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Opsonized Virulent *Brucella abortus* Replicates within Nonacidic, Endoplasmic Reticulum-Negative, LAMP-1-Positive Phagosomes in Human Monocytes

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Cells in the *Brucella* spp. are intracellular pathogens that survive and replicate within host monocytes. *Brucella* maintains persistent infections in animals despite the production of high levels of anti-*Brucella*-specific antibodies. To determine the effect of antibody opsonization on the ability of *Brucella* to establish itself within monocytes, the intracellular trafficking of virulent *Brucella abortus* 2308 and attenuated *hfq* and *bacA* mutants was followed in the human monocytic cell line THP-1. Early trafficking events of *B. abortus* 2308-containing phagosomes (BCP) were indistinguishable from those seen for control particles (heat-killed *B. abortus* 2308, live *Escherichia coli* HB101, or latex beads). All phagosomes transiently communicated the early-endosomal compartment and rapidly matured into LAMP-1$^+$, cathepsin D$^+$, and acidic phagosomes. By 2 h postinfection, however, the number of cathepsin D$^+$ BCP was significantly lower for live *B. abortus* 2308-infected cells than for either *Brucella* mutant strains or control particles. *B. abortus* 2308 persisted within these cathepsin D$^-$, LAMP-1$^+$, and acidic vesicles; however, at the onset of intracellular replication, the numbers of acidic *B. abortus* 2308 BCP decreased while remaining cathepsin D$^-$ and LAMP-1$^+$. In contrast to *B. abortus* 2308, the isogenic *hfq* and *bacA* mutants remained in acidic, LAMP-1$^+$ phagosomes and failed to initiate intracellular replication. Notably, markers specific for the host endoplasmic reticulum were absent from the BCPs throughout the course of the infection. Thus, opsonized *B. abortus* in human monocytes survives within phagosomes that remain in the endosomal pathway and replication of virulent *B. abortus* 2308 within these vesicles corresponds with an increase in intraphagosomal pH.

Brucella species are bacterial intracellular pathogens of mammals that maintain chronic infections by surviving and replicating within host monocytes and macrophages (34). This intracellular niche is critical for *Brucella* pathogenesis, as demonstrated by the attenuated nature of mutants defective in in vitro intracellular survival assays and the importance of cell-mediated immunity in controlling *Brucella* infections (2, 3, 10). In general, successful intracellular pathogens either escape from the phagosome soon after internalization or remain in the phagosome and disrupt the complex host cell trafficking machinery to avoid delivery to the phagosome/lysosomal compartment (21). The maturation of phagosomes into the destructive lysosomal compartment is a highly regulated process that involves the endosomal, phagosomal, and lysosomal trafficking pathways (36). *Brucella* spp. do not escape from phagosomes, and intracellular survival is achieved by inhibiting phagosome-lysosome fusion (9, 25, 27).

In the absence of opsonizing antibody, internalization of the brucellae into host macrophages is facilitated by the binding of the bacteria to lipid rafts on the host cell plasma membrane (23, 29, 39, 40). There is a considerable amount of experimental evidence, however, that opsonic entry plays an important role in the early stages of *Brucella* infections (12, 14, 16, 42). Although opsonization of the brucellae with specific immunoglobulin G (IgG) enhances the brucellacidal activity of cultured macrophages, virulent strains of *Brucella* can still resist killing by these phagocytes and eventually demonstrate net intracellular replication (1, 14, 15, 42). This ability to replicate after opsonin-mediated phagocytosis likely plays an important role in the ability of the brucellae to persist for prolonged periods in the host. It is well established that *Brucella*-specific IgG levels become elevated during the early stages of infection in both natural hosts and humans, but there is no correlation between the appearance of these serologic responses and resolution of the infection (12, 15, 32). In fact, elevated IgG levels are considered an indicator of active *Brucella* infections in humans and animals (37).

Studies with cultured murine macrophages have shown that following nonopsonic entry, virulent *Brucella* strains are trafficked to intracellular compartments that are favorable for intracellular survival and replication and that these intracellular compartments are enriched in membrane components originating from the endoplasmic reticulum (ER) (6, 17). Studies examining the interactions of *Brucella melitensis* and *B. suis* strains with cultured human peripheral blood monocytes, on the other hand, demonstrated that the *Brucella*-containing phagosomes avoid fusion with lysosomes in these host cells, but no association was detected between the *Brucella*-containing phagosomes and the ER of the human peripheral blood monocytes (30). The experiments described in this report were performed to gain a better understanding of the trafficking pat-

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terns of virulent *B. abortus* 2308 in the human monocytic cell line THP-1 after IgG-mediated phagocytosis and to assess the interaction of the *B. abortus* 2308-containing phagosomes (BCPs) with components of the host cell ER after entry of the brucellae into these phagocytes via this route. *B. abortus hfq* and *bacA* mutants derived from virulent strain 2308 were also included in this study because experimental evidence obtained in the mouse model suggests that the inability of the *hfq* and *bacA* mutants to resist killing by macrophages following IgG-mediated entry plays an important role in their inability to maintain chronic infection in experimental hosts (20, 31).

MATERIALS AND METHODS

Bacterial culture. All chemicals were obtained from Sigma-Aldrich unless otherwise stated. Virulent *B. abortus* laboratory strain 2308 was cultured on trypticase soy agar (TSA; Difco) supplemented with 5% bovine blood (BA) at 37°C under 5% CO_2. Attenuated *B. abortus* mutants Hfq3 (2308 *hfq*) (31) and KL7 (2308 *bacA*) (20) were grown on BA supplemented with 50 μg/ml of kanamycin. *Escherichia coli* HB101 was grown on TSA at 37°C under 5% CO_2. A green fluorescent protein (GFP)-expressing derivative of the broad-host-range plasmid pBBR1MCS (designated pBBR1MCS6-Y) (22) was introduced into the *B. abortus* strains and *E. coli* HB101 by electroporation, and the bacterial strains carrying this plasmid were maintained on BA or TSA supplemented with 6 μg/ml of chloramphenicol. Heat-killed *B. abortus* cells were prepared by incubating cell suspensions at 70°C in water for 30 min. Loss of viability was confirmed by plating portions of the heated cell suspension on BA and subsequent incubation at 37°C for 4 days.

THP-1 culture and differentiation into adherent monocytes. The human monocytic cell line THP-1 was cultured in RPMI 1640 medium with 2 mM glutamine supplemented with 1.5 g/ml sodium bicarbonate (Mediatech) and 10% fetal bovine serum (Gemini Bioproducts). Suspension cultures of THP-1 cells were maintained at a cell density between 5×10^5 and 2×10^6 cells/ml of cell culture medium and split two to three times per week. Cell culture viability was monitored by hemocytometer and trypan blue dye exclusion. THP-1 cells growing in suspension were harvested at a density of 1×10^6 cells/ml, resuspended in fresh medium supplemented with 5 nM phorbol myristic acid (PMA) to terminally differentiate the cells into adherent monocytes (35), and placed into 12- or 24-well tissue (Costar, NY) culture plates containing sterile #1 glass coverslips in each well. After overnight culture in the presence of PMA, adherent cells were washed three times gently with phosphate-buffered saline (PBS; pH 7.4) and incubated for an additional 24 h in complete RPMI 1640 medium with no PMA. The additional 24-h incubation following PMA differentiation allowed the cells to increase attachment to the glass surface and develop morphology characteristics similar to those of monocytes.

Intracellular survival and replication of the *B. abortus* strains in THP-1 cells. THP-1 cells used for the evaluation of the intracellular survival and replication profiles of the *B. abortus* strains were treated in a similar fashion but plated in 96-well flat-bottom tissue culture plates. Bacterial suspensions were prepared and generated by scraping 48-h cultures of the *B. abortus* strains grown on BA into screw-cap microfuge tubes containing PBS. *E. coli* cultures were grown on TSA medium for 24 h prior to harvesting. Pellets of bacteria were resuspended by vigorous vortexing, and numbers of bacteria present in the suspensions were determined by optical density at 600 nm measurements. These suspensions were used to generate dilute *Brucella* and *E. coli* preparations using complete RPMI 1640 medium whereby the bacterial density was adjusted to desired levels to account for variations in the numbers of target monocytes. Opsonization took place within these dilute suspensions containing either rabbit anti-*Brucella* (Difco) or anti-*E. coli* (Molecular Probes) IgG. Concentrations of antibody necessary to mediate opsonization without agglutinating the bacterial suspensions were achieved using antibody dilutions ranging from 1/2,000 to 1/5,000. Bacterial cell suspensions and antisera were incubated together either at 37°C in a shaking water bath for 20 min or at room temperature for 30 min followed by brief vortexing. Suspensions of opsonized bacteria were added to monocyte monolayers at a multiplicity of infection (bacteria/monocyte ratio) of 10:1 for *B. abortus* KL7 and *E. coli* HB101 and 20:1 for *B. abortus* 2308 and Hfq3. Tissue culture plates were gently agitated by hand and then centrifuged at 4°C for 10 min at 270 × *g*. Monolayers were washed gently with cold PBS to remove nonadherent bacteria and then incubated in fresh medium for 20 min at 37°C with 5% CO_2 to allow for phagocytosis of adherent bacteria. Monolayers were washed three times with PBS to remove any remaining nonadherent bacteria. Fresh media containing 100 μg/ml gentamicin was added following the last washing step to kill adherent, extracellular bacteria. For experiments lasting longer than 2 h, the 100 μg/ml-gentamicin-supplemented medium was replaced with medium containing 10 μg/ml gentamicin after 1 h and the bacteria remained in this medium for the duration of the experiment. Viability of intracellular *Brucella* was determined by lysing monocytes with 0.1% deoxycholate, diluting suspensions in PBS, and plating aliquots in triplicate on BA medium (31). Percentages of bacterial survival at 24 and 48 h were calculated based on the number of internalized bacteria detected at 1 h postinfection which represents 100% of internalized bacteria. Statistical comparisons were made using Student's *t* test.

Antibodies and reagents for fluorescence microscopy. Primary antibodies used for immunofluorescence microscopy were as follows: mouse anti-LAMP-1 monoclonal, anti-mannose-6-phosphate receptor (Iowa State Hybridoma), mouse anti-EEA1, anti-calnexin, anti-BiP/GRP74, anti-p115, anti-p230, anti-SRP54, anti-Rab5 (Transduction Laboratories), mouse anti-transferrin receptor (Molecular Probes), rabbit anti-*Brucella* antibody (Difco), rabbit anti-*E. coli* antibody (Molecular Probes), and mouse and rabbit anti-cathepsin D (Oncogene). Primary antibodies were used routinely at the concentration of 1/100 except for the following: mouse anti-LAMP-1, 1/20; mouse anti-mannose-6-phosphate receptor, 1/5. Slow-fade and Pro-long antifade mounting solution, $DiOC_6$, and Lysotracker Red DND-99 were also purchased from Molecular Probes.

Immunofluorescence microscopy. Coverslips harboring the adherent and infected monocytes were fixed with freshly prepared 4% paraformaldehyde in PBS (pH 7.4) at room temperature for 20 min. Following fixation, adherent and extracellular bacteria were differentiated from internalized bacteria by differential antibody staining performed prior to permeabilizaing monocytes for antibody staining of cellular antigens for time course experiments under 2 h. Differential staining was initiated by washing monolayers with cold PBS, followed by incubating coverslips in PBS at 1 h at 4°C successively with rabbit anti-*Brucella* antibody (1/500), followed with IgG-specific anti-rabbit secondary antibodies conjugated with either AMCA or Alexa 350 fluorochromes. Monolayers were routinely permeabilized with bovine serum albumin-PBS solution containing a final concentration of 0.1% saponin (BSP). However, the preferred permeabilization method for monolayers to be incubated either with anti-cathepsin D or with anti-LAMP-1 was treatment with ice-cold methanol for 1 min, followed by multiple washes with ice-cold PBS. Incubations with primary and secondary antibodies (at concentrations indicated above) were at room temperature on a shaking platform for 1 h, at which time coverslips were washed three times with cold BSP and affixed to a glass slide with Pro-long or Slow-fade mounting solutions. Immunofluorescence microscopy was performed with either an Olympus BTX upright microscope equipped with DAPI (4′,6′-diamidino-2-phenylindole), eGFP, and Texas Red filter sets, with a cooled charge-coupled device image sensor and MetaView software (Fig. 2 through 5) or a Bio-Rad Radiance 2000 inverted scanning laser confocal microscope equipped with Ar/HeNe/Red Diode lasers and AGR-3 filter configuration (Bio-Rad Lasersharp software) (Fig. 6 through 11). Postacquisition image processing was performed with ImageJ v1.33g (http://rsb.info.nih.gov/ij/index.html).

RESULTS

Human THP-1 monocytes accurately model chronic *B. abortus* intracellular infection. The infection protocol employed for these experiments was designed to follow the maturation of newly formed *Brucella* containing phagosomes into vesicles harboring replicating bacteria. Through opsonization, a relatively low multiplicity of infection of 20 bacteria per monocyte can be used, which allows for a sufficient number of monocytes to be infected with a single bacterium, though conditions are needed to study the trafficking of phagosomes containing individual bacteria. Synchronization of bacterial uptake was achieved by low-speed centrifugation at 4°C, followed by rapid warming to 37°C for 20 min to allow for phagocytosis to be completed. Using this protocol with an multiplicity of infection of 20:1, approximately 10% of the monocytes were infected, of which there were typically between 1 and 10 bacteria per macrophage (data not shown).

The intracellular survival and replication profile exhibited by

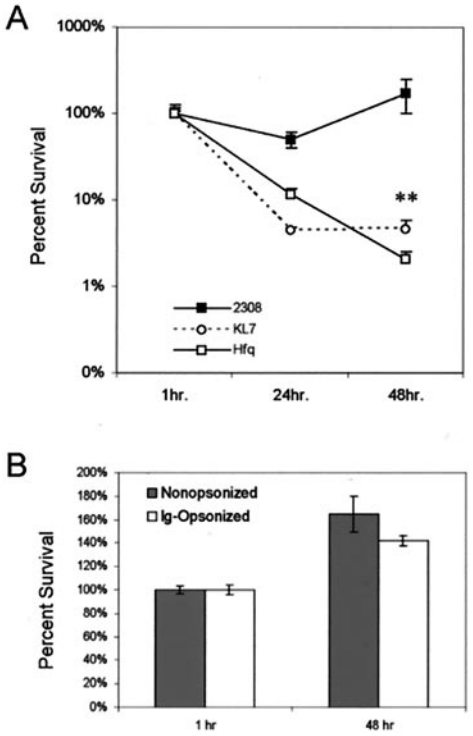

FIG. 1. Survival and replication of *B. abortus* 2308 (■), KL7 (2308 *bacA*) (○), and Hfq3 (2308 *hfq*) (□) in THP-1 cells. (A) PMA-differentiated THP-1 cells were infected with IgG-opsonized *B. abortus* and incubated for the indicated times whereby monocytes were lysed and intracellular bacteria were enumerated by growth on BA plates following serial dilution. Results are presented as percent survival, which was calculated by dividing the number of intracellular brucellae present at the times indicated postinfection by the number of CFU at 1 h postinfection and multiplying by 100. The number of intracellular brucellae present at each time point represents the average of three wells of THP-1 cells infected with each individual strain. Results presented are of a single representative experiment chosen from three independent experiments. (B) Similar experiments were performed by infecting THP-1 cells with either Ig-opsonized or nonopsonized *B. abortus* 2308. Ig-opsonized bacteria were internalized at a level 10 times that observed for nonopsonized bacteria, and the difference in uptake was accounted for by calculating percent survival. Statistical significance was calculated using Student's *t* test analysis (**, $P \leq 0.001$).

B. abortus 2308 in THP-1 cells under these experimental conditions resembles that displayed by this strain when it is introduced into cultured murine macrophages after opsonization with IgG (3, 12, 16). Specifically, the number of viable intracellular *B. abortus* 2308 cells decreased between 1 and 24 h postinfection in the THP-1 cells, but net intracelluar replication of strain 2308 was observed in these phagocytes between 24 and 48 h postinfection (Fig. 1A). Likewise, *B. abortus* Hfq3 (2308 *hfq*) and KL7 (2308 *bacA*) displayed significant attenuation in the THP-1 cultures compared to the parental 2308 strain (Fig. 1A), which is consistent with the attenuation displayed by the *B. abortus hfq* and *bacA* mutants in cultured murine macrophages and experimentally infected mice (20, 31). Genetic complementation of the *hfq* mutation in *B. abortus* Hfq3 with a plasmid-borne copy of *hfq* (31) and directed reversion of the *bacA* mutation in *B. abortus* KL7 through reconstruction of the *bacA* locus (20) restored virulence of these mutants in THP-1 cells (data not shown). Similar viability experiments performed with the parental *B. abortus* 2308 strain compared the intracellular survival of Ig opsonized or nonopsonized in THP-1 monocytes. The multiplicity of infection of 20:1 was used for both opsonized and nonopsonized and the same method described above for infection monocytes was employed for these experiments. As expected, the number of bacteria recovered at 1 h postinfection was a log higher for the opsonized bacteria than that for nonopsonized (nonopsonized, 2.8 ± 0.10 $\log_{10}$ CFU; opsonized, 4.2 ± 0.17 $\log_{10}$ CFU). Percent survival calculations account for the differences in internalization between these two groups, and the results show that both opsonized and nonopsonized bacteria are equally capable of replicating within differentiated THP-1 monocytes (Fig. 1B).

Nascent *Brucella* phagosomes communicate with early and late endosomes. Previous reports have shown that murine monocytes internalize nonopsonized *Brucella* into phagosomes that communicate with the early endosomes, but not late endosomes/lysosomes (1, 5, 30). To determine if this is also the case for opsonized *Brucella* internalized by human monocytes, fluorescence microscopy was used to examine the acquisition of various endosomal and phagosomal markers by phagosomes containing either live *B. abortus* 2308, Hfq3, or KL7; heat-killed *B. abortus* 2308; live *E. coli* HB101; or 0.8-μm latex beads. Phagosomes were scored for the presence of these vesicular markers on a per-phagosome basis; thus, a vesicle containing several bacteria would count as one phagosome. For early time points at >1 h, adherent extracellular bacteria were excluded from analysis by performing differential staining that fluorescently labels extracellular bacteria (see Materials and Methods).

(i) Interactions of *Brucella* containing phagosomes with the early-endosomal compartment. Newly formed phagosomes containing live *B. abortus* or *E. coli* strains, heat-killed *B. abortus* 2308 (Fig. 2), or latex beads (data not shown) rapidly acquired the early-endocytic marker EEA1. EEA1 colocalization was transient and levels dropped rapidly after peaking at 20 min. Additional early-endosomal markers, Rab5 and TfR also transiently associated with phagosomes containing live *B. abortus* or *E. coli* strains, heat-killed *B. abortus* 2308 or latex beads at similar rates to that observed for EEA1 (data not shown). These experimental findings are consistent with earlier reports stating that nascent *Brucella* phagosomes communicate with early endosomes (1, 5, 30).

(ii) Interactions of *Brucella* containing phagosomes with the late-endosomal compartment. Maturing phagosomes eventually replace early-endocytic components on the vesicle membrane with those associated with late endosomes and lysosomes such as the mannose-6-phosphate receptor, LAMP-1, the vacuolar ATPase complex, and Rab7 (reviewed in reference 38). This transition is critical not only for general phagosome trafficking but it also represents a key juncture in the development of the replicative niche of the intracellular brucellae since acidification of this compartment during the early stages of infection appears to be necessary for the intracellular replication of these bacteria (18, 28). At 40 min postinfection, phagosomes containing either live *B. abortus* 2308 or *E. coli* HB101 or heat-killed *B. abortus* 2308 were found to be heavily enriched with LAMP-1 (Fig. 3). Maximal levels of LAMP-1

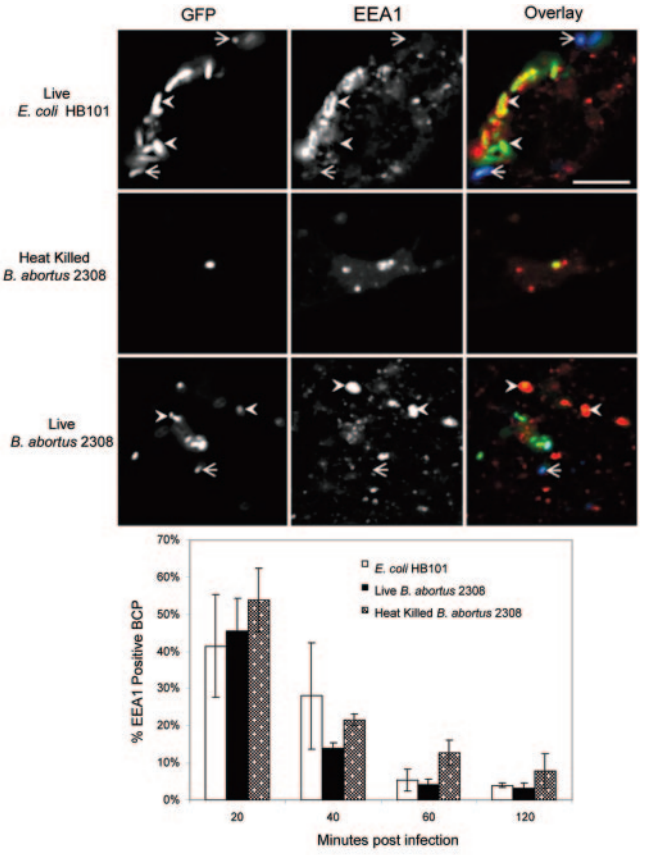

FIG. 2. Transient association of *Brucella*-containing phagosomes (BCP) with the early-endosomal marker EEA1. Monocytes were infected with opsonized bacteria, fixed at times indicated, and scored for the percent localization of EEA1 antigen on BCP following immunofluorescence staining. Representative images of BCP from monocytes infected with either control particles (heat-killed *B. abortus* or *E. coli* HB101) or virulent live *B. abortus* cells are shown from the 20-min time point coinciding with the highest level of EEA1 colocalization. Identical magnification of images was used to illustrate the size difference between *Brucella* and *E. coli* (bar, 10 μ). Intracellular bacteria (solid arrow, only green fluorescence) were differentiated from extracellular bacteria (line arrow, green and blue fluorescence) by differential antibody staining (see Materials and Methods). Percent colocalization for EEA1 was performed by scoring BCP for the presence or absence of EEA1, and results shown represent the averages and standard deviations from three independent experiments. All three BCP transiently associated with EEA1 soon after internalization, followed by rapid loss of this marker. No statistical differences were detected among the different BCP. The kinetics for the acquisition and loss of endocytic markers Rab5 and transferrin receptor followed the same transient pattern described here for EEA1 (data not shown).

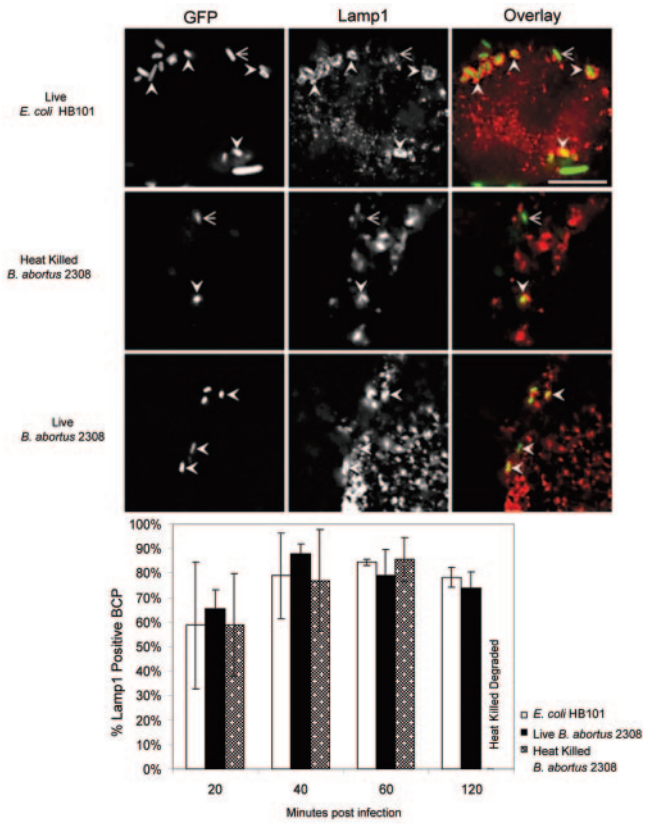

FIG. 3. Rapid acquisition of phagosomal marker LAMP-1. Monocytes were infected with opsonized bacteria, and at times indicated in the graph, BCP were scored for the presence (indicated by solid arrow) or absence (indicated by line arrow) of LAMP-1. Representative images are shown at 60 min postinfection coinciding with peak LAMP-1 acquisition for all BCP types (bar, 10 μm). A significant reduction in detectable GFP for heat-killed BCP occurred at 2 h due to the fixation process (methanol) needed for optimal anti-LAMP-1 antibody staining. As a result, the numbers of detectable heat-killed *Brucella* cells visualized at 2 h were significantly lower than those for live virulent *Brucella* and *E. coli* and thus, colocalization percentages were not calculated for heat-killed bacteria at 2 h. Percent colocalization and standard deviations were calculated using results from three independent experiments. No statistical differences were detected among the different BCP types at any time point.

colocalization for all of these phagosomes were observed by 60 min postinfection. In addition to the arrival of LAMP-1, components of the proton pump ATPase were also delivered to these phagosomes, causing the rapid acidification of the lumen of these vesicles. Vesicle acidification was detected using the acidotropic dye Lysotracker (Molecular Probes), which is retained in vesicles with a pH of ≤5.5 (Fig. 4). Addition of the proton-pump-specific inhibitor bafilomycin A abolished all Lysotracker staining of the *B. abortus*- and *E. coli*-containing phagosomes, demonstrating that the observed vesicle acidity was the direct result of an active proton pump complex present in the phagosomal membrane (data not shown). These experimental findings indicate that phagosomes containing *B. abortus* 2308 do not deviate from the normal early-phagosomal trafficking pathway in THP-1 cells.

Interactions of *Brucella*-containing phagosomes with lysosomes. Fusion of phagosomes with lysosomes represents the end point of phagosome maturation. Experiments performed with nonopsonized *Brucella* in murine monocytes demonstrated that phagosomes containing virulent *Brucella* do not fuse with lysosomes. This altered trafficking is detected as early as 1 h postinfection for live and heat-killed *B. suis* in the murine macrophage-like cell line J774.A1 (29), while Celli et al. reported cathepsin D levels below 40% throughout the first 24 h postinfection for phagosomes containing *B. abortus* 2308 in cultured bone marrow-derived macrophages from C57BL6 mice (5). In contrast, we did not see a statistically significant difference between the number of phagosomes containing live

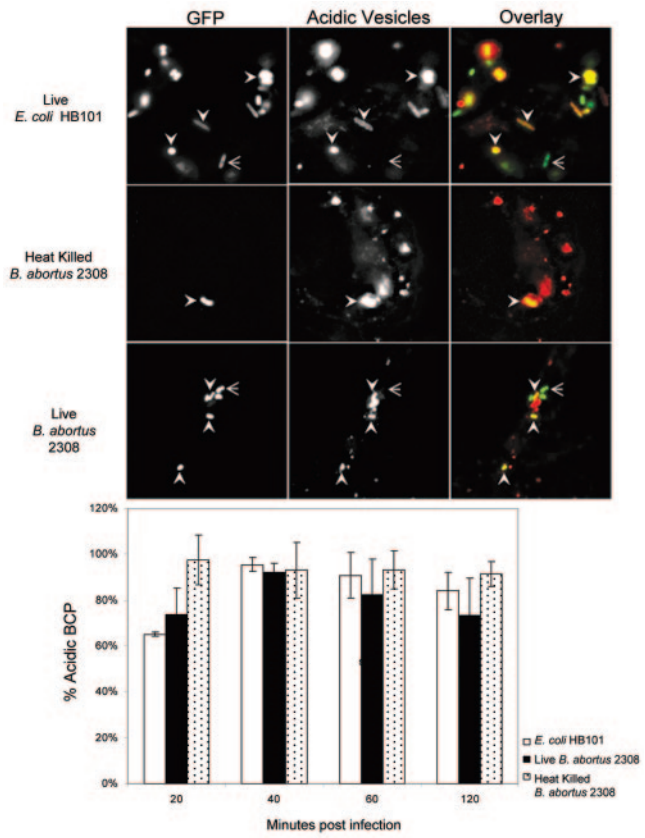

FIG. 4. Early *B. abortus*-containing phagosomes are acidic. Monocytes were infected with bacteria for the times indicated in the graph and subsequently treated with the acidotropic dye Lysotracker Red prior to fixation. GFP-positive phagosomes were then scored on the basis of accumulation of the red acidotropic dye to calculate percent acidic BCP (positive, solid arrow; negative, line arrow). Visualization of acidotropic dye does not require methanol treatment that reduced the detection of dead *Brucella* GFP; therefore, adequate amounts of dead bacteria were able to be visualized at the 2-h time point, unlike samples analyzed for LAMP-1 or cathepsin D colocalization. Representative images are shown from samples collected at the 60-min time point. Colocalization percentages and standard deviations were calculated using results from three independent experiments. No statistical differences were detected among the different BCP types at any time point.

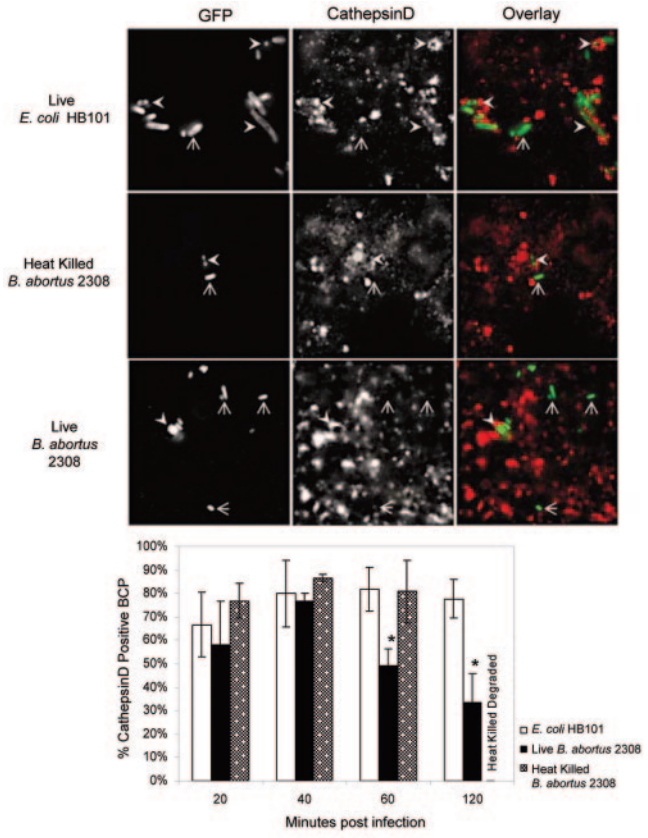

FIG. 5. Early but not late *B. abortus*-containing phagosomes associate with lysosomes. Monocytes were incubated at the indicated times following infection with either *E. coli*, dead *B. abortus*, or live *B. abortus* bacteria and then fixed with 4% paraformaldehyde for 10 min and permeabilized with ice-cold methanol for 1 min. After washing with cold PBS, monolayers were incubated with mouse anti-cathepsin D in BSP, followed with secondary anti-mouse Cy3-conjugated antibodies (red). Representative images shown are from 60 min postinfection. BCP were scored for the presence (solid arrows) or absence (line arrows) of cathepsin D to calculate percent colocalization. Results from three independent experiments were used to calculate the average and standard deviation for each time point. Statistical comparisons of live *B. abortus* to *E. coli* (60 and 120 min postinfection) and dead *B. abortus* (60 min postinfection) were significant by two-tailed Student's *t* test analysis (*, $P \leq 0.05$).

or heat-killed *B. abortus* 2308 or live *E. coli* HB101 that stained cathepsin D positive in THP-1 cells until 60 min postinfection, when these bacteria were introduced by the opsonic route (Fig. 5). The kinetics of cathepsin D delivery to these phagosomes coincided with the arrival of LAMP-1 (Fig. 3) and acidification of the lumen of the phagosome (Fig. 4). In noninfected cells, cathepsin D-positive lysosomes were always found to be acidic and LAMP-1 positive (data not shown). Thus, a significant number of phagosomes containing live *B. abortus* 2308 matured into phagolysosomes in the THP-1 cells that were indistinguishable from those containing heat-killed *B. abortus* 2308 or live *E. coli* HB101 phagosomes at 20 and 40 min postinfection. As time progressed, however, the percentage of cathepsin D-positive phagosomes containing live *B. abortus* 2308 phagosomes began to decrease, although the levels of LAMP-1-positive and acidic vesicles containing live *B. abortus* 2308 remained elevated (Fig. 3 and 4).

***B. abortus* 2308 replicates in LAMP-1-positive, deacidified vesicles in THP-1 cells.** As shown in Fig. 1, the majority of the brucellacidal activity occurs within the initial 24 h postinfection and net intracellular replication of the brucellae is observed between 24 and 48 h postinfection. Accordingly, microscopic analysis of infected THP-1 cells revealed that after 24 h, the number of phagosomes containing multiple brucellae increases, as does the total number of bacteria observed within individual phagosomes as the infection progresses. The increase in the number of bacteria within these vesicles was attributed to bacterial replication, and vesicles harboring these replicating bacteria were deemed replicative vesicles. Interestingly, not all of the intracellular brucellae observed in the THP-1 cells at 24 or 48 h postinfection were found in these

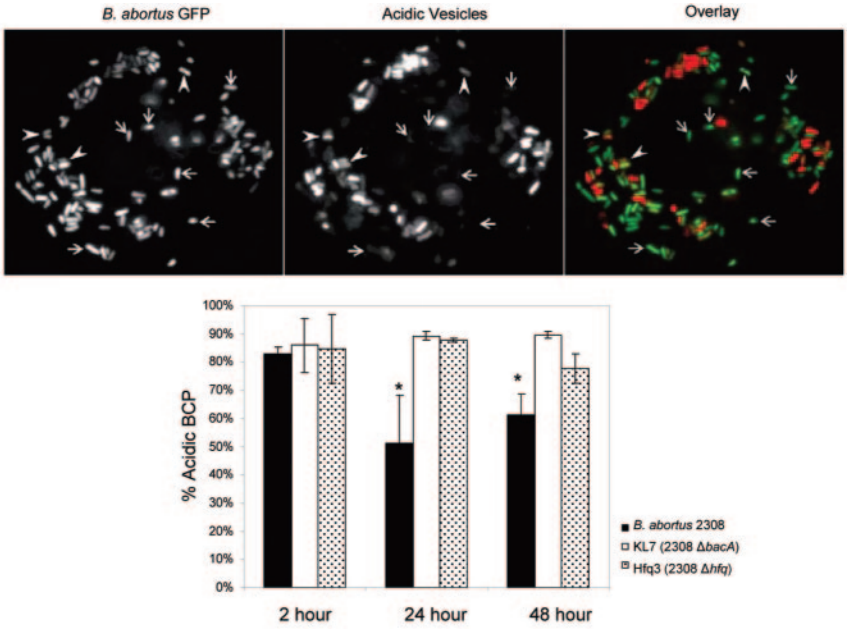

FIG. 6. *B. abortus* 2308 resides predominately in nonacidic compartments at 24 and 48 h postinfection in THP-1 cells, while isogenic *bacA* and *hfq* mutants remain largely confined to acidified phagosomes within these phagocytes. Only phagosomes containing single bacteria (BCP) were scored for the presence of the acidic marker (see text). Illustrated are panels from a representative macrophage scored for acidic (solid arrows) and nonacidic (line arrows) *B. abortus* 2308-containing phagosomes at 24 h postinfection. The results presented are averages from multiple independent experiments for each stain. *, $P \leq 0.05$ for comparisons of phagosomes containing *B. abortus* 2308 with those containing *B. abortus* KL7 or Hfq3 using the Student's *t* test.

replicative vesicles. In fact, many phagosomes containing a single bacterium were seen late in infection in THP-1 cells that also harbored large replicative vesicles containing many brucellae (Fig. 6, 7, and 9).

We suspected that the transition of brucellae from a nonreplicating physiological state into a replication competent state corresponded with a change in the nature of the intraphagosomal environment. To examine this possibility, phagosomes containing live *B. abortus* 2308 were characterized at various intervals between 2 and 48 h postinfection in the THP-1 cells.

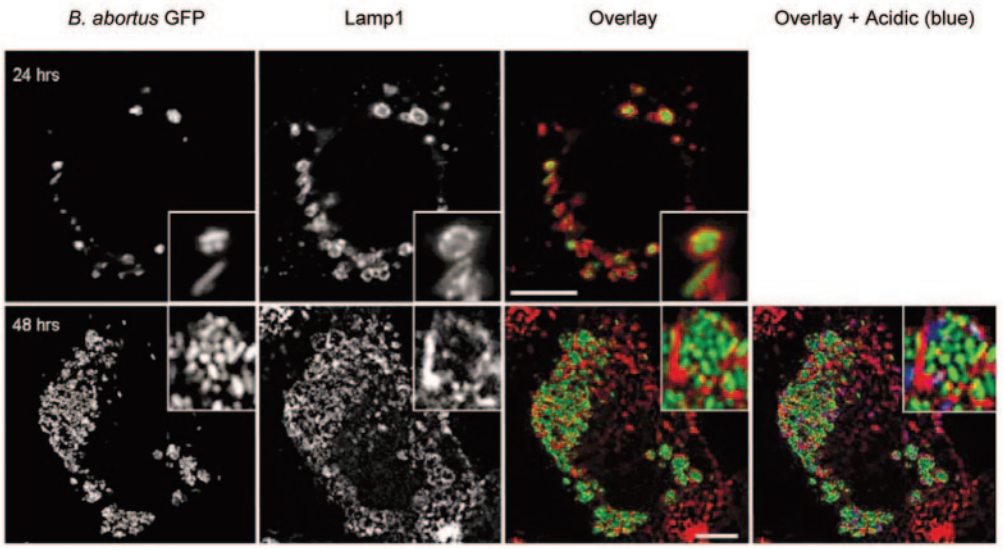

FIG. 7. Replication of *B. abortus* 2308 within LAMP-1-positive vesicles that are largely in THP-1 cells at 48 h postinfection. Representative microscopic images of the infected THP-1 monolayers are shown during the replicative phase of the intracellular life cycle of *B. abortus* 2308 in these phagocytes. Acidified vesicles (blue) were labeled prior to fixation by treating cells with Lysotracker (Molecular Probes). Images were collected using a Bio-Rad 2000 scanning laser microscope. LAMP-1 primary antibodies were visualized using Cy5-conjugated anti-mouse secondary antibody (red fluorescence). Bar, 10 μm.

During this period, the phagosomes containing *B. abortus* 2308 displayed the same colocalization patterns as BCPs observed at 2 h postinfection (LAMP-1$^+$ and cathepsin D$^-$). For example, the *B. abortus* 2308-containing phagosomes were largely LAMP-1 positive (80.0% ± 3.6% positive) and cathepsin D negative (46.3% ± 16%) at 24 h postinfection in these phagocytes. This pattern of high LAMP-1 (Fig. 7 and 9) and low cathepsin D on *Brucella* phagosomes remained constant for the remainder of the experiment and was also detected surrounding large vesicles that contained numerous densely packed, replicating brucellae.

Despite the constant colocalization of LAMP-1 on phagosomes harboring *B. abortus* 2308, microscopic analysis of these vesicles at 24 (Fig. 6) and 48 (Fig. 7) h postinfection indicated that these replicative vesicles were not highly acidic, as they did not stain with the fluorescent acidotropic marker. A more detailed analysis focused on phagosomes that contained only single bacteria in the THP-1 cells, since those that harbor multiple bacteria were assumed to have already undergone the transition into a replication-permissive vesicle and to have become nonacidic. Between 2 and 24 h postinfection, the number of single *B. abortus* 2308 cells residing in acidified (e.g., Lysotracker-positive) phagosomes decreased from 83.1% ± 2% at 2 h to 51% ± 17% by 24 h (Fig. 6). These results demonstrate a positive correlation between the deacidification of single *Brucella*-containing phagosomes with the ability of *Brucella* to form large replicative phagosomes.

Attenuated *B. abortus* mutants Hfq3 (2308 *hfq*) and KL7 (2308 *bacA*) remain confined to acidic phagosomes. Unlike *B. abortus* 2308, the isogenic *hfq* mutant Hfq3 and *bacA* mutant KL7 do not exhibit net intracellular replication in THP-1 cells (Fig. 1). Microscopic analysis of infected THP-1 monolayers also showed that in contrast to their parent strain, *B. abortus* Hfq3 and KL7 remain largely confined to acidic phagosomes for the duration of their intracellular residence in these phagocytes (Fig. 6, graph). These experimental findings are particularly interesting given the fact that the colocalization patterns of Hfq3- and KL7-containing phagosomes with EEA1-, LAMP-1-, and cathepsin D-specific markers were nearly identical to those displayed by *B. abortus* 2308-containing phagosomes in THP-1 cells at 20 min, 1, 2, 24, 48, and 72 h postinfection. Also, Hfq3- and KL7-containing phagosomes acidified at the same rate in these phagocytes as those containing *B. abortus* 2308 (data not shown). As shown in Fig. 3 and 5, methanol treatment of infected monolayers for fixation decreases the GFP signal detected for heat-killed *B. abortus* 2308 cells due to the delivery of these BCP to the degradative phagolysosomal compartment. The persistence of fluorescent *B. abortus* Hfq and KL7 in acidic phagosomes in THP-1 cells (Fig. 6) strongly suggests that the *B. abortus* *hfq* and *bacA* mutants are able to maintain viability, but are unable to initiate replication, in these intracellular compartments.

Ig-opsonized *B. abortus* 2308 cells do not colocalize with the ER in THP-1 cells. Markers specific for the membrane of the ER (calnexin, Sec61β, and SRP54) and the ER lumen (Bip/GRP74), as well as the ER-tropic fluorescent dye $DIOC_6$, were used in microscopic analysis to examine the *B. abortus* 2308-containing phagosomes for evidence of interaction with the ER in THP-1 cells. Throughout the course of infection of these phagocytes, the *B. abortus* 2308-containing phagosomes (including the large replicative vesicles containing multiple bacteria) either were found to be devoid of ER markers entirely or appeared to be located in closed proximity to, but physically separated from, ER-positive structures (Fig. 8 and 9). Specifically, close microscopic inspection of these areas of apparent ER association revealed that the ER-specific markers were more distant from the intracellular brucellae than LAMP-1-specific markers; moreover, the ER markers were contiguous with ER structures in the THP-1 cells and not wholly integrated with the *B. abortus* 2308-containing phagosomes. It is also noted that latex beads and the attenuated *B. abortus hfq* and *bacA* mutants exhibited this same pattern of limited association with the ER in THP-1 cells at 48 h postinfection (Fig. 10). In contrast, THP-1 cells infected with nonopsonized bacteria were frequently found to harbor replicating *Brucella* within ER-positive compartments (Fig. 11). The distinct ER-positive *Brucella*-containing vesicles were found only within the monolayers infected with nonopsonized bacteria, although some nonopsonized *Brucella* cells were also negative for ER markers.

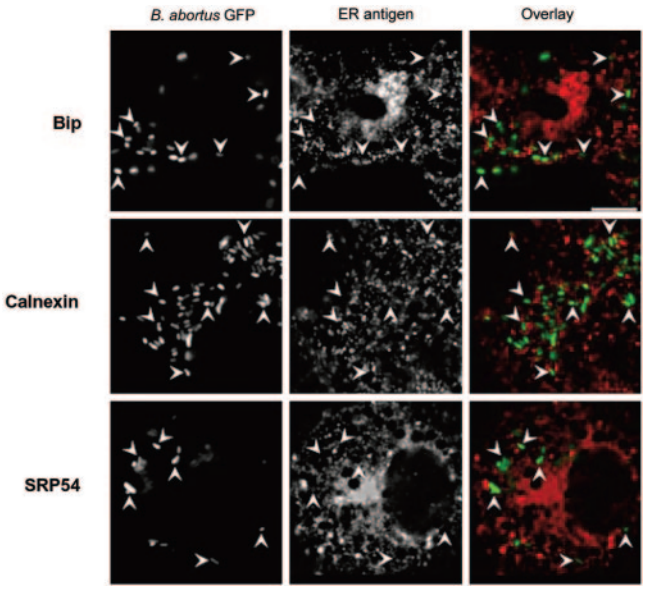

FIG. 8. Proximal staining of ER markers and *Brucella*-containing phagosomes. Monocytes were fixed 2 h postinfection and stained for ER-specific antigens Bip, SRP54, and calnexin (red). Although few *Brucella* vesicles were seen positively colocalized with the host ER, the majority of *Brucella*-containing phagosomes were observed adjacent to areas of intense ER staining that was characterized as "proximal ER staining" and not positive colocalization of *Brucella* within the host ER network. Similar staining patterns, for both positive and proximal ER staining, were observed for latex beads, heat-killed *Brucella*, and avirulent *E. coli* (data not shown). Arrows note the position of *Brucella*-containing phagosomes to aid in comparing their location to ER markers. Bar, 5 μm.

DISCUSSION

These experiments were undertaken to the effect that antibody opsonization had on the ability of *Brucella abortus* 2308. Intramacrophage survival is the cornerstone of *Brucella* pathogenesis and affords the brucellae the ability to cause chronic

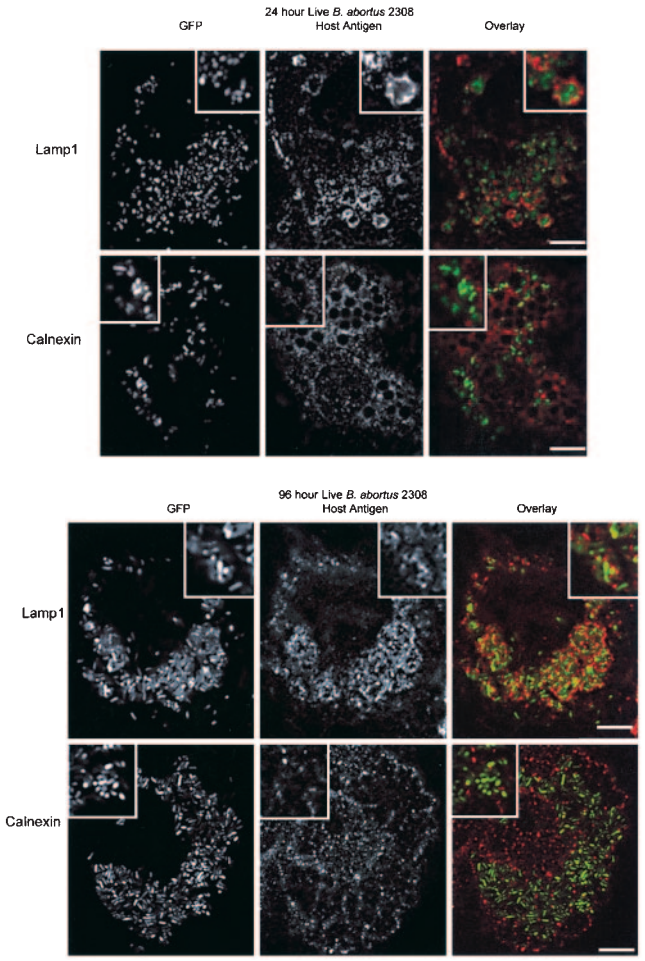

FIG. 9. Comparison of LAMP-1 and ER colocalization with *Brucella*-containing phagosomes in THP-1 monocytes at 24 and 96 h postinfection. Antibody-opsonized *B. abortus* 2308 cells (GFP) were used to infect monolayers for 24 and 96 h, fixed, and then stained for either LAMP-1 or calnexin host antigen (red). Phagosomes containing either single or multiple *Brucella* cells colocalized extensively with LAMP-1 at both 24 and 96 h and were identical to those obtained earlier with monolayers infected at 48 and 72 h postinfection with live *B. abortus* (Fig. 7). The tight and continuous opposition of LAMP-1 to intracellular *Brucella* is in contrast to the punctuated and more proximal staining pattern observed for the ER marker calnexin. A typical cluster from each micrograph was magnified to further illustrate the differences in staining patterns between LAMP-1 and calnexin markers (panel inserts). Staining for other ER-specific antigens (Bip, Sec61β, and SRP54) revealed similar patterns of ER proximal to *Brucella* phagosomes as well as latex beads (not shown). Bar, 10 μm.

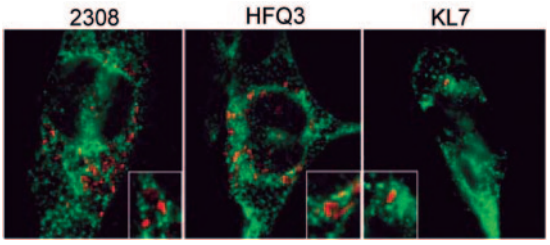

FIG. 10. Association of late-infection *Brucella*-containing phagosomes with the endoplasmic reticulum of THP-1 cells. THP-1 cells infected with *B. abortus* 2308, Hfq3 (2308 *hfq*), or KL7 (2308 *bacA*) were fixed at 48 h postinfection and stained for host ER elements using anti-Bip-specific antibody. Grayscale images were pseudocolored to make the GFP-labeled brucellae appear red, and Cy3-labeled Bip appears green. Consistent with the results obtained from brucellacidal assays employing these phagocytes (Fig. 1), considerably more brucellae were seen in THP-1 cell monolayers infected with virulent *B. abortus* 2308 at 48 h postinfection than were seen in THP-1 cells infected with *B. abortus* Hfq3 or KL7. A monocyte with a moderate bacterial burden of *B. abortus* 2308 was shown for a more accurate comparison to ER staining patterns observed for the attenuated mutants. No differences were detected, however, between these interactions of phagosomes containing these strains with the ER of THP-1 cells. Clear colocalization of ER markers with *Brucella*-containing vesicles was visible for some vesicles (filled arrows); however, the majority of the *Brucella*-containing phagosomes appear to be in close proximity to, but not contiguous with, the ER of THP-1 cells (line arrows) (see text). Arrows note the positions of *Brucella*-containing phagosomes to aid in comparing their location to ER markers. Bar within large panel, 10 μm; bar within panel insert, 1 μm.

disease in a wide range of host animals (32). Brucellosis in humans presents a chronic debilitating disease known as undulant or Malta fever (41). *Brucella*-specific immunoglobulin is produced during infection; however, these antibodies appear to promote infection by giving the noninvasive bacteria a rapid means to enter new monocytes and macrophages (1, 14, 15, 42) Experimental evidence suggests that survival of the brucellae within the host cell is achieved by bacterial interference of intracellular trafficking events whereby phagosomes containing *Brucella* avoid fusion with lysosomes (9, 25, 27). In doing so, this highly adapted intracellular pathogen is not exposed to toxic hydrolases, lipases, and defensins and is allowed to survive and replicate within phagocytes. The *virB* operon encoding the type IV secretion machinery is necessary for the development of the replicative phagosome within which the brucellae reside in cultured murine macrophages (5), HeLa cells (7), and the human monocytic cell line THP-1 (4) after nonopsonic entry into these cells. The effector molecule or molecules transported by the type IV secretion apparatus and how they influence the trafficking patterns of *Brucella*-containing vacuoles in these host cells, however, is presently unknown.

The binding of immunoglobulin-opsonized bacteria to the Fc receptor complex on host monocytes and macrophages results in rapid phagocytosis (8). Once the bacteria are engulfed via this route, plasma membrane components are rapidly removed from the nascent phagosomes and these vesicles acquire membrane components associated with early endosomes (24). Thus, it was not surprising to find that virulent *B. abortus* 2308 cells internalized through IgG-mediated phagocytosis were found within phagosomes that communicated with the early-endocytic compartment in THP-1 cells (Fig. 12). Newly formed *B. abortus* 2308-containing phagosomes transiently associated with the early-endosomal marker EEA1 soon after internalization by the THP-1 cells. The rates of association and intensity of phagosome staining for EEA1 were equivalent for live or heat-killed *B. abortus* 2308 and live *E. coli* HB101 cells. The association of the *B. abortus* 2308- and *E. coli* HB101-containing phagosomes with the early-endosomal compartment in the THP-1 cells was further supported by the observation that these phagosomes also exhibit transient colocalization with the transferrin receptor and the endosomal

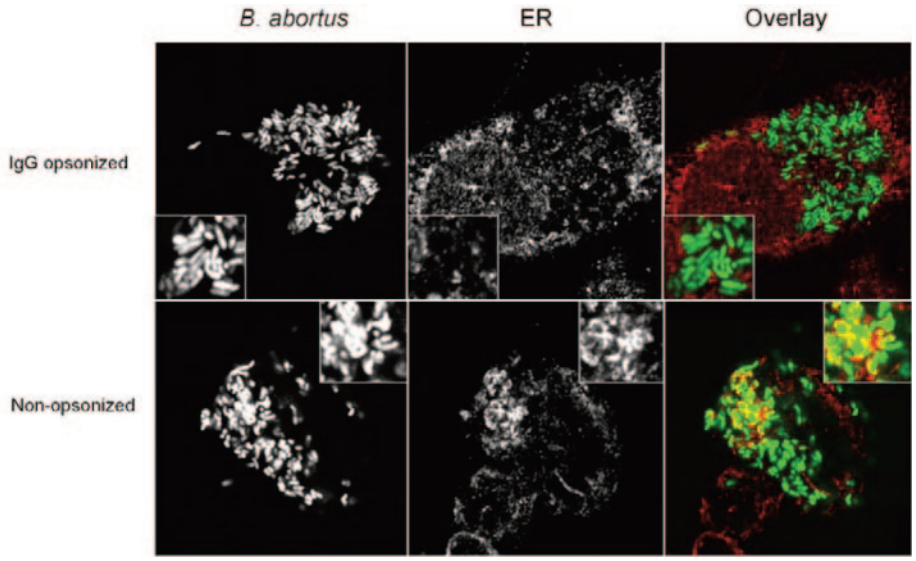

FIG. 11. Localization of nonopsonized *B. abortus* 2308 with the endoplasmic reticulum. THP-1-differentiated monocytes were infected with either Ig-opsonized or nonopsonized *B. abortus*. At 76 h postinfection, the monolayers were fixed and stained for the endoplasmic reticulum using anti-calnexin antibodies followed with Cy3-conjugated secondary antibodies (red). In the representative images shown, the Ig-opsonized bacteria were primarily found in vesicles that did not colocalize with the ER marker. In contrast, monocytes infected with nonopsonized *B. abortus* did contain an appreciable number of *Brucella* cells colocalizing with the ER marker. Although not all of the vesicles containing nonopsonized bacteria were positive for the ER, the presence of any distinct ER colocalization was absent from monocytes infected with opsonized bacteria.

membrane-trafficking protein Rab5 during analysis by immunofluorescence microscopy (data not shown).

Following the loss of endosomal components, phagosomes containing either live or dead *B. abortus* 2308 or live *E. coli* HB101 simultaneously acquired the late-endosomal/lysosomal marker LAMP-1 and were acidified by the delivery of the proton pump (V^+ATPase), marking the successful transition of these vesicles into the late-endosomal compartment. The majority of these phagosomes also appeared to be destined to form phagolysosomes, as indicated by their early acquisition of the lysosomal hydrolase cathepsin D. As late as 40 min postinfection, most of the phagosomes containing live virulent *B. abortus* 2308 had fused with lysosomes. Following the 40-min time point, however, the percentage of cathepsin D-positive phagosomes containing *B. abortus* 2308 decreased significantly at 60 min and was further reduced by 120 min postinfection. No such reduction in cathepsin D levels was observed for phagosomes containing either heat-killed *B. abortus* 2308 or live *E. coli* HB101. There are two possible explanations for these results. Either the cathepsin D is lost from the phagosomes containing the live *B. abortus* 2308 during the period between 40 and 60 min postinfection in the THP-1 cells or a significant number of the *B. abortus* 2308 cells residing in the cathepsin D-positive phagosomes were killed and subsequently degraded, resulting in an enrichment for the *B. abortus* 2308-containing phagosomes that were cathepsin D negative at earlier times. The second scenario appears more plausible since the selective removal or destruction of cathepsin D within these vesicles would appear mechanically difficult to perform while retaining other late-endosomal/lysosomal characteristics (e.g., remaining LAMP-1 positive).

Another interesting and potentially informative observation was that there appears to be a distinct temporal correlation between the deacidification of LAMP-1-positive, cathepsin D-negative phagosomes containing single bacteria and the onset of net intracellular *Brucella* replication. Others have proposed that a rise in intraphagosomal pH beyond a threshold level is necessary before the brucellae can initiate replication in these intracellular compartments (17). Such a requirement is also consistent with the fact that although *Brucella* strains are generally quite resistant to in vitro exposure to acidic conditions down to pH values of 4 and below (11, 19, 31), these strains are capable of replicating when cultured at pH 5 and above. The mechanism underlying the rise in intraphagosomal pH in the *B. abortus* 2308-containing phagosomes is unknown, but it is apparently dependent upon the viability of the intracellular brucellae, since a corresponding rise in intravesicular pH was not observed for the phagosomes containing individual heat-killed *B. abortus* 2308 cells.

Although the brucellae end up residing in an intracellular compartment in host macrophages in which they can survive and replicate, there is considerable evidence that they encounter a variety of environmental stresses during their intracellular residence, including exposure to reactive oxygen intermediates, acidic pH, and nutrient deprivation (17, 32). Genetic evidence suggests that the product of the *hfq* gene (an RNA binding protein known as host factor I, or HF-I) is required for efficient stationary-phase gene expression in *B. abortus* 2308. Moreover, the generalized increase in resistance to environmental stresses that accompanies the transition into stationary phase appears to be critical for allowing the brucellae to appropriately adapt to the environmental conditions encountered in the phagosomal compartments of host macrophages. The phenotype displayed by the *B. abortus hfq* mutant Hfq3 in THP-1 cells is consistent with the HF-I-dependent stationary-phase gene expression playing a role in successful adaptation to the environ-

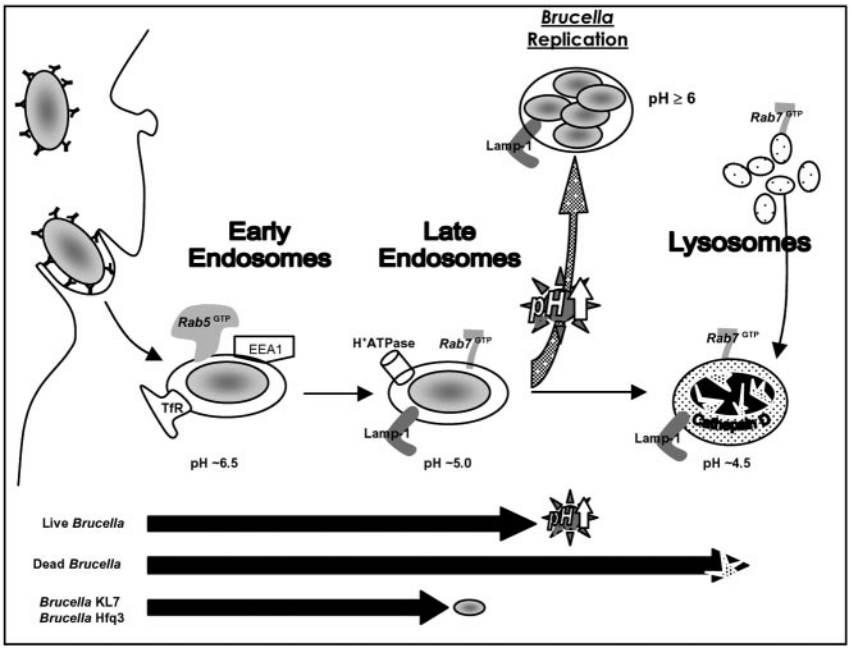

FIG. 12. Schematic representation of the intracellular trafficking patterns of IgG-opsonized *B. abortus* 2308, KL7 (2308 *bacA*), and Hfq3 (2308 *hfq*) in the human monocytic cell line THP-1. The black arrows at the bottom of the figure denote the extent to which phagosomes containing the different *B. abortus* strains progress down the endolysosomal pathway within these host cells. All *Brucella* cells were trafficked rapidly through the early- and late-endosomal compartments regardless of viability or virulence status. Progression of phagosomes beyond the late-endosomal compartment (LAMP-1$^+$ and acidic) correlated with the viability/virulence status of the bacteria used for infection (see Discussion). Remaining viable *Brucella* cells were found within phagosomes that were no longer acidic (pH $\geq$ 6) while remaining LAMP-1 positive and cathepsin D negative. Replicating *Brucella* cells were also observed within this same nonacidic compartment, demonstrating a correlation between the rise in intraphagosome replication and the shift in bacterial physiology from survival to intracellular replication (speckled arrow). By contrast, phagosomes containing attenuated *Brucella* Hfq3 and KL7 were unable to progress to either fuse with lysosomes or take on the characteristics of a replicative vesicle while phagosomes containing heat-killed *Brucella* proceeded to fuse with lysosomes unabated at the same rates as phagosomes containing avirulent *E. coli* and latex beads.

mental conditions encountered in the replicative phagosome, since this mutant is trafficked to the same acidic, LAMP-1-positive, cathepsin D-negative intracellular compartment in these phagocytes as the parental 2308 strain but does not replicate in these phagosomes.

Although the precise function of the *bacA* gene product in *B. abortus* 2308 remains unresolved, genetic evidence suggests that it plays a role in modifying the fatty acid composition of the lipid A moiety of the lipopolysaccharide in this bacterium (13). The *B. abortus bacA* mutant KL7 also ends up in acidic, LAMP-1-positive, cathepsin D-negative phagosomes in THP-1 cells when it enters these cells via the opsonic route, but like the *hfq* mutant, KL7 does not appear to be able to replicate within this intracellular compartment. The altered cell envelope of the *B. abortus bacA* mutant makes this strain more susceptible than its parental strain to a number of environmental stresses, including exposure to acidic pH and membrane-damaging agents such as deoxycholate and sodium dodecyl sulfate (33). Consequently, the intracellular behavior of the *B. abortus bacA* mutant in THP-1 cells suggests that the lipid A modifications made by BacA play a critical role in allowing the brucellae to resist the environmental conditions encountered in the replicative phagosome.

Studies with cultured murine macrophages and HeLa cells have shown that there is a clear correlation between entry into a replicative phagosome that is enriched in membrane components arising from the host cell ER and intracellular replication of virulent *Brucella* strains in these host cells (5–7, 26). Thus, it was interesting to find that *B. abortus* 2308 demonstrated extensive intracellular replication in phagosomes that showed no clear association with ER components when introduced into THP-1 cells via IgG opsonization. It is quite possible that the route of entry into these host cells plays a major role in the divergent nature of these experimental findings. In the earlier studies showing the association between intracellular replication of the brucellae and entrance into the ER-derived replicative phagosome in murine macrophages and HeLa cells, the brucellae were not opsonized and likely entered these cells via their interactions with lipid rafts on the host cell surface (23, 29, 39, 40). Indeed, we observed a significant portion of nonopsonized *Brucella* in replicative compartments that were positive for ER constituents. It is also important to note that Rittig et al. reported that *B. suis*- and *B. melitensis*-containing phagosomes in cultured human peripheral blood monocytes were lacking ER components for both opsonized and nonopsonized brucellae (30). Nevertheless, the studies described here demonstrate that the primary intracellular niche for *B. abortus* internalized by opsonin-mediated phagocytosis is in phagosomes that are devoid of ER components. Although we predict that internalization by this method is the key contributing factor to the differences between our observations and those in other published reports, there is also

considerable likelihood that other variables in the host-pathogen interaction contribute to the outcome of *Brucella* infection. These contributing factors would include the type and activation status of the monocyte/macrophage population, differences in *Brucella* spp. pathogenesis, and types of bacterial internalization, as well as possible variation among host species. Further experiments are needed to determine if this intracellular niche reached by opsonized *Brucella* in the human THP-1 cell line is also observed within human peripheral blood monocytes or within murine monocyte/macrophage cell lines and primary monocytes. Preliminary results with human peripheral blood monocytes support our observations within THP-1 cells whereby opsonized *B. abortus* cells were found viable in single LAMP-1-positive, ER-negative vesicles at 36 h postinfection.

In summary, the results of the studies described in this report indicate that the membrane composition of the replicative phagosome within which the brucellae survive and replicate within host macrophages differs based upon their route of entry into these phagocytes. Moreover, they show that entrance into an ER-enriched intracellular compartment in these host cells is not an absolute requirement for intracellular survival and replication of the brucellae. These experimental findings have the most relevance with regard to the interactions of the brucellae with host macrophages during the period after the onset of a specific humoral immune response. Although strong IgG responses are induced during *Brucella* infections both in natural hosts and in humans, there does not appear to be a strong correlation between the responses and resolution of the infection (12, 15, 32). In fact, it has been postulated that *Brucella*-specific antibodies may actually help the brucellae gain entry into their intracellular niche in host macrophages (12, 14, 16, 42). The capacity of the brucellae to resist killing by host macrophages when taken up by IgG-mediated phagocytosis would certainly be predicted to help these bacteria maintain chronic infections in their hosts. The fact that the brucellae appear to be able to replicate within phagosomes that have progressed to different stages along the endocytic pathway provides further evidence of how well adapted the brucellae are for their intracellular lifestyle in the host.

ACKNOWLEDGMENTS

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