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Brucella abortus Rough Mutants Are Cytopathic for Macrophages in Culture

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Rough mutants of *Brucella* spp. are attenuated for survival in animal models. However, conflicting in vitro evidence has been obtained concerning the intracellular survival of rough mutants. Transposon-derived rough mutants isolated in our laboratory were previously shown to exhibit small but significant reductions in intracellular survival in a 12-h in vitro assay. Several recent publications report that rough mutants exhibited increased macrophage uptake relative to their smooth parental strains, and a reduction in numbers at the end of the assay has been interpreted as intracellular killing. In an effort to explore the role of O antigen in the interaction between *Brucella abortus* and macrophages, we have monitored the uptake of rough mutants and survival in vitro by using the murine macrophage cell line J774.A1. The results confirm a 10- to 20-fold-increased uptake of rough mutants over that of smooth organisms under standard conditions. Recovery of the rough mutants persisted up to 8 h postinfection, but at the point when intracellular replication of the smooth organisms was observed, the number of rough organisms recovered declined. Fluorescence microscopy revealed the intracellular multiplication of both smooth and rough organisms, and assays performed in the absence of antibiotic confirmed the replication of the rough organisms. Examination by phase-contrast microscopy revealed the lytic death of macrophages infected with the rough mutants, which was confirmed by the release of lactate dehydrogenase (LDH) from the cell cytoplasm. Thus, the decline in the number of rough organisms was the result of the lysis of macrophages and not from intracellular killing. The cytopathic effect is characterized as necrotic rather than apoptotic cell death based on early LDH release, annexin V and propidium iodide staining, morphological changes of infected cells and nuclei, and glycine protection. The cytopathic effect was observed with macrophages at multiplicities of infection (MOIs) of as low as 20 and was not observed with epithelial cells at MOIs of as high as 2000. These findings suggest a role for O antigen during the early stages of host-agent interaction that is essential in establishing an intracellular niche that maintains and supports persistent intracellular infection resulting in disease.

Brucella spp. are facultative intracellular bacteria that cause brucellosis in a variety of animals and undulant fever in humans (3). Brucellosis is a worldwide zoonosis characterized by persistence of the organism in the reticuloendothelial system in secondary hosts and in the reproductive system in primary hosts. The ability of these organisms to survive in professional and nonprofessional phagocytic cells is the basis for disease (18). The gene products and mechanisms that are essential for the intracellular lifestyle are currently under investigation. Among these, the O antigen has been classified as a major virulence determinant of *Brucella* and is essential for survival of the classical species in the host. Although it is well accepted that rough variants and mutants of the naturally occurring smooth species exhibit reduced virulence in the host, the role of O antigen specifically in intracellular survival remains controversial. Rough mutants lacking O antigen are rapidly cleared and do not cause disease (1, 24, 49). However, the naturally occurring rough species *Brucella ovis* and *Brucella canis* are virulent in their natural hosts (rams and dogs, respectively) (11). Early studies showed that intracellular survival of naturally occurring rough variants was drastically reduced relative to that of the smooth parental strains (30, 41, 42). More recently, transposon-derived and genetically engineered rough strains have shown that intracellular survival is either unaffected or only slightly reduced (1, 23, 24, 40, 49). The improved genetic definition of the latter and the virulence of naturally occurring rough species has led most investigators to support the idea that O antigen may not be required for intracellular survival. Recently, evidence has been presented that suggests that O antigen is critical in inhibiting programmed cell death (apoptosis) and that a rough derivative of *Brucella melitensis* defective in the expression of glycosyl transferase (*wboA*) fails to block apoptosis, resulting in death of the cells along with any internalized bacteria (17).

As the first line of defense of the immune system, the macrophage is not only an important component of innate immunity but also a critical coordinator of adaptive immunity. Macrophages are professional phagocytes and antigen-presenting cells, and the cytokines produced by macrophages are critical modulators for adaptive immunity (54). In order to establish an infection, many pathogens have the ability to subvert the macrophage's functions by inducing either apoptosis (26, 37, 53) or necrosis (4, 45). However, recent studies show that some intracellular bacterial infections utilize different strategies to prevent apoptosis (8, 27, 35). Inhibition of apoptosis may prevent release of the pathogen into the extracellular environment, in which the organism exhibits reduced replication and is

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exposed to antibody and complement-mediated inactivation (1, 27). Although *Brucella* invasion, intracellular trafficking, and growth in professional phagocytes and epithelial cells have been widely investigated (2, 12–14, 25, 39), only antiapoptotic effects of *Brucella* infection on phagocytes have been reported (17, 22, 27). The cytopathic effects (CPE) of *Brucella* infection have been overlooked (19–21).

In this study, it is demonstrated that *Brucella abortus* rough mutants are taken up in greater numbers by macrophages than the smooth parental strains and that this increased uptake and replication coincide with necrotic cell death of the macrophages. In contrast to several recent publications that support a role of O antigen in intracellular survival, the data reported suggest that O antigen may be important for the proper uptake of the organism and persistence in the host cells that results in disease either through attainment of the proper intracellular niche or by directly inhibiting cytopathic cell death. In the absence of O antigen *B. abortus* replicates efficiently within cells, but the resulting necrotic death of the macrophage may lead to the death of the organism by extracellular bactericidal mechanisms.

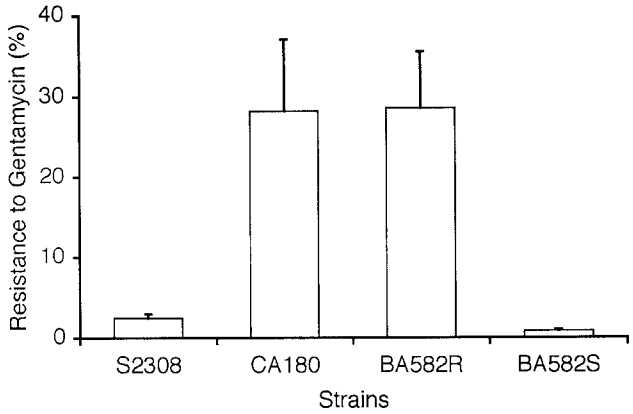

FIG. 1. *B. abortus* rough mutants efficiently invade macrophages. Murine J774.A1 macrophages cultured in 24-well plate were infected with *B. abortus* S2308 and rough mutants (CA180 and BA582R) at an MOI of 200 as described in Materials and Methods. The cells were washed with Peptone saline and lysed with 0.5% Tween 20 following 1 h incubation in DMEM supplemented with 100 μg of gentamicin per ml. *B. abortus* uptake represents the percentage of added CFU protected from gentamicin killing. Data represent the means ± standard deviations from three independent experiments.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacteria used in this experiment include the *B. abortus* virulent S2308 strain, an S2308-derived *cydB* mutant (BA582) (15), a natural rough derivative of BA582 (BA582R), and transposon-derived rough mutants of S2308 (CA180, CA353, CA533, and CA613) (1). The bacteria were grown on tryptic soy agar (TSA) plates with or without 100 μg of kanamycin per ml at 37°C, and this was used to inoculate tryptic soy broth or tryptic soy broth supplemented with kanamycin. Twenty-four-hour bacterial cultures (late log phase) were used to infect cells.

Cell culture. Murine macrophage-like J774.A1 (ATCC TIB-67), BHK-21 (ATCC CCL-10), Vero (ATCC CCL-81), and primary bovine epithelial cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal bovine serum, 1 mM L-glutamine, and 1 mM nonessential amino acids. For J774.A1 cells, passages 4 through 15 were used in these experiments. Human monocyte THP-1 cells (ATCC TIB-202) were maintained in RPMI 1640 with 10% (vol/vol) fetal bovine serum, 1 mM L-glutamine, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 0.05 mM β-mercaptoethanol. Monocytic differentiation of the THP-1 cells was induced with 100 nM phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (Sigma, St. Louis, Mo.) (48). Bovine macrophages derived from peripheral blood monocytes were prepared as described previously (43). In *B. abortus* invasion and replication experiments, 2.5×10^5 cells were seeded into each well of a 24-well plate. For CPE assay, 5×10^4 cells were seeded into each well of a 96-well plate. The cells were cultured overnight before infection with *B. abortus*.

Macrophage infection and survival assay. Monolayers of cells were cultured in 24-well plates and typically infected with *Brucella* at a multiplicity of infection (MOI) of 20, 200, or 2,000 CFU per cell. To synchronize the infection, the infected plates were centrifuged at $200 \times g$ for 5 min at room temperature. Following a 20-min incubation at 37°C in an atmosphere containing 5% (vol/vol) CO_2, the cells were washed three times with peptone saline (1% [wt/vol] Bacto peptone and 0.85% [wt/vol] NaCl). To evaluate macrophage invasion, the cells were incubated for 1 h at 37°C in fresh DMEM supplemented with 100 μg of gentamicin per ml to kill extracellular bacteria. After 1 h (time zero), the monolayers were washed with DMEM to remove gentamicin, and then the cells were lysed with 0.5 ml of 0.5% (vol/vol) Tween 20 in sterile water. The CFU per well were obtained by plating dilutions on TSA plates or TSA plates supplemented with 100 μg of kanamycin per ml. The percent bacterial uptake or invasion was calculated as the number of bacteria recovered divided by the number of bacteria inoculated into each well. To assess intracellular growth of the bacteria, the concentration of gentamicin was reduced to 20 μg/ml (time zero). Infected cells were lysed as described above at selected time points postinfection (p.i.). Bacterial recovery was determined as described above. All invasion and survival assays were performed with triplicate wells, and the results presented represent the averages from at least three separate experiments.

Indirect immunofluorescence assay. J774.A1 cells were seeded in 24-well plates with 0.17-mm-diameter glass coverslips (Fisher Scientific, Pittsburgh, Pa.) at 10^5 cells/well 1 day before the infection. The cells were infected at an MOI of 20 as described above. The coverslips were recovered, and the cells were fixed with 3.7% (vol/vol) formaldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 30 min at room temperature. The cells were washed with PBS plus 0.05% (vol/vol) Tween 20 (PBS-T) and incubated with mouse anti-*B. abortus* serum diluted (1:500) in PBS-T containing 0.05% (vol/vol) Triton X-100 (PBS-TT) for 1 h at room temperature. Donkey anti-mouse immunoglobulin G (IgG) Alexa Fluor 594 conjugate (Molecular Probes, Eugene, Oreg.) diluted in PBS-TT (1:500) was added and incubated at room temperature for 1 h after three washes. The coverslips were washed and mounted on slides. *B. abortus* growth was observed by Ultima confocal microscopy (Meridian Instruments, Okemos, Mich.).

For differential staining of intracellular and extracellular bacteria, the fixed cells on coverslips were incubated for 1 h with mouse anti-*Brucella* serum (1:500) in PBS and washed three times with PBS before being permeabilized with PBS-TT. The cells were incubated with goat anti-*Brucella* serum (1:500) in PBS-TT for 1 h and washed with PBS-T before being stained with Alexa Fluor 594 donkey anti-mouse IgG (1:500) and Alexa Fluor 488 donkey anti-goat IgG (1:500) in PBS-TT. The extracellular bacteria (red) and intracellular bacteria (green) were observed with an IX70 fluorescence microscopy.

Quantitation of cytopathic cell death. Cells cultured in 96-well plates were infected with *B. abortus* in triplicate wells as described above. The culture supernatants were collected at various time points p.i., and the lactate dehydrogenase (LDH) released was determined by a CytoTox 96 nonradioactive cytotoxicity assay (Promega, Madison, Wis.) according to the manufacturer's instructions with minor modifications. To reduce the LDH background from fetal bovine serum, the supernatants were diluted 1:1 with PBS before the assay. Cytopathic cell death is expressed as a percentage of maximum LDH release, i.e., $100 \times$ (optical density at 490 nm [OD_{490}] of infected cells $-$ OD_{490} of uninfected cells)/(OD_{490} of lysed uninfected cells $-$ OD_{490} of uninfected cells). Phase-contrast microscopy was also used to visualize morphological comparisons of infected and uninfected cells (Olympus IX70 microscope). These assays were performed in triplicate wells, and the data presented represent the average ± standard deviation from at least three separate experiments.

Evaluation of apoptotic and necrotic cell death. Apoptotic macrophages were detected by using three approaches. In the first approach, cells were stained with annexin V and propidium iodide (PI) by using the annexin V-FLUOS staining kit (Roche Diagnostics Corporation, Indianapolis, Ind.). *B. abortus*-infected macrophages were incubated with annexin V and PI in staining buffer for 20 min at room temperature and observed by fluorescence microscopy (Olympus IX70 microscope). The images were obtained with an RT Slide Spot digital camera

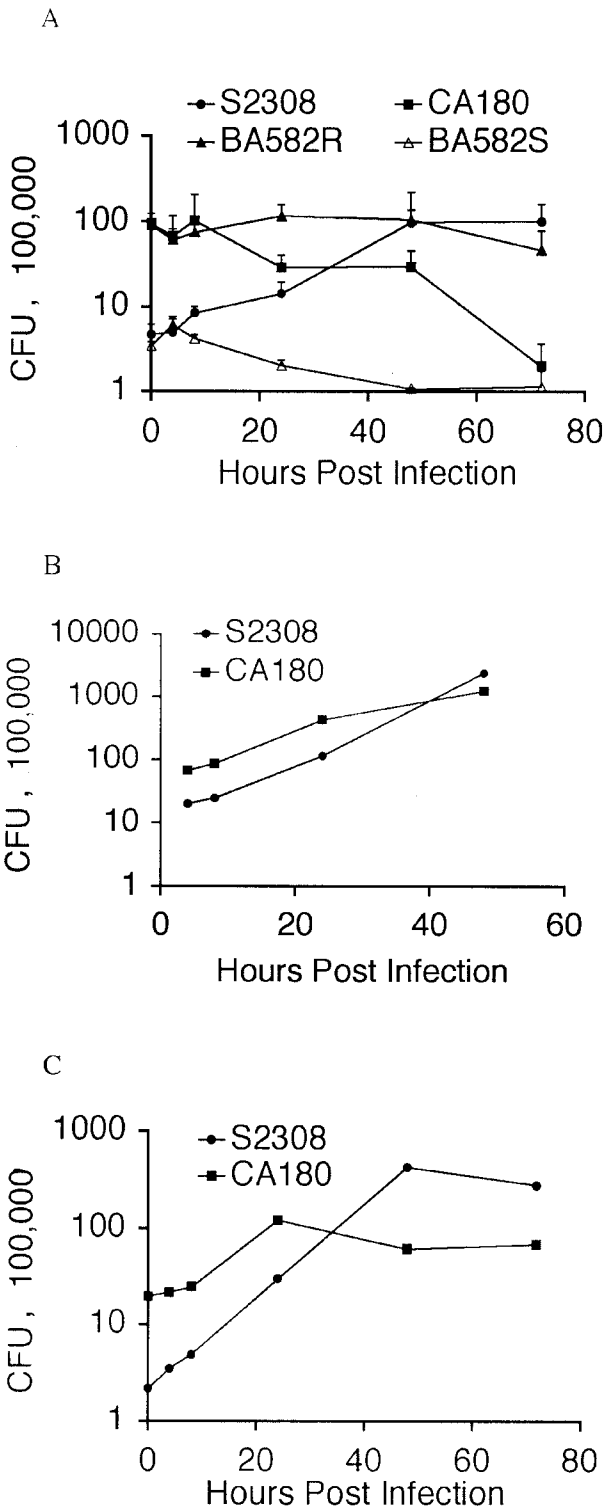

FIG. 2. *B. abortus* rough mutants replicate in macrophages efficiently. (A) Murine macrophages were infected with S2308 and rough mutants at an MOI of 200 as described in the legend to Fig. 1. After 1 h, the medium was removed and replenished with complete medium containing 20 μg of gentamicin per ml. At selected time points, the medium was removed and the cells were washed prior to lysis of the cell monolayer. Each point represents the mean ± standard deviation from three to five independent experiments. (B) Murine macrophages were infected with S2308 and rough mutant CA180 at an MOI of 200. The infected cells were cultured in complete medium without gentamicin. Results of a representative experiment are shown (C) Murine macrophages were infected with S2308 and rough mutant CA180 at an MOI of 20. The infected cells were cultured in complete medium with 20 μg of gentamicin per ml. In each experiment the cells were lysed with 0.5% Tween 20 at the indicated time points, and the CFU in each well were determined by plating serial dilutions on TSA or TSA containing kanamycin. Results of a representative experiment are shown.

and analyzed with Spot software (Diagnostic Instruments, Inc.). Apoptotic and necrotic cell numbers were counted in representative fields containing at least 600 cells. In addition, nuclear morphology was examined following annexin V and PI staining. In a second approach, the morphologies of the *B. abortus*-infected cells and control cells were observed by phase-contrast microscopy. The third approach is a glycine protection assay (4). Briefly, the J774.A1 macrophages were treated with 5 μM glycine in complete medium 30 min before infection and 24 h thereafter. The LDH released in the supernatant at 24 h p.i. was detected, and the cytotoxicities of glycine-treated cells and untreated cells were determined as described above. Gliotoxin (Sigma)-treated cells were employed as a positive control for apoptotic cell death, and *t*-butyl-hydroperoxide (TBH) (Sigma)-treated cells were used as a positive control for necrotic cell death.

RESULTS

***B. abortus* rough mutants efficiently invade and replicate in macrophages.** Previous studies suggested that S2308-derived rough mutants exhibit reduced survival in macrophages by 12 h p.i (1, 49). To more carefully characterize the survival and invasiveness of rough mutants in tissue culture, the murine macrophage-like cell J774.A1 was infected with *B. abortus* strain CA180, a rough mutant with a Tn*5* transposon insertion in the *manB* gene of S2308 (1). In order to rule out any effect of transposon Tn*5*, a second mutant, BA582, which has a Tn*5* transposon inserted in *cydB* (15), was used as a control. A spontaneously arising rough variant, BA582R, identified by crystal violet staining and acriflavin agglutination in passaged BA582 cultures was used as an additional control. As shown in Fig. 1, 28.2% ± 8.80% of inoculated rough *B. abortus* CA180 (MOI = 200) was taken up by the macrophages. However, only 2.45% ± 0.48% and 0.86% ± 0.16% of the inoculated smooth *B. abortus* S2308 and BA582, respectively, were similarly taken up. Consistent with enhanced uptake of rough mutants, 28.6% ± 6.92% of the BA582R cells invaded macrophages. To rule out the possibility that the observed differences in uptake were due to increased resistance of rough *Brucella* to gentamicin, in vitro experiments demonstrated that the MICs of gentamicin for S2308 and CA180 were identical (data not shown).

To determine whether the cells were equally infected, J774.A1 macrophages cultured on glass coverslips were infected with S2308 and CA180 at an MOI of 20. The cells were fixed at 1 h p.i., and the internalized bacteria were detected by double antibody staining as described in Materials and Methods. Only 7% of the cells were infected with S2308, while 90% of the cells were infected with CA180. In S2308-infected cells, 89.7% of the cells had one to two organisms, and 10.3% of the cells contained three or more bacteria. In contrast, 62.8% of the CA180-infected cells had 4 to 10 bacteria, 18.2% of the cells contained 1 to 3 organisms, and 19% of the infected cells had more than 10 bacteria.

Although the rough organisms are consistently taken up by macrophages at higher levels, sensitivity to macrophage killing could explain their reduced virulence, so bacterial survival was

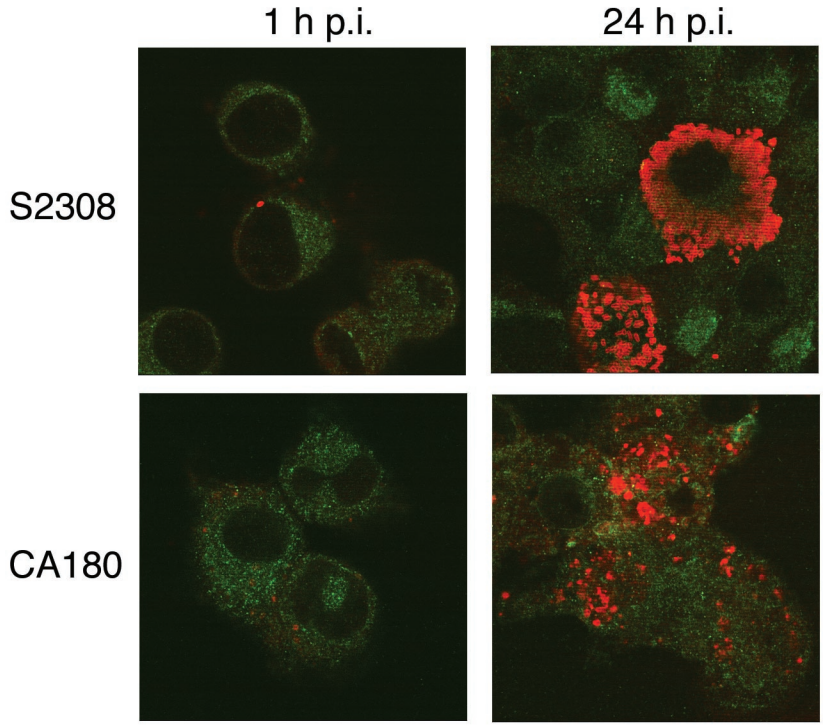

FIG. 3. Intracellular replication of *B. abortus* in murine macrophages. J774.A1 cells were infected with S2308 and CA180 at an MOI of 20. The cells were fixed at 1 and 48 h p.i. and stained by indirect immunofluorescence assay. The intracellular bacteria (red) were observed by confocal microscopy. Individual smooth organisms fluoresce more intensely due to elevated levels of O-antigen antibody in the sera used.

determined at various times p.i. (Fig. 2). When infection was performed at an MOI of 200, the macrophage content of smooth *B. abortus* S2308 increased twofold by 8 h p.i., reaching a plateau (20-fold increase) by 48 h p.i. (Fig. 2A). In contrast, the macrophage content of CA180 changed little over the first 8 h p.i. and then decreased sharply between 24 and 48 h p.i., and by 72 h p.i., only 2% of the invading CA180 (time zero) were recoverable (Fig. 2A). The continued recovery of BA582R was difficult to explain, since survival of this double mutant was expected to be reduced, due to both the rough phenotype and the transposon insertion in the *cyd* locus (15) (Fig. 2A). Since the decline in recovery of the rough mutants may also be explained by death of the cells and exposure of the organism to the bactericidal effect of gentamicin in the culture medium, survival assays were performed in the absence of gentamicin (Fig. 2B). These results revealed that the replication of CA180 was similar to that of the smooth strain over the first 48 h. Persistence and/or replication of the rough mutant could also be observed in assays using a reduced MOI of 20 (Fig. 2C). In order to confirm these results, intracellular bacteria were visualized following fixation with 3.7% (vol/vol) formaldehyde by indirect immunofluorescence assay at 1 and 48 h p.i., starting with an MOI of 20 (Fig. 3). The results revealed large numbers of intracellular smooth and rough organisms. These results demonstrated that *B.* abortus rough mutants survive and multiply in murine macrophages.

***B. abortus* rough mutants are cytopathic for macrophages.** Although *B. abortus* rough mutants were shown to invade the macrophages more efficiently than their smooth parental strains (Fig. 1), the subsequent decline in CA180 was at first interpreted to result from increased sensitivity to intracellular killing within macrophages (30, 41, 42). However, these results did not explain the increased numbers of organisms recovered when medium lacking gentamicin was used or when intracellular organisms were observed by fluorescence microscopy. In order to explain this reduced recovery, infected macrophages were visualized over time by using phase-contrast microscopy. In these experiments, it became evident that reduced recovery of CA180 at 24 h p.i. was associated with a marked CPE in CA180 infected cells by 24 h p.i. (Fig. 4). Since differences in recovery of the two rough strains, CA180 and BA582R, were observed (Fig. 2A), additional experiments were performed with a panel of previously described rough mutants to see whether the CPE was a general property of infection in J774.A1 cells (1). The infected cells were evaluated by phase-contrast microscopy at different time points (8, 12, 24, and 48 h) following infection, and the CPE was found to be dose dependent at each of three MOIs (20, 200, and 2,000) (Table 1). The morphological change was clearly visible by 8 h p.i. with CA180-infected cells and was dramatic by 12 h p.i. at an MOI of 200. By 24 h p.i., more than 80% of the cells were dead (Fig. 4 and Table 1). However, if an MOI of 2,000 was used in the infection, the CPE was observed much earlier. More than 75% of the cells were dead by 8 h p.i. with CA180 (Table 1). We also observed that these rough mutants differed in their cytopathogenesis, with the order CA180 > CA163 > CA353 > CA533. The variability in CPE induced by different rough mutants has not been defined, but it may be related to the different genes inactivated and, as a result, the structure of rough lipopolysaccharide (R-LPS) in these mutants (1). The persistent recovery

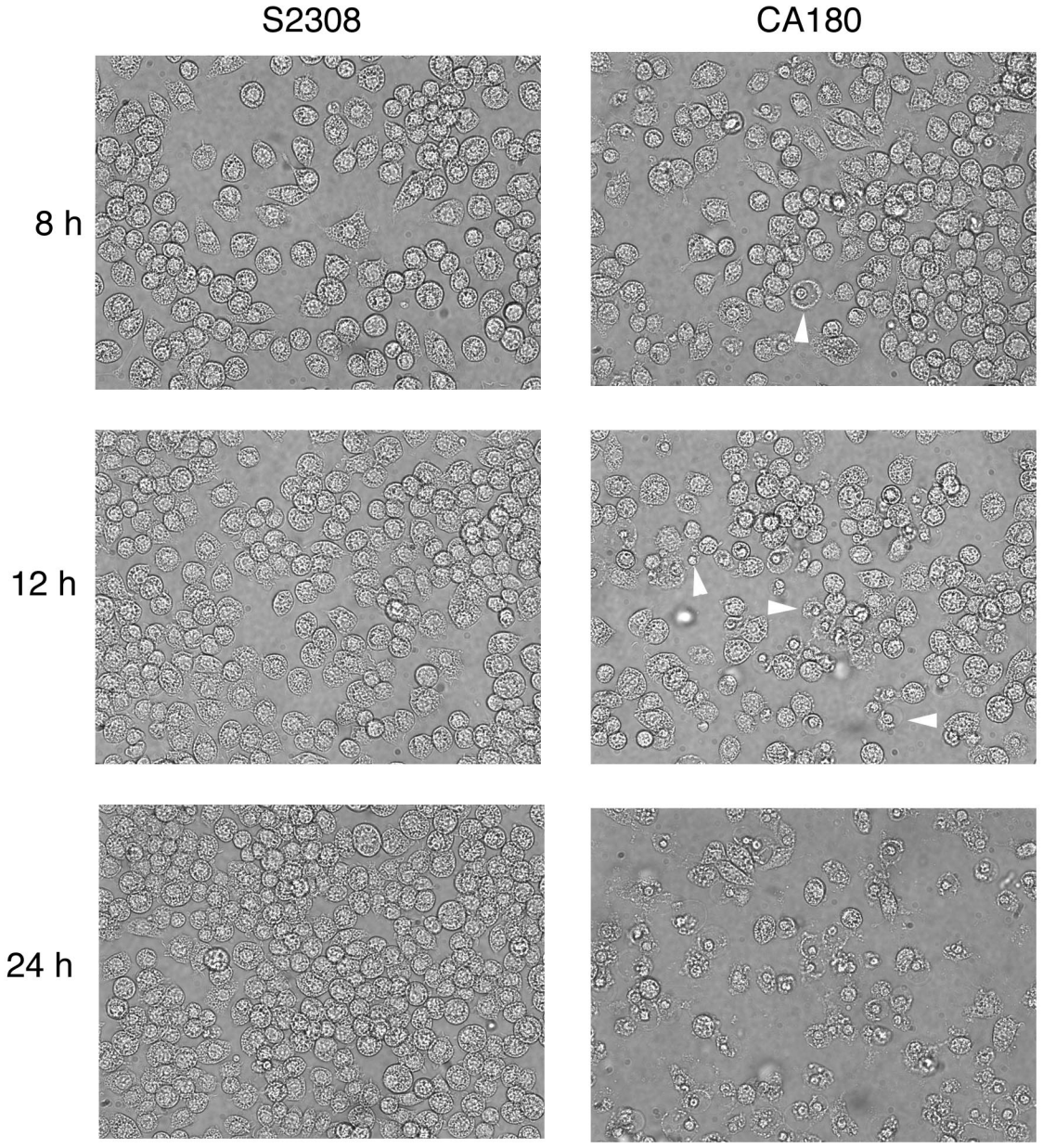

FIG. 4. Morphology of *B. abortus*-infected macrophages. J774.A1 macrophages in 24-well tissue culture plates were infected with *B. abortus* S2308 or CA180 at an MOI of 200. Infected cells were observed at the times indicated by phase-contrast microscopy. Magnification, ×200. Arrowheads indicate dead cells.

of BA582R (Fig. 2A) may be attributed to the reduced CPE observed in monolayers infected with this strain (data not shown) and is not unusual among spontaneously derived rough mutants (see below).

To determine the kinetics of the CPE of rough mutants, J774.A1 was infected with CA180 and S2308 at an MOI of 200. The cell culture supernatants were collected at various time points, and the LDH released by the cytopathic cells was detected by a CytoTox 96 nonradioactive cytotoxicity assay (Fig. 5). Using this assay, CPE was detected as early as 6 h p.i., and the LDH release reached 14.0% ± 9.0% by 8 h p.i. By 24 h p.i., 87.8% ± 14.9% of LDH release was detected. In contrast, only 4.4% ± 2.63% of the LDH was released from S2308-infected cells at 24 h p.i. (Fig. 5).

To determine the cellular specificity of the CPE, THP-1, Vero, BHK, bovine monocyte-derived macrophage, and primary bovine epithelial cells were infected with strains CA180 and S2308 at an MOI of 200. LDH released by the infected cells was detected at 24 h p.i. by the CytoTox96 nonradioactive cytotoxicity assay. The results revealed that CA180 is cytopathic to J774.A1, THP-1, and bovine monocyte-derived macrophages but not to BHK, Vero, and primary bovine epithelial

TABLE 1. CPE induced by *B. abortus* rough mutant infection in J774.A1 cells

MOI	h p.i.	CPE[a] with strains				
		S2308	CA180	CA353	CA533	CA613
2,000	8	−	++++	++	+	+++
	12	−	++++	++++	++	++++
	24	−	++++	++++	++++	++++
	48	−	++++	++++	++++	++++
200	8	−	+	+	−	+
	12	−	++	+	+	++
	24	−	++++	++	+	++
	48	−	++++	+++	+++	+++
20	8	−	−	−	−	−
	12	−	+	−	−	−
	24	−	+	−	−	−
	48	−	++	+	+	+

[a] ++++, 75 to 100% CPE; +++, 50 to 75% CPE; ++, 25 to 50% CPE; +, <25% CPE; −, no CPE.

cells (Fig. 6). Since the lack of cytopathic effect shown by CA180 infection in Vero, BHK, and bovine epithelial cells may be attributable to a reduced rate of uptake by nonprofessional phagocytes (7), *B. abortus* uptake and LDH release at 24 h p.i. were determined by using BHK and J774.A1 cells infected with CA180 at MOIs of 2,000 and 200, respectively. Although similar amounts of CA180 were taken up by BHK and J774.A1 cells ($2.4 \times 10^6 \pm 0.6 \times 10^6$/well in BHK cells versus $3.9 \times 10^6 \pm 2.2 \times 10^6$/well in J774.A1 cells), the BHK cells did not exhibit any CPE. The LDH released at 24 h p.i. in BHK and J774.A1 cells was 1.85% ± 1.33% and 66.34% ± 9.75%, respectively. In addition, Vero cells were infected with CA180 at MOIs of 2,000 and 10,000. LDH release was not detected at 48 h p.i. (<1.5%). These results demonstrated that *B. abortus* rough mutant infection was noncytopathic for epithelial cells. Therefore, the CPE induced by *B. abortus* rough mutants was specific for macrophages in culture.

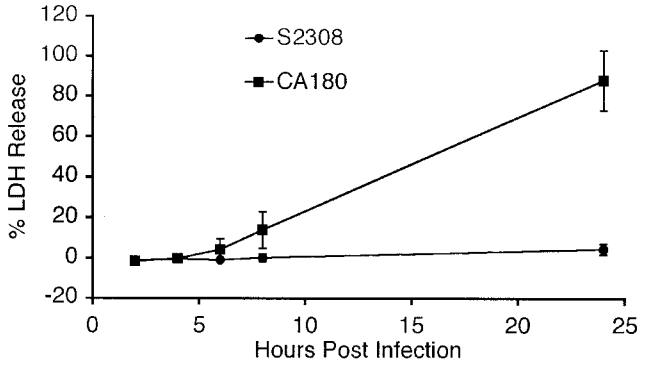

FIG. 5. *B. abortus* rough mutants are cytopathic to J774.A1 macrophages. Macrophages cultured in 96-well plate were infected with S2308 and CA180 at an MOI of 200. The supernatants were collected at the indicated time points, and the LDH released by the infected cells was detected by CytoTox 96 nonradioactive cytotoxicity assay. Each point indicates the mean ± standard deviation from three independent experiments.

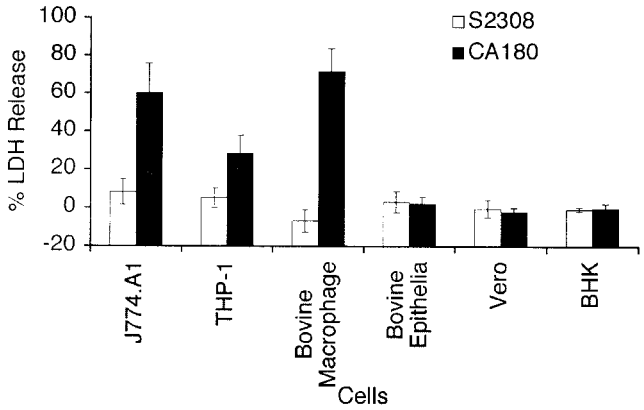

FIG. 6. *B. abortus* rough mutants are cytopathic to professional phagocytes but not epithelial cells. J774.A1, THP-1, bovine macrophage, Vero, BHK, and primary bovine epithelial cells were infected with S2308 and CA180 at an MOI of 200. The supernatants were collected at 24 h p.i., and the LDH was detected by CytoTox 96 nonradioactive cytotoxicity assay. Each point indicates the mean ± standard deviation from three independent experiments.

***B. abortus* rough mutants induce necrosis, not apoptosis, in murine macrophages.** Infection with smooth *Brucella* has been reported to inhibit programmed cell death (apoptosis) (17, 22, 27). Rough derivatives of *Brucella* strains exhibit attenuated survival in infected hosts (1), and reduced replication in vitro in cell culture has been suggested to result from a failure to prevent apoptosis (17). Early reports (19–21) revealing a CPE induced specifically by rough mutants are in agreement with the results reported here that infection by rough mutants results in macrophage death (Fig. 3 and 5). The release of LDH is a nonspecific measure of cell death during both necrosis and late-stage apoptosis. Therefore, several methods were used to characterize cell death to determine whether the CPE induced by infection with rough *B. abortus* mutants resulted from necrosis or apoptosis.

One characteristic of the early stage of apoptosis is the translocation of phosphatidylserine from the inner cell membrane to the outer cell membrane, which is easily detected with fluorescein-conjugated annexin V (47). PI will stain DNA only during late-stage apoptosis. In contrast, necrotic cells stain strongly with both annexin V and PI due to early permeabilization of the cell membrane (47). S2308- and CA180-infected cells were stained with annexin V and PI at 24 h p.i. (Fig. 7). Consistent with the results of the LDH assay (Fig. 5), more than 90% of the CA180-infected cells were necrotic or apoptotic (Fig. 7E and F), while only about 5% of the S2308-infected cells were necrotic or apoptotic at 24 h p.i. (Fig. 7C and D). For apoptotic cells, gliotoxin-treated macrophages were used as a positive control (Fig. 7G and H) (4). To determine whether cell death resulted from necrosis or apoptosis, the number of apoptotic cells (annexin V positive) and necrotic cells (annexin V and PI positive) were counted by using a fluorescence microscope at various times p.i. (Fig. 8). Necrotic cells could be detected as early as 6 h p.i. and increased to 15% by 8 h p.i. By 24 h p.i., more than 90% of the CA180-infected J774.A1 cells were necrotic (Fig. 7F and 8). The percentage of necrotic cells matched the cytopathogenesis detected by the

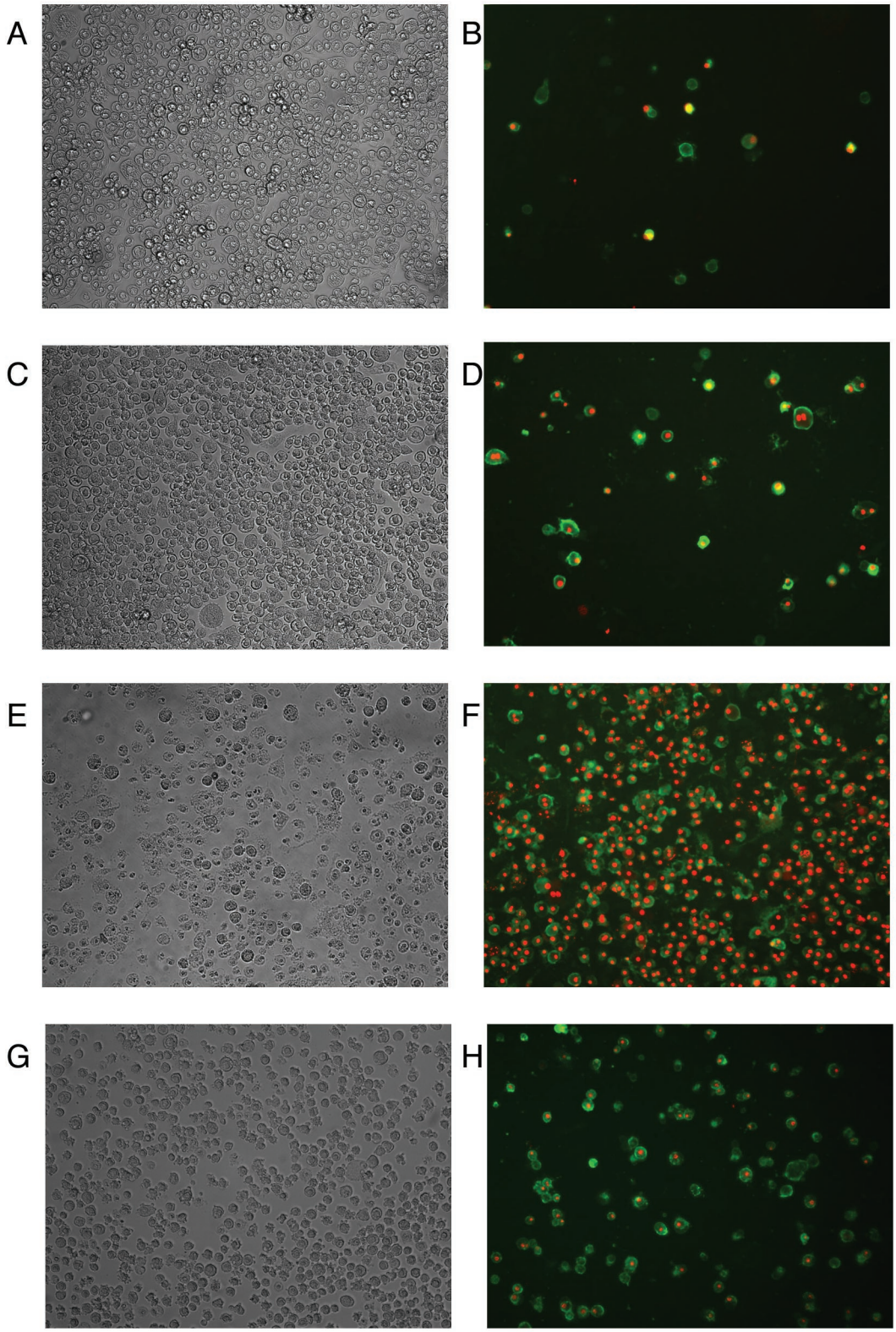

FIG. 7. Necrotic cell death in macrophages associated with *B. abortus* rough mutant infection. J774.A1 macrophages cultured in 96-well plate were uninfected (A and B), infected with S2308 (C and D) or CA180 (E and F) at an MOI of 200 for 24 h, or treated with 5 μM gliotoxin for 5 h (G and H). The cells were stained with annexin V (green) and PI (red) and observed by phase-contrast (A, C, E, and G) or fluorescence (B, D, F, and H) microscopy. Magnification, ×200.

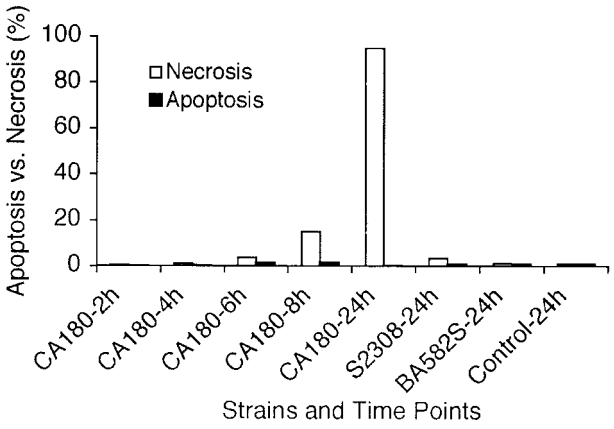

FIG. 8. CPE of CA180 infection correlate with the number of necrotic cells. J774.A1 macrophages cultured in 96-well plates were infected with either S2308, CA180, or BA582 at an MOI of 200. The cells were stained with annexin V and PI at the indicated time points. The necrotic cells (annexin V and PI positive) and apoptotic cells (annexin V positive and PI negative) were observed and counted by fluorescence microscopy. The data represent the percentages of necrotic and apoptotic cells in representative fields having at least 600 cells.

CytoTox 96 nonradioactive cytotoxicity assay (Fig. 5 and 8). Apoptotic cells never exceeded 1.6% over the course of the experiment (Fig. 8). These results suggest that the CPE induced following infection with rough *B. abortus* mutants resulted from necrotic cell death and not from apoptotic cell death.

Apoptotic and necrotic cells can be differentiated by morphological changes and nuclear condensation (53). To further confirm that cell death did not result from apoptosis, gliotoxin-treated cells and rough-mutant-infected cells were stained with annexin V and PI and observed by fluorescence microscopy under higher magnification (Fig. 9). Gliotoxin-treated cells showed typical signs of apoptosis at 6 h posttreatment, including cell shrinkage, membrane blebbing, and nuclear condensation (Fig. 9A and C). However, CA180-infected cells were swollen and lysed, and their nuclei were not condensed (Fig. 9B and D), indicating that the cells were not apoptotic.

It has been demonstrated that glycine treatment limits necrotic cell death by blocking nonspecific ion fluxes through the outer membrane but has limited effects during apoptosis (4, 45). Therefore, glycine protection assays were used to differentiate necrosis from apoptosis. J774.A1 cells were treated with 5 μM glycine for 30 min at 37°C prior to CA180 and S2308

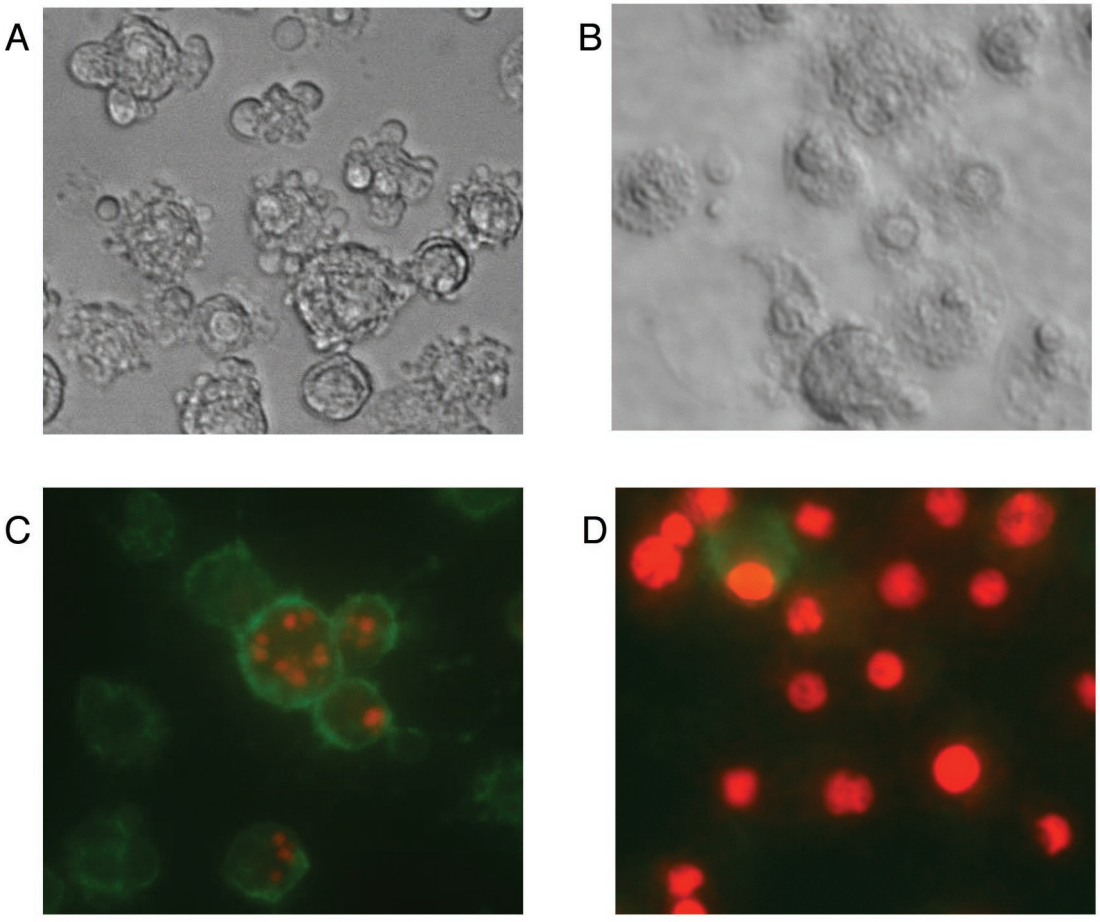

FIG. 9. Morphological characteristics of CA180-infected cells. (A and B) J774.A1 macrophages treated with gliotoxin for 6 h (A) and infected with CA180 for 24 h (B). The morphology of the cells was observed by phase-contrast microscopy. Magnification, ×600. (C and D) The nuclei of the cells treated with gliotoxin (C) or infected with CA180 (D) were visualized after annexin V and PI staining.

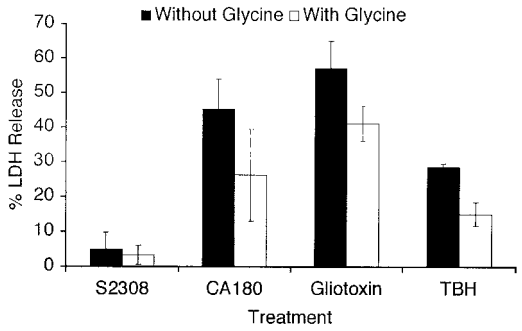

FIG. 10. Effect of glycine treatment on the CPE induced by CA180 infection. J774.A1 cells were infected with CA180 for 24 h at an MOI of 200 or treated with 200 μM TBH or 5 μM gliotoxin for 20 h in the presence or absence of 5 μM glycine. CPE was determined by LDH release. Each point indicates the mean ± standard deviation from two or three independent experiments.

infection. The glycine-treated cells were also incubated with 5 μM gliotoxin or 100 μM TBH, a necrosis-inducing reagent (45). The same level of glycine was maintained in the culture medium after *B. abortus* infection (24 h) and gliotoxin or TBH treatment (20 h). Glycine treatment inhibited CA180-induced LDH release and TBH treatment induced LDH release, by 42.2 and 47.4%, respectively (Fig. 10). As expected, glycine had a reduced effect (28.2% protection) on gliotoxin-induced LDH release (4). These results are consistent with *B. abortus* rough-mutant-induced necrotic cell death of macrophages.

DISCUSSION

Our results have confirmed that infection by *B. abortus* rough mutants induces a CPE that is restricted to macrophages, including the macrophage-like cell lines J774.A1 (murine) and monocyte-derived THP-1 (human) and primary monocyte-derived macrophages (bovine). The CPE observed was characterized most fully in J774.A1 cells, was MOI dependent, and varied in severity depending on the gene interruption. In addition to recent studies suggesting that apoptotic cell death may be blocked by O antigen but continues unabated in cells infected with rough mutants (17), the cell death induced by the rough mutants described here appeared to be necrotic based on early LDH release, annexin V and PI staining, morphological appearance of infected cells, lack of nuclear condensation, and glycine protection. In agreement with the work of Freeman et al. (19–21), the CPE observed was restricted to live bacteria, as it was eliminated by both heat treatment and chloramphenicol pretreatment of the *B. abortus* (data not shown). Furthermore, these data are in accordance with the work by Freeman and coworkers (19–21), and more recently by others (24, 40, 49), describing the ability of rough organisms to replicate within macrophages and/or monocytes.

In light of the conflicting results in the literature, it is important to note that there are several important differences among the experiments described here and by others, including the mutations causing the rough phenotype, the genetic background of the mutants, and the host cells employed. In this work, we examined five transposon-derived rough derivatives of *B. abortus* S2308, i.e., CA180, CA353, CA533, and CA613 (1) and BA184 (31), and each of these rough mutants induced necrotic cell death in J774.A1 cells. However, the severity of the CPE induced varied, which may be related to differences in R-LPS structure (1). We have also examined two spontaneously occurring rough variants of *B. abortus*, BA582R and RB51 (both derived from S2308), as well as the naturally occurring rough species *B. canis* and *B. ovis*. BA582R caused a limited CPE, as demonstrated by microscopic observation of the cells (unpublished results) and the persistent recovery of organisms in a macrophage killing assay (Fig. 2A). RB51, a naturally occurring rough isolate of *B. abortus* that has recently been employed as a vaccine strain in cattle (44), caused a reduced CPE in macrophages that was detected only by an LDH release assay (data not shown). These results may be explained by differences in R-LPS structure or may be attributable to a low level of O-antigen expression, notably in RB51 (9). The genetic defect responsible for the reduced O-antigen production in RB51 has not been completely defined (50), but a small amount of O-antigen expression remaining may be sufficient to alter host-agent interaction. This has also been assumed to be the case for the naturally occurring rough species (46). *B. canis* and *B. ovis*, like the classical smooth species, cause no detectable CPE but invade host cells like other rough mutants at elevated efficiency (6; J. Pei and T. A. Ficht, unpublished data). Although differences in R-LPS may explain these observations, one alternative is that enhanced uptake and the CPE may be controlled by separately encoded functions. Differences in genetic background or expression from *B. canis* and *B. ovis* could explain this distinction, while the multiple passages used to select RB51 may account for multiple genetic changes. According to the latter explanation, differences in R-LPS would not be directly responsible for the CPE.

A second possible explanation for differences in the results among research laboratories is the use of different cell types, i.e., macrophages (including primary cells and cell lines) and epithelial cells. In this study, CA180 infection induced cell death in a murine macrophage-like cell line, a human macrophage-like cell line, and primary bovine macrophages. However, infection in Vero, BHK-21, and bovine epithelial cells in our laboratory did not induce CPE up to 48 h p.i. (Fig. 6), suggesting that the CPE is cell dependent. Detilleux et al. (14) showed that rough mutants were taken up more readily by Vero cells than smooth strains, and the rough strains grew well. However, no CPE were reported. The variability of CPE in host cells may be attributable to differences in invasion and intracellular trafficking of *B. abortus* in different cell types (7, 13, 14, 25).

Survival and replication of the rough strains prior to the CPE calls into question the role for smooth LPS during intracellular survival. It has been previously reported that internalization mechanisms may determine the bacterial destination in the host cell (29, 40, 51). The data presented may begin to describe an alternate uptake pathway for rough organisms preventing intracellular trafficking along the normal pathway (40). Along these lines, it has been shown that *Brucella* uptake via lipid rafts is associated with successful invasion of host cells, while failure to be taken up by caveolin-associated compartments results in nonproductive infection (51). Several studies have focused on the role of the type IV secretion system in controlling uptake and trafficking (10, 12, 51, 52), but the

results reported here suggest that O antigen may play a key role in proper uptake and the ultimate outcome of infection.

It has been reported that *B. abortus* BA582, a *cydB*::Tn*5* mutant, is attenuated for intracellular survival in macrophages (15), and this was confirmed in the assays performed here. However, BA582R not only was found to invade the cells more efficiently than the parental BA582S (Fig. 1) but also persisted in the macrophages for longer times than observed for *B. abortus* CA180 (Fig. 2A). These results demonstrate that rough *B. abortus* mutants invade and persist within macrophages, suggesting efficient uptake and resistance to intracellular killing mechanisms, even in the case of mutations attenuating survival of smooth strains.

R-LPS from *B. abortus* has been shown to stimulate tumor necrosis factor alpha (TNF-α) production via the same mitogen-activated protein kinase signaling pathways (ERK and JNK) as for *Escherichia coli* LPS in RAW 264.7 macrophage-like cells (32), and it may be that this has caused the observed CPE. It is possible that the binding of *Brucella* to the macrophage enhances the ability of R-LPS to activate TNF-α production by the host cells. Another possibility is that, like rough *Salmonella* (34), *B. abortus* rough mutants shed more LPS and stimulate TNF-α production. It is interesting that *Brucella* releases a factor, shown to be Omp25, that prevents expression of TNF-α (5, 33). Synthesis of Omp25 and its role in the CPE induction after CA180 infection need to be further investigated. These possibilities, along with the ability of smooth LPS and R-LPS to act directly to inactivate or activate cell signaling pathways, remain to be examined.

Since rough organisms fail to cause disease in any host (except the naturally occurring rough species), one may conclude that the CPE induced by rough derivatives is not part of natural infection and that natural rough species either do not cause a CPE in vivo or, if they do, are rapidly cleared. However, differences among *Brucella* spp. prevent generalized conclusions. Our working hypothesis is that spontaneously appearing rough mutants are important for the cell-to-cell spread of the organism but in the absence of smooth strains cannot by themselves sustain infection. Preliminary results with mixed infections reveal an accelerated release of smooth and rough organisms which limits intracellular replication (Pei and Ficht, unpublished results). The possibility exists that the CPE is the result of a second, undefined mechanism that is more readily apparent in rough mutants (R-LPS, reduced Omp25, etc). This mechanism may function under normal conditions to provide a gradual release of smooth organisms from the host cell. Interestingly, the CPE (or in vitro virulence) of the genetically engineered rough mutants defined in this report was inversely proportional to their virulence in mice (1). This is reminiscent of the situation with *Salmonella*, in which modifications to the O antigen alter survival (28, 38). It has been reported for *Salmonella* that uptake rate variation resulting from LPS structural changes correlated inversely with virulence (36). In the present study, the uptake rates of the genetically engineered rough mutants were not compared. However, comparison of one of the rough mutants, CA180, with *B. abortus* S2308 indicated that the virulence in mice correlated inversely with the uptake rate. Because CA180 killed macrophages rapidly, we have also postulated that the rough mutants are killed when exposed to complement and other bactericidal factors after the infected macrophages lyse. However, reports of resistance of *B. melitensis* rough mutants to complement-mediated lysis (16) suggests a different possible fate for such organisms. The fact that rough organisms are rarely identified in clinical specimens suggests that spontaneously occurring rough derivatives may be similar to *B. abortus* RB51 or *B. canis*, but characterization of such mutants does not support this contention. In fact, the in vivo appearance of rough mutants mirrors their frequency of appearance in vitro (J. Turse and T. A. Ficht, unpublished results).

Finally, it is important to note that the O-antigen structures for these mutants or any of those described in the literature are incomplete. The differences in the results obtained in several laboratories underscores the need to physically and chemically characterize the O antigen and even the complete phenotype of each mutant to obtain a better understanding of the contribution of LPS to the virulence of *Brucella* species.

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