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Necrotic Death of *Rhodococcus equi*-Infected Macrophages Is Regulated by Virulence-Associated Plasmids†

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***Rhodococcus equi* is a gram-positive intracellular pathogen that can cause severe bronchopneumonia in foals and AIDS patients. It has been reported that advanced infection of foals is characterized by tissue necrosis, coinciding with the presence of degenerate bacteria-laden macrophages. Here, we report that the possession of the VapA-expressing plasmid, which has been previously correlated with a high level of virulence for foals and mice, strongly increases cytotoxicity of *R. equi* for murine macrophage-like (J774E) cells. Isolates containing different, VapB-expressing plasmids are less virulent and also have a lower cytotoxic potential. Isogenic strains lacking either plasmid are avirulent and have a very low cytotoxic potential. We show, using fluorescence-activated cell sorter analysis (annexin V/7-amino-actinomycin D and sub-G_1-analysis), Western blotting [poly-(ADP-ribose) polymerase processing analysis], and electron microscopy (macrophage and nucleus morphologies) that the deaths of murine macrophages are the result of necrotic rather than apoptotic events. We demonstrate that the bacteria must be alive in order to act cytotoxic. Therefore, one effect of the virulence-associated plasmids during infection with *R. equi* is the promotion of necrotic damage to the host.**

Rhodococcus equi is a nocardioform gram-positive coccobacillus and an important foal pathogen producing severe pyogranulomatous pneumonia in very young horses (24, 38). Infection usually occurs via the respiratory tract. In addition to being a foal pathogen, *R. equi* can infect AIDS patients, and more than 100 cases in which patients showed symptoms and histopathology similar to those seen in infected foals and leading to death in ~50% of the individuals have been documented (8, 29, 59). Furthermore, a low number of cases that occurred in immunocompetent humans with an ~11% mortality rate have been reported (29). In addition to their own importance in human and veterinary medicine, these bacteria have a close phylogenetic relationship to mycobacteria and, hence, to such important pathogens as *Mycobacterium tuberculosis* (causing human tuberculosis) and *Mycobacterium leprae* (causing leprosy). Members of both genera, *Mycobacterium* and *Rhodococcus*, are largely soil inhabitants and possess complex wax-like cell walls (4) enriched in lipoarabinomannans (41), in mycolic acids, and in mycolic-acid derived compounds such as cord factor (trehalose dimycolate). Also, both *M. tuberculosis* and *R. equi* can produce caseous granulomas and cavitary pneumonia, demonstrating not only phylogenetic but also pathogenetic relatedness. Not surprisingly, some human infections with rhodococci have been mistaken for tuberculosis (55).

Due to its capability to survive and multiply in murine and equine macrophages, *R. equi* has been classified as a facultative intracellular bacterium (25). Accordingly, *R. equi* is found frequently in macrophages in vivo (24), although it does not seem to enter pulmonary epithelial cells. It has been reported that *R. equi* interferes with the maturation of its phagosomes to phagolysosomes in primary foal alveolar macrophages (20, 62), that some virulent *R. equi* bacteria are able to destroy host cells (20, 37), and that virulent or avirulent *R. equi* induces similar levels and types of cytokines in infected murine macrophages (17). Still, almost nothing is known about the molecular mechanisms underlying the pathogenicity of *R. equi*.

Major progress was made recently when it was found that almost all clinical *R. equi* isolates from foals contain a virulence-associated plasmid, i.e., an ~80- to 90-kbp extrachromosomal element which is associated with full virulence for horses and mice and which mediates the expression of VapA (mnemonic for virulence-associated plasmid protein A), a secreted lipoprotein and a marker of this kind of plasmid (18, 44, 57). When isogenic strains of *R. equi* with plasmids and their cured partner strains were compared in a mouse killing model, the plasmid-harboring isolates had a 50% lethal dose (LD_{50}) of 10^6 per mouse, whereas the plasmid-cured derivatives did not kill, even at 10^8 bacteria per mouse, which was the highest dose tested (48). For foals, the minimum infective dose for the plasmid-containing strain was 10^4 bacteria, and for the cured partner strain it was $>10^9$ bacteria, demonstrating the importance of the plasmid for virulence and also the usefulness of the murine model in virulence research (60). Two VapA-expressing plasmids from two independent foal isolates have recently been sequenced, and their sequences turned out to be almost identical (53).

A second type of plasmid, the VapB-expressing plasmid (75 to 95 kbp), is associated with intermediate levels of virulence in mice ($LD_{50} = 10^7$) and in foals, and it is not usually associated

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with foal disease (51, 54). This plasmid contains the gene for VapB, which is structurally homologous to VapA (7), but it does not have a VapA gene. The plasmid occurs in asymptomatic pigs and has also been isolated from AIDS patients (48). In addition to VapA- and VapB-expressing plasmids, several types of cryptic plasmids that have so far not been associated with any pathogenic effects exist (47, 50).

Interestingly, there is no strict correlation between possession of certain kinds of plasmids and infection of humans; although many isolates from AIDS patients express either VapA or VapB, most isolates from immunocompromised (but not AIDS) patients contain neither plasmid (34, 36, 48, 49). Environmental samples usually do not contain the VapA-expressing plasmid; Takai et al. (52) analyzed more than 200 soil and sand samples from more than 200 locations in Japan for the presence of *R. equi*. They isolated *R. equi* from almost three-quarters of the samples, but none of the more than 1,000 resulting bacterial isolates expressed VapA.

In this study, we investigated the cytotoxic effect of *R. equi* infection for murine macrophages. Killing of host cells by pathogens occurs by necrosis, apoptosis, or a combination of both. Apoptosis is a particular form of programmed cell death to which the host cell actively contributes and which involves a finely tuned cascade of factors and events that finally lead to cell death. The key factors in this sophisticated machinery are caspases, a family of cysteine proteases which specifically activate or inactivate a number of proteins along the apoptotic cascade of events (56). Necrosis (or oncosis [43]), on the other hand, is a more passive form of cell death, usually triggered by physical insults to the cell such as hypoglycemia, hypoxia, or toxin exposure (43). Its characteristics are ATP depletion, high intracellular sodium concentrations, organelle swelling, and typical changes in intracellular calcium levels (3). The overall process is probably not as finely tuned as apoptotic cell death is.

Apoptosis seems to be the more frequently used method of host cell killing by pathogens (35). The best-understood cases of apoptotic killing are those by the enterobacteria *Salmonella*, *Shigella*, or *Yersinia*. *Shigella*-induced apoptosis requires the host cell caspase 1 (21) and involves tripeptidyl peptidase II (22). For *Salmonella* it is not definitively clarified if killing is by apoptosis or necrosis, because the killing shares aspects of both processes (6). Pathogens do not necessarily deal with all types of host cells in the same way: mycobacteria can induce necrosis in infected epithelial cells (12), a process possibly caused by mycobacterial lipids, such as the polyketide of *Mycobacteria ulcerans* (16). In macrophages, on the other hand, *M. tuberculosis* infection can cause apoptosis by a tumor necrosis factor alpha-dependent mechanism (1, 27, 28).

Importantly, apoptosis is usually an immunologically silent kind of cell death with professional phagocytes recognizing and removing apoptotic cells without mounting an immune response. In contrast to apoptosis, cell necrosis is strongly proinflammatory (33).

In this study, we show not only that virulent *R. equi* is cytotoxic for murine macrophages but also that this process is strongly up-regulated by the presence of VapA-expressing plasmids and, less strongly, by VapB-expressing plasmids, that cytotoxicity requires bacterial viability, and that cell death occurs by necrosis rather than by apoptosis.

MATERIALS AND METHODS

Reagents and bacterial strains. All reagents were of the highest purity available and were obtained from the following companies unless specified otherwise: Sigma (Taufkirchen, Germany), Fisher (Schwerte, Germany), and Roth (Karlsruhe, Germany). *R. equi* strains 85F and 85F(−) (5) were obtained from A. Hartke (Laboratoire de Microbiologie de l'Environnement, IRBA, Université de Caen, Caen, France), and *Listeria innocua* strain 6 isolates (32) were from the strain collection of the Lehrstuhl für Mikrobiologie, University of Würzburg, Würzburg, Germany. *R. equi* strains ATCC 33701, ATCC 33701(−), 2, 2(−), 11, and 11(−) have been described previously (48).

Cultivation of bacteria and host cells. *R. equi* and *L. innocua* were grown aerobically in brain heart infusion (BHI) broth at 37°C in a rotatory shaker at 190 rpm for 16 h. Cultures to be used in these experiments were from early stationary phase (at an optical density at 600 nm of approximately 3.0) and were almost exclusively coccoid (15) as determined by light microscopical observation. All cultures that were not used directly in an experiment were kept at 30°C or less, as increased temperatures can lead to a spontaneous loss of the VapA-expressing plasmid (46). Bacteria to be used directly in experiments were grown at 37°C, because temperatures of >32°C are necessary for expression of *VAP* genes (45). Bacterial cell numbers were determined by measuring the optical absorbance at 600 nm (A_{600}) of the cultures and calculating live cell counts by the following formula: 1 A_{600} unit is equivalent to 1.5×10^8 *R. equi* bacteria or 2×10^8 *L. innocua* bacteria. The murine macrophage-like cell line J774E (13) was grown in RPMI 1640 (Gibco Life Technologies, Karlsruhe, Germany)–2 mM glutamine–10% 56°C-treated fetal calf serum (Sigma) at 37°C and 5% CO_2, and split 1 to 2 every 2 to 3 days.

Infection of host cells with bacteria and determination of infection levels. To infect macrophages, phosphate-buffered saline (PBS)-washed bacteria were added to the host cells in 12-well-culture dishes in a 30-μl volume per ml of medium at a final multiplicity of infection (MOI) of 30, unless stated otherwise. Macrophages were grown to approximately 80% confluency, and the medium was changed the day before the experiment. Infection occurred during 1 h at 37°C, followed by the removal of noninternalized bacteria and thorough washing (three times) with PBS at 37°C. Fresh culture medium that was supplemented with 10 μg of gentamicin sulfate (Sigma)/ml was added to prevent bacteria from multiplying in the culture (our preliminary experiments had demonstrated that rhodococci and listeriae multiply well in media without added antibiotics). Infection was continued for the indicated periods of time.

The number of infecting bacteria was determined by two methods. (i) The bacterial life count from newly infected macrophages was determined as follows. J774E cells were cultivated in 12-well-plates, infected at a MOI of 30 for 1 h, and then thoroughly washed three times with 37°C PBS. Fresh culture medium that included 10 μg of gentamicin/ml was added. After 1 h of incubation, the medium was discarded and macrophages were scraped off each well into 1 ml of ice-cold PBS by use of a rubber policeman. Macrophages were washed twice with PBS and lysed by passing 15 times through a 27-gauge needle, and serial dilutions in PBS were plated on BHI agar and counted after a 24-h incubation at 37°C. (ii) Alternatively, infection levels were determined by fluorescence microscopy (particular in the experiments with pretreated, dead bacteria). For this, bacteria were prelabeled with 5- (and 6-) carboxytetramethylrhodamine, succinimidyl ester [5-(6)-TAMRA, SE] (catalogue no. C-1171; Molecular Probes, Leiden, The Netherlands) as previously described for *Afipia* (32). After 1 h of J774E infection with the labeled and washed bacteria, noningested bacteria were removed by repeated washing, and the numbers of ingested bacteria were determined by fluorescence microscopy. Based on the results from these experiments, the number of particles per macrophage (MOI) was adjusted in each experiment after counting of macrophages to obtain the same level of infection (i.e., total number of ingested particles) regardless of the pretreatment. Adjusted MOIs were as follows according to the treatment to which *R. equi* 33701 was subjected: no treatment and heat treatment, 30; UV treatment, 40; PFA and amikacin plus tetracycline treatment, 50; gramicidin S treatment, 60.

Analysis of cell death by using trypan blue. To quantify dead macrophages (i.e., cells whose plasma membranes have disintegrated to an extent that they cannot exclude small hydrophilic molecules), the classical trypan blue exclusion assay was employed (in this assay, live cells do not stain and dead cells stain blue [11]). Macrophages were grown in 12-well-plates (Nunc, Wiesbaden, Germany) in 1 ml of medium per well. At the start of the infection experiment, the cell number per well was $8 \times 10^5 \pm 2 \times 10^5$ (mean ± standard deviation) macrophages; infection was done as above. The time of addition of gentamicin was defined as 0 min. At 5 and 24 h, macrophages were carefully scraped off by using a rubber policeman, and all the material from the wells (medium plus macrophages, including nonadherent cells) was centrifuged in a Hettich Universal 32R

centrifuge (1,000 rpm, 10 min, 4°C). For the experiments whose results are shown in Fig. 3, 4, and 5, the supernatant was removed and the pellet was resuspended in ice-cold PBS. The cells were stored on ice until the number of trypan blue-positive macrophages was determined from at least 100 macrophages per sample and time. For the experiments whose results are shown in Fig. 2, the supernatant was removed and the pellet was resuspended in 0.5% trypan blue in PBS, briefly and gently vortexed, and spun again. Nonbound trypan blue was removed as much as possible. Alternatively, and with similar results, the trypan blue-treated macrophages were fixed in 200 μl of 3% paraformaldehyde in PBS at 4°C overnight. The fixed cells were centrifuged, the pellet was carefully taken up in 1 ml of PBS, and the percentage of trypan blue-positive macrophages was determined for each sample type and each time in each experiment; at least 200 macrophages were counted, and the proportion of blue cells was noted.

Cytotoxicity of bacterial broth supernatant. To determine the cytotoxicity potential of bacterial culture supernatants, bacteria were grown in liquid BHI as above (for 24 or 48 h) and were then collected by centrifugation in a Hettich Mikro20 centrifuge at 15,000 rpm and ambient temperature for 15 min. The supernatant was mixed with various amounts of macrophage growth media yielding up to one-third of the total media as spent bacterial broth. Incubation was for 22 h at 37°C (5% CO_2), after which time the level of cytotoxicity was determined with the trypan blue exclusion assay as described above. Alternatively, bacteria were added at an MOI of 30 to macrophage media alone and were kept for 1 h at 37°C and 5% CO_2; bacteria were separated from media as above, and the supernatant was used to replace growth media of J774E cells and left for 24 h; then, trypan blue exclusion was determined.

Treatment of bacteria before infection. To determine whether dead rhodococci still have a cytotoxic potential, we treated 10^8 bacteria in a 200-μl volume of PBS either for 1 h at 37°C with gramicidin S (125 μg/ml), for 5 h at 37°C with amikacin (500 μg/ml) plus tetracycline (250 μg/ml), or for 1 h with UV at ambient temperature, or the bacteria were treated with heating (30 min, 70°C) or with paraformaldehyde fixation (3% paraformaldehyde in PBS for 20 min at 23°C, followed by a brief wash in 50 mM ammonium chloride in PBS to quench unreacted paraformaldehyde). All samples were washed twice with PBS. Levels of infection with dead bacteria were determined by fluorescence microscopy as stated above.

Differentiation of cell death by necrosis and cell death by apoptosis. Almost confluent layers of J774E macrophages were infected for 1 h with *R. equi* at a MOI or 30. The medium was discarded, and the cells were washed three times with warm PBS. Macrophages were incubated for the indicated time periods in fresh culture medium including 10 μg of gentamicin/ml. The cells were scraped off by using a rubber policeman and were washed twice with PBS (1,000 rpm, 4°C, 10 min). To distinguish apoptosis (programmed cell death) from necrosis (nonprogrammed cell disintegration), we used the following methods: (i) annexin V/7-AAD staining, (ii) subG_1 analysis, (iii) PARP processing analysis, (iv) electron microscopy observation, and (v) infection of macrophages in the presence of caspase 3 or 7 inhibitor I.

(i) Annexin V/7-AAD staining. One hundred thousand infected macrophages were washed with annexin V-binding buffer (1,000 rpm, 4°C, 10 min). Cell pellets were incubated with 100 μl of annexin V-binding buffer (10 mM HEPES [pH 7.4], 140 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$), 3 μl of 7-amino actinomycin D (BD PharMingen, Heidelberg, Germany), and 3 μl of annexin V-PE (BD PharMingen) for 15 min at room temperature in the dark. After addition of 200 μl of annexin V-binding buffer, 5,000 cells were analyzed by using a FACScan (Becton Dickinson, San Jose, Calif.).

(ii) SubG_1 analysis. For sub-G1 analysis (23), 5×10^5 infected cells were placed in a 10-ml glass tube. Three milliliters of a −20°C fixation buffer (70% ethyl alcohol, 20% PBS, 10% distilled water) was slowly added with vortexing. Tubes were sealed with parafilm and incubated at 4°C overnight. Samples were spun for 10 min at 1,000 rpm at 23°C, and the supernatant was aspirated and discarded. Macrophages were resuspended in 1 ml of extraction buffer (45 mM Na_2HPO_4, 2.5 mM citric acid, 0.1% [wt/vol] Triton X-100, 0.01% [wt/vol] sodium azide, pH 7.8), and placed in fluorescence-activated cell sorter (FACS) tubes. After 20 min of incubation at room temperature, cells were spun as above and the supernatant was discarded. Cell pellets were taken up in 400 μl of staining buffer {10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 0.1 M NaCl, 2 mM $MgCl_2$, 0.1% [wt/vol] Triton X-100, 0.02% [wt/vol] sodium azide, pH 6.8}, 25 μl of RNase solution (10 mg/ml of deionized water), and 20 μl of propidium iodide solution (1 mg/ml). After 30 min in the dark at 23°C, samples were analyzed in a FACScan.

(iii) PARP processing analysis. Six million infected J774E macrophages were centrifuged (8,000 rpm, 15 min, 4°C), and the pellets were resuspended in 150 μl of Kyriakis Lysis Buffer Modified containing 10 U of benzonase (catalogue no. 101653; VWR Scientific, Darmstadt, Germany), incubated for 30 min on ice, and vortexed every 10 min during this incubation time. After centrifugation in a Hettich Mikro20 centrifuge (14,000 rpm, 15 min, 4°C), each of the supernatants was mixed with 150 μl of 2× Laemmli buffer. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and PARP was detected by using a monoclonal antibody (catalogue no. P76420; BD/Transduction Laboratories, Heidelberg, Germany) and enhanced chemiluminescence technology (Amersham, Freiburg, Germany). This monoclonal antibody recognizes the full-length 113-kDa PARP and its caspase-dependent 24-kDa fragment, but not the 89-kDa fragment.

(iv) Electron microscopy observation. Infected macrophages were fixed and further treated and stained as described by Lührmann et al. (32).

(v) Infection of macrophages in the presence of a potent caspase inhibitor. For infection of macrophages, a 25 μM concentration of caspase 3/7 inhibitor I (5-[(S)-(-)-2-(methoxymethyl)pyrrolidino]–sulfonylisatin [catalogue no. 218826; Calbiochem]) was added to macrophages 30 min before infection (37°C), during infection, and at all subsequent steps. Parallel samples were treated with the inhibitor only. After 24 h of infection, samples were processed for trypan blue staining as above.

RESULTS

When infecting the murine J774E macrophage-like cell line with *R. equi* (strain 85F) for 24 h, we observed that most of the infected macrophages detached from the culture dish and clumped, suggesting that some of the macrophages had died. Such cytotoxicity has previously been described for alveolar macrophages from foals (20, 62) or mice (24) and has been proposed to be a typical feature for infections with "virulent" *R. equi* strains, although virulence was then a phenotypical feature rather than a molecular description of a specific bacterial property. As this strain possesses the VapA-expressing plasmid, we set out to analyze whether cytotoxicity was dependent on the presence of this particular plasmid.

***R. equi* strains harboring the VapA-expressing virulence-associated plasmid are much more cytotoxic than their plasmid-cured partner strains.** We infected J774E cells either with the 85F strain or with its plasmid-cured derivative 85F(−) (5). After overnight infection, we observed dramatic damage to macrophages infected with the plasmid-bearing strain, whereas macrophages containing plasmid-cured isogenic bacteria seemed barely more damaged than the noninfected control macrophages or J774E cells infected with the nonpathogenic gram-positive bacterium *L. innocua* (used here as a negative control) (Fig. 1). Using the microscopic trypan blue exclusion assay, we quantified the cytotoxic effect of these bacteria and found that the plasmid-bearing strain killed some 79% of the macrophages during a 24-h infection (MOI = 30) whereas the plasmid-cured strain killed only 9% and spontaneous cell death under these conditions or cell death in the sample infected with *L. innocua* was approximately 1%.

These results strongly suggested that the possession of the virulence-associated plasmid was required for cytotoxicity, but they also raised the question as to whether the variation in cytotoxicity was really dependent on the presence of the virulence-associated plasmid or whether the effect was due to chromosomal mutations that occurred only in the plasmid-cured strain. To address this question, we used a second pair of isogenic plasmid-bearing and plasmid-cured *R. equi*, termed ATCC 33701 and ATCC 33701(−) (44). Strains 85F and ATCC 33701 were isolated from horses by different groups of researchers in two different geographic areas (Japan and Canada). The same cytotoxic pattern in the plasmid-harboring and in the plasmid-cured strains was observed (Fig. 2). Although *R.*

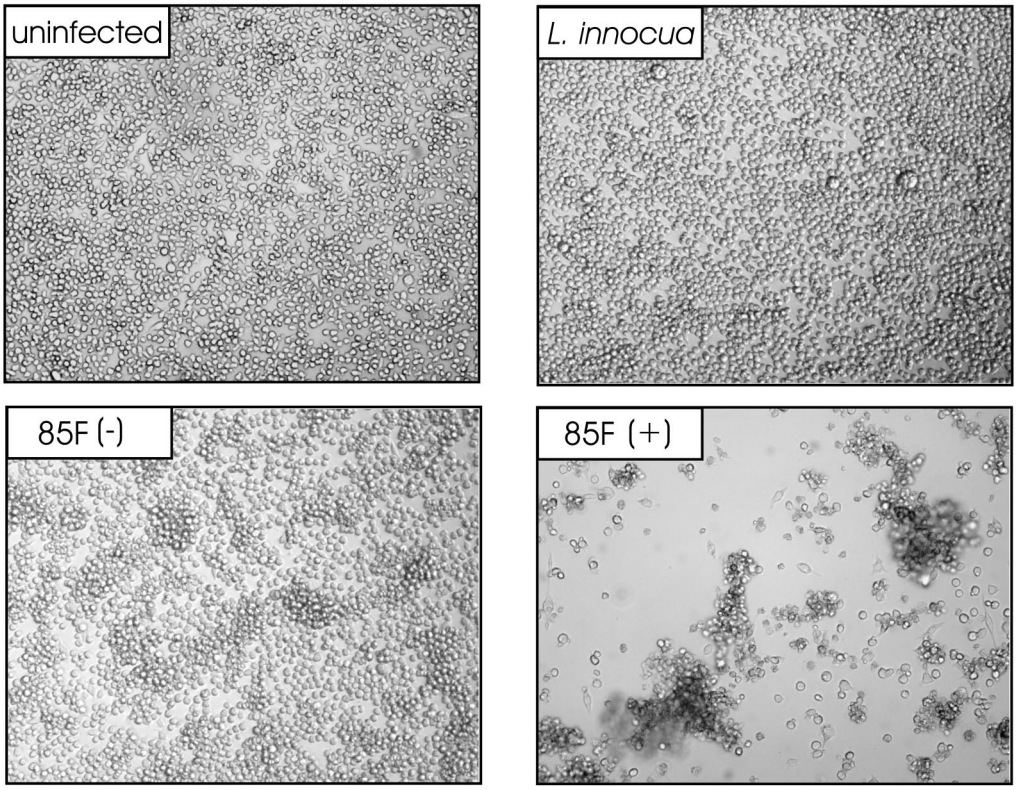

FIG. 1. Infection of J774E cells with *R. equi* carrying the VapA-expressing plasmid leads to detachment and clumping of the host cells, whereas infection with their isogenic cured derivative does not. J774E macrophages were infected with various *R. equi* preparations at a MOI of 30 for 1 h, followed by a removal of noningested bacteria and a 24-h incubation at 37°C and 5% CO_2. Shown are portions of the culture dish bottoms observed through an inverted light microscope. 85F(+), plasmid-bearing *R. equi*; 85F(−), cured derivative; *L. innocua*, J774E cells infected with apathogenic *L. innocua*; uninfected, no bacteria added.

equi 85F produces stronger cytotoxic effects than ATCC 33701 does, we still used the latter strain in subsequent experiments, because it is among the most frequently used strains in research on *R. equi* pathogenesis.

In another experiment, we determined cytotoxicity after a constant time period of infection (24 h) and plotted it against the MOI (number of bacteria per macrophage added to the macrophages for 1 h before the chase). Cytotoxic effects of VapA-expressing *R. equi* increased with increasing MOIs (Fig. 3). Similarly, increasing the time of infection leads to increased severity of cytotoxicity (Fig. 2). Therefore, the extent of cytotoxicity was a function of infection rate and infection duration. The average percentage of infected macrophages at a MOI of 30 was 62% (±12%), and the average number of bacteria in each infected macrophage was 3.7 (±1.4). Incubation of infected macrophages for more than 24 h (44 or 72 h) led to a decrease in the number of trypan blue-positive macrophages in each sample (data not shown). This was probably due to the killing of rhodococci that had been released from the necrotic macrophages and were therefore in contact with the externally added gentamicin and due to phagocytosis, and therefore removal, of burst macrophages by viable ones.

The VapB-expressing plasmids only moderately increase cytotoxicity. As VapB-expressing *R. equi* strains are only moderately virulent in foals and mice compared to VapA-expressing strains (54), we examined whether this differential behavior would also be reflected in their cytotoxic potential. We employed two independent pairs of VapB plasmid-containing strains and their corresponding cured derivatives, named *R. equi* strains 2 and 2(−) and *R. equi* strains 11 and 11(−) (48). Using the trypan blue exclusion assay, we determined that both plasmid-positive strains were more cytotoxic than their corresponding isogenic partner strains but that the plasmid-mediated increase in cytotoxicity was much lower in these strains than in the strain harboring the VapA-expressing plasmid (Fig. 4). It should also be noted that the cured strains can still possess some cytotoxic potential [compare, e.g., strain 2(−) with the uninfected control sample in Fig. 4], indicating that a cytotoxic potential is not absolutely dependent on the presence of a plasmid.

Importantly, the observed differences in cytotoxicity are not due to different infection rates that occur with the different bacterial strains. Using live-cell count determination, we observed that the number of intracellular bacteria varies among experiments and among all the strains used by an average of only 10%. Additionally, this low degree of variability was not correlated with a certain genotype, so that infection rates were higher and again lower among experiments for all the strains used [ATCC 33701 and ATCC 33701(−), 2 and 2(−), and 11 and 11(−); see Materials and Methods].

Viability of rhodococci is required for cytotoxicity. To analyze whether viability is required for bacterial cytotoxicity, we

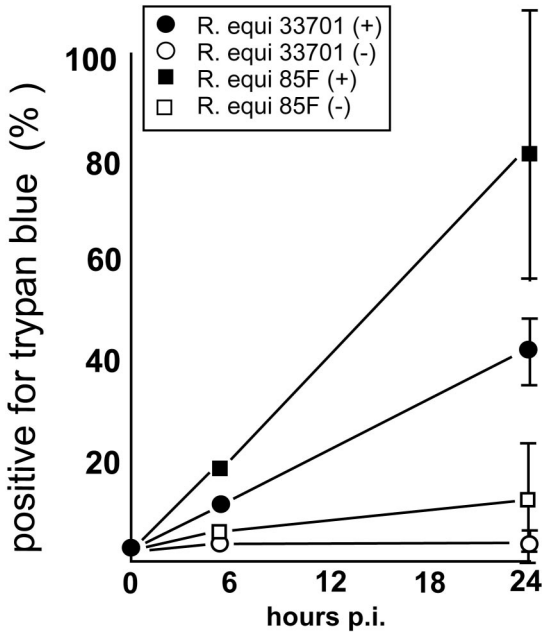

FIG. 2. *R. equi* strains carrying the VapA-expressing plasmid are strongly cytotoxic, whereas their isogenic cured derivatives are not. J774E cells were infected at a MOI of 30 for 1 h, followed by washes to remove noningested bacteria, fixed either immediately or at 5 or 24 h postinfection, and analyzed for their membrane integrity by using the trypan blue exclusion assay. (+), plasmid-bearing *R. equi*; (−), cured derivatives. Results are from five independent experiments with at least 200 macrophages counted per sample and experiment. Standard deviations for samples infected for 5 h were omitted for clarity and did not exceed 9%.

killed the bacteria before infection. We used a variety of killing mechanisms to make sure that it is the killing of the bacteria itself that would change cytotoxic potential and not, e.g., exclusively thermal inactivation of surface protein activities by heat killing.

The various killing mechanisms used in our experiments (Fig. 5), i.e., treatment with antibiotics (tetracycline plus amikacin or gramicidin S), formaldehyde, heat, or UV radiation, work via completely different mechanisms (protein synthesis inhibition, membrane permeabilization, protein cross-linking, denaturation of macromolecules and their assemblies, and

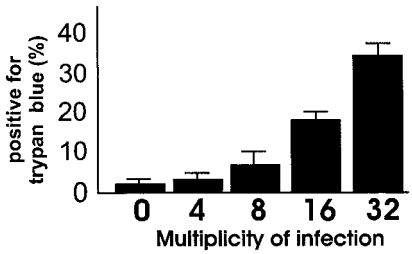

FIG. 3. Cytotoxicity depends on MOI. J774E cells were infected with *R. equi* ATCC 33701 for 1 h with MOIs of 4, 8, 16, or 32 or were left uninfected (0), washed three times to remove noningested bacteria, and incubated for 24 h. Analysis was done as described in Materials and Methods. Results are from three independent experiments with at least 100 macrophages counted per sample and experiment.

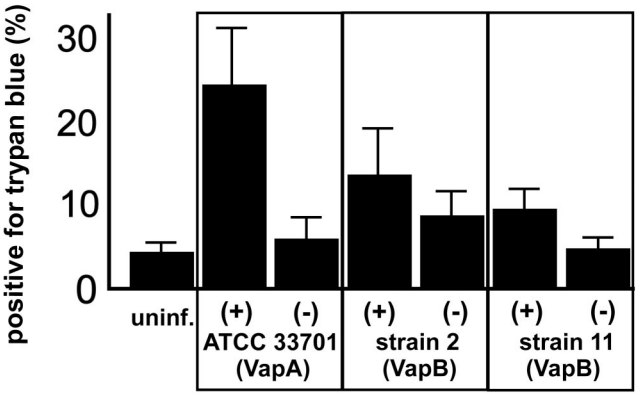

FIG. 4. VapB-expressing plasmids only moderately increase cytotoxic activities. Experiments were done as explained for Fig. 3 but at a MOI of 20. Two independent isolates of *R. equi* expressing VapB and their isogenic cured derivatives [strain 2 or 2(−) and strain 11 or 11(−)] were used to infect J774E macrophages as described in the legend to Fig. 3. Strains ATCC 33701 and its cured derivative were used as control bacteria. Cytotoxicity was assessed as described for Fig. 3 (using 24 h). Results are from three independent experiments with at least 100 macrophages counted per sample and per experiment.

DNA and phospholipid damage). Under these conditions 100% of the treated bacteria were killed, with the exception of the treatment with tetracycline plus amikacin, which killed 94% of the bacteria. The fact that all of these treatments led to a dramatic decrease in cytotoxicity for infected J774E cells (approximately 1 to 4% versus 31% in all samples) strongly suggests that it is bacterial viability that is required for this process.

To further ensure that the differences in cytotoxicity were really due to the differences in viability of the bacteria and not due to differences in the extent of uptake of bacteria that were

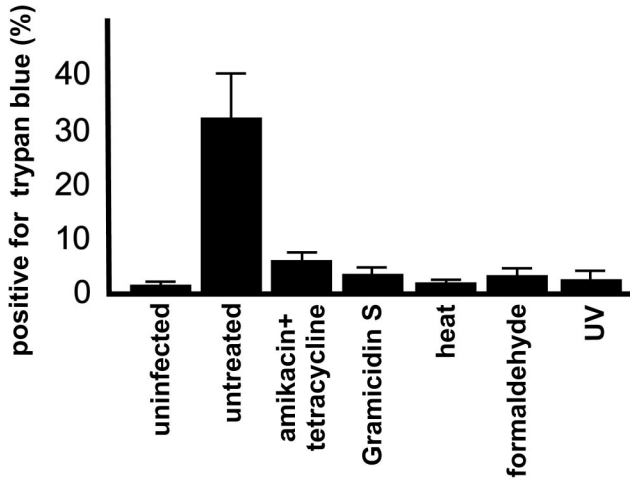

FIG. 5. Cytotoxicity by *R. equi* requires bacterial viability. *R. equi* ATCC 33701 bacteria were pretreated as described in Materials and Methods with either buffer only (untreated), tetracycline plus amikacin, gramicidin S, heat, paraformaldehyde (formaldehyde), or UV. Uninfected J774E were analyzed in parallel. Infection and quantification of the percentage of dead macrophages were as in Fig. 3. Results are from three independent experiments performed in duplicate with at least 100 macrophages counted per sample and experiment.

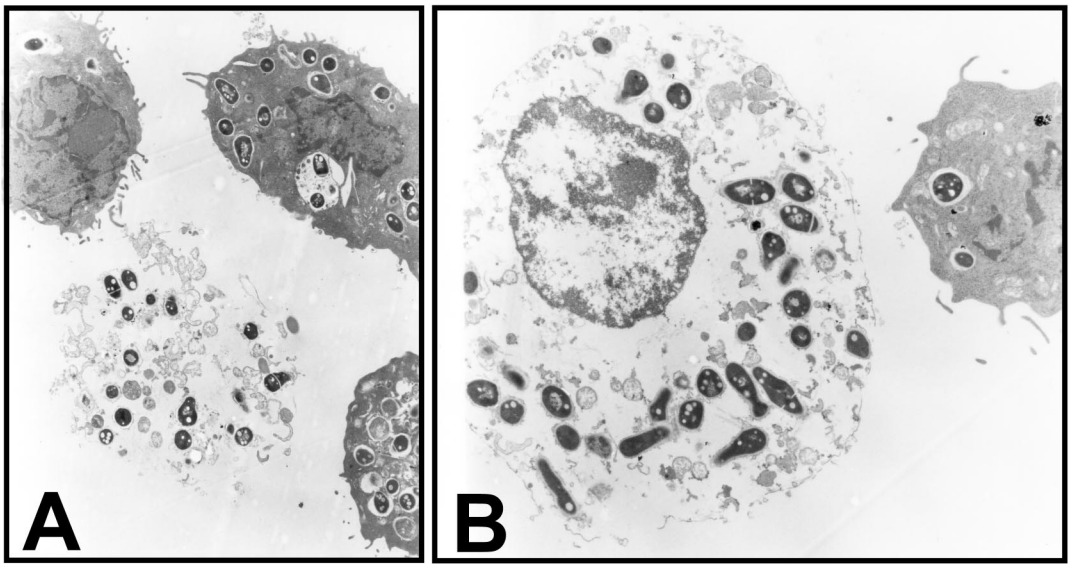

FIG. 6. Electron microscopic analysis of infected J774E macrophages and "ghosts." J774E cells were infected with *R. equi* ATCC 33701 for 1 h at a MOI of 25, chased for 24 h, and prepared for transmission electron microscopy. Note the ghost-like cell next to the infected, but morphologically intact cells. Magnifications, ×3,200 (A) and ×4,400 (B). Although the ghost cell in panel A is almost completely disintegrated, and although almost all organelles have been lost, the nucleus is still intact and chromatin distribution appears normal.

treated differently, we determined the infection rates with these bacteria and found them to be different. As outlined in Materials and Methods, we have corrected for such altered infection rates by varying the number of added bacteria so that in all samples similar numbers of bacteria (three per cell) were found intracellularly.

***R. equi*-mediated cytotoxicity has necrotic rather than apoptotic characteristics.** To determine whether host cell death occurred by necrosis or by apoptosis, we used several independent methods for analysis. The first was to use transmission electron microscopy with infected macrophages, as apoptotic cells are characterized by the appearance of very densely packed chromatin in the nucleus periphery (leading to highly electron-dense areas), by membrane blebbing, and by the falling apart of the nucleus into several micronuclei (30). We were not able, however, to detect any of these microstructural features in our samples. Shown in Fig. 6 are typical dead *R. equi*-infected cells, which demonstrate the appearance of ghost-like host cells in infected samples that invariably harbored several apparently undamaged *R. equi* bacteria and that often possessed nuclei of normal appearance in spite of severe overall damage. In a second approach, we analyzed the presence of phosphatidylserine in the outer membrane leaflet of the plasma membrane, which is a hallmark of early apoptotic cells. We quantified the percentage of cells containing phosphatidylserine by using FACS and analyzing the binding of a phosphatidylserine affinity probe, fluorescent annexin V (30). Membrane-impermeable fluorescent 7-amino-actinomycin D (7-AAD) was used as a control to make sure that annexin V-positive cells did not have disrupted plasma membranes and, therefore, would be classified as necrotic rather than early apoptotic.

Our data (Fig. 7) showed that approximately 29% of the macrophages containing *R. equi* ATCC 33701 were positive for 7-AAD at 6 h after infection. No significant population of macrophages was simultaneously positive for annexin V and negative for 7-AAD (a hallmark of early apoptotic cells) at any of the times tested, suggesting that the event is necrotic rather than apoptotic from the onset of infection. Macrophages infected with ATCC 33701(−) showed identical characteristics,

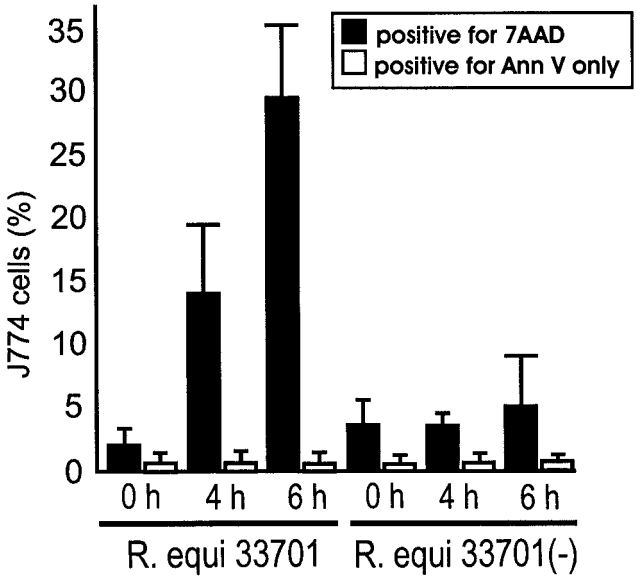

FIG. 7. Infected J774E cells become simultaneously positive for annexin V and for 7-AAD, suggesting that they are necrotic and not apoptotic. Infection at a MOI of 30 was for 1 h, followed by either no chase (0 h) or a 4- or 6-h chase. Macrophages that were only annexin V positive are represented by the open bars; macrophages that were positive for 7-AAD or positive for both annexin V and 7-AAD are represented by the black bars. For details, see the text. Data are from three independent experiments.

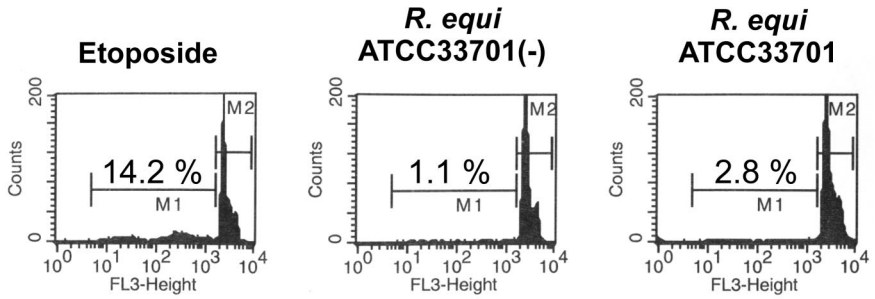

FIG. 8. Sub-G_1 analysis of infected J774E cells demonstrates the absence of apoptotic events. Infection at a MOI of 20 was for 1 h, followed by a 6-h chase. Cells were fixed and treated as described in Materials and Methods and subjected to FACS analysis. The M2 region represents living and necrotic macrophages in the G_0, G_1, M, and S phases, and the M1 region represents apoptotic cells with a reduced DNA content (subG_1 peak). This FACS analysis shows macrophages either treated with 300 μM etoposide for 6 h (Etoposide) or infected with *R. equi* ATCC 33701 or ATCC 33701(−).

although the number of cells positive for 7-AAD was much smaller (Fig. 7).

As a third method to analyze the mode of cell death in *R. equi* infection, we have used sub-G_1 analysis of infected macrophages (26, 58). In this protocol, infected cells are stained with a fluorescent DNA stain (such as propidium iodide or DAPI [4′,6′-diamidino-2-phenylindole]). Due to the action of endogenous endonucleases in apoptotic cells, the DNA is cleaved into endonucleosomal fragments of typical sizes. These DNA fragments are extracted from the cells. This loss of DNA is detectable by FACS analysis, as the reduced nuclear staining of apoptotic cells results in a novel (sub-G_1) fluorescence peak to the left of the regular fluorescence peak representing diploid cells (Fig. 8). This signal is diagnostic for apoptotic cells and absent in necrotic cell populations. Macrophages treated with the apoptosis-inducing topoisomerase II inhibitor, etoposide (61), showed this characteristic sub-G_1 peak, and around 14% of the cells were apoptotic (Fig. 8, left panel). In contrast, neither the infection of J774E macrophages with *R. equi* ATCC 33701 nor that with ATCC 33701(−) led to an induction of apoptosis, as demonstrated by the absence of a subG_1 peak (Fig. 8, middle and left panels). The low percentage of apoptotic cells (1 to 3%) in infected macrophages is inherent to these cells and was also seen in sub-G_1 analysis of uninfected J774E macrophages (data not shown). It should be noted that the concentration of etoposide used in these experiments (300 μM) is relatively high, indicating that our host cells have relatively little sensitivity to etoposide. After a 4-h incubation with etoposide, only approximately 1% of the macrophages are killed by etoposide; yet, after 24 h 57% and after 44 h 91% have died. Staurosporine, a broad-range protein kinase inhibitor and another pharmacological inducer of apoptosis (42) was more potent, killing 5% of J774E cells at 4 h, 86% by 24 h, and 100% by 74 h after infection when added at a concentration of 0.4 μM. Both etoposide and staurosporine induced apoptosis efficiently in J774E cells. The word "killing" applies in this context to macrophages possessing ruptured plasma membranes, this rupture following apoptotic death.

A further characteristic of apoptotic cells is the specific proteolytic cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). Cysteine proteinases such as caspase 3 and caspase 7 create diagnostic 89- and 24-kDa protein fragments from the 113-kDa full-length protein early in the apoptotic cascade (61). As a fourth method to discriminate between apoptotic and necrotic cell death, we performed analyses of the molecular state of PARP in infected J774E cells that showed that there was virtually no processing in cells infected either with plasmid-bearing or with plasmidless *R. equi* (Fig. 9). Macrophages treated in parallel with either of the inducers of apoptosis, etoposide or staurosporine, on the other hand, showed pronounced PARP processing at all times tested (61) (Fig. 9). Additional signals between 24 and 113 kDa of molecular mass are due to less abundant, necrosis-dependent cleavage products (40). Together these data clearly indicated that *Rhodococcus*-induced cell death was by necrotic rather than by apoptotic mechanisms. Finally, caspase 3/7 inhibitor I,

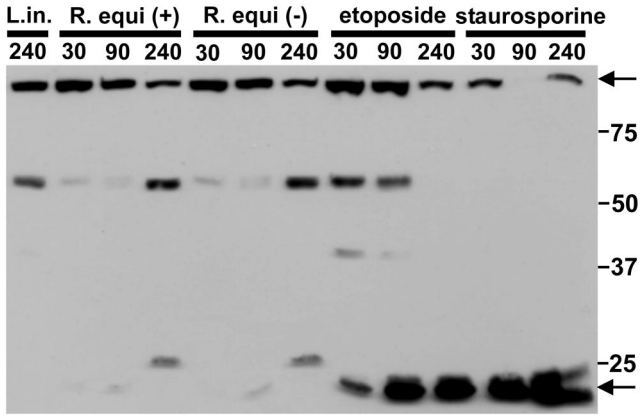

FIG. 9. The lack of 24-kDa PARP cleavage product in macrophages infected with *R. equi* suggests the absence of apoptotic events. During early apoptosis, full-length 113-kDa PARP is specifically cleaved by caspases to yield 89- and 24-kDa processed fragments. The monoclonal antibody used in this immunoblot recognizes both the full-length protein and the 24-kDa fragment, but not the 89-kDa fragment. Appearance of the 24-kDa fragment indicates apoptosis. Either the J774E cells were infected with *R. equi* ATCC 33701, ATCC 33701(−), or nonpathogenic *L. innocua* (L. in.) for the indicated times (30, 90, or 240 min), or the apoptosis-inducing agents etoposide or staurosporine were added at 300 or 4 μM, respectively, for the same periods of time. Whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and PARP was visualized (see Materials and Methods). Molecular masses (in kDa) are indicated on the right margin, and arrows indicate the running positions for full-length PARP and the 24-kDa fragment.

an isatin sulfonamide-based potent inhibitor of apoptosis (31) used at a concentration of 25 μM did not inhibit *R. equi*-mediated cytotoxicity either at 4 or at 24 h postinfection, whereas the compound alone was not toxic (data not shown). This further indicates that infected macrophages die by necrosis rather than by apoptosis.

The cytotoxic factor is not a singly acting substance contained in the culture medium. One possibility for how the macrophages were damaged was the release of a constitutively produced cytotoxic bacterial component leading to cell death, in a process similar to what was observed with apoptotic killing by *Staphylococcus aureus* alpha-toxin (2). To address this question, two types of samples were analyzed: (i) rhodococci were incubated with the macrophage culturing media alone for 1 h (corresponding to the initial 1-h infection period), and the bacteria were separated by centrifugation followed by addition of the spent media to noninfected macrophages for up to 48 h; (ii) spent 48-h BHI broth from which the bacteria had been removed by centrifugation was added to noninfected macrophages for 24 h. In either case, no cytotoxic effect on the macrophages was detected, not even at a high concentration of bacterial broth supernatant (one-third of the total macrophage media volume), and this was true for all eight different isolates tested, three of which were VapA positive (data not shown). In parallel, J774E cells were infected with *R. equi*(+), which was cytotoxic to the normal degree, demonstrating that the macrophages were able to undergo necrosis. These data suggest that the cytotoxic factor is not a constitutively produced and released rhodococcal factor.

DISCUSSION

In this study, we show that *R. equi* can be highly cytotoxic for the murine macrophage cell line J774E. There have been reports that mentioned (20, 25, 38, 62) or demonstrated (25, 37) the presence of a cytotoxic effect on mammalian cells of some "virulent" *R. equi* versus a lack thereof in "avirulent" ones, but the correlation between possession of a virulence-associated plasmid and cytotoxic potential has not been described. We now present evidence that the cytotoxic potential is regulated by virulence-associated plasmids. Either VapA- or VapB-expressing plasmids can increase the cytotoxic activity of *R. equi*. The presence of the VapA-expressing plasmid in *R. equi* increased the percentage of killed macrophages in a standard assay by approximately 20 to 70% over the cytotoxic effect of the respective cured partner strains. VapB expression increased macrophage killing by approximately 5% only. This is in agreement with the differences in the LD_{50} doses (numbers of bacteria required to kill 50% of intravenously infected mice) of rhodococci. Approximately 10 times more bacteria expressing VapB than those expressing VapA are required to kill 50% of the mice (48). This correlation suggests that cytotoxic activity may be a major virulence determinant of *R. equi.*

We have analyzed the nature of death of the infected cells by using several methods: sub-G_1 FACS analysis, annexin V/7-AAD staining, PARP processing, and analysis of nuclear morphology. All of the results obtained with these methods agree with the interpretation that *R. equi* kills its host macrophages by necrosis (sometimes referred to as oncosis) rather than in an apoptotic process. In fact, our kinetic data clearly suggest a black-and-white situation in which there is not even a small subpopulation of host cells that enter the apoptotic pathway.

Necrotic effects of *R. equi* infection for mammalian cells are not just an in vitro observation but are also observed in infected mice and foals. In biopsy samples taken from such animals, morphologically intact bacteria can be seen within macrophages, and more-advanced stages of *R. equi*-caused disease are characterized by necrosis coinciding with the presence of degenerate macrophages full of bacteria (24, 38). However, it is not clear from these pathohistological studies whether the observed necrotic effect of *R. equi* in vivo is primary or rather a secondary event following apoptotic cell death.

The degree of necrosis is likely to be limited in vivo through macrophage activation (39). Gamma interferon and phagocytosis of rhodococci cause production of superoxide radicals plus nitric oxide by murine macrophages, which leads to killing of ingested *R. equi* via peroxinitrite production (9, 23). Therefore, not surprisingly, gamma interferon seems to play a key role in limiting the infection (e.g., knockout mice defective in production of gamma interferon or of inducible nitric oxide synthetase are particularly susceptible to infection [9]). Future studies will have to analyze whether activation of macrophages can limit necrosis in vitro, too.

It is as yet unknown which molecular factors are involved in the killing of macrophages by virulent *R. equi*. The 80-kbp VapA-expressing plasmid has recently been sequenced (53), but nucleotide sequence analysis did not reveal any obvious candidate for a cytotoxic factor such as an archetype hemolysin, a phospholipase, or the like. Furthermore, De La Pena-Moctezuma et al. (10) analyzed several *R. equi* strains, including an isogenic pair with or without the virulence-associated plasmids for 239 phenotypes including sensitivity to various antibiotics, sugar utilization pattern, peptidase activities, testosterone degradation and more, and they did not find any difference between the strains other than the possession or lack of VapA expression. Previous studies have suggested that necrosis may be caused by rhodococcal glycolipids (49), in agreement with the finding that local injection of rhodococcal cord factor (trehalose dimycolate) also leads to formation of necrotic foci in mice (19). Similarly, purified mycolactone, a mycobacterial polyketide, is cytotoxic for mammalian cells (16). But at present there exists no experimental evidence that there is a correlation between glycolipid composition and presence of the VapA-expressing plasmid. We did not find any cytotoxic activity to be associated with broth in which virulent *R. equi* was cultivated, suggesting that constitutively released glycolipids may not be crucial in induction of necrosis. Similarly, no such correlation was found when testing whether necrosis seen after infection of lung epithelial cells by *M. tuberculosis* could be reproduced by spent bacterial media alone (12). Obviously, unbiased genetic dissection of the virulence-associated plasmids is needed in order to identify a factor(s) responsible for the cytotoxic potential of *R. equi* and to determine whether it is a directly acting cytotoxic factor or whether cytotoxicity is a more indirect effect, e.g., through upregulation of production of a low-abundance cytotoxic glycolipid.

There are very few examples of virulence-associated plasmids contributing to host cell death. The most prominent example is *Shigella flexneri*, which kills host cells by apoptosis (14, 35). The increase in cytotoxicity potential by possession of the

plasmid is as pronounced in *S. flexneri* infections as it is in *R. equi* (VapA+) infections (i.e., an approximately tenfold increase). Generally, cell death seems to occur much earlier for cells infected with *Shigella* than it does in *R. equi* infection, possibly reflecting the fast multiplication of the enterobacteria in their host cells compared to the much slower multiplication reported for *R. equi* (25).

We found that *R. equi* must be alive in order to be cytotoxic. All *R. equi* isolates possessing the VapA-expressing plasmid but treated with paraformaldehyde, UV, the cyclic decapeptide gramicidin S, tetracycline plus amikacin, or by heat (70°C for 30 min) were strongly inhibited in their cytotoxic potency. It is reasonable to assume that there is not a single component in the bacterial cell that is similarly inactivated by all of these treatments and, hence, that it is viability per se that is required.

We hypothesize that viability is the crucial component for macrophage killing, although intracellular multiplication may be required to a limited degree. Hondalus and Mosser (25) have shown that virulent *R. equi* multiplies in murine peritoneal macrophages or in (gamma-irradiated) murine J774A.1 cells. However, the initial lag period of 6 to 12 h described by the authors of that study, followed by a slow increase in the total number of bacteria per macrophage, suggests that bacterial multiplication, if any, is not crucial for relatively early necrosis (seen at 4 and 24 h postinfection).

The experimental system described here seems to be exquisitely suited to study necrotic short-term effects in moderately infected macrophages (the maximum average number of intracellular bacteria per infected macrophage was 3.7 in our experiments). The strong necrotic phenomena seen in this system will allow the molecular dissection of the molecular events leading to macrophage necrosis and are likely to be of importance to the understanding of necrosis in the natural host, the foal, as well. However, due to a strong cytotoxic effect of infection, we are faced with technical obstacles to monitor intracellular multiplication (if any) in this particular system over prolonged periods of time: gentamicin has to be added to the macrophage growth media to keep rhodococci from fast extracellular multiplication. However, as soon as host macrophages become leaky due to necrosis, they will expose the enclosed bacteria to gentamicin and, hence, it will likely be bacteria from the most heavily infected macrophages that die first by the action of the antibiotic.

One could expect that macrophages infected with *R. equi*(+) at a MOI of 3 (i.e., on average, much less than one bacterium per macrophage) would show signs of necrosis after prolonged infection and, possibly, by multiplication of bacteria. We do, however, observe death of approximately two-thirds of these bacteria (data not shown) over a 24 h-period and no significant necrosis, a phenomenon which could be due to any or all of the following effects: (i) killing of intracellular bacteria by gentamicin entering phagosomes via the endocytic pathway, (ii) killing of the infected macrophages and subsequent contact of bacteria with gentamicin, coupled with (iii) ingestion of necrotic macrophages by intact phagocytes (a process which we can occasionally observe by use of the microscope).

In summary, we hypothesize that at least two factors act in concert in the production of cytotoxicity: one factor which is necessary for low levels of necrosis (e.g., cell wall-associated mycolic glycolipids) and which is present in most if not all *R. equi* bacteria and a second, virulence-associated plasmid-dependent factor(s), which is needed for the expression of full cytotoxic potential. It also still remains to be determined whether cytotoxic potential is a virulence factor of *R. equi*, i.e., directly increases virulence in the infected animal, or whether it is rather a consequence of the action of other virulence factors and infection could proceed without it. The latter could be the case if, e.g., the bacteria killed the macrophages by the constant production and release of glycolipids, which can only happen with live bacteria; thus, every bacterial factor that prevented bacterial death would promote host cell necrosis even if this would not be needed for further development of the infection. Again, genetic and functional dissection of the virulence-associated plasmid will help to answer these questions.

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