucella-containing phagosomes and the endocytic pathway. Lysosomes were marked with 60 nm colloidal gold particles, which were incorporated into the macrophages for 60 min. Afterwards their access to lysosomes was allowed by incubation during 3 h at 37°C. Strain S19 was internalized during 15 min at 37°C. After rinsing, the macrophages were incubated at 37°C for 20 h. Early endosomes were marked with 20 nm colloidal gold particles, through their internalization during 15 min before fixing the cells. Presence of endosome and lysosome markers was monitored by transmission electron microscopy. (X15,000).
non-professional phagocytes. Despite the above, some authors assume that “the events described in the intracellular traffic of Brucella in HeLa, Vero, trophoblasts and 3T3 fibroblasts, are also valid for the traffic in macrophages.”

In macrophages incubated with Brucella up to 2 h after internalization, 60% of the bacteria are localized in compartments which can be identified as phagolysosomes, but the rest of them localize in phagosomes that do not interact with either endosomes or lysosomes. After 20 h the bacterium is observed in two types of well-defined compartments: (a) a large one with characteristics of phagolysosomes, where a high percentage of bacteria remain morphologically intact; and another one (b) a small one, barely larger than of the bacterium, which does not interact with lysosomes or endosomes, and where Brucella shows no evident morphological alterations.

It is interesting to note that we find differences in the transport of gold from endosomes to lysosomes in macrophages infected with virulent Brucella, suggesting that the virulent strain not only occupies two different kinds of compartments, but also alters the endocytic pathway of the cell it parasitizes. Other authors have reported these two well-defined types of compartments in monocytes-macrophages from human beings. According to Rittig et al., the morphological heterogeneity of these two types of compartments has functional consequences: the spacious compartment would lead to the destruction of the bacterium whereas the small one would represent a niche that allows the bacterium to survive by being less fusogenic with lysosomes.

In addition, formation of the small compartment also occurs, but with much lower frequency, when using heat-killed bacteria or reduced virulence. Unlike what was observed by Rittig et al., in our experiments the morphology of the bacterium turns out to be still intact in large compartments after 20 h of incubation, suggesting that, in spite of being in phagolysosomes, it resists degradation. Strains S19 and the RB51 were observed in general in spacious vesicle compartments even after 45 min of incubation.

The vaccine strains of Brucella abortus showed an interesting behavior inside the macrophage, which justifies their use for further comparisons with virulent strain 2308. We can say that S19 and RB51 enter acidic compartments more efficiently than the virulent strain, in general occupy phagolysosomes which appear to be more fusogenic and interact with de novo formed endosomes. Like strain 2308, the vaccine strains travel neither the autophagic pathway nor the one to the ER.

Concerning intracellular trafficking, strains S19 and RB51 do not behave as inert particles but, on the other hand, they do not affect the endocytic pathway of the infected macrophage in the same way as the virulent strain does. This suggests that the disturbances they can induce within the cell are circumscribed to the phagosomes containing them.

The mechanisms involved in membrane trafficking determine the maturation process of the phagosome. The intracellular pathogen develops the most diverse strategies to affect this trafficking so as to be able to survive and multiply inside the cell it infects.

Reconstruction of the events occurring within the phagocytic pathway will allow identification of what pathogenic factors are involved in the resistance posed by pathogens to being destroyed by phagocytosis, a natural mechanism to elicit a highly efficient innate response. Provided that intracellular trafficking is a complex mechanism that involves participation of membrane vesicle reservoirs, elements of the cytoskeleton and dynamic exchanges between phagosome components and intracellular compartments, it is obvious that the precise mechanisms used by different microorganisms will also turn out to be very complex.

We hope that the experimental models presented in this study will be useful for the clarification of the molecular mechanisms involved and contribute to the struggle against diseases caused by intracellular parasites.

**Experimental Procedures**

**Reagents, materials and solutions.** LysoSensor (L7535), LysoTracker (L-7528), BCECF AM [29,79-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; acetoxy-methyl ester; B1170],
TAMRA [5-(and-6) carboxytetramethylrhodamine; succinimidyl ester; C1171] and DiOC6 (3,3-dihexyloxacarbocyanine iodide; D273) were from Molecular Probes, Eugene, OR. Unless specified otherwise, all other reagents were from Sigma Chemical Co., St. Louis, MO. A polyclonal mouse anti-Brucella antibody was generated in our laboratory, and an immunoglobulin G (IgG) fraction was purified from ascites fluid. Rabbit anti-mouse IgG was obtained from Cappel Organon Teknika Corp., Malvern, PA. Bovine serum albumin (BSA) was mannosylated as previously described. Colloidal gold particles were obtained using the citrate reducing method and coated with mannosylated BSA as described previously. Eagle basic medium containing 20 mM HEPES-NaOH, pH 7 and supplemented with 5 mg of BSA per ml or 5% fetal calf serum (FCS), was used for short incubations of macrophages (BME).

**Bacterial strains.** *B. abortus* 2308, a smooth virulent strain; *B. abortus* smooth vaccine strain S19, *B. abortus* rough vaccine strain RB51, were grown at 37°C in Brucella agar (Merck Diagnostica for Microbiology) with 10% CO₂ for 48 h to stationary phase, resuspended in phosphate-buffered saline (PBS), washed, resuspended in the same buffer (approximately 10⁹ CFU/ml) and used immediately. Bacterial numbers were determined by comparing the optical density at 600 nm with a standard curve. Direct bacterial counts (CFU) were determined by plating a serial dilution on Brucella agar and incubating the plate at 37°C for 3 days. For some experiments, Brucella was opsonized with a polyclonal mouse anti-Brucella antibody (8 x 10⁷ bacteria were incubated with 2 mg of the antibody in 40 ml of BME for 1 h at 20°C and washed three times with BME). For light microscopy, Brucella was labeled with tetramethylrhodamine [8 x 10⁷ bacteria were incubated with 5 mg of TAMRA in 50 ml of PBS (pH 8) for 1 h at 20°C and washed five times with BME]. To label only live bacteria, Brucella was loaded with BCECF (8 x 10⁷ Brucella bacteria were incubated with 10 mM BCECF AM in 200 ml of BME for 1 h at 25°C and washed five times with BME). Labeling the bacteria with antibodies, TAMRA or BCECF, did not affect the CFU of the preparation (data not shown).

**Bacterium uptake by macrophages.** J-774-E clone cells, a murine macrophage cell line, were grown in minimum essential medium containing Earle’s salts supplemented with 10% FCS in a 5% CO₂ atmosphere. To label endocytic compartments with colloidal gold particles, the cells were washed with BME and in the same medium resuspended colloidal gold particles coated with mannosylated BSA. Lysosomes were marked with 60-nm colloidal gold particles, which were incorporated into the macrophages for 60 min. After repeated rinses, their access to lysosomes was allowed by incubation during 3 h at 37°C. After rinsing, the macrophages were incubated at 37°C for 20 h. Early endosomes were marked with 20-nm colloidal gold particles, through their internalization during 15 min before fixing the cells. Presence of endosome and lysosome markers was monitored by transmission electron microscopy. Phagosomes containing lysosome marker (60-nm gold particles) or endosome marker (20-nm gold particles) and those with no markers were counted. Macrophages were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7); and processed for transmission electron microscopy. For the 20 h time point, 5% FCS replaced BSA in the BME.

To assess accessibility of newly internalized gold particles to preexisting Brucella-containing phagosomes, a protocol similar to the one described above was used. In brief, after a 5 min

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**Figure 8.** Morphological alterations of Brucella abortus strains. The morphological alterations observed in the bacteria (heat-killed and live *B. abortus* 2308 and *B. abortus* S19) were quantified, after 20 h of internalization, in which early and late endosomes were marked with colloidal gold particles of different size. Morphological alterations were monitored by transmission electron microscopy.

**Figure 9.** Endosome-lysosome fusion in macrophages infected with *Brucella abortus*. Using the previous experiments we performed a count of the fusion of lysosome compartments (60 nm gold particles) with endosomes (20 nm gold particles). The count was performed in macrophages which contained at least one phagosome with *Brucella abortus* (infected macrophage) and in macrophages containing no bacteria (non-infected macrophage), using always the same sample. Macrophages were incubated for 20 h with *B. abortus* 2308 and S19 (as a live vaccine strain).
uptake of live opsonized *B. abortus* bacteria, the microbes were chased for 45 or 120 min at 37°C. Cells were then incubated with colloidal gold particles for 15 min and chased for 0 or 60 min.

**Phagosome acidification.** Macrophages were plated for 24 h on coverslips and incubated with opsonized *Brucella* labeled with TAMRA or BCECF-labeled *Brucella*, respectively. Each slide was incubated with 1 mM LysoTracker for experiments carried out with TAMRA and processed with Paint Shop Pro program (Jasc Software, Inc., Eden Prairie, MN).

**Autophagosome and ER labeling.** Macrophages were plated for 24 h on coverslips and incubated with opsonized *Brucella* labeled with TAMRA for 1 h at 20°C (100 *Brucella* bacteria/cell). Cells were then washed with BME and chased for different periods of time at 37°C. To label autophagosomes, the coverslips were mounted in BME containing 40 µg of gentamicin per ml and 50 µM monodansylcadaverine (MDC). Slides were analyzed for up to 15 min as described above using a set of filters for MDC (excitation, 330 to 380 nm; barrier, 420 nm). To label the ER, the coverslips were fixed for 5 min in 0.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.1 M sucrose. After several washes with the sucrose-phosphate buffer, the coverslips were incubated for 10 s with 2.5 µg of DiOC6 per ml in the same buffer. The coverslips were then washed in PBS-sucrose and analyzed as described above using a set of filters for fluorescein (450 to 490 nm; barrier, 520 nm).

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