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Intracellular Trafficking of *Brucella abortus* in J774 Macrophages

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Brucella abortus is a facultative intracellular bacterium capable of surviving inside professional and non-professional phagocytes. The microorganism remains in membrane-bound compartments that in several cell types resemble modified endoplasmic reticulum structures. To monitor the intracellular transport of *B. abortus* in macrophages, the kinetics of fusion of phagosomes with preformed lysosomes labeled with colloidal gold particles was observed by electron microscopy. The results indicated that phagosomes containing live *B. abortus* were reluctant to fuse with lysosomes. Furthermore, newly endocytosed material was not incorporated into these phagosomes. These observations indicate that the bacteria strongly affect the normal maturation process of macrophage phagosomes. However, after overnight incubation, a significant percentage of the microorganisms were found in large phagosomes containing gold particles, resembling phagolysosomes. Most of the *Brucella* bacteria present in phagolysosomes were not morphologically altered, suggesting that they can also resist the harsh conditions prevalent in this compartment. About 50% colocalization of *B. abortus* with LysoSensor, a weak base that accumulates in acidic compartments, was observed, indicating that the *B. abortus* bacteria do not prevent phagosome acidification. In contrast to what has been described for HeLa cells, only a minor percentage of the microorganisms were found in compartments labeled with monodansylcadaverine, a marker for autophagosomes, and with DiOC6 (3,3′-dihexyloxacarbocyanine iodide), a marker for the endoplasmic reticulum. These results indicate that *B. abortus* bacteria alter phagosome maturation in macrophages. However, acidification does occur in these phagosomes, and some of them can eventually mature to phagolysosomes.

The facultative intracellular parasite *Brucella abortus* causes abortion and infertility in cattle and undulant fever in humans. The bacterium is endemic in many underdeveloped countries and responsible for large economic losses and chronic infections in human beings (30). *Brucella* infects its hosts through mucosae and wounds and initially is incorporated into professional phagocytes where it survives and reproduces (14). Afterwards, the bacterium infects several types of nonprofessional phagocytic cells including those of endocardium, brain, joints, and bones. *Brucella* has a special tropism for reproductive organs, causing a high rate of abortion in pregnant animals (28).

The intracellular survival of *Brucella* has been documented for several cell types. According to multiple observations, *B. abortus* is incorporated into phagosomes and remains in membrane-bound compartments until the host cell dies. In nonprofessional phagocytes, *Brucella* is located in structures that resemble the endoplasmic reticulum (ER) (6). Recent evidence indicates that *Brucella* is transported through the autophagic pathway before accumulating in the ER (22, 23).

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 have monitored the intracellular transport of a virulent strain of *B. abortus* in J774 macrophages, a well-characterized murine cell line. The normal maturation process of phagosomes has been extensively studied with these macrophages (2). As soon as new phagosomes are formed, they exchange material with early endosomes. This active process permits the recycling of membrane-associated proteins and soluble proteins to the cell surface. As the composition of the phagosomal membrane changes, it becomes fusogenic with late endocytic compartments and the phagosome interacts with lysosomes, acquiring a complex cocktail of hydrolytic enzymes (4, 21, 25).

The aim of the present work was to monitor the interaction of phagosomes containing dead and live *B. abortus* bacteria with different endocytic compartments in macrophages. The results indicate that, soon after internalization, *Brucella* alters the transport to hydrolytic compartments and prevents fusion with newly formed endosomes. However, the bacterium does not prevent phagosome acidification and survives in vesicles that do not resemble ER structures.

MATERIALS AND METHODS

Reagents, materials, and solutions. LysoSensor (L7535), LysoTracker (L-7528), BCECF AM [2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; acetoxymethyl ester; B1170], TAMRA [5-(and-6)-carboxytetramethylrhodamine; succinimidyl ester; C1171], and DiOC6 (3,3′-dihexyloxacarbocyanine iodide; D273) were from Molecular Probes, Eugene, Oreg. Unless specified, 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., and labeled with ^{125}I using chloramine T (final activity, 3×10^6 cpm/μg) (29). Bovine serum albumin (BSA) was mannosylated as previously described (7). Colloidal gold particles were obtained using the citrate reducing method and coated with mannosylated BSA as described previously (17). 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).

Bacteria. *B. abortus* 2308, a virulent smooth strain, was grown at 37°C in *Brucella* agar (Merck Diagnostica for Microbiology) with 10% CO_2 for 48 h to stationary phase, resuspended in phosphate-buffered saline (PBS), washed, and resuspended in the same buffer (approximately 10^{10} 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. When required, the microbes were killed by heating them to 60°C for 60

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min. No bacterial growth was observed during 10 days after plating these preparations at 37°C. For some experiments, *Brucella* was opsonized with a polyclonal mouse anti-*Brucella* antibody (8×10^7 bacteria were incubated with 2 μg of the antibody in 40 μl of BME for 1 h at 20°C and washed three times with BME). A radiolabeled rabbit anti-mouse IgG antibody was used as a secondary antibody to assess hydrolysis (8×10^7 opsonized bacteria were incubated with 0.3 μg of ^{125}I-labeled rabbit anti-mouse antibody in 40 μl of BME for 1 h at 20°C and washed three times with BME). For light microscopy, *Brucella* was labeled with tetramethylrhodamine (8×10^7 *Brucella* bacteria were incubated with 5 μg of TAMRA in 50 μl 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 (8×10^7 *Brucella* bacteria were incubated with 10 μM BCECF AM in 200 μl 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.

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% CO_2 atmosphere. To label endocytic compartments with colloidal gold particles, the cells were washed with BME and resuspended in the same medium containing 20-nm colloidal gold particles coated with mannosylated BSA. After a 15-min uptake at 37°C, the cells were washed to eliminate noninternalized ligand and incubated at 37°C for 60 min to chase the gold particles into lysosomes. *B. abortus* (dead or alive, opsonized or not) bacteria were incubated with the macrophages (100 *Brucella* bacteria/macrophage) for 5 min at 37°C. Cells were then washed five times with BME to remove nonadherent bacteria. Macrophages were then incubated at 37°C for 0, 15, and 45 min and 2 and 24 h; fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7); and processed for transmission electron microscopy. For the 24-h time point, 5% FCS replaced BSA in the BME.

To assess the 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 uptake of dead or live opsonized *B. abortus* bacteria, the microbes were chased for 45 or 120 min at 37°C. The cells were then incubated with colloidal gold particles for 15 min and chased for 0 or 60 min.

Bacterium digestion. Macrophages were grown in six-well plates for 24 to 48 h. The medium was then removed, and cells were inoculated with 1 ml of BME containing opsonized *Brucella* labeled with radioactive rabbit anti-mouse antibody (200 bacteria/cell, 0.1 cpm/bacterium). Culture plates were centrifuged for 10 min at $170 \times g$ at 20°C and washed three times with BME to remove nonadherent bacteria. Monolayers were incubated with 1 ml of BME. The medium was replaced at 0, 15, 30, and 45 min and 1.5, 2, 3, and 20 h. After the first 15 min, the medium was supplemented with gentamicin (40 μg/ml) in order to kill extracellular *Brucella*. For the 20-h time point, 5% FCS replaced BSA in the BME. The conditioned media were precipitated with 5% trichloroacetic acid (TCA), and the radioactivity in the pellets and supernatants was measured. At the end of the experiment, the cells were solubilized in 0.5% Triton X-100 and the radioactivity was counted. The percentage of total TCA-soluble radioactivity released into the medium at each time point was calculated. The total amount of counts was obtained by adding the radioactivity of pellets and supernatants and the cell-associated radioactivity at the end of the experiment.

Phagosome acidification. Macrophages were plated for 24 h on coverslips and incubated with opsonized *Brucella* labeled with TAMRA or BCECF 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. The coverslips were mounted in BME containing 40 μg of gentamicin per ml and 5 μM LysoSensor or 1 μM Lyso-Tracker for experiments carried out with TAMRA- or BCECF-labeled *Brucella*, respectively. Each slide was finally analyzed for up to 30 min in an Eclipse TE300 Nikon microscope equipped with a Hamamatsu Orca 100 camera operated with the Metaview software (Universal Imaging Corp., West Chester, Pa.). Images were taken with two sets of filters (excitation, 510 to 560, and barrier, 590, for TAMRA; and excitation, 450 to 490, and barrier, 520, for LysoSensor) and processed with the 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; barrier, 420). 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; barrier, 520).

RESULTS

Colocalization of *Brucella* and colloidal gold particles loaded in lysosomes. To monitor by electron microscopy the intracellular transport of *B. abortus* to preformed lysosomes,
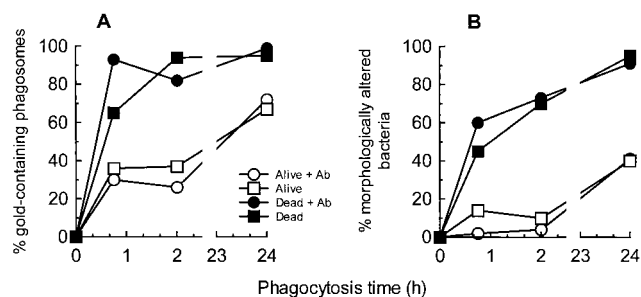

FIG. 1. Fusion of *Brucella*-containing phagosomes with preformed lysosomes. Colloidal gold particles (20 nm) coated with mannosylated BSA were internalized in J774 macrophages for 15 min at 37°C and chased into lysosomes by an additional 60-min incubation. *B. abortus* (opsonized [squares] or not opsonized [circles]) was incubated with the macrophages (100 *Brucella* bacteria/macrophage) for 5 min at 37°C. Cells were then washed five times with BME to remove nonadherent bacteria. Macrophages were incubated at 37°C for different times, fixed, and processed for transmission electron microscopy. (A) The percentage of phagosomes containing gold particles was calculated by counting at least 100 phagosomes for each condition. (B) The percentage of digested *Brucella* was estimated by counting at least 150 intraphagosomal bacteria. The results are from one of three independent experiments performed.

late endocytic compartments from J774 macrophages were labeled with colloidal gold particles (15-min internalization, 60-min chase). Heat-killed and live bacteria were then internalized for 5 min and chased for up to 24 h. At different time points, the cells were fixed and the colocalization of bacteria and gold particles was quantified by electron microscopy. The results showed that dead bacteria accumulated in gold-containing phagosomes soon after internalization, whereas most live *Brucella* bacteria remained in gold-free phagosomes for more than 2 h (Fig. 1A). However, after 24 h of uptake about 60% of the phagosomes formed with live *Brucella* contained colloidal gold particles.

B. abortus exhibited a very distinct profile in the electron microscope (Fig. 2, inserts). Dead bacteria were digested by macrophages very efficiently, evincing morphological alterations soon after internalization (Fig. 1B and 2b). At 24 h of incubation, most of the *Brucella* bacteria were digested and it was difficult to find unequivocal *Brucella* profiles in macrophages (Fig. 1B and 2f). In contrast, most of the live bacteria presented an intact morphology even at the latest time point. Interestingly, it was common to observe morphologically intact *Brucella* bacteria even in phagosomes containing colloidal gold particles (Fig. 2e). Viability of *B. abortus* assessed by the number of CFU at different times of internalization indicated that there was a two- to fourfold decrease in live *Brucella* bacteria during the first 12 h of uptake. After this initial decrease, the bacteria started to grow and reached maximum CFU at 30 h of internalization. Afterwards, the macrophages began to die and the CFU decreased abruptly (data not shown).

To assess whether opsonization would affect the intracellular destination of *B. abortus*, the bacteria were coated with a mouse anti-*Brucella* antibody. The uptake was more efficient under these conditions (0.9 *Brucella* bacterium/cell with antibody versus 0.3 *Brucella* bacterium/cell without antibody); however, the kinetics of fusion with gold-containing compartments and the digestion of live and dead *Brucella* bacteria were not significantly altered (Fig. 1). Also, the CFU of *Brucella* after 24 h of uptake was not significantly affected by the presence of the antibody (data not shown).

Representative images of live and dead *Brucella* bacteria at different times after internalization are shown in Fig. 2. Forty-five minutes after internalization of live *Brucella* bacteria, a
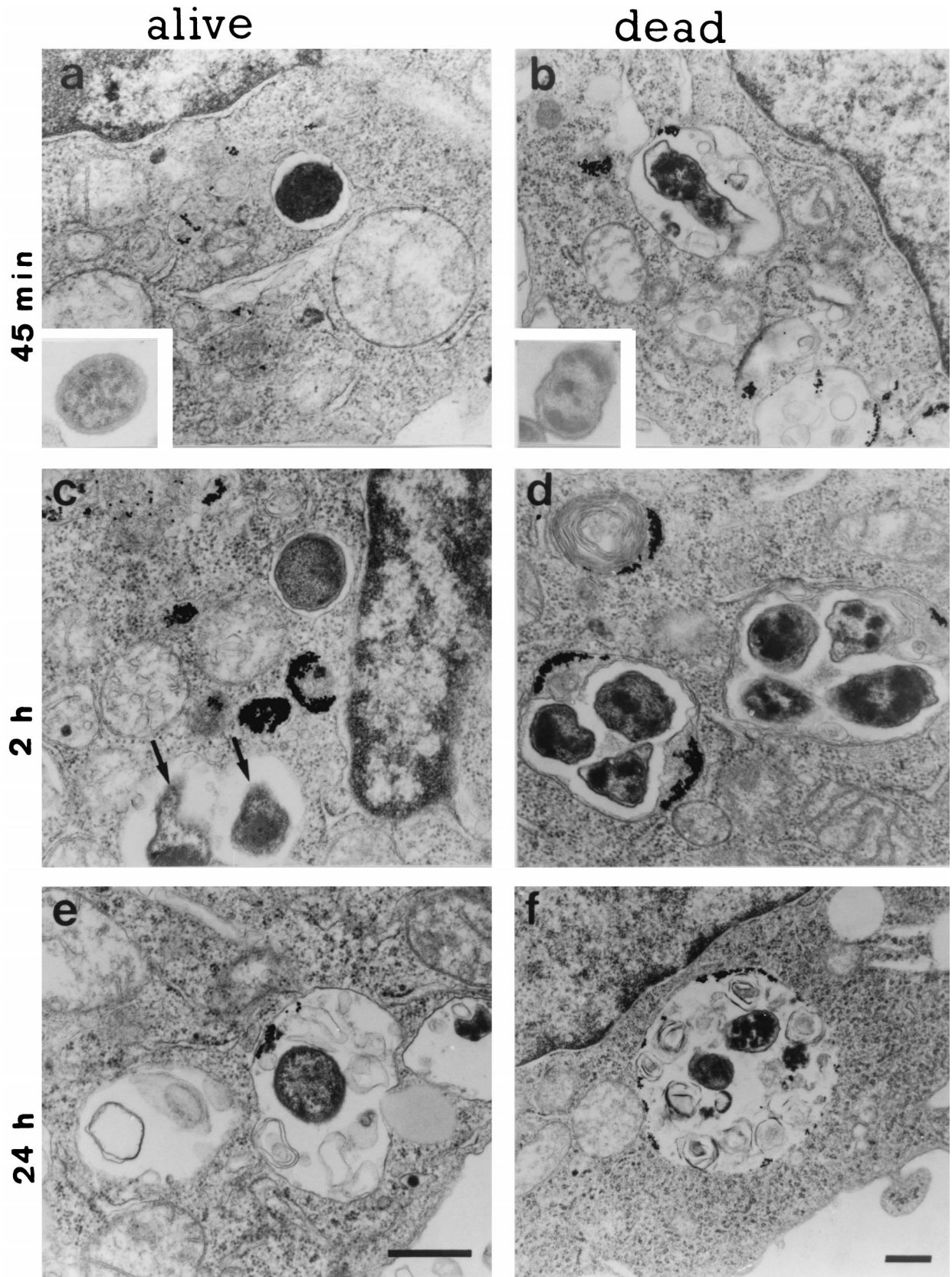
FIG. 2. Images of the fusion of *Brucella*-containing phagosomes with preformed lysosomes. Lysosomes were loaded with 20-nm colloidal gold particles as described in the Fig. 1 legend. Live (a, c, and e) and heat-killed (b, d, and f) *Brucella* bacteria were internalized for 5 min and chased for 45 min (top panels), 2 h (middle panels), and 24 h (bottom panels). Gold-containing compartments are abundant in these cells. Live *Brucella* bacteria are located in small, gold-free phagosomes, except in the 24-h phagosome. The *Brucella* shown at this time point (e) is not digested in spite of being located in a phagolysosome containing gold particles. Two partially digested *Brucella* bacteria are shown in panel c (arrows). Colloidal gold particles are present in all phagosomes containing heat-killed *Brucella* (b, d, and f). The phagosome in panel f is especially large and presents several membranous bodies that may represent highly digested *Brucella*. For morphological comparison, extracellular live and dead *Brucella* bacteria are shown as inserts in panels a and b, respectively. Bars, 1 μm.
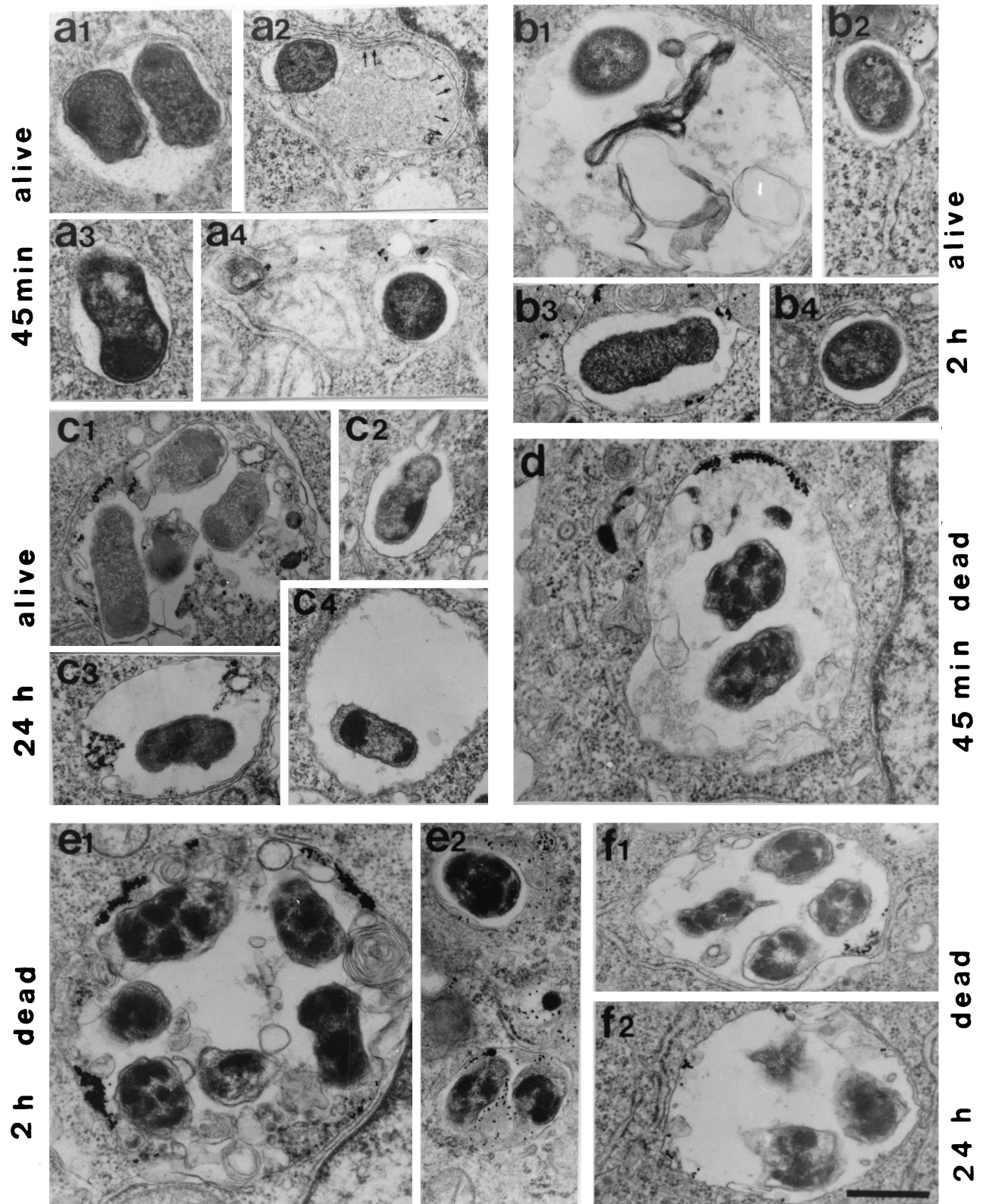
FIG. 3. Gallery of phagosomes containing live (a to c) and heat-killed (d to f) *Brucella* bacteria. Panels a and d correspond to 45-min phagosomes, panels b and e correspond to 2-h phagosomes, and panels c and f correspond to 24-h phagosomes. Small phagosomes were prevalent with live *Brucella* (a1, a3, a4, b2, b4, and c2). However, large phagosomes were also observed. A large phagosome with a cytoplasm-like content and double membrane (arrows) resembling an autophagosome is shown in panel a2. The phagosomes in panels b1, b3, and c1 present abundant internal membranes and resemble phagolysosomes. The phagosome in panel c4 is spacious but contains little intravesicular content. Phagosomes containing heat-killed *Brucella* generally present characteristics of phagolysosomes, i.e., they present abundant intravesicular membranes and gold particles (d to f). Bar, 1 μm.
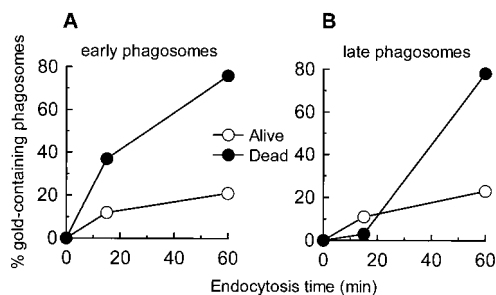
FIG. 4. Accessibility of internalized gold particles to preformed phagosomes containing live and heat-killed *Brucella* bacteria. Live and heat-killed *Brucella* bacteria were internalized by J774 macrophages for 5 min and chased for 45 min (A) or 120 min (B). Colloidal gold particles were then internalized for 15 min and chased for 0 to 60 min. Colocalization of gold particles with *Brucella* was quantified for all conditions in at least 100 phagosomes. The results are from one of the two independent experiments performed.
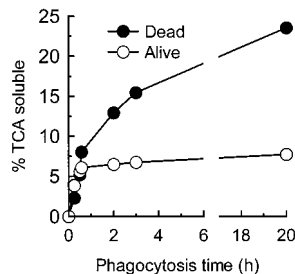

FIG. 5. Hydrolysis of a radiolabeled antibody attached to live or heat-killed *Brucella*. *Brucella* bacteria opsonized with a mouse anti-*Brucella* antibody were incubated with a rabbit anti-mouse IgG antibody labeled with ^{125}I. The *Brucella* bacteria were then bound to J774 macrophages, and the release of TCA-soluble radioactivity into the medium was assessed after different times of internalization. The values are expressed as percentages of the total radioactivity bound to the cells, and they are the means of duplicate samples. Four independent experiments were performed with similar results.

bacterium is observed in a small vesicle, without gold particles (Fig. 2a). In contrast, a dead bacterium is already present in a gold-containing phagosome and is partially digested (Fig. 2b). After 2 h of uptake of live bacteria, several *Brucella* bacteria were still in small vesicles (Fig. 2c). In the same micrograph, two partially digested *Brucella* bacteria are observed in a separate phagosome. At the same time point, several heat-killed *Brucella* bacteria are observed inside large phagosomes containing gold particles (Fig. 2d). After 24 h, an intact *Brucella* is shown inside a large phagosome containing gold particles and several internal vesicles (Fig. 2e). At this internalization time, dead *Brucella* bacteria were hard to distinguish. In the large phagosome shown in Fig. 2f, only two partially digested *Brucella* bacteria can be recognized. Several other membranous bodies may represent highly digested bacteria.

A gallery of different kinds of phagosomes formed by the internalization of live and heat-killed *Brucella* bacteria is shown in Fig. 3. Phagosomes containing live *Brucella* were generally small and devoid of intravesicular membranes even after 24 h of uptake (Fig. 3a1, 3a3, 3a4, 3b2, 3b4, and 3c2). However, large phagosomes were not rare (Fig. 3b1, 3c1, and 3c4). A few large phagosomes could represent autophagosomes (Fig. 3a2) because of a cytoplasm-like content and a visible double membrane. Most of the phagosomes containing heat-killed *Brucella* were easily recognized as phagolysosomes at the earliest time point analyzed. These phagolysosomes were rich in gold particles and intraphagosomal vesicles.

Accessibility of *Brucella*-containing phagosomes to newly internalized gold particles. The above results indicate that phagosomes containing live *Brucella* mature with different kinetics than those of normal phagosomes. The question remains whether such phagosomes stay as early phagosomes or whether they represent a different compartment. It is well known that phagosomes are accessible to newly endocytosed markers and that early phagosomes receive newly internalized markers faster than do late phagosomes. In order to assess the accessibility of endocytic markers to phagosomes containing *Brucella*, heat-killed and live bacteria were internalized for 45 min (early phagosomes) or 120 min (late phagosomes). Afterwards, the cells were incubated with colloidal gold particles and the kinetics of the arrival of gold in the phagosomes was monitored for 60 min. Colloidal gold particles reached early phagosomes loaded with heat-killed bacteria after 15 min of uptake, whereas a 60-min uptake was necessary to reach late phagosomes (Fig. 4). Conversely, phagosomes formed by the inter-

nalization of live *Brucella* were reluctant to incorporate gold particles at either of the time points assessed (Fig. 4).

Digestion of *Brucella*-associated proteins. The above results imply that live *Brucella* hampers transport to lysosomes whereas the heat-killed bacterium is readily transported to these hydrolytic organelles. To study the arrival of *Brucella* in proteolytic compartments, opsonized live and heat-killed *Brucella* bacteria were coated with a radiolabeled antibody. The bacteria were bound to the macrophages and internalized for different periods of time. The release of TCA-soluble radioactivity into the medium was used as an indication of the arrival of the bacteria in a protease-rich compartment. Proteolysis of heat-inactivated *Brucella* was evident after a few minutes of internalization. The kinetics of digestion of live *Brucella* was not very different during the first minutes of uptake, but afterwards the rate of release of TCA-soluble radioactivity into the medium was much lower than that with the heat-inactivated bacterium. These results indicate that there was a significant delay in the transport of live bacteria to proteolytic compartments (Fig. 5).

Acidification of *Brucella*-containing phagosomes. As phagosomes mature, the intravesicular pH decreases from 6.5 in newly formed phagosomes to about 4 in mature phagolysosomes. It was important to assess whether the presence of live *Brucella* could alter the acidification of phagosomes. The pH of phagosomes containing heat-killed and live *Brucella* was monitored by colocalization with LysoSensor, a weak base probe that accumulates in acidic compartments and fluoresces at acidic pH ($pK_a = 5.2$). About 40% of the live bacteria colocalize with LysoSensor after 60 min of uptake (Table 1). This percentage had not decreased even after 20 h, indicating that the initial acidification of *Brucella*-containing phagosomes was not due to the presence of altered bacteria facing digestion (Table 1 and Fig. 6a). In agreement with this observation, 50% of live *Brucella* bacteria labeled with BCECF—a fluorescent marker that accumulates in and is retained exclusively by live bacteria—were present in acidic compartments (Table 1 and Fig. 6b). The percentage of colocalization of heat-killed *Brucella* was similar or lower than that observed for live *Brucella*. After 20 h of uptake, heat-killed *Brucella* was difficult to recognize inside macrophages. The results indicate that *Brucella* does not abrogate phagosome acidification and that it can survive under low-pH conditions inside macrophages.

Limited colocalization of *B. abortus* with MDC, an autophagosomal marker, and DiOC6, an ER marker. In HeLa cells, *Brucella* is found transiently in autophagosomes before local-

TABLE 1. Percentages of heat-killed and live *Brucella*-containing phagosomes that colocalize with markers of acidic compartments[a]

Time	% Heat-killed *Brucella*	% Live *Brucella*	*Brucella* marker	Acidic compartment marker
30 min	NE	39 (33)	TAMRA	LysoSensor
1 h	29 (55)	43 (218)	TAMRA	LysoSensor
2 h	44 (131)	44 (189)	TAMRA	LysoSensor
20 h	NE	50 (202)	TAMRA	LysoSensor
20 h	NE	51 (85)	BCECF	LysoTracker

[a] J774 macrophages were incubated for 1 h at 20°C with opsonized heat-killed or live *B. abortus* labeled with TAMRA or BCECF. Cells were then washed with BME and chased for 1, 2, or 20 h at 37°C. The coverslips were mounted in BME containing 5 μM LysoSensor or 1 μM LysoTracker for experiments carried out with TAMRA- or BCECF-labeled *Brucella*, respectively. Colocalization of *Brucella* with acidic compartment markers was evaluated from images recorded in three independent experiments. Numbers are the percentages of colocalization and, in parentheses, the total numbers of *Brucella* bacteria counted. After 20 h of uptake, most of the heat-killed *Brucella* bacteria were digested and could not be recognized for evaluation. BCECF is incorporated only by live bacteria. NE, not evaluated.

izing to the ER (22, 23). According to the electron microscopy observations, a small percentage of phagosomes containing live *Brucella* present morphological characteristics of autophagosomes in J774 macrophages. To assess whether live and heat-killed *Brucella* bacteria were transported to autophagosomal compartments in macrophages, the transport of TAMRA-labeled *Brucella* was monitored in cells stained with MDC, a fluorescent marker for autophagosomes. The results indicate that most of the *Brucella*-containing phagosomes did not colocalize with MDC as they matured (Table 2 and Fig. 6c). During the first hour of internalization, the percentage of colocalization was similar to that observed for heat-killed bacteria. At later internalization times, the colocalization with MDC decreased for live *Brucella* and did not change for dead bacteria. To label the ER, the cells were fixed and incubated with DiOC6. Colocalization with the ER marker was very rare for live and heat-killed *Brucella* bacteria after 2 or 20 h of internalization. According to these results, although a small percentage of *B. abortus* transiently travel through autophagosomal compartments, most of them survive inside structures not related to autophagosomes or the ER.

DISCUSSION

Professional phagocytes are central effector cells in defense against microbial pathogens that kill a variety of microorganisms by ingesting them in phagosomes where they are exposed to a harmful environment. The bactericidal condition inside the vacuole is related to the presence of reactive oxygen species, hydrolytic enzymes, and specialized antimicrobial proteins and peptides. Low pH and depletion of nutrients and cations have also been proposed as part of the toxic conditions in phagosomes (20). Not all microbes that enter into a professional phagocyte are exposed to the same toxic environment. For example, the oxidative burst is triggered by a complex mechanism that depends in part on the receptor engaged in the phagocytic process (19). Also, fusion with specific granules in neutrophils is determined by the receptor involved in the uptake (13). Moreover, some microbes are not really phagocytosed by phagocytes. They invade cells and form a specialized vacuole with characteristics that are controlled by the microbe and not by the phagocyte (18).

It is now known that intracellular microbes have developed a series of strategies to survive inside cells (27). Alteration of the normal process of phagosome maturation has been described for several microorganisms such as *Mycobacterium*, *Legionella*, *Chlamydia*, and *Listeria* spp. (1, 27). In the case of *Brucella*, inhibition of phagosome-lysosome fusion has been reported by several authors (9, 22, 23). However, phagosome maturation is a complex process that involves a series of fusions with different endocytic compartments and recycling of membranes and proteins by means of tubular connections and budding of transport vesicles (2). We have monitored by electron microscopy the fusion of newly formed phagosomes with preexisting late endocytic compartments labeled with colloidal gold particles. Additionally, the entrance of newly endocytosed colloidal gold particles into preformed phagosomes containing *Brucella* was assessed. The results show that *Brucella* significantly delays fusion with preformed lysosomes and prevents the interaction with newly formed endosomes. Alteration in the intracellular transport of *Brucella* is also supported by the observation that the arrival of the bacterium in proteolytic compartments was very slow. However, at late time points, a significant percentage of *Brucella* were found in gold-containing phagosomes with morphological characteristics of phagolysosomes. The presence of *B. abortus* in phagolysosomes of professional phagocytes has been reported by other authors (5, 11). In contrast to what has been described for other cell types, we observed a very limited colocalization of *B. abortus* with markers of the autophagosomal pathway and the ER. Moreover, there was no preferential colocalization of the live versus the heat-killed bacterium with these markers.

In other cell types, *Brucella* also hampers fusion with lysosomes, but is found first in autophagosomal vacuoles and later in vesicles that correspond to specialized regions of the ER (22, 23). According to what is presently known, the differences observed between the intracellular transport of *B. abortus* in professional phagocytes and that in nonprofessional phagocytes may be a consequence of the same survival strategy. *Brucella* can—by means of a still-unknown mechanism—delay the fusion of newly formed phagosomes with late endocytic compartments. In HeLa cells, the lack of fusion may allow the interaction of the phagosome with early autophagic vesicles

TABLE 2. Percentages of heat-killed and live *Brucella*-containing phagosomes that colocalize with an autophagosome marker (MDC) or an ER marker (DiOC6)[a]

Marker and time (h)	% Heat-killed *Brucella*	% Live *Brucella*
Autophagosome (MDC)		
1	14 (63)	15 (34)
2	14 (49)	6 (35)
4	NE	5 (62)
20	NE	7 (179)
ER (DiOC6)		
2	6 (167)	5 (233)
20	NE	4 (200)

[a] J774 macrophages were incubated for 1 h at 20°C with opsonized heat-killed or live *B. abortus* labeled with TAMRA. 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 MDC. To label the ER, the coverslips were fixed in 0.25% glutaraldehyde and incubated for 10 s with 2.5 μg of DiOC6 per ml. Colocalization of *Brucella* with MDC and DiOC6 was evaluated from images recorded in three independent experiments. Numbers are the percentages of colocalization and, in parentheses, the total numbers of *Brucella* counted. After 20 h of uptake, most of the heat-killed *Brucella* bacteria were digested and could not be recognized for evaluation. NE, not evaluated.
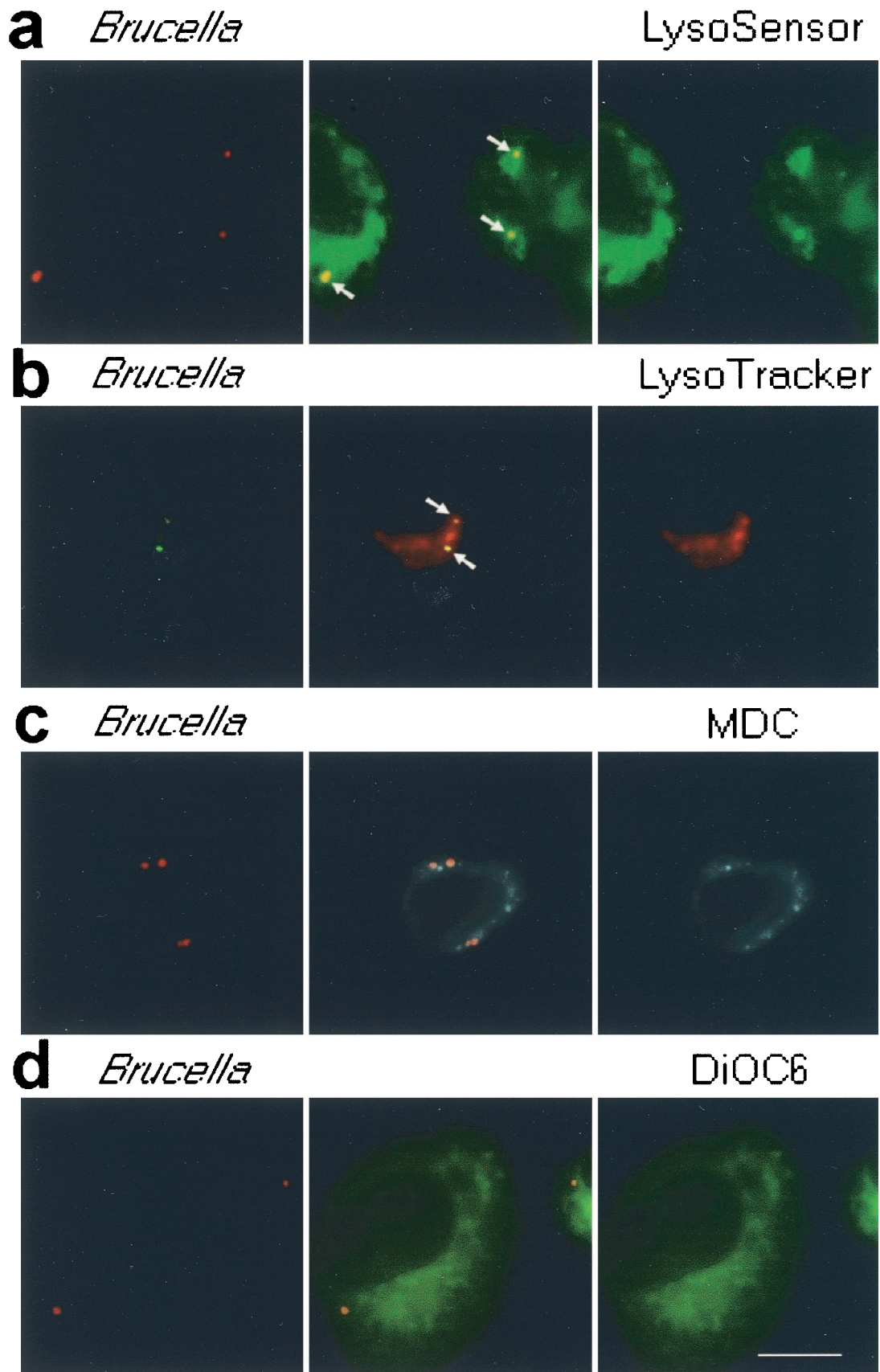
FIG. 6. Colocalization of live *Brucella* with probes that label acidic compartments, autophagosomes, and the ER. Live *Brucella* bacteria were labeled with TAMRA (a, c, and d, left and middle panels) or BCECF (b, left and middle panels) and incubated with J774 macrophages for 20 h. Cells were labeled with LysoSensor, a green fluorescent weak base that accumulates in acidic compartments (a, right and middle panels); LysoTracker, a red fluorescent weak base that accumulates in acidic compartments (b, right panel); MDC, a marker for autophagosomal compartments (c, right and middle panels); and DiOC6, a marker for the ER (d, right and middle panels). Arrows, *Brucella* bacteria present in acidic compartments. Bar, 15 μm.

that normally fuse with endosomes (15) and presumably with other related endosomal structures such as newly formed phagosomes. The presence of the microbe in the autophagic vesicle would render this vesicle less fusogenic with late endocytic compartments and hamper the maturation of the autophagosome to an autophagolysosome. The fact that autophagosomes have an ER origin (8) may allow the interaction of the *Brucella*-containing autophagosome with some regions of the ER. In macrophages, which have a more active endocytic route, the mechanism employed by *Brucella* to delay fusion may not be sufficient to permanently prevent fusion with a late compartment. Hence, phagosomes will eventually mature to phagolysosomes. Autophagosomes interacting with *Brucella*-containing phagosomes will also mature to autophagolysosomes and not to ER-derived vacuoles. It has been shown that *B. abortus* expresses specific proteins after phagocytosis, oxidative stress, and acidic pH (26). It would be interesting to know whether the differences between the intracellular destinations of *B. abortus* in HeLa cells and that in macrophages are reflected in differential patterns of protein expression.

Opsonization of *B. abortus* bacteria did not affect their ability to prevent fusion with other endocytic compartments. Also, survival inside the macrophage was not significantly affected by entering through the Fc receptor. Similar observations have been made for *Brucella suis* (24). However, opsonization may affect survival in interferon-treated J774 macrophages (10). The concentration and type of antibody used may also modify the effect of opsonization on *Brucella* survival (12). Although antibodies may have a role in the relationship between *Brucella* and the cell host, our results indicate that *B. abortus* can alter intracellular transport independently of opsonization with specific antibodies.

The delayed fusion with late endocytic compartments does not prevent acidification of the phagosomes. A large percentage of live *B. abortus* bacteria were present in acidic vacuoles after an overnight incubation. Porte et al. (24) have reported that acidification of phagosomes may favor the survival of *B. suis* inside J774 macrophages. Acidic intravesicular pH may trigger the expression of several proteins necessary for intracellular survival of the microbe (26). Acidification is important for some transport steps in the endocytic pathway, and alkalinization of vesicles may prevent fusion with late compartments (3). However, a low pH seems to be necessary but not sufficient for fusion, and acidic phagosomes containing *Brucella* evinced attenuated fusion with late compartments.

Localization of *B. abortus* to vesicles resembling phagolysosomes was frequent after 24 h of internalization. However, most of the *Brucella* bacteria within these phagosomes presented an intact morphology, suggesting that they were resistant to the lysosomal environment. It has been reported that the outer membrane of *B. abortus* is resistant to bactericidal cationic peptides (16) and that phagocytosis induces the production of specific proteins (26). Hence, it is possible that *Brucella* can resist digestion inside phagolysosomes. Normally, maturation of newly formed phagosomes to phagolysosomes is a fast process. The delayed maturation observed for *Brucella* phagosomes may be very important to prevent early digestion and to allow the bacteria to express new genes necessary for intracellular survival.

In the future, it will be important to understand at the molecular level the alteration in the mechanism of intracellular transport caused by *Brucella* and the genes in the bacteria responsible for this remarkable effect.

REFERENCES

1. **Alvarez-Dominguez, C., R. Roberts, and P. Stahl.** 1997. Internalized *Listeria monocytogenes* modulates intracellular trafficking and delays maturation of the phagosome. J. Cell Sci. **110:**731–743.
2. **Beron, W., C. Alvarez-Dominguez, L. Mayorga, and P. D. Stahl.** 1995. Membrane trafficking along the phagocytic pathway. Trends Cell Biol. **5:**100–104.
3. **Clague, M. J., S. Urbe, F. Aniento, and J. Gruenberg.** 1994. Vacuolar ATPase activity is required for endosomal carrier vesicle formation. J. Biol. Chem. **269:**21–24.
4. **Desjardins, M., L. A. Huber, R. G. Parton, and G. Griffiths.** 1994. Biogenesis of phagolysosomes proceeds through a sequential series of interactions with the endocytic apparatus. J. Cell Biol. **124:**677–688.
5. **Detilleux, P. G., N. F. Cheville, and B. L. Deyoe.** 1988. Pathogenesis of *Brucella abortus* in chicken embryos. Vet. Pathol. **25:**138–146.
6. **Detilleux, P. G., B. L. Deyoe, and N. F. Cheville.** 1990. Entry and intracellular localization of *Brucella* spp. in Vero cells: fluorescence and electron microscopy. Vet. Pathol. **27:**317–328.
7. **Diaz, R., L. Mayorga, and P. Stahl.** 1988. In vitro fusion of endosomes following receptor-mediated endocytosis. J. Biol. Chem. **263:**6093–6100.
8. **Dunn, W. A., Jr.** 1990. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. J. Cell Biol. **110:**1923–1933.
9. **Frenchick, P. J., R. J. Markham, and A. H. Cochrane.** 1985. Inhibition of phagosome-lysosome fusion in macrophages by soluble extracts of virulent *Brucella abortus.* Am. J. Vet. Res. **46:**332–335.
10. **Gross, A., S. Spiesser, A. Terraza, B. Rouot, E. Caron, and J. Dornand.** 1998. Expression and bactericidal activity of nitric oxide synthase in *Brucella suis*-infected murine macrophages. Infect. Immun. **66:**1309–1316.
11. **Harmon, B. G., L. G. Adams, and M. Frey.** 1988. Survival of rough and smooth strains of *Brucella abortus* in bovine mammary gland macrophages. Am. J. Vet. Res. **49:**1092–1097.
12. **Hoffmann, E. M., and J. J. Houle.** 1995. Contradictory roles for antibody and complement in the interaction of *Brucella abortus* with its host. Crit. Rev. Microbiol. **21:**153–163.
13. **Joiner, K. A., T. Ganz, J. Albert, and D. Rotrosen.** 1989. The opsonizing ligand on *Salmonella typhimurium* influences incorporation of specific, but not azurophil, granule constituents into neutrophil phagosomes. J. Cell Biol. **109:**2771–2782.
14. **Liautard, J. P., A. Gross, J. Dornand, and S. Kohler.** 1996. Interactions between professional phagocytes and *Brucella* spp. Microbiologia **12:**197–206.
15. **Liou, W., H. J. Geuze, M. J. Geelen, and J. W. Slot.** 1997. The autophagic and endocytic pathways converge at the nascent autophagic vacuoles. J. Cell Biol. **136:**61–70.
16. **Martinez de Tejada, G., J. Pizarro-Cerda, E. Moreno, and I. Moriyon.** 1995. The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. Infect. Immun. **63:**3054–3061.
17. **Mayorga, L. S., R. Diaz, and P. D. Stahl.** 1988. Plasma membrane-derived vesicles containing receptor-ligand complexes are fusogenic with early endosomes in a cell-free system. J. Biol. Chem. **263:**17213–17216.
18. **Mordue, D. G., and L. D. Sibley.** 1997. Intracellular fate of vacuoles containing *Toxoplasma gondii* is determined at the time of formation and depends on the mechanism of entry. J. Immunol. **159:**4452–4459.
19. **Mosser, D. M.** 1994. Receptors on phagocytic cells involved in microbial recognition. Immunol. Ser. **60:**99–114.
20. **Nathan, C. F., and J. Gabay.** 1992. Antimicrobial mechanisms of macrophages, p. 259–267. *In* R. van Furth (ed.), Mononuclear phagocytes: biology of monocytes and macrophages. Kluwer Academic Publishers, Boston, Mass.
21. **Pitt, A., L. S. Mayorga, P. D. Stahl, and A. L. Schwartz.** 1992. Alterations in the protein composition of maturing phagosomes. J. Clin. Investig. **90:**1978–1983.
22. **Pizarro-Cerda, J., S. Meresse, R. G. Parton, G. van der Goot, A. Sola-Landa, I. Lopez-Goni, E. Moreno, and J. P. Gorvel.** 1998. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect. Immun. **66:**5711–5724.
23. **Pizarro-Cerda, J., E. Moreno, V. Sanguedolce, J. L. Mege, and J. P. Gorvel.** 1998. Virulent *Brucella abortus* prevents lysosome fusion and is distributed within autophagosome-like compartments. Infect. Immun. **66:**2387–2392.
24. **Porte, F., J. P. Liautard, and S. Kohler.** 1999. Early acidification of phagosomes containing *Brucella suis* is essential for intracellular survival in murine macrophages. Infect. Immun. **67:**4041–4047.
25. **Rabinowitz, S., H. Horstmann, S. Gordon, and G. Griffiths.** 1992. Immunocytochemical characterization of the endocytic and phagolysosomal compartments in peritoneal macrophages. J. Cell Biol. **116:**95–112.
26. **Rafie-Kolpin, M., R. C. Essenberg, and J. H. Wyckoff III.** 1996. Identification and comparison of macrophage-induced proteins and proteins induced un-

der various stress conditions in *Brucella abortus*. Infect. Immun. **64:**5274–5283. (Erratum, **65:**1137, 1997.)

27. **Sinai, A. P., and K. A. Joiner.** 1997. Safe haven: the cell biology of nonfusogenic pathogen vacuoles. Annu. Rev. Microbiol. **51:**415–462.

28. **Smith, L. D., and T. A. Ficht.** 1990. Pathogenesis of *Brucella*. Crit. Rev. Microbiol. **17:**209–230.

29. **Stahl, P. D., P. H. Schlesinger, E. Sigardson, J. S. Rodman, and Y. C. Lee.** 1980. Receptor-mediated pinocytosis of mannose-glycoconjugates by macrophages: characterization and evidence for receptor recycling. Cell **19:**207–215.

30. **Zavala, I., A. Nava, J. Guerra, and C. Quiros.** 1994. Brucellosis. Infect. Dis. Clin. N. Am. **8:**225–241.

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