INFECTION AND IMMUNITY, May 2006, p. 2667–2675 Vol. 74, No. 5
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.5.2667–2675.2006

Brucella abortus Rough Mutants Induce Macrophage Oncosis That Requires Bacterial Protein Synthesis and Direct Interaction with the Macrophage

Jianwu Pei, Joshua E. Turse, Qingmin Wu, and Thomas A. Ficht*

Department of Veterinary Pathobiology, Texas A&M University and Texas Agricultural Experiment Station, College Station, Texas 77843-4467

Received 21 November 2005/Returned for modification 2 January 2006/Accepted 1 February 2006

Previous studies suggest that smooth *Brucella* organisms inhibit macrophage apoptosis. In contrast, necrotic cell death of macrophages infected with rough *Brucella* organisms in vitro has been reported, which may in part explain the failure of some rough organisms to thrive. To characterize these potential macrophage killing mechanisms, J774.A1 murine macrophages were infected with *Brucella abortus* S2308-derived rough mutant CA180. Electron microscopic analysis and polyethylene glycol protection assays revealed that the cells were killed as a result of necrosis and oncosis. This killing was shown to be unaffected by treatment with carbenicillin, an inhibitor of bacterial cell wall biosynthesis and, indirectly, replication. In contrast, chloramphenicol treatment of macrophages infected at multiplicities of infection exceeding 10,000 prevented cell death, despite internalization of large numbers of bacteria. Similarly, heat-killed and gentamicin-killed CA180 did not induce cytopathic effects in the macrophage. These results suggested that killing of infected host cells requires active bacterial protein synthesis. Cytochalasin D treatment revealed that internalization of the bacteria was necessary to initiate killing. Transwell experiments demonstrated that cell death is not mediated by a diffusible product, including tumor necrosis factor alpha and nitric oxide, but does require direct contact between host and pathogen. Furthermore, macrophages preinfected with *B. abortus* S2308 or pretreated with *B. abortus* O polysaccharide did not prevent rough CA180-induced cell death. In conclusion, *Brucella* rough mutant infection induces necrotic and oncotic macrophage cell death that requires bacterial protein synthesis and direct interaction of bacteria with the target cells.

Brucella spp. are facultative intracellular bacteria that cause brucellosis in a variety of animals and undulant fever in humans. The disease is one of the most widespread zoonoses in the world, especially in developing countries (7). Six species have been described, but only *Brucella melitensis*, *B. abortus*, and *B. suis* pose a threat to public health. Therefore, these species have been classified as category B agents that can be used as biological weapon (35). Although the disease has been described in humans for more than 100 years, its virulence mechanisms remain largely undefined.

Macrophages are the primary target cells in which *Brucella* organisms multiply and cause persistent infection. In addition, the bacteria infect a variety of nonprofessional phagocytes, including NIH 3T3, HeLa, Vero, DMCK, and BHK cells (50). The bacteria also invade trophoblast cells and cause abortion in ruminants (5, 45). It is well accepted that survival of *Brucella* in host macrophages determines virulence and contributes to disease pathogenesis (41). The majority of rough *Brucella* mutants are attenuated for virulence due to reduced survival in host cells, but *B. canis* and *B. ovis* are two exceptions (11). Recent studies have revealed that rough derivatives of *Brucella* may survive and multiply in host cells (25, 26, 48, 56). The significance of these observations may reside in the nature of the genetic defects responsible for rough character. Associated with these differences are numerous studies showing that rough *Brucella* infection results in macrophage death (17, 21–23, 48). However, it is unclear how macrophages are killed and how the killing mechanisms contribute to disease pathogenesis.

As the front line of the innate immune response, macrophages ingest and kill invading pathogens, produce various cytokines, and perform antigen presentation to develop adaptive immunity. Successful pathogens must develop strategies to avoid innate and adaptive immune responses. One key strategy used by pathogens is production of specific factors that control the fate of the infected host. Some pathogens, such as *Salmonella* (8, 10, 33, 42, 46), *Shigella flexneri* (32, 61), and *Mycobacterium tuberculosis* (13), induce macrophage cell death via apoptosis or necrosis, presumably favoring bacterial spread in the host. Other pathogens, such as *Brucella* (17, 24, 29, 55, 59), *Chlamydia* (19, 20), *Bartonella henselae* (36), low-dose *Mycobacterium tuberculosis*, and *Mycobacterium bovis* (BCG) (15, 38) inhibit host cell apoptosis, favoring bacterial survival by escaping host immune surveillance.

Virulence factors that mediate cell death have been identified in some bacterial pathogens (28). The *Salmonella* pathogenicity island 1-encoded SipB protein is responsible for the induction of macrophage apoptosis after *Salmonella* infection (31, 52). In *Shigella flexneri*, IpaB protein secreted by a type III secretion system binds to and activates caspase 1, which induces host cell apoptosis (32, 60, 61). Unlike the case for other pathogenic bacteria, classical virulence factors such as exotoxins, cytolysins, capsules, fimbriae, flagella, virulence plasmids, lysogenic phages, resistance forms, and antigenic variation are

* Corresponding author. Mailing address: Department of Veterinary Pathobiology, Texas A&M University, College Station, TX 77843-4467. Phone: (979) 845-4118. Fax: (979) 862-1088. E-mail: tficht@cvm.tamu.edu.

missing from *Brucella* (27). Our recent studies have confirmed previous reports describing cell death of macrophages infected with rough *Brucella* organisms in vitro (17, 21, 23), and cell death appears to result from necrosis, not apoptosis (48). However, the mechanisms and virulence factors that mediate macrophage cell death have not been identified, and it is not clear whether this is a property that is restricted to rough organisms or is enhanced as a result of changes in the bacterial membrane.

To characterize the mechanisms and factors responsible for macrophage cell death, we used murine J774.A1 macrophages infected with the rough *Brucella* mutant CA180 as a model and demonstrated that macrophages were killed by necrotic and oncotic cell death. Macrophage killing by rough *Brucella* mutants is shown to require bacterial protein synthesis and direct interaction of live bacteria with macrophages.

MATERIALS AND METHODS

Bacterial strains. The bacteria used in these experiments include *B. abortus* virulent strain S2308; rough mutant CA180, derived from S2308 by transposon insertion in *manA*, encoding phosphomannomutase (BMEII0899) (3); CA180 complemented by pBBR1MCS6-Y::ManBA, expressing phosphomannomutase; and CA180 with empty vector pBBR1MCS6-Y. Bacteria were grown on tryptic soy agar plates with or without antibiotic, as appropriate, and prepared for inoculation as described previously (48). Killed bacteria were obtained by incubating cultures at 60°C in a water bath for 2 h or by incubating for 1 h at room temperature in medium supplemented with 50 μg/ml gentamicin. The viability of the bacteria was evaluated by growth on tryptic soy agar at 37°C for 5 days.

Cell culture and reagents. Murine macrophage-like cell line J774.A1 (ATCC TIB-67) was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 0.1 mM nonessential amino acids (complete DMEM) as previously described (48). The cells were passaged every 3 to 5 days and discarded after passage 15. Cytochalasin D and the inducible nitric oxide synthase inhibitor, L-NAME (*N*-ω-nitro-L-arginine methyl ester), were purchased from Sigma (St. Louis, MO). Goat anti-murine tumor necrosis factor alpha (TNF-α) and goat immunoglobulin G (IgG) control were purchased from PeproTech Inc. (Rocky Hill, NJ). CytoTox 96 nonradioactive cytotoxicity assay kits were purchased from Promega (Madison, Wis.). *B. abortus* O polysaccharide (OPS) was kindly provided by J. W. Cherwonogrodzky (Department of National Defense, Alberta, Canada).

Macrophage infection. Monolayers of J774.A1 macrophages cultured in 24-well plates were infected with *B. abortus* at a multiplicity of infection (MOI) of 100 as described previously (48). For mixed infections, J774.A1 cells were infected with S2308 and CA180 at a ratio of 1:1 and an MOI of 100 for each strain. For superinfection, J774.A1 macrophages were infected with S2308 at an MOI of 100. After 24 h, the cells were washed twice with DMEM to remove gentamicin and then superinfected with CA180 at an MOI of 100.

Treatment of macrophages with antibiotics and other reagents. J774.A1 macrophages cultured in 24-well plates were infected with CA180 and incubated with DMEM containing 50 μg/ml gentamicin for 1 h to kill extracellular bacteria (48). The media were replaced with complete DMEM containing chloramphenicol (30 μg/ml) or carbenicillin (50 μg/ml) before the plates were incubated for another 24 h. To inhibit bacterial uptake, macrophages were treated with cytochalasin D (2 μg/ml) 1 h prior to and during *Brucella* infection. To determine the roles of TNF-α and nitric oxide in cell death, CA180-infected macrophages were treated with L-NAME (5 to 20 mM) or goat anti-mouse TNF-α IgG (5 to 20 μg/ml). Similar concentrations of goat IgG were used as controls (34). To determine the effects of smooth *Brucella* OPS on the cytopathic effect of rough *Brucella* infection in macrophages, J774.A1 cells were treated with smooth *B. abortus* OPS (10 μg/ml) for 2 h prior to the infection with CA180 and over the course of the infection. Cytopathic cell death was determined via measurement of lactate dehydrogenase (LDH) release in the cell culture supernatants as previously described (48).

PEG protection assay. J774.A1 cells cultured in 24-well plates were infected with CA180 at an MOI of 100 as described above. Following a 1-hour incubation, growth medium was replaced with complete DMEM containing 50 μg/ml gentamicin and 30 mM of polyethylene glycol (PEG) 3350 or PEG 8000. Sucrose (30 mM) was included as a negative control. Supernatants were collected at 24 h postinfection (p.i.), and LDH release was determined.

Quantitation of cytopathic cell death. Lactate dehydrogenase released into cell culture supernatants was detected using the CytoTox 96 nonradioactive cytotoxicity assay as described previously (48). Cell death was expressed as a percentage of LDH release, which is calculated with the following formula: percentage of LDH release = 100 × (test LDH release − spontaneous release)/(maximum release − spontaneous release).

Transmission electron microscopy (TEM). J774.A1 cells cultured in six-well plates were infected with S2308 and CA180 as described above. The infected cells were fixed at various times following infection with 2% (wt/vol) glutaraldehyde, 2% (wt/vol) paraformaldehyde, 2% (wt/vol) acrolein, 1.5% (vol/vol) dimethyl sulfoxide in 0.133 mM cacodylate buffer (pH 7.4) for 16 h in 4°C. The samples were postfixed in 1% (wt/vol) osmium tetroxide and processed at the Image Analysis Laboratory in the College of Veterinary Medicine and Biomedical Sciences, Texas A&M University. Infected cells were visualized using electron microscopy (EM 10 CA high-resolution electron microscope; Carl Zeiss, Inc., Oberkochen, Germany).

Transwell experiments. J774.A1 macrophages cultured in 24-well plates were infected with CA180 at an MOI of 100. Transwells (VWR, Sugar Land, TX) were inserted and seeded with macrophages at 10^5 cells/well without infection. At 24 h p.i., culture medium was removed. Cells remaining in the 24-well plate and transwells were lysed with 1% (vol/vol) Triton X-100. The lysates were diluted with phosphate-buffered saline (pH 7.4), and LDH levels were determined as described above and previously (48). The cell number remaining was estimated using a standard curve obtained from lysates of various concentrations of J774.A1 macrophages.

Statistical analysis. Statistical significance was determined using Student's *t* test for data with two groups and one-way analysis of variance for multiple group comparison; a P value of <0.05 was considered significant.

RESULTS

Morphological features of macrophage necrosis and oncosis induced by *B. abortus* CA180 infection. Our previous studies have demonstrated that *Brucella* rough mutant infection in macrophages results in what is best described as necrotic cell death (48). To further characterize cell death, J774.A1 macrophages infected with *Brucella* strains S2308 and CA180 were visualized using TEM. S2308 multiplying in the cell was found surrounded by a clear zone. Infection with S2308 did not cause cell death despite massive bacterial multiplication (Fig. 1A). In contrast, CA180 infection resulted in macrophage cell death in the absence of large numbers of intracellular bacteria. Characteristics of CA180-infected dying cells included swollen mitochondria, leaking cytoplasmic content, and rarefied nuclei, which are classical signs of necrosis (Fig. 1B and C). In addition, we observed that some dead cells exhibited cell and organelle swelling, which are characteristics of oncosis (18, 44) (Fig. 1D, E, and F). These results confirmed our previous report that rough *Brucella* infection induces macrophage cell death through necrosis rather than apoptosis or physical destruction induced by bacterial multiplication (48). Also, in contrast to smooth infection, multiple rough bacteria appear to be contained within single membrane-bound compartments (Fig. 1B, inset), while smooth organisms were singly encased (Fig. 1A, inset) during the steps preceding cell death.

Pore formation contributes to cell death. Osmotic cell lysis caused by oncosis can be prevented by addition of carbohydrates of different sizes to the cell culture medium (43). PEGs of different molecular weights have been used to estimate the pore size on the membrane of cells infected with pathogens, such as *Legionella pneumophila* (37), *Shigella flexneri* (6), *Pseudomonas aeruginosa* (12), and *Burkholderia pseudomallei* (54). Our previous study showed that CA180 infection induced

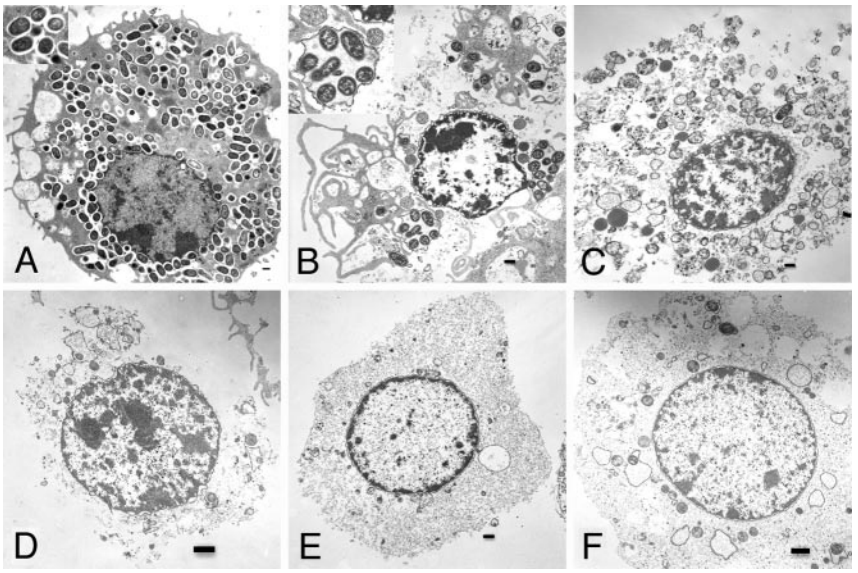

FIG. 1. Ultrastructure of macrophages infected with *Brucella*. (A) J774.A1 macrophages infected with S2308 at an MOI of 10 and fixed at 48 h p.i. (B) J774.A1 macrophages infected with CA180 at an MOI of 10 and fixed at 48 h p.i. (C to F) J774.A1 macrophages infected with CA180 at an MOI of 100 and fixed at 24 h p.i. The samples were observed using transmission electron microscopy. Bar, 1 μm.

rapid LDH release from macrophages, especially when elevated MOIs were used (48), suggesting pore formation in the host cell membrane. Transmission electron microscopic observation suggested that some of the infected cells died from oncosis (Fig. 1E and F). To confirm this hypothesis, CA180-infected J774.A1 macrophages were treated with 30 mM of either sucrose, PEG 3350, or PEG 8000. LDH release was significantly decreased with PEG 3350 treatment ($P < 0.001$) and was completely blocked in the presence of PEG 8000 ($P < 0.001$). In contrast, sucrose treatment had no effect on macrophage death (Fig. 2). These data support the hypothesis that CA180 infection induces pore formation in macrophage membranes, resulting in oncotic cell death.

Cytotoxicity induced by CA180 infection is prevented by expression of phosphomannomutase by *trans* complementation. The disruption of *manBA*, encoding phosphomannomutase, by Tn*5* transposon insertion in CA180 prevents core oligosaccharide biosynthesis (3). In an effort to restore wild-type lipopolysaccharide production, CA180 was transformed with pBBR1MCS6-Y::manBA expressing phosphomannomutase. Expression of O antigen was confirmed by Western blotting, colonial crystal violet staining, and acriflavin agglutination (data not shown). *trans* complementation of CA180 with pBBRMCS6-Y::manBA restored the smooth phenotype and prevented cytotoxicity ($P < 0.001$), while infection with CA180 transformed with the empty vector released levels of LDH identical to those released by CA180-infected macrophages ($P > 0.05$) (Fig. 3). These results demonstrate that the cytotoxicity of CA180 to macrophages is due to the failure to express phosphmannomutase in the transposon mutant.

Macrophage cell death requires viable bacteria and bacterial protein synthesis. Since macrophage cell death did not accompany massive bacterial multiplication (48), we wanted to determine if cell death was a result of direct interaction with

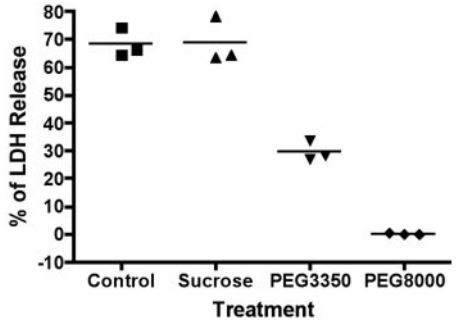

FIG. 2. CA180 infection induces pore formation on macrophage membranes. J774.A1 macrophages were infected with CA180 at an MOI of 100. The infected cells were treated with complete DMEM containing 30 mM sucrose, PEG 3350, or PEG 8000. The LDH release was detected at 24 h p.i. The results are from three independent experiments.

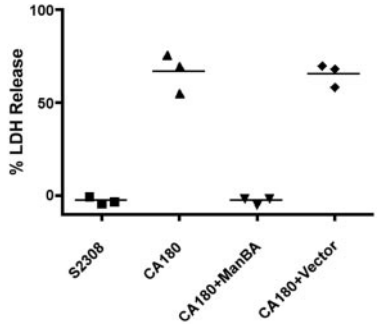

FIG. 3. Complementation of cytotoxicity induced by CA180 infection. J774.A1 macrophages were infected with S2308, CA180, complemented CA180-ManBA, and CA180 with empty vector (CA180-vector) at an MOI of 100. Cell death caused by the infection was determined by LDH release. The results are from three independent experiments.

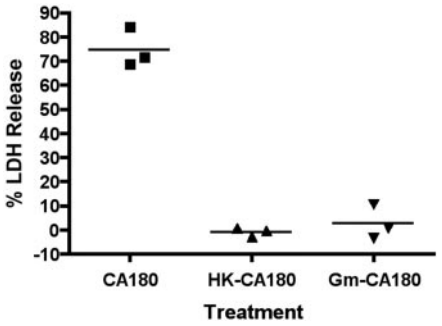

FIG. 4. Killed *Brucella* organisms do not induce macrophage cell death. J774.A1 macrophages were inoculated with heat-killed (HK-) and gentamicin-killed (Gm-) CA180 at an MOI of 100. The supernatants were collected at 24 h postinoculation, and LDH release was detected by CytoTox 96 nonradioactive cytotoxicity assay. The results are from three independent experiments.

the *Brucella* rough mutant. J774.A1 cells were inoculated with heat-killed and gentamicin-killed CA180 at an MOI of 100. Although the killed bacteria were internalized as efficiently as live bacteria as visualized by microscopic immunofluorescence (data not shown), the macrophages did not exhibit any cytopathic effect (Fig. 4). These results indicate that stimulation of the host cell does not occur due to internalization of a large number of dead bacteria; instead, the bacteria must be viable. Therefore, it was hypothesized that bacterial factors responsible for cell death are actively produced during infection.

To characterize the factors, J774.A1 macrophages were infected with CA180 at an MOI of 10 and treated with chloramphenicol and carbenicillin. Chloramphenicol treatment inhibits bacterial protein synthesis and, therefore, bacterial multiplication. Carbenicillin inhibits bacterial cell wall synthesis and replication but not protein synthesis. The use of both antibiotics helps to rule out replication as the critical factor causing cytotoxic cell death. Bacterial replication was evaluated at 24 h p.i. based on viable bacterial (CFU) recovery. In either chloramphenicol- or carbenicillin-treated cells, CA180 multiplication was undetectable (Fig. 5A). These results were confirmed by immunofluorescence staining of the infected cells (data not shown). LDH levels in cell culture supernatants revealed that chloramphenicol treatment, which inhibits bacterial protein synthesis and replication, prevented macrophage cell death (Fig. 5B). However, carbenicillin treatment, which inhibits bacteria cell wall synthesis and replication but not protein synthesis, did not completely inhibit macrophage cell death; 18.9% of

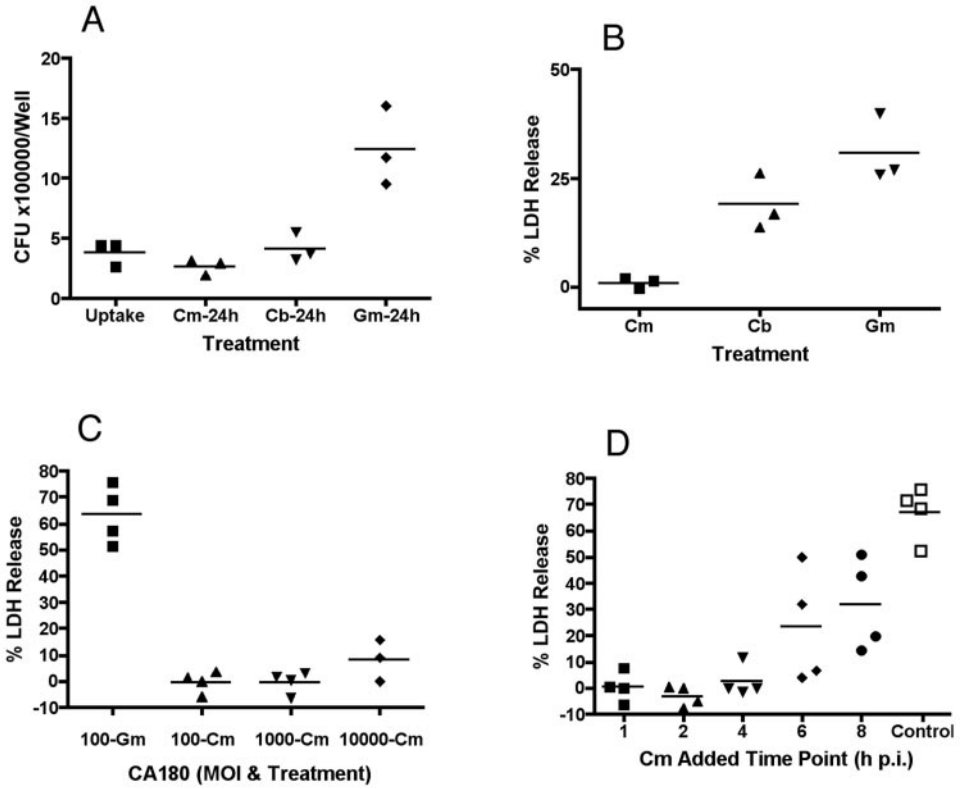

FIG. 5. Macrophage killing by CA180 infection requires bacterial protein synthesis, not multiplication. J774.A1 macrophages infected with CA180 at an MOI of 100 were incubated for 1 h with DMEM containing 40 μg/ml of gentamicin (Gm), and then the media were replaced with DMEM containing chloramphenicol (Cm) (30 μg/ml) or carbenicillin (Cb) (50 μg/ml). (A) Bacterial multiplication was determined by CFU recovery at 24 h p.i. (B) LDH levels were detected by CytoTox 96 nonradioactive cytotoxicity assay at 24 h p.i. (C) J774.A1 macrophages were infected with CA180 at an MOI of 100, 1,000, or 10,000, and the media were replaced with DMEM containing 30 μg/ml of chloramphenicol. LDH release was detected at 24 h p.i. (D) J774.A1 macrophages were infected with CA180 at an MOI of 100, and the media were replaced with DMEM containing 30 μg/ml of chloramphenicol at the indicated time points p.i.; LDH release was detected at 24 h p.i. The results are from three to four independent experiments.

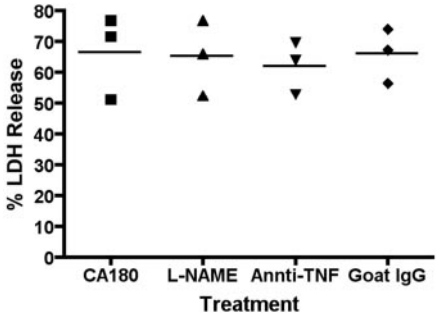

FIG. 6. Roles of TNF-α and nitric oxide in macrophage cell death induced by CA180 infection. J774.A1 macrophages were infected with CA180 at an MOI of 100 and treated with L-NAME and goat anti-mouse TNF-α. The viability of the macrophages was determined by detecting LDH release at 24 h p.i. The results are from three independent experiments.

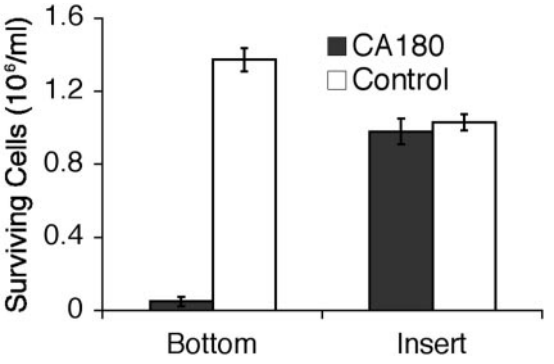

FIG. 7. Macrophage cell death induced by CA180 infection requires direct interaction of bacteria and macrophages. J774.A1 macrophages cultured in a 24-well plate were infected with CA180 at an MOI of 100. A transwell seeded with J774.A1 without infection was inserted. The surviving cells in the 24-well plate and transwells were lysed with 1% Triton X-100 at 24 h p.i. LDH release was detected, and the corresponding cell numbers were determined with a standard curve obtained from a serially diluted cell suspension with known concentrations. The data shown are representative of two independent experiments with similar results. Error bars indicate standard deviations.

LDH release was detected in carbenicillin-treated cells, compared with 30.9% in untreated cells ($P > 0.05$), in experiments performed at reduced MOIs of 10 to limit the rate at which lysis occurs (Fig. 5B). The difference in cytotoxicity observed is attributable to reduced numbers of bacteria for *Brucella* treated with carbenicillin (Fig. 5A). To further characterize the importance of protein synthesis, J774.A1 cells were infected with CA180 at an MOI of 100, 1,000, or 10,000 and treated with chloramphenicol, and LDH release in the supernatants was determined at 24 h p.i. Once again, chloramphenicol treatment prevented the cytopathic effect of CA180 infection in murine macrophages, even when cells were infected at an MOI of 10,000 (Fig. 5C). In order to rule out effects of chloramphenicol on macrophages (40), J774.A1 cells were pretreated with chloramphenicol (60 μg/ml) for 24 h and washed with DMEM prior to CA180 infection. LDH release detected at 20 h p.i. revealed that pretreatment of macrophages with chloramphenicol did not affect CA180-induced macrophage cell death (data not shown). Taken together, these data demonstrated that bacterial protein synthesis is critical for cytotoxicity.

To provide preliminary insight regarding the nature of the bacterial component responsible and the timing of its expression, chloramphenicol (30 μg/ml) was added at different time points following infection with CA180 at an MOI of 100. LDH release detected at 24 h p.i. was blocked completely by adding chloramphenicol within 4 h p.i. An increased release of LDH (23.2%) was detected when chloramphenicol was added at 6 h p.i.; however, this value is not significantly different from LDH release at the earlier time points ($P > 0.05$). Chloramphenicol addition at 8 h p.i. resulted in 32.0% release of LDH, which is significantly different from release at earlier time points ($P < 0.05$) but not significantly different from LDH release from gentamicin-treated control macrophages ($P > 0.05$) (Fig. 5D). These results suggest that the factor(s) was produced or accumulated to effective levels by approximately 6 to 8 h p.i., a time corresponding to a point when virulent *Brucella* organisms have theoretically adjusted to the intracellular environment (9).

Macrophage activation is not the cause of cell death. It has been shown that rough *Brucella* infection activates macrophages and induces proinflammatory cytokine production, including that of nitric oxide and TNF-α (34, 51). TNF-α and nitric oxide are produced as a result of CA180 infection in J774.A1 macrophages (data not shown). The possibility existed that necrotic cell death was mediated by TNF-α induced by CA180 infection in an autocrine/paracrine fashion or that cell death was due to hyperactivation of the macrophages by nitric oxide. To rule out these possibilities, J774.A1 cells were infected with CA180 at an MOI of 100 and treated with L-NAME or goat anti TNF-α (34). LDH release detected at 24 h p.i. revealed that L-NAME and anti-TNF-α antibody treatments did not affect macrophage cell death (Fig. 6), suggesting that TNF-α and nitric oxide were not involved in the cytotoxicity.

Cytotoxic cell death induced by *Brucella* requires macrophage uptake. The previous sections reveal that macrophage killing induced by CA180 infection required bacterial protein synthesis but not TNF-α and nitric oxide. To determine whether the unknown protein factors were released from the infected cells, a transwell study was performed (Fig. 7). The numbers of live cells recovered from infected and uninfected bottom wells were $0.052 \times 10^6 \pm 0.027 \times 10^6$/ml and $1.377 \times 10^6 \pm 0.065 \times 10^6$/ml, respectively; 96% of the cells were killed by CA180 infection. However, the live cell numbers in the insert wells with infected and uninfected bottom wells were $0.983 \times 10^6 \pm 0.07 \times 10^6$/ml and $1.033 \times 10^6 \pm 0.045 \times 10^6$/ml, respectively ($P > 0.05$) (Fig. 7). These results demonstrate that the unknown macrophage killing factors were not able to diffuse from the infected cell layer. This result was consistent with the observation of individual dead cells in monolayers infected with CA180 at an MOI of 10 (data not shown).

It has been well established that cytochalasin D inhibits actin polymerization and therefore bacterial invasion of mammalian cells (2, 30). Invasion of macrophages by *Brucella* can be similarly inhibited by cytochalasin D treatment (39). To determine whether the killing mechanisms require internalization of the bacteria, J774.A1 cells were pretreated with cytochalasin D (2 μg/ml) for 1 h before CA180 infection at an MOI of 50 or 500.

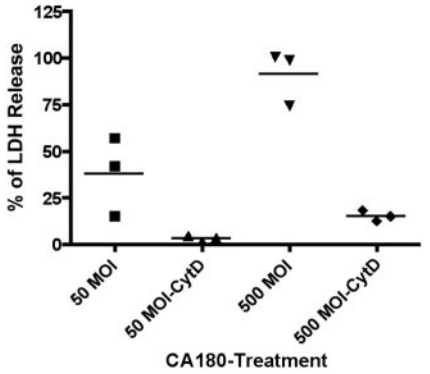

FIG. 8. Internalization of bacteria is necessary for macrophage cell death. J774.A1 cells were treated with cytochalasin D (2 μg/ml) for 1 h prior to infection with CA180 at an MOI of 50 or 500. LDH release was detected at 24 h p.i. The data shown are from three independent experiments.

The infected cells were incubated with DMEM containing gentamicin (40 μg/ml) for 24 h, and LDH release in the supernatants was detected. Cytochalasin D treatment dramatically reduced cell death, showing that bacterial internalization is required to initiate the killing mechanisms (Fig. 8).

Smooth *Brucella* infection and smooth *Brucella* OPS do not inhibit CA180 infection-induced macrophage cell death. It has been reported that smooth *Brucella* infection inhibits apoptosis in macrophages in vitro and in vivo (17, 24, 29, 55, 59). Although antiapoptotic activity of *Brucella* in J774.A1 macrophages has not been reported, infection with an MOI of 100 of S2308 did not result in J774.A1 cell death (48). Our recent study also showed that smooth *Brucella* organisms do not activate macrophages in terms of NF-κB p65 translocation and cytokine and nitric oxide production (unpublished results). To determine whether smooth *Brucella* infection prevents induction of cell death by rough *Brucella* infection, J774.A1 macrophages were infected with a mixture of S2308 and CA180 at an MOI of 100 for each strain. Previous results indicate that 40% of the cells are infected with smooth organisms under these conditions and 100% of the cells are infected with rough organisms. Infection with either organism has no detectable effect on the uptake of reinfecting or superinfecting organisms (data not shown). LDH detection at 24 h p.i. revealed that S2308 infection did not prevent CA180-induced cell death (Fig. 9A). Because S2308 invades macrophages in reduced numbers compared with CA180 (48), many macrophages may be killed by CA180 before the smooth organisms multiply. To overcome this potential problem, we infected J774.A1 cells with S2308 at an MOI of 100 and 24 h later superinfected the cells with CA180. The results demonstrate that smooth *Brucella* multiplication did not inhibit rough *Brucella*-induced cell death (Fig. 9B). Similarly, *Brucella* OPS treatment of the macrophages prior to infection with CA180 did not interfere with cell death (Fig. 9C).

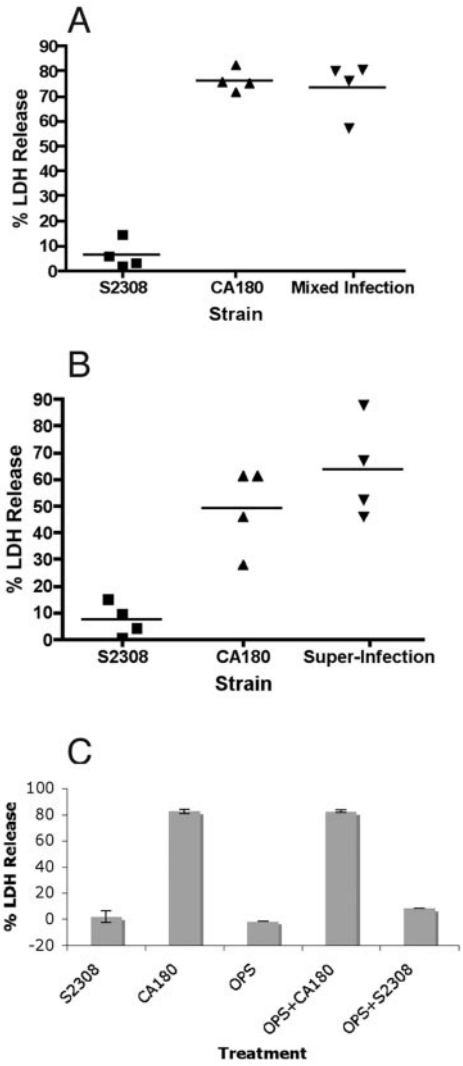

FIG. 9. Smooth *Brucella* infection and multiplication in murine macrophages or *Brucella* OPS treatment does not prevent CA180 infection-induced cell death. J774.A1 macrophages cultured on 24-well plates were infected with a mixture of S2308 and CA180 at an MOI of 100 (A), infected with S2308 and superinfected with CA180 at 24 h p.i. (B), or treated with *B. abortus* OPS (10 μg/ml) and infected with CA180 (C). LDH release was detected at 24 h p.i. The data shown are from four independent experiments. Error bars indicate standard deviations.

DISCUSSION

It has been demonstrated that some rough derivatives of *B. abortus*, *B. suis*, and *B. melitensis* are cytotoxic to macrophages (17, 21–23, 48). However, the mechanisms behind this killing have not been investigated. Unlike other pathogenic bacteria, *Brucella* appears to have few, if any, classical virulence factors (27). Although putative virulence factors have been identified as a result of *Brucella* genomic sequence analysis, most remain to be verified (14). In this report, we demonstrate for the first time that *Brucella* rough mutant CA180 infection in macrophages produces a factor(s) that causes necrotic and oncotic cell death. This factor is not readily diffusible based on transwell experiments and microscopic observation of infected cultures. Infection with heat-killed or gentamicin-killed bacteria did not cause cell death, suggesting that the factor is actively produced by viable bacteria. Infection of macrophages with CA180 at elevated MOIs (up to 10,000) followed by treatment

with chloramphenicol revealed that the killing mechanism(s) requires active bacterial protein synthesis. Macrophages were not killed if bacterial protein synthesis was inhibited within the first 4 h p.i., indicating that factors induced by *Brucella* infection within 4 h are not sufficient to kill the cells or that the factors are not synthesized until later in infection. Treatment of infected cells with carbenicillin did not significantly prevent macrophage cell death, while bacterial multiplication was inhibited, suggesting a direct link between cell death and bacterial protein synthesis. The difference in LDH release between carbenicillin-treated and gentamicin-treated cells was attributable to bacterial growth in gentamicin-treated controls. Furthermore, cytochalasin D pretreatment significantly inhibits cell death, demonstrating that bacterial internalization is necessary for the killing observed.

Recent studies show that chloramphenicol prevents cell apoptosis through a p21-dependent pathway (40). Our data demonstrate that CA180-infected macrophages were killed by oncosis and necrosis, not by apoptosis. However, to rule out the possibility that chloramphenicol antiapoptotic effects prevent macrophage killing, CA180 was pretreated with chloramphenicol (60 μg/ml) and washed with PBS before infecting macrophages. Since chloramphenicol is a bacteriostatic reagent, the bacteria will be revived after removal. Therefore, chloramphenicol-treated CA180 still induces macrophage cell death (data not shown). In the macrophage pretreatment experiment, chloramphenicol has to be removed to ensure proper CA180 infection. However, antiapoptotic effects should be prolonged, since the half-life of p21 mRNA is 18 h after chloramphenicol is removed (40). Under the current experimental conditions, CA180-induced cell death can be detected in chloramphenicol-pretreated cells within 24 h or less, depending on the MOI, and is not significantly different from that of untreated cells, suggesting that chloramphenicol antiapoptotic activity is not involved in the cell killing mechanisms.

Four pathways leading to cell death have been described to date: apoptosis, autophagy, oncosis, and pyroptosis (18). Oncosis is a prelethal pathway leading to cell death characterized by cell and organelle swelling, cell blebbing, and increased membrane permeability (18, 44). A variety of pathogenic microorganisms have been shown to cause host cell oncosis, including bacteria (6, 12, 37, 54) and viruses (49). In the current study, TEM studies showed that, in addition to necrosis, CA180-infected macrophages underwent oncosis with characteristics similar to those described above, which confirmed previous reports that infected cells were not killed via apoptosis (48). PEG protection assays suggested that CA180 infection induces pore formation in the macrophage membrane. These data revealed for the first time that *Brucella* rough mutants, or at least those defective in phosphomannomutase, induced pore formation in murine macrophages and caused oncotic cell death.

CA180 is a deep rough mutant derived from S2308 with a Tn*5* insertion in the gene encoding phosphmannomutase, a key enzyme involved in O-antigen biosynthesis (3). Without O-antigen expression, some structures of the bacterial surface may be exposed or altered. Consequently the interaction between bacteria and host cells may be altered. However, the results shown demonstrate that contact of the rough bacteria with host cells was insufficient to kill the cells; instead, the organisms must be internalized. Furthermore, if bacterial protein synthesis is inhibited with chloramphenicol, despite the presence of a large number of internalized organisms, the macrophage will not be killed. These data indicate that cell death did not result from the interaction of preexisting bacterial factors or structural components with J774.A1 macrophages; the rough bacteria must actively synthesize proteins after internalization. The obvious questions are whether smooth *Brucella* organisms produce such factors and whether they are important for virulence. One possibility is that production of the factor is suppressed or masked in smooth *Brucella* as a result of O-antigen expression. Therefore, the factor may be produced only by rough *Brucella* and be directly related to the structure of O antigen. This hypothesis is supported by the observation that different rough *Brucella* mutants have different levels of cytotoxicity which are related to their rough character (48). Proteomic studies and microarray analysis will help to address this possibility. A second possibility is that the factor is produced by smooth *Brucella* but O antigen inhibits the macrophage killing mechanisms. This hypothesis is supported by the facts that smooth *Brucella* organisms do not activate macrophages (data not shown), but they do inhibit macrophage or monocyte apoptosis (17, 24, 29, 55, 59). Although there have been no reports of antiapoptotic activity in J774.A1 after infection with smooth *Brucella*, our studies have shown that S2308 infection at an MOI of 100 does not induce J774.A1 macrophage cell death (48). In contrast, mixed infection and superinfections with S2308 or exposure to purified OPS demonstrated no direct effect of smooth organisms or OPS on cell death induced by rough organisms. These observations are consistent with the idea that the factor is neither produced in macrophages infected with smooth *Brucella* nor inhibited by their presence. These results support the hypothesis that spontaneous rough mutant generation may induce cell death and promote *Brucella* spread.

To rule out the possible role of transposon in macrophage cell death and demonstrate that the cell death is due to gene inactivation, we complemented CA180 in *trans* with the phosphmannomutase-expressing plasmid pBBR1MCS6-Y. The complemented CA180 did not induce cell death, indicating that phosphmannomutase gene knockout-induced roughness is responsible for the cell killing.

Although some rough strains of *B. abortus*, *B. suis*, and *B. melitensis* have been demonstrated to be cytotoxic to macrophages in culture (17, 21–23, 48), we found that not all rough *Brucella* mutants are cytotoxic to macrophages (data not shown). The outcomes of infection are most likely determined by the balance between cell killing mechanisms and survival ability of rough *Brucella* in host cells. If the organisms can reach replication niches and survive, the host cells will be killed. Otherwise, the bacteria will be cleared by the host cells.

In the current report, we hypothesize that *Brucella* rough mutant-caused cell death is mediated by a nonsecreted factor(s) induced following macrophage infection. Expression of this factor is not prevented by smooth *Brucella* infection and multiplication. Perhaps it is important that the factor is not secreted to avoid massive destruction of macrophages and induction of strong immune responses. In this case infected macrophage will not be lysed simultaneously; only a few cells containing sufficient rough organisms will be lysed. Therefore, an

appropriate balance between pathogen and host will be maintained.

It is generally accepted that *Brucella* rough mutants are attenuated in animal hosts because they are sensitive to killing factors in body fluids, with the obvious exceptions of the natural rough species *B. ovis* and *B. canis*, which are virulent in their natural hosts (11). In addition, Fernandez-Prada et al. have also demonstrated that rough *B. melitensis* organisms are not sensitive to complement-mediated lysis (16, 17). In the TEM study of *Brucella*-infected cells, we observed that rough organisms were enclosed within a membrane prior to cell lysis, suggesting that rough *Brucella* may be resistant to killing factors in vivo. The reason for the observed attenuation of rough *Brucella* mutants in animal models may be that, after intraperitoneal inoculation, the bacteria are killed by factors in ascitic fluid prior to invasion (unpublished result). Furthermore, bacteria wrapped in cellular membrane may be easily recognized as cell debris and taken up quickly by macrophages, initiating another round of infection.

Salmonella infection induces macrophage and dendritic cell death by a mechanism that depends on SipB and the type III secretion system (57, 58). Although possible type III secretion gene homologs have been identified in *Brucella* recently, it is not clear whether they are functional (1). It has been demonstrated that a type IV secretion system is present in *Brucella* (47); the virulence factors secreted by the type IV secretion system have not yet been identified. Pore formation is a characteristic property of the Dot/Icm system in *Legionella* and causes a similar oncotic cell death in macrophages (4, 53). However, there are no reports of enhanced activity in the absence of *Legionella* O antigen. The significance of this difference is unknown; however, cytotoxicity is not consistently observed with all rough *Brucella* organisms. Thus, it appears likely that other factors are involved and that their expression and character may change independently of changes in lipopolysaccharide structure.

ACKNOWLEDGMENTS

We thank Allison Rice-Ficht for her critical reading of the manuscript and Helga Sittertz-Bhatkar from Image Analysis Laboratory for her excellent technical help with electron microscopy analysis.

This work was supported by grants to T.A.F. from USDA/CSREES (99-35204-7550) and NIH/AID (AI48496).

REFERENCES

1. **Abdallah, A. I., N. J. Commander, M. J. Woodward, S. Spencer, C. A. Hart, and C. Winstanley.** 2003. Type III secretion homologs are present in *Brucella melitensis*, *B. ovis*, and *B. suis* biovars 1, 2, and 3. Curr. Microbiol. **46:**241–245.
2. **Aderem, A., and D. M. Underhill.** 1999. Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. **17:**593–623.
3. **Allen, C. A., L. G. Adams, and T. A. Ficht.** 1998. Transposon-derived *Brucella abortus* rough mutants are attenuated and exhibit reduced intracellular survival. Infect. Immun. **66:**1008–1016.
4. **Alli, O. A., L. Y. Gao, L. L. Pedersen, S. Zink, M. Radulic, M. Doric, and Y. Abu Kwaik.** 2000. Temporal pore formation-mediated egress from macrophages and alveolar epithelial cells by *Legionella pneumophila*. Infect. Immun. **68:**6431–6440.
5. **Anderson, T. D., and N. F. Cheville.** 1986. Ultrastructural morphometric analysis of *Brucella abortus*-infected trophoblasts in experimental placentitis. Bacterial replication occurs in rough endoplasmic reticulum. Am. J. Pathol. **124:**226–237.
6. **Blocker, A., P. Gounon, E. Larquet, K. Niebuhr, V. Cabiaux, C. Parsot, and P. Sansonetti.** 1999. The tripartite type III secreton of *Shigella flexneri* inserts IpaB and IpaC into host membranes. J. Cell Biol. **147:**683–693.
7. **Boschiroli, M. L., V. Foulongne, and D. O'Callaghan.** 2001. Brucellosis: a worldwide zoonosis. Curr. Opin. Microbiol. **4:**58–64.
8. **Brennan, M. A., and B. T. Cookson.** 2000. *Salmonella* induces macrophage death by caspase-1-dependent necrosis. Mol. Microbiol. **38:**31–40.
9. **Celli, J., C. de Chastellier, D. M. Franchini, J. Pizarro-Cerda, E. Moreno, and J. P. Gorvel.** 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. **198:**545–556.
10. **Chen, L. M., K. Kaniga, and J. E. Galan.** 1996. *Salmonella* spp. are cytotoxic for cultured macrophages. Mol. Microbiol. **21:**1101–1115.
11. **Corbel, M. J., and E. L. Thomas.** 1985. Use of phage for the identification of *Brucella canis* and *Brucella ovis* cultures. Res. Vet. Sci. **38:**35–40.
12. **Dacheux, D., J. Goure, J. Chabert, Y. Usson, and I. Attree.** 2001. Pore-forming activity of type III system-secreted proteins leads to oncosis of *Pseudomonas aeruginosa*-infected macrophages. Mol. Microbiol. **40:**76–85.
13. **Danelishvili, L., J. McGarvey, Y. J. Li, and L. E. Bermudez.** 2003. *Mycobacterium tuberculosis* infection causes different levels of apoptosis and necrosis in human macrophages and alveolar epithelial cells. Cell Microbiol. **5:**649–660.
14. **DelVecchio, V. G., V. Kapatral, R. J. Redkar, G. Patra, C. Mujer, T. Los, N. Ivanova, I. Anderson, A. Bhattacharyya, A. Lykidis, G. Reznik, L. Jablonski, N. Larsen, M. D'Souza, A. Bernal, M. Mazur, E. Goltsman, E. Selkov, P. H. Elzer, S. Hagius, D. O'Callaghan, J. J. Letesson, R. Haselkorn, N. Kyrpides, and R. Overbeek.** 2002. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc. Natl. Acad. Sci. USA **99:**443–448.
15. **Durrbaum-Landmann, I., J. Gercken, H. D. Flad, and M. Ernst.** 1996. Effect of in vitro infection of human monocytes with low numbers of *Mycobacterium tuberculosis* bacteria on monocyte apoptosis. Infect. Immun. **64:**5384–5389.
16. **Fernandez-Prada, C. M., M. Nikolich, R. Vemulapalli, N. Sriranganathan, S. M. Boyle, G. G. Schurig, T. L. Hadfield, and D. L. Hoover.** 2001. Deletion of *wboA* enhances activation of the lectin pathway of complement in *Brucella abortus* and *Brucella melitensis*. Infect. Immun. **69:**4407–4416.
17. **Fernandez-Prada, C. M., E. B. Zelazowska, M. Nikolich, T. L. Hadfield, R. M. Roop II, G. L. Robertson, and D. L. Hoover.** 2003. Interactions between *Brucella melitensis* and human phagocytes: bacterial surface O polysaccharide inhibits phagocytosis, bacterial killing, and subsequent host cell apoptosis. Infect. Immun. **71:**2110–2119.
18. **Fink, S. L., and B. T. Cookson.** 2005. Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells. Infect. Immun. **73:**1907–1916.
19. **Fischer, S. F., C. Schwarz, J. Vier, and G. Hacker.** 2001. Characterization of antiapoptotic activities of *Chlamydia pneumoniae* in human cells. Infect. Immun. **69:**7121–7129.
20. **Fischer, S. F., J. Vier, S. Kirschnek, A. Klos, S. Hess, S. Ying, and G. Hacker.** 2004. *Chlamydia* inhibit host cell apoptosis by degradation of proapoptotic BH3-only proteins. J. Exp. Med. **200:**905–916.
21. **Freeman, B. A., D. J. Kross, and R. Circo.** 1961. Host-parasite relationships in brucellosis. II. Destruction of macrophage cultures by *Brucella* of different virulence. J. Infect. Dis. **108:**333–338.
22. **Freeman, B. A., G. Pearson, and W. D. Hines.** 1964. Host-parasite relationships in brucellosis. III. Behavior of avirulent *Brucella* in tissue culture monocytes. J. Infect. Dis. **114:**441–449.
23. **Freeman, B. A., and B. H. Rumack.** 1964. Cytopathogenic effect of *Brucella* spheroplasts on monocytes in tissue culture. J. Bacteriol. **88:**1310–1315.
24. **Galdiero, E., C. Romano Carratelli, M. Vitiello, I. Nuzzo, E. Del Vecchio, C. Bentivoglio, G. Perillo, and F. Galdiero.** 2000. HSP and apoptosis in leukocytes from infected or vaccinated animals by *Brucella abortus*. New Microbiol. **23:**271.
25. **Godfroid, F., A. Cloeckaert, B. Taminiau, I. Danese, A. Tibor, X. de Bolle, P. Mertens, and J. J. Letesson.** 2000. Genetic organisation of the lipopolysaccharide O-antigen biosynthesis region of *Brucella melitensis* 16M (wbk). Res. Microbiol. **151:**655–668.
26. **Godfroid, F., B. Taminiau, I. Danese, P. Denoel, A. Tibor, V. Weynants, A. Cloeckaert, J. Godfroid, and J. J. Letesson.** 1998. Identification of the perosamine synthetase gene of *Brucella melitensis* 16M and involvement of lipopolysaccharide O side chain in *Brucella* survival in mice and in macrophages. Infect. Immun. **66:**5485–5493.
27. **Gorvel, J. P., and E. Moreno.** 2002. *Brucella* intracellular life: from invasion to intracellular replication. Vet. Microbiol. **90:**281–297.
28. **Grassme, H., V. Jendrossek, and E. Gulbins.** 2001. Molecular mechanisms of bacteria induced apoptosis. Apoptosis **6:**441–445.
29. **Gross, A., A. Terraza, S. Ouahrani-Bettache, J. P. Liautard, and J. Dornand.** 2000. In vitro *Brucella suis* infection prevents the programmed cell death of human monocytic cells. Infect. Immun. **68:**342–351.
30. **Gruenheid, S., and B. B. Finlay.** 2003. Microbial pathogenesis and cytoskeletal function. Nature **422:**775–781.
31. **Hersh, D., D. M. Monack, M. R. Smith, N. Ghori, S. Falkow, and A. Zychlinsky.** 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. Proc. Natl. Acad. Sci. USA **96:**2396–2401.
32. **Hilbi, H., J. E. Moss, D. Hersh, Y. Chen, J. Arondel, S. Banerjee, R. A. Flavell, J. Yuan, P. J. Sansonetti, and A. Zychlinsky.** 1998. *Shigella*-induced apoptosis is dependent on caspase-1 which binds to IpaB. J. Biol. Chem. **273:**32895–32900.
33. **Jesenberger, V., K. J. Procyk, J. Yuan, S. Reipert, and M. Baccarini.** 2000.

Salmonella-induced caspase-2 activation in macrophages: a novel mechanism in pathogen-mediated apoptosis. J. Exp. Med. **192:**1035–1046.
34. **Jimenez De Bagues, M. P., A. Terraza, A. Gross, and J. Dornand.** 2004. Different responses of macrophages to smooth and rough *Brucella* spp.: relationship to virulence. Infect. Immun. **72:**2429–2433.
35. **Kaufmann, A. F., M. I. Meltzer, and G. P. Schmid.** 1997. The economic impact of a bioterrorist attack: are prevention and postattack intervention programs justifiable? Emerg. Infect. Dis. **3:**83–94.
36. **Kempf, V. A., A. Schairer, D. Neumann, G. A. Grassl, K. Lauber, M. Lebied-ziejewski, M. Schaller, P. Kyme, S. Wesselborg, and I. B. Autenrieth.** 2005. *Bartonella henselae* inhibits apoptosis in Mono Mac 6 cells. Cell Microbiol. **7:**91–104.
37. **Kirby, J. E., J. P. Vogel, H. L. Andrews, and R. R. Isberg.** 1998. Evidence for pore-forming ability by *Legionella pneumophila*. Mol. Microbiol. **27:**323–336.
38. **Kremer, L., J. Estaquier, E. Brandt, J. C. Ameisen, and C. Locht.** 1997. *Mycobacterium bovis* bacillus Calmette Guerin infection prevents apoptosis of resting human monocytes. Eur. J. Immunol. **27:**2450–2456.
39. **Kusumawati, A., C. Cazevieille, F. Porte, S. Bettache, J. P. Liautard, and J. Sri Widada.** 2000. Early events and implication of F-actin and annexin I associated structures in the phagocytic uptake of *Brucella suis* by the J-774A.1 murine cell line and human monocytes. Microb. Pathog. **28:**343–352.
40. **Li, C. H., S. L. Tzeng, Y. W. Cheng, and J. J. Kang.** 2005. Chloramphenicol-induced mitochondrial stress increases p21 expression and prevents cell apoptosis through a p21-dependent pathway. J. Biol. Chem. **280:**26193–26199.
41. **Liautard, J. P., A. Gross, J. Dornand, and S. Kohler.** 1996. Interactions between professional phagocytes and *Brucella* spp. Microbiologia **12:**197–206.
42. **Lindgren, S. W., I. Stojiljkovic, and F. Heffron.** 1996. Macrophage killing is an essential virulence mechanism of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **93:**4197–4201.
43. **Lobo, A. L., and R. A. Welch.** 1994. Identification and assay of RTX family of cytolysins. Methods Enzymol. **235:**667–678.
44. **Majno, G., and I. Joris.** 1995. Apoptosis, oncosis, and necrosis. An overview of cell death. Am. J. Pathol. **146:**3–15.
45. **Meador, V. P., and B. L. Deyoe.** 1989. Intracellular localization of *Brucella abortus* in bovine placenta. Vet. Pathol. **26:**513–515.
46. **Monack, D. M., B. Raupach, A. E. Hromockyj, and S. Falkow.** 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. Proc. Natl. Acad. Sci. USA **93:**9833–9838.
47. **O'Callaghan, D., C. Cazevieille, A. Allardet-Servent, M. L. Boschiroli, G. Bourg, V. Foulongne, P. Frutos, Y. Kulakov, and M. Ramuz.** 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol. Microbiol. **33:**1210–1220.
48. **Pei, J., and T. A. Ficht.** 2004. *Brucella abortus* rough muntants are cytopathic for macrophages in culture. Infect. Immun. **72:**440–450.
49. **Perez, J. F., M. E. Chemello, F. Liprandi, M. C. Ruiz, and F. Michelangeli.** 1998. Oncosis in MA104 cells is induced by rotavirus infection through an increase in intracellular Ca2+ concentration. Virology **252:**17–27.
50. **Pizarro-Cerda, J., E. Moreno, and J. P. Gorvel.** 2000. Invasion and intracellular trafficking of *Brucella abortus* in nonphagocytic cells. Microbes Infect. **2:**829–835.
51. **Rittig, M. G., A. Kaufmann, A. Robins, B. Shaw, H. Sprenger, D. Gemsa, V. Foulongne, B. Rouot, and J. Dornand.** 2003. Smooth and rough lipopolysaccharide phenotypes of *Brucella* induce different intracellular trafficking and cytokine/chemokine release in human monocytes. J. Leukoc Biol. **74:**1045–1055.
52. **Santos, R. L., R. M. Tsolis, A. J. Baumler, R. Smith III, and L. G. Adams.** 2001. *Salmonella enterica* serovar Typhimurium induces cell death in bovine monocyte-derived macrophages by early SipB-dependent and delayed SipB-independent mechanisms. Infect. Immun. **69:**2293–2301.
53. **Sexton, J. A., H. J. Yeo, and J. P. Vogel.** 2005. Genetic analysis of the *Legionella pneumophila* DotB ATPase reveals a role in type IV secretion system protein export. Mol. Microbiol. **57:**70–84.
54. **Sun, G. W., J. Lu, S. Pervaiz, W. P. Cao, and Y. H. Gan.** 2005. Caspase-1 dependent macrophage death induced by *Burkholderia pseudomallei*. Cell Microbiol. **7:**1447–1458.
55. **Tolomeo, M., P. D. Carlo, V. Abbadessa, L. Titone, S. Miceli, E. Barbusca, G. Cannizzo, S. Mancuso, S. Arista, and F. Scarlata.** 2003. Monocyte and lymphocyte apoptosis resistance in acute and chronic brucellosis and its possible implications in clinical management. Clin. Infect. Dis. **36:**1533–1538.
56. **Ugalde, J. E., C. Czibener, M. F. Feldman, and R. A. Ugalde.** 2000. Identification and characterization of the *Brucella abortus* phosphoglucomutase gene: role of lipopolysaccharide in virulence and intracellular multiplication. Infect. Immun. **68:**5716–5723.
57. **van der Velden, A. W., S. W. Lindgren, M. J. Worley, and F. Heffron.** 2000. *Salmonella* pathogenicity island 1-independent induction of apoptosis in infected macrophages by *Salmonella enterica* serotype Typhimurium. Infect. Immun. **68:**5702–5709.
58. **van der Velden, A. W., M. Velasquez, and M. N. Starnbach.** 2003. *Salmonella* rapidly kill dendritic cells via a caspase-1-dependent mechanism. J. Immunol. **171:**6742–6749.
59. **Wang, M., N. Qureshi, N. Soeurt, and G. Splitter.** 2001. High levels of nitric oxide production decrease early but increase late survival of *Brucella abortus* in macrophages. Microb. Pathog. **31:**221–230.
60. **Zychlinsky, A., B. Kenny, R. Menard, M. C. Prevost, I. B. Holland, and P. J. Sansonetti.** 1994. IpaB mediates macrophage apoptosis induced by *Shigella flexneri*. Mol. Microbiol. **11:**619–627.
61. **Zychlinsky, A., M. C. Prevost, and P. J. Sansonetti.** 1992. *Shigella flexneri* induces apoptosis in infected macrophages. Nature **358:**167–169.

Editor: D. L. Burns