Pathogenic Brucellae Replicate in Human Trophoblasts

Suzana Pinto Salcedo,1,2,3,a Nicolas Chevrier,1,2,3,4 Thais Lourdes Santos Lacerda,1,2,3 Amira Ben Amara,5 Sarah Gerart,1,2,3 Vilma Arce Gorvel,1,2,3 Chantal de Chastellier,1,2,3 José Maria Blasco,6 Jean-Louis Mege,5 and Jean-Pierre Gorvel1,2,3

1Aix-Marseille Université UM 2, Centre d’Immunologie de Marseille-Luminy, 2Institut National de la Santé et de la Recherche Médicale U 1104, 3Centre National de la Recherche Scientifique UMR 7280, Marseille, France; 4Faculty of Arts and Sciences Center for Systems Biology, Harvard University, Cambridge, Massachusetts; 5Unité de Recherche sur les Maladies Infectieuses Tropicales et Emergentes, Aix-Marseille Université, Marseille, France; and 6Unidad de Sanidad Animal, Centro de Investigación y Tecnología Agroalimentaria de Aragón/Gobierno de Aragón, Zaragoza, Spain

(See the editorial commentary by O’Callaghan on pages 1034–6.)

Brucellae replicate in a vacuole derived from the endoplasmic reticulum (ER) in epithelial cells, macrophages, and dendritic cells. In animals, trophoblasts are also key cellular targets where brucellae efficiently replicate in association with the ER. Therefore, we investigated the ability of Brucella spp. to infect human trophoblasts using both immortalized and primary trophoblasts. Brucella extensively proliferated within different subpopulations of trophoblasts, suggesting that they constitute an important niche in cases where the fetal-maternal barrier is breached. In extravillous trophoblasts (EVTs), B. abortus and B. suis replicated within single-membrane acidic lysosomal membrane-associated protein 1–positive inclusions, whereas B. melitensis replicated in the ER-derived compartment. Furthermore, B. melitensis but not B. abortus nor B. suis interfered with the invasive capacity of EVT-like cells in vitro. Because EVT are essential for implantation during early stages of pregnancy, the nature of the replication niche may have a central role during Brucella-associated abortion in infected women.

Keywords. Brucella; trophoblasts; trafficking.
the presence of the lysosomal membrane–associated protein 1 (LAMP1). Acidification of the BCVs is essential [15] because it enables intracellular expression of the genes encoding the VirB type IV secretion system (TASS) [16]. Brucella then controls fusion with endoplasmic reticulum (ER) membranes in a VirB T4SS-dependent manner [17, 18]. Brucella replicates within ER-derived compartments in both phagocytes and nonprofessional phagocytes [13, 18, 19], including trophoblasts of infected animals [20–22].

To date no work has been done on human trophoblasts. Because epidemiological data from endemic regions suggests that Brucella can breach the fetal–maternal barrier, we investigated for the first time the ability of Brucella to survive and replicate within human trophoblasts.

**MATERIALS AND METHODS**

**Culture of Trophoblastic Cell Lines and Term Placenta Trophoblasts**

Culture conditions for the JEG-3 (American type culture collection clone HTB-36), BeWo (clone CCL-98 from ATCC), JAR (from Dr Ashley Moffet), and HTR8 cells (from Dr Charles Graham) are detailed in Supplementary Methods. To obtain in vitro artificial syncytiotrophoblasts, BeWo cells were incubated with 1 mM 8-Bromoadenosine 3′,5′-cyclic monophosphate for 2 days prior to infection [33].

Term placentas from uncomplicated full-term pregnancies were obtained from the Maternity of Hôpital Nord in Marseille, with the ethical agreement (number 08–012) of the ethical board of Institut Fédératif de Recherche 48 (Marseille, France). Upon receipt of the placenta, tissue was cut into small portions (1 µm²), washed in phosphate-buffered solution (PBS), and incubated for 45 minutes at 37°C with gentle agitation in digestion solution (for 10 g of tissue: 25 mM Hepes, 0.6 mM magnesium sulfate, 0.25% trypsin, and 0.2 mg/mL DNase I in Hank’s balanced salt solution). Suspension was filtered in a cell strainer (10 µm), fetal calf serum (FCS) was added to a final concentration of 5% and centrifuged at 1000 g for 15 minutes. Cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM) F-12 Ham media with 10% FCS, and 10 mL of cell suspension was layered on a Percoll gradient (15 mL of 25% density and 15 mL of 60% density) and centrifuged for 20 minutes at 1200 g. The white ring layer was recovered, resuspended, and pelleted. Finally, positive selection with magnetic rat anti-mouse immunoglobulin G2 (IgG2)–coated microbeads (MiltenyiBiotec) and anti-epidermal growth factor receptor antibody (EGFR) antibody (Santa Cruz) was performed. Cells were incubated with anti-EGFR antibody for 30 minutes and then with IgG2a-coated beads for 45 minutes at 4°C. Cells were then resuspended in DMEM F-12 Ham media with 10% FCS and penicillin/streptomycin and seeded for experiments. Cells were washed and media changed 2 hours prior to infection to remove the antibiotics. Approximately 3 × 10⁷ EGFR² cells were obtained from 30 g of placenta. When necessary, cells were seeded in fibronectin-coated coverslips for 24 hours prior to infection.

**Bacterial Strains**

In this study the reference wild-type smooth virulent *B. melitensis* 16 M, *B. suis* 1330, and *B. abortus* 2308 strains and their corresponding isogenic virB9⁻ mutants [18] were used. Green fluorescent protein (GFP)–expressing derivatives contain a pBBR1MCS-2 expressing the gfp-mut3 gene under the control of the lac promoter. For infection, strains were grown on tryptic soy broth at 37°C for 16 hours (early stationary phase), and the absence of dissociation of the inoculi was assessed by plating and crystal violet staining of the isolated colonies. The Spanish strains *B. abortus* 558 (isolated from the vaginal excretion of a cow following abortion) and *B. abortus* 283 (isolated from a human brucellosis patient) both belong to biovar 3, the most prevalent in Northern Spain.

**Infection and Intracellular Replication Assays**

Cells grown in 24-well tissue culture plates were seeded at 2 × 10⁴ cells per well and inoculated at a multiplicity of infection (MOI) of 100:1. Replication in inclusion was observed at MOIs of 50, 100, and 300. Bacteria were centrifuged onto trophoblasts at 400 g for 5 minutes and then incubated for 30 minutes at 37°C in a 5% carbon dioxide atmosphere. Cells were extensively washed, incubated for 1 hour in medium with 100 µg/mL gentamycin to kill extracellular bacteria, and further cultured with 20 µg/mL gentamycin for the indicated times of infection. To monitor bacterial intracellular survival, infected cells were lysed with 0.1% Triton X-100 in water, and serial dilutions of the lysates were plated on trypticase soy broth agar to enumerate colony-forming units (CFUs). Human chorionic gonadotropin (hCG) was measured from supernatants using an enzyme-linked immunosorbent assay kit (Abzyme).

**Immunofluorescence Microscopy**

Cells were fixed in 3% paraformaldehyde, pH 7.4, at 37°C for 15 minutes and then processed for immunofluorescence labeling as previously described [18]. Samples were examined on a Zeiss LSM 510 or on a Leica SP5 confocal microscope for image acquisition. Images were then assembled using Adobe Photoshop. Antibodies used are detailed in Supplementary Methods. JEG-3 cells were transfected using the FuGENE 6 transfection reagent (Roche). The HLA-G expression vector was pRC-RSV-HLA-G1 (Joel LeMaoult, CEA, Paris). Autophagy was induced by amino acid and serum starvation. JEG-3 cells were infected with *B. abortus*, transfected with Microtuble-associated protein light chain 3 (LC3) a-RFP constructs at 2 hours after infection and simultaneously incubated under normal or starvation conditions (Earle’s balanced salt solution.

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after 1 PBS wash) for 24 hours. Samples were processed for transmission electron microscopy as described previously [18] (Supplementary Methods).

Invasion Assay

Infected JEG-3 cells were collected at 24 hours and resuspended in DMEM with 0.5% bovine serum albumin. They were seeded onto the pre-prepared Matrigel invasion chamber (BD Biosciences) in which the lower chamber had DMEM with 5% FCS. Chambers were incubated for 22 hours in a carbon dioxide atmosphere, and invasion was estimated by counting the cells by microscopy. Cells were labeled with cytokeratin 7 (CK7) to visualize trophoblasts and bacteria were detectable by GFP expression. The invasion index is the ratio between the percentage of invasion of infected cells to the percentage of invasion of noninfected control cells and corresponds to 3 independent experiments.

Statistical Analysis

The unpaired 2-tailed Student t test was performed to determine the statistical differences between experimental datasets.

RESULTS

B. abortus Replication Within LAMP1 and CD63-Positive Acidic Vacuoles in JEG-3 Human Trophoblasts

We first investigated the ability of Brucella to infect and replicate in the human trophoblastic cell line JEG-3, which has some phenotypic features comparable to EVTs [23–25]. JEG-3 trophoblasts were infected with B. abortus 2308 wild-type reference strain and intracellular bacterial counts were determined. B. abortus significantly replicated within JEG-3 cells (Figure 1A). Surprisingly, this replication was not fully dependent on the VirB type IV secretion system (T4SS) because a virB mutant was also able to replicate, albeit at a lower level (Figure 1A).

Confocal immunofluorescence microscopic observation of infected JEG-3 trophoblasts confirmed the intracellular replication of the wild-type B. abortus (Figure 1B and 1C). However, it revealed an unusual replication compartment: B. abortus were mostly found in large vacuoles (Figure 1B). These were negative for the ER marker calnexin (Figure 1D), which is normally present on BCVs from 12 hours post-infection onward [18]. Instead, these vacuoles were positive for LAMP1 at 24 hours post-infection (Figure 1B). LAMP1 was retained on the vacuole harboring wild-type bacteria throughout the infection. Large LAMP1-positive vacuoles, which contained high numbers of bacteria at 48 hours post-infection, were also observed for the virB mutant, confirming its ability to replicate within JEG-3 trophoblasts (Figure 1E).

We also investigated whether these large vacuoles were autophagic because autophagy markers have been described on BCVs [13, 26]. These vacuoles were not labeled with the autophagy marker LC3 (Figure 1F). Using electron microscopy, we confirmed that these vacuoles contained a single membrane (Figure 1G). In addition, LC3-RFP ectopically expressed in JEG-3 cells was not recruited to the BCVs at 24 hours post-infection unless autophagy was induced by starvation from the beginning of the infection (Supplementary Figure S1A). Therefore, we concluded that this atypical BCV does not constitute the recently described autophagic BCV [26]. Hereafter we designate these vacuoles as Brucella inclusions because they morphologically resemble Chlamydia infections. However, in contrast to Chlamydia, B. abortus inclusions were acidic, as evidenced by the presence of lysotracker at both 24 and 48 hours post-infection (Figure 1H and 1I), and were positive for the late-endosomal/multivesicular body marker CD63 (Figure 1H and 1I). The lysosomal enzyme cathepsin D was not detected in association with B. abortus inclusions (Supplementary Figure S1B).

Brucella spp. Replication in Human Trophoblasts of Different Lineages

We then compared B. abortus infection of other trophoblastic cell lines. The BeWo cell line presents numerous differences when compared with the JEG cells that are indicative of different lineage, including low expression of HLA class I transcripts HLA-E and -G and higher expression of placental growth hormones [24]. This is also the case for the JAR trophoblastic cell line that was derived from a gestational choriocarcinoma, principally regarding HLA-1 expression profiles [23]. In both BeWo and JAR trophoblasts, B. abortus was not found in inclusions and displayed an ER-like distribution (Figure 2A and 2B). Similar results were obtained in the placental cell line HTR8/SVneo, which was derived from first trimester villous explants (Figure 2A and 2B). Microscopic analysis confirmed the replication within BCVs positive for calnexin (Figure 2C and data not shown). Because there is no cell line available that corresponds to syncytiotrophoblasts, we infected BeWo pretreated with 8-Br-cAMP to induce cell fusion and formation of a syncytiotrophoblast-like culture. B. abortus replicated in perinuclear location, and no inclusions were observed (Figure 2D). Therefore, B. abortus inclusions were specific to JEG-3 trophoblasts, suggesting a specific intracellular trafficking within EVTs.

Because a key feature of JEG-3 trophoblasts is their specific expression of HLA-G, we ectopically expressed this HLA-I molecule in BeWo, JAR, and HTR8 (all are HLA-G negative) and then infected the transfected cells with B. abortus. Surface expression of HLA-G did not alter the trafficking of B. abortus in BeWo, JAR, or HTR8. Bacteria replicated in calnexin-positive BCVs and inclusions were rarely observed (Supplementary Figure S2), suggesting that HLA-G surface expression alone is not sufficient to induce the atypical intracellular trafficking observed in JEG-3 trophoblasts.

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Figure 1. *Brucella abortus* replication in lysosomal membrane-associated protein 1 (LAMP1) and CD63-positive acidic inclusions in JEG-3 trophoblastic cells. A, JEG-3 cells were infected with *B. abortus* wild-type or virB9 mutant and lysed. Intracellular colony-forming unit counts enumerated at 2, 24, 48, and 60 hours after inoculation. Data are means ± standard error of the mean (SEM) from 1 representative experiment performed in triplicate. B, Representative confocal micrographs of JEG-3 cells infected with green fluorescent protein–expressing wild type for 12, 24, 48, and 72 hours and labeled for LAMP1 (red). C, The number of bacteria per inclusion was counted at 2 and 24 hours after inoculation. Data are means ± SEM from 4 independent experiments. JEG-3 cells infected with wild-type *B. abortus* for 24 hours were also labeled for the endoplasmic reticulum with anti-calnexin (D) and autophagic compartments with anti-LC3 (F) antibodies. E, Representative micrograph of virB mutant inclusion at 48 hours after inoculation and labeled for LAMP1 (red). G, Electron micrograph representative of a *B. abortus* wild-type inclusion at 24 hours after infection. Mitochondria (M), nucleus (N), and bacteria (Ba) are indicated. H, The percentage of inclusions positive for LAMP1, lysotracker, or CD63 was quantified by microscopy for both wild-type and virB mutant *B. abortus* at 24 and 48 hours after infection. Data are means ± SD (N = 3). I, Representative images of JEG-3 cells infected with wild-type *B. abortus* for 24 hours processed for labeling of LAMP1, lysotracker, and CD63. Scale bars correspond to 5 µm for B, D, E, F, and G and 0.5 µm for C.
Figure 2. Brucella spp. infection of different trophoblastic cell lines. A, Representative confocal micrographs of HTR8 and BeWo cells infected with B. abortus for 24 hours and labeled for lipopolysaccharide (LPS; green) and lysosomal membrane-associated protein 1 (LAMP1; red). B, Enumeration of the percentage of JEG-3, JAR, HTR8, and BeWo cells with B. abortus inclusions after 24 hours of infection (***P < .0005 compared with JEG-3). C, BeWo trophoblastic cells infected for 24 hours with green fluorescent protein (GFP)-expressing B. abortus labeled for calnexin (red). D, BeWo cells were induced to form an artificial syncytium by pretreatment with 8-Br-cAMP. They were then infected with GFP-expressing B. abortus for 24 hours and labeled for cytokeratin 7 (CK7, red) and 4-diamidino-2-phenylindole as a nuclear stain. E, Representative confocal images of JEG-3 B. abortus 558 cells infected with B. abortus 558 (field bovine isolate) and B. suis 1330 for 24 hours and labeled with LPS (green) and LAMP1 (red). F, Comparison of the percentage of inclusions in JEG-3 cells infected with different Brucella spp. for 24 hours (***P < .0005). All microscopy counts correspond to means ± SD (N = 3). Scale bars represent 10 µm for all except C (5 µm). G, Representative micrograph of JEG-3 cells infected with B. melitensis for 24 hours and labeled for calnexin (red). H, Enumeration of calnexin-positive labeling on B. abortus inclusions and B. melitensis-containing vacuoles at 24 hours after infection (*P < .05). I, JEG-3 cells were infected with B. abortus or B. melitensis wild-type reference strains and lysed. Intracellular colony-forming unit counts were then enumerated at different times after inoculation (*** P < .0005). Data are means ± standard error of the mean from 1 representative experiment performed in triplicate.
We then analyzed the intracellular replication in JEG-3 trophoblasts of a *B. abortus* clinical isolate 283 obtained from an infected patient and a *B. abortus* field isolate 558 obtained from an infected bovine, both from the same northern region of Spain. The 2 *B. abortus* clinical isolates replicated extensively in JEG-3 cells and at significantly higher numbers than the wild-type reference *B. abortus* 2308 strain (Supplementary Figure S3A); this replication occurred within LAMP1-positive inclusions (Figure 2E and 2F).

Human brucellosis is normally associated with *B. abortus*, *B. suis*, and *B. melitensis*. We therefore investigated their intracellular trafficking in JEG-3 trophoblasts. *B. suis* 1330 was found to replicate within LAMP1-positive inclusions (Figure 2E and 2F), whereas *B. melitensis* 16M did not (Figure 2F). Instead, *B. melitensis* 16M or H38 (not shown) BCVs were positive for calnexin (Figure 2G and 2H). *B. abortus* replication within inclusions was slower than that of *B. melitensis* (Figure 2I), suggesting that the ER-derived BCVs are a more suited niche for brucelle intracellular replication. As expected, *B. melitensis* replication in JEG-3 cells was fully dependent on VirB (Supplementary Figure S3B). *B. melitensis* virB mutants did not replicate and they did not form inclusions, which was observed for the *B. abortus* 2308 virB mutant.

Trophoblasts have important immunological and endocrine functions throughout pregnancy. We therefore investigated whether infection of JEG-3 cells with the different *Brucella* strains has an impact on hCG secretion, a hormone essential during the early stages of pregnancy. JEG-3 cells secreted significantly higher numbers of hCG after 48 hours of culture (Figure 3A). The level and kinetics of hCG secretion were not affected when we infected JEG-3 cells with *B. abortus*, *B. suis*, or *B. melitensis* (Figure 3A). We then assayed the ability of infected JEG-3 cells to migrate and invade a Matrigel basement membrane matrix using a standard in vitro assay. Microscopic counts revealed that JEG-3 cells infected with *B. melitensis* were significantly less invasive than JEG-3 cells infected with either *B. abortus* or *B. suis* (Figure 3B). To exclude the possibility that these results were due to higher levels of cytotoxicity in *B. melitensis*-infected JEG-3 cells, we measured lactate dehydrogenase (LDH) release. We found lower LDH release in JEG-3 cells infected with *B. melitensis* when compared with *B. abortus* and *B. suis* at both 24 and 48 hours after infection (data not shown; maximum percentage of cytotoxicity observed was below 11%). In conclusion, only *B. melitensis* can interfere with specific trophoblast functions.

**B. abortus** and **B. suis** Inclusions Are Specific to EVT Placental Trophoblasts

Although immortalized trophoblastic cells are often used to study trophoblast function in vitro, all available cell lines significantly differ from placental trophoblasts [23, 27]. For this reason, we isolated primary trophoblasts from term placentas to compare the intracellular trafficking of *B. abortus* and *B. melitensis*, which is epidemiologically the most prevalent strain in pregnant women with brucellosis. Trophoblasts were obtained by magnetic separation of EGFR*+* cells and their phenotype confirmed by labeling for CK7, a standard marker for trophoblasts. Cells were not kept in culture for longer than 4 days; it is at this time that more than 90% of cells present in culture were viable and were CK7high/interm (high and intermediate levels of expression), indicating they were trophoblasts. In 40% of preparations, both *B. abortus* and *B. melitensis* failed to replicate in either EGFR*+* or EGFR*−* cells and were
efficiently eliminated, suggesting that some women may be inherently resistant to *Brucella* infection. In the other preparations, *Brucella* replicated very efficiently and reached very high bacterial numbers in a VirB-dependent manner (Figure 4A). The kinetics of loss of LAMP1 from BCVs (Figure 4B) was equivalent to that described for cultured epithelial cells, macrophages, and dendritic cells [13, 18, 19]. In CK7\textsuperscript{high/interm} cells both *B. abortus* and *B. melitensis* replicated in ER-derived compartments (Figure 4C and 4D).

At term, the vast majority of trophoblasts recovered are cytotrophoblasts that do not express surface HLA-G. In 2 of our preparations we observed a very small percentage of cells (<0.1%) that were CK7\textsuperscript{high}, in which *B. abortus* was found within inclusions (Figure 4E). These cells also expressed HLA-G (Figure 4F). In the case of *B. melitensis*, no inclusions were found. Due to the rare presence of EVTs in our trophoblast preparations obtained from term placentas, it was not possible to quantify the intracellular trafficking of brucellae. Instead, we cultured term cytotrophoblasts on fibronectin-coated coverslips for 24 hours to induce their differentiation in EVTs. Microscopic examination confirmed that approximately 50% of cells expressed HLA-G. This is also the method of choice for obtaining EVTs from first-trimester placentas, which is when they are most prevalent [28]. We then infected fibronectin-cultured placenta trophoblasts with *B. abortus* and analyzed for the presence of *Brucella* inclusions. The majority of *B. abortus* in HLA-G\textsuperscript{+} trophoblasts were found in inclusions (Figure 5). This was also the case for *B. suis* (65% infected cells with inclusions) but not for *B. melitensis*, which had 12% of cells with inclusions observed in HLA-G–expressing cells at 48 hours post-infection. These results confirm that *B. abortus* and *B. suis*, but not *B. melitensis*, replicate within inclusions in EVTs.

**DISCUSSION**

To our knowledge, this is the first study of *Brucella* infection of human trophoblasts. *Brucella* spp. extensively replicate in human trophoblasts but display striking differences in the nature of their replicative compartment. In JEG-3 trophoblastic cells and EVTs, *B. abortus* and *B. suis* fail to reach the normal ER-derived niche. Nonetheless, they are able to replicate in a LAMP1-positive acidic inclusion without affecting...
the invasion properties of infected JEG-3 cells. In contrast, \textit{B. melitensis} is able to reach its normal ER-derived compartment, resulting in reduced invasion capability of infected cells. This highlights the first biological difference between these \textit{Brucella} spp. All 3 strains express VirB, which is necessary for the establishment of an ER-derived replication niche. However, it appears as if \textit{B. melitensis} is better equipped to reach this compartment, perhaps by encoding specific virulence factors or better regulating their expression. In JEG-3 cells the \textit{virB} mutant was able to replicate but not to the same level as the wild-type strain in JEG-3 cells, suggesting the VirB T4SS may have a role in replication within inclusions that is independent of mediating fusion with the ER. These cells may provide an interesting model to study novel functions of the VirB.

In most cell types analyzed to date, \textit{B. abortus} has been shown to replicate within an ER-derived compartment. However, in 1 study \textit{Brucella} was found within nonacidic LAMP1-positive large vacuoles in THP-1 human monocytes [29]. At the late timepoints analyzed, \textit{B. abortus} is most likely in autophagic BCVs [26] because at earlier stages, such as 24 hours post-infection, the bacteria are dispersed around the nucleus and not in tight clusters as was observed in JEG-3 cells and EVTs. Furthermore, others have shown that mutants lacking a functional VirB T4SS are highly attenuated in THP-1 cells [30].

Interestingly, \textit{Listeria monocytogenes} is not able to escape from the vacuole in first trimester EVTs, as it does in most other cell types. Instead, bacteria remain confined in an acidic vacuole that is positive for LAMP1 and are not able to spread to other cells [31]. Our results are consistent with the idea that EVTs provide a key barrier to the spread of bacteria to the remaining placenta and the fetus. Both \textit{B. abortus} and \textit{B. suis}, which normally replicate within an ER-derived phagosome, are found in an acidic compartment in EVTs but surprisingly are still able to replicate and form atypical inclusions. In contrast, \textit{B. melitensis} escapes this containment; it reaches the ER where it replicates in its typical compartment and interferes with the invasive capabilities of JEG-3 cells. This could have important consequences during pregnancy because impairment of EVT invasion of the maternal decidua would hamper implantation or the regulation of blood supply to the fetus and subsequently contribute to abortion. \textit{B. melitensis} is the most virulent strain for humans and is the one most often associated with reports of abortion in pregnant women with brucellosis in endemic regions, occurring mainly in the first trimester of pregnancy [6–9]. However, consumption of fresh goat milk products in endemic areas could explain the predominance of \textit{B. melitensis}-associated abortions in humans. It would be interesting to perform a worldwide epidemiological study to determine if there is increased incidence of abortion in women infected with \textit{B. melitensis} compared with \textit{B. abortus} or \textit{B. suis}. It is possible that the EVTs constitute a point of entry for \textit{Brucella} during the first trimester if bacteria arrive from the decidua (where EVTs are in contact with maternal tissue and immune cells). By surviving and replicating within these cells, \textit{Brucella} could breach the fetal–maternal barrier and, in some cases, lead to abortion. Interestingly, \textit{Chlamydia pneumoniae}, which is another pathogen associated with adverse pregnancy outcome, has also been shown to reduce EVT invasion capabilities [32]. It remains to be demonstrated if EVT function is impaired in brucellosis-linked abortions. This will be difficult to investigate because samples can be obtained only after abortion, the endpoint of the infection where both the placenta and uterus are full of bacteria. Nonetheless, it is clear that human trophoblasts provide a

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**Figure 5.** \textit{Brucella abortus} replication in inclusions in extravillous trophoblasts. Trophoblasts purified from term placentas were cultured on fibronectin and then infected with green fluorescent protein–expressing \textit{B. abortus} for 24 and 48 hours and processed for labeling of human leukocyte antigen-G (blue). \(\text{A, The percentage of trophoblasts with inclusions was quantified by microscopy and data are means ± SD of 3 independent experiments with trophoblasts purified from 3 different placentas (\(***P < .0005\)). B, Representative micrograph. Scale bar corresponds to 10 µm.}\)
Replication niche for *Brucella* and could therefore be important during clinical brucellosis in endemic regions, when in many cases the fetal–maternal barrier is breached and *Brucella* infection leads to abortion in pregnant women.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copylefted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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