Rhodococcus equi can survive a phagolysosomal environment in macrophages by suppressing acidification of the phagolysosome

Kiminori Toyooka, 1 † Shinji Takai 2 and Teruo Kirikae 1

1 Department of Infectious Diseases, Research Institute, International Medical Center of Japan, Toyama 1-21-1, Shinjuku, Tokyo 162-8655, Japan
2 Department of Animal Hygiene, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034-8628, Japan

Rhodococcus equi is one of the most important causes of pneumonia in foals and has emerged as a significant opportunistic pathogen of immunosuppressed hosts such as human immunodeficiency virus-infected patients. Virulent R. equi harbouring an 85 kb plasmid, but not the avirulent form lacking the plasmid, has the ability to survive in macrophages. However, the survival mechanism is not known. In the present study, morphological interactions were observed between virulent or plasmid-cured avirulent R. equi and phagolysosomes in murine macrophage-like J774.1 cells by immunocytological methods. The J774.1 cells phagocytosed virulent and avirulent bacteria to a similar extent, and both bacteria replicated in single membrane vacuoles at similar rates up to 6 h after infection. Thereafter, the virulent bacteria continued to grow, whereas the avirulent bacteria stopped growing. When the infected cells were stained with phagosomal and lysosomal markers and observed with a confocal fluorescence microscope, the majority of phagosomes containing these bacteria were fused with lysosomes. Neither R. equi organism has the ability to hinder phagosome-lysosome fusion. The acidity in phagolysosomes containing R. equi was examined by staining with LysoTracker Red DND-99, an acidotropic probe. The phagolysosomes containing virulent organisms were not acidic as compared with avirulent organisms. Over 90 % of the phagolysosomes containing avirulent R. equi were stained with LysoTracker 6 h after infection, whereas less than 50 % of those containing virulent R. equi were stained. Furthermore, when the supernatant obtained from a virulent R. equi culture was added to the cell cultures, the acidity of acidic compartments in macrophages was reduced. The authors conclude that some substance(s) produced by virulent R. equi suppress acidification in phagolysosomes, and help R. equi survival and replication in the bactericidal environment.

INTRODUCTION

Rhodococcus equi is a Gram-positive, facultative intracellular coccobacillus and an important pulmonary pathogen of foals aged 1–3 months (Prescott, 1991; Takai, 1997; Meijer & Prescott, 2004). It has been identified as an increasingly common opportunistic pathogen of immunocompromised hosts such as human immunodeficiency virus (HIV)-infected patients (Gradon et al., 1992; Verville et al., 1994; Sirisanthantana & Supparatpinyo, 1996). Intracellular pathogens, including R. equi, are able to survive and replicate within phagocytic cells of the host, and have evolved mechanisms to manipulate the cell organelles. These organisms are capable of membrane trafficking in several distinct ways (reviewed by Russell, 2000; Sinai, 2000). For example, Shigella, Listeria and Rickettsia rapidly lyse their plasma membrane-derived phagosome and replicate in the cytoplasm. Legionella and Brucella replicate within autophagosome-like or ER-like compartments (Pizarro-Cerda et al., 1998; Arenas et al., 2000; Sturgill-Koszycki & Swanson, 2000). Mycobacteria replicate within an early phagosomal compartment (Clemens & Horwitz, 1996). Leishmania, Coxella and possibly Salmonella survive the phagolysosomal environment (Akporiaye et al., 1983; Russell, 2000). R. equi is able not only to persist in macrophages but also to replicate within them (Honda 2000).

R. equi strains isolated from pneumonic foals typically contain a large plasmid of about 85–90 kb with a 27 kb pathogenicity island containing seven virulence-associated

In this study, we sought to analyse the difference in adaptation to phagolysosomes in murine macrophage-like cell line J774.1 between a wild-type \textit{R. equi} strain and its plasmid-cured strain.

**METHODS**

**Bacteria.** \textit{R. equi} ATCC 33701 (virulent) and its plasmid-cured strain ATCC 33701P (avirulent) were used in this study. Pure cultures of these strains were obtained in brain heart infusion broth (BHI, Difco). The plasmid-cured isogenic strain was obtained by several passages (Takai et al., 1991). Cultures were incubated in a rotary shaker at 100 r.p.m. at 38 °C for 48 h and then stored as a cell suspension in 20 % glycerol at −80 °C. Although we found that the virulent strain did not lose the virulence plasmid at least after five passages, aliquots (1 ml) were thawed for each experiment, and the organisms were incubated in BHI broth for 24 h at 37 °C, washed by centrifugation (16 000 g for 15 min) and suspended (approximately 1 \times 10^8 organisms ml^{-1}) in PBS (pH 7.4). Viable colony-forming units were quantified by plating of serial dilutions on BHI-agar plates.

**Cells and cell culture.** Murine macrophage-like J774.1 cells were kindly provided by Dr T. Suzuki, University of Kansas Medical Center. The cells were grown in flasks with RPMI 1640 medium (Nakarai) containing fetal bovine serum (FBS; 10 %) and 4 mM L-glutamine. The plasmid-cured isogenic mutant was obtained by several passages (Takai et al., 1991). Cultures were incubated in a rotary shaker at 100 r.p.m. at 38 °C for 48 h and then stored as a cell suspension in 20 % glycerol at −80 °C. Although we found that the virulent strain did not lose the virulence plasmid at least after five passages, aliquots (1 ml) were thawed for each experiment, and the organisms were incubated in BHI broth for 24 h at 37 °C, washed by centrifugation (16 000 g for 15 min) and suspended (approximately 1 \times 10^8 organisms ml^{-1}) in PBS (pH 7.4). Viable colony-forming units were quantified by plating of serial dilutions on BHI-agar plates. Heat-killed cells were prepared by boiling the organisms suspended in PBS for 15 min.

**In vitro infection and estimation of intracellular bacterial number.** J774.1 cells cultured overnight (2 \times 10^5 cells) were infected with either virulent or avirulent organisms (1 \times 10^8 organisms ml^{-1}, 20 times more than the number of the J774.1 cells) for 30 min. Cells were then washed with PBS three times to remove uninfected organisms. Washed cells were cultured in RPMI 1640-FBS supplemented with 1 \mu g gentamicin ml^{-1} to kill remaining extracellular bacteria. At specified times after infection, the number of intracellular organisms was determined by microscopic observation or viable counts in agar culture. For microscopic observation, cells on coverslips were fixed in 2 % paraformaldehyde in PBS for 30 min, washed in PBS and permeabilized in 0-1 % saponin in PBS for 30 min at room temperature. After application of Gram stain, bacteria in the cells (at least 100 cells) were viewed with a light microscope and counted. For the colony-forming units assay, infected cells in the 24-well cultures were lysed with 0.1 % saponin in PBS for 30 min at room temperature. Coverslips washed in PBS and permeabilized in 0-1 % saponin in PBS for 30 min at room temperature. Coverslips washed in PBS and permeabilized in 0-1 % saponin in PBS for 30 min at room temperature.

**Bacterial growth in calcium-depleted medium.** To examine growth of \textit{R. equi} in calcium-deprived broth, virulent and avirulent organisms were inoculated at 1 \times 10^7 organisms ml^{-1} into 24-well plates in Luria–Bertani (LB) medium and supplemented with 10, 300 or 600 \mu M EGTA, and then incubated at 37 °C. After incubation, the numbers of organisms were determined both by the optical density at 600 nm and by the colony-forming units assay.

**Antibodies.** Rat monoclonal antibody 1D4B against lysosome-associated membrane protein-1 (LAMP-1) was obtained from Pharmingen, BD Biosciences. Rabbit polyclonal antibody against early endosomal antigen 1 (EEA1) was obtained from Affinity BioReagents. Rabbit polyclonal antibody against cathepsin D was obtained from Wako Pure Chemical Industries. Secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG, anti-rabbit IgG and anti-rat IgG, and Alexa Fluor 568 goat anti-mouse IgG and anti-rabbit IgG obtained from Molecular Probes. To prepare mouse polyclonal antibodies to \textit{R. equi}, the heat-killed avirulent \textit{R. equi} organisms (1 \times 10^8 organisms ml^{-1}), emulsified with an equal volume of Freund’s complete adjuvant (Wako), were injected intraperitoneally into female BALB/c mice (0-5 ml of the mixture per mouse). Injections were repeated three times at 2-week intervals. Two weeks after the last injection, mouse serum was collected for the anti-\textit{R. equi} antibody.

**Immunofluorescence microscopy and electron microscopy.** Cells were seeded on glass coverslips and infected as described above. After incubation, cells were fixed with 2 % paraformaldehyde for 30 min, washed in PBS and permeabilized in 0-1 % saponin in PBS for 30 min at room temperature. Cells on coverslips were soaked in primary antibody in PBS (diluted 1 : 200) for 30 min at room temperature, washed with PBS and soaked with secondary antibody in PBS (diluted 1 : 500). The coverslips with the cells were then mounted on glass slides and viewed with a confocal laser scanning microscope system (LSM 510; Carl Zeiss). In colocalization analysis, cross talk was prevented by means of multi-tracking and a dual direction scan. The results were scored by analysing more than 100 phagosomes from three separate monolayers for each time point.

For electron microscopy, J774.1 cells infected with virulent or avirulent \textit{R. equi} were fixed with 4 % paraformaldehyde and 2 % glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.4) for 2 h at room temperature. The cells were transferred to a secondary fixative containing 2 % osmium tetroxide in 100 mM potassium phosphate buffer for 2 h at room temperature. After washing, the cells were dehydrated in a methanol series and embedded in Epon resin. Ultrathin sections mounted on nickel grids were stained with 4 % aqueous uranyl acetate and 1 % aqueous lead citrate. The grids were examined and photographed with a transmission electron microscope (model 1010EX; JEOL) at 80 kV.

**Detection of the intracellular location of \textit{R. equi} with acidotropic dye.** LysoTracker Red DND-99 (Molecular Probes) served as an acidotropic dye. It consists of a weak base conjugated to a red fluorophore and is used as a marker of phagosomal acidification and maturation (Via et al., 1998). LysoTracker was diluted in RPMI 1640 (1 : 20 000) and added to J774.1 cell cultures for 2 h. Cells were infected with virulent or avirulent organisms (1 \times 10^8 organisms ml^{-1}) for 30 min. Cells were then washed repeatedly to remove unphagocytosed organisms and cultured again with 50 nM LysoTracker in RPMI 1640-FBS. At 0-5 and 6 h post-infection, cells were washed, fixed in 2 % paraformaldehyde for 30 min, washed again in PBS and permeabilized in 0-1 % saponin in PBS for 30 min at room temperature. Coverslips loaded with the cells were soaked in anti-\textit{R. equi} antibody in PBS (diluted 1 : 500) for 30 min at room temperature, washed with PBS and soaked again in secondary antibody in PBS (diluted 1 : 500). The coverslips were mounted on glass slides, and more than 100 phagosomes from three separate monolayers were analysed for each time point with a confocal laser scanning microscope system.
Assay of acidity inside phagolysosomes in cells exposed to bacterial culture supernatant. Culture supernatants were prepared by centrifugation (16,000 g for 15 min) from the BHI-broth cultures, with virulent or avirulent R. equi, of organisms grown for 24 h at 37 °C. Supernatants were adjusted to pH 7.4 with PBS. The supernatants (100 µl) were added to J774.1 cell cultures (1 × 10⁶ cells per 2-ml well). The cells were incubated for 30 min. An equal volume of 100 nM LysoTracker in RPMI 1640-FBS was added and cells were incubated at 37 °C in 95% air/5% CO₂ for 30 min. Some of the cultures were mixed with 100 nM bafilomycin A₁ (Sigma), an inhibitor of the proton ATPase (H⁺-ATPase), and others were mixed with BHI-broth medium. After the incubation, the cells were washed with PBS three times and plated into 96-well plates for fluorescence assay. The fluorescence was measured with a Cytofluor 4000 multi-well fluorescence plate reader (Applied Biosystems) at 560 nm/620 nm and 590 nm/620 nm. The fluorescence of cells not exposed to LysoTracker was subtracted from the fluorescence of cells exposed to LysoTracker. Each experiment was repeated at least three times.

RESULTS AND DISCUSSION

Survival of R. equi organisms in macrophage-like J774.1 cells in vitro

Initially, the numbers of virulent and avirulent organisms phagocytosed by the cells were almost the same (Figs 1 and 2a). The number of virulent R. equi in the cells increased gradually during the first 12 h and then began to decrease. The number of avirulent R. equi increased for the first 6 h but decreased thereafter (Figs 1 and 2a). Heat-killed organisms disappeared quickly after being phagocytosed. The different fates of the two living R. equi organisms in the cells were confirmed with a colony-forming assay (Fig. 2b). Although more than 98% of the cells on glass coverslips infected with either virulent or avirulent R. equi were not stained with trypan blue 6–36 h after infection, the rapid decline of living virulent R. equi after the first 12 h might have been due to the gentamicin penetrating into the damaged cells, and the death of J774.1 cells might have been the result of necrotic events (Luhrmann et al., 2004).
J774.1 cells easily recognized both virulent organisms with virulence plasmid and plasmid-cured avirulent \( R. \) equi organisms, and phagocytosed them to the same extent (Figs 1 and 2). These findings suggest that surface components related to phagocytosis of both virulent and avirulent organisms are similar. The virulence plasmid contains 64 ORFs, including a virulence-associated protein A (VapA) gene, which encodes a surface-expressed antigen (Takai et al., 2000). Thus, the VapA antigen seems not to affect phagocytosis. After phagocytosis, virulent organisms multiplied constantly in the cells for 12 h, whereas the growth of avirulent organisms declined after 6 h of incubation (Fig. 2). We conclude that avirulent organisms cannot establish a milieu to grow continuously in the cells, but virulent organisms can.

**Replication of \( R. \) equi organisms in phagosomes**

To detect the precise location of the organisms in J774.1 cells, cells infected with virulent and avirulent organisms were observed with a transmission electron microscope at 1 and 24 h after infection. The photographs show that virulent \( R. \) equi were in phagosomal organelles 1 h post-infection (Fig. 3a), and that they had multiplied within single membrane vacuoles 24 h post-infection (Fig. 3b, c). Avirulent organisms were observed in phagosomal organelles 1 h post-infection (data not shown), but had not multiplied 24 h post-infection (Fig. 3d). Virulent organisms grew to form clusters in the vacuoles. No bacteria were observed in cytoplasm.

The macrophages infected with virulent organisms were damaged by the growing organisms at 24 h (Fig. 3b, c). Then, gentamicin in the medium could permeate through the macrophage membrane. The decline of the colony-forming units of virulent organisms at 24 and 36 h after infection was due to the killing action of gentamicin and/or to drop-off of the cells with the organisms from the coverslips (Fig. 2b).

**Change in the phagosomal antigens**

EEA1 is a membrane-bound protein component specific to the early endosome. It is essential for fusing early endocytic vesicles (Mills & Finlay, 1998) and for phagosomal maturation (Fratti et al., 2001). LAMP-1 is located on the membranes of lysosomes and phagolysosomes, and cathepsin D is in these organelles (Blum et al., 1991; Fukuda, 1991). Therefore, we used anti-EEA1 antibody to detect early phagosomes, and anti-LAMP-1 and anti-cathepsin D antibodies to detect phagolysosomes and lysosomes. Confocal fluorescence microscopy analysis of these cells is shown in Fig. 4.

![Fig. 3. Electron micrographs of J774.1 cells infected with \( R. \) equi. (a) Virulent \( R. \) equi are seen in phagosomal organelles 1 h after infection. (b) Virulent \( R. \) equi have replicated into single membrane vacuoles 24 h after infection. (c) Higher magnification image of area outlined in (b). (d) Avirulent \( R. \) equi organisms have not replicated in the cell 24 h after infection. Bars, (a) 500 nm, (b, d) 2 μm, (c) 200 nm.](image-url)
Although the fluorescent images of EEA1 were colocalized with the fluorescent images of virulent or avirulent \( R. \) equi 30 min post-infection, there was little colocalization 6 h post-infection (Fig. 4a, d). The percentages of virulent or avirulent \( R. \) equi colocalized with EEA1 at 30 min post-infection were 48.6% and 51.2%, respectively (Fig. 4g), whereas at 6 h post-infection the percentages dropped to 1.9% and 2.5%, respectively (Fig. 4g). In contrast, the fluorescent images of LAMP-1 showed colocalization with virulent and avirulent organisms 6 h post-infection (Fig. 4b, e). The percentages of virulent and avirulent organisms colocalized with LAMP-1 at 30 min post-infection were about 50%, and had increased at 6 h post-infection to 95.0% and 98.0%, respectively (Fig. 4h). Results similar to those with LAMP-1 were obtained for colocalization of the organisms and cathepsin D (Fig. 4c, f, i). Thus, at 30 min, half the phagosomes containing either virulent or avirulent \( R. \) equi organisms were early phagosomes and the others were phagolysosomes. At 6 h, the majority of the phagosomes having either virulent or avirulent \( R. \) equi organisms were phagolysosomes. Collectively the results indicate that phagosomes containing the organisms are rapidly fused with lysosomes to mature into phagolysosomes.

Zink et al. (1987) and Hietala & Ardans (1987) observed in electron micrographs that morphologically intact \( R. \) equi organisms were present and replicating in phagosomes of equine alveolar macrophages in the early stage of infection.

**Fig. 4.** Characteristics of \( R. \) equi phagosomes viewed with a laser confocal microscope. J774.1 cells infected with virulent or avirulent \( R. \) equi and fixed with formaldehyde at 0.5 h (a, b, c) or 6 h (d, e, f) after infection. (a, d) Cells labelled with antibody to EEA1. (b, e) Cells labelled with antibody to LAMP-1. (c, f) Cells labelled with antibody to cathepsin D. Bars, 10 μm. (g–i) Percentage of \( R. \) equi colocalized with EEA1 (g), LAMP-1 (h) and cathepsin D (i). Data are mean ± SD. Grey and white bars designate avirulent and virulent \( R. \) equi, respectively.
Their findings suggested that \textit{R. equi} organisms block the maturation of endosomes to phagosomes and the succeeding phagolysosome formation in the same manner as \textit{Mycobacterium tuberculosis} (Clemens & Horwitz, 1996). However, we did not observe the active replication of \textit{R. equi} organisms in phagosomes of murine macrophage-like J774.1 cells (Fig. 4). \textit{R. equi} multiplication was seen in phagosomal organelles (Fig. 3), suggesting that phagosomes containing the organisms convert quickly to phagolysosomes and that the organisms can replicate there. Although the discrepancy between the earlier observations and ours may be due to the different host cells and different detection methods, the modern method of confocal microscopy with immunostaining used in this study is more reliable than the methods used by others.

Some kinds of pathogens, such as \textit{M. tuberculosis} (Clemens & Horwitz, 1996; Via et al., 1988), block phagosome-lysosome fusion, which results in escape from bactericidal substances in the lysosome and the hindrance of acidification of the phagosome. Other pathogens, such as \textit{Shigella} (Sansonetti et al., 1986) and \textit{Listeria} (Portnoy et al., 1988), lyse the phagosomal membrane and take sanctuary in cytoplasm. However, \textit{R. equi} behaves differently. In J774.1 cells, phagosomes containing virulent \textit{R. equi} organisms, as well as avirulent \textit{R. equi}, fuse with lysosomes, and the organisms start to grow in the phagolysosomes (Fig. 4). Although the growth of avirulent \textit{R. equi} in phagolysosomes did not persist, that of virulent \textit{R. equi} lasted for a while (Figs 1, 2 and 3). Virulent \textit{R. equi} are tolerant of bactericidal substances in the phagolysosomal environment.

A protein for sensing and binding calcium ions is necessary for the survival of \textit{Salmonella} (Garcia Vescovi et al., 1997). A calcium-binding protein (CBP1) is associated with a virulence factor of \textit{Histoplasma capsulatum}, an intracellular fungal pathogen (Sebghati et al., 2000). These pathogens can survive within the calcium-poor environment of macrophage phagosomes by secreting a calcium-binding protein and robbing calcium ions from the surroundings. We examined the growth of \textit{R. equi} in calcium-deprived broth, and no difference could be seen in the growth between virulent and avirulent \textit{R. equi} organisms (data not shown).

\section*{Acidity in phagosomes having virulent or avirulent organisms}

Infected cells treated with LysoTracker are shown 30 min and 6 h post-infection in Fig. 5. At 6 h, the majority of avirulent organisms were colocalized with acidic substances (Fig. 5a, c). In contrast, only a few virulent organisms were colocalized (Fig. 5b, c). At 30 min, less than 40 % of either organism were colocalized with acidic substances in the cells. These results indicate that in the early stage of infection, the milieu surrounding both virulent and avirulent \textit{R. equi} are not acidic. Thereafter, the milieu of avirulent organisms becomes acidic and that of virulent organisms does not.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.jpg}
\caption{Colocalization of \textit{R. equi} with the acidotropic dye LysoTracker}
\end{figure}

\textbf{Blocking to acidify phagosomes by bacterial products}

Results from the antibody colocalization experiments suggested that trafficking to phagolysosomes occurred for both virulent and avirulent \textit{R. equi} organisms. Results from LysoTracker experiments, however, showed that phagolysosomes containing virulent \textit{R. equi} organisms did not acidify. We hypothesized that virulent \textit{R. equi} organisms in phagolysosomes secrete inhibitors to hinder phagolysosomal acidification. To confirm this, culture supernatants of the organisms and LysoTracker were added together to the J774.1 cell culture, and the fluorescence intensity of the cells was assessed (Fig. 6). When the fluorescence intensity of cells incubated with PBS was taken as 100 %, the intensity of the cells exposed to virulent supernatant was 67.9 %. The fluorescence intensities of avirulent supernatant, medium supplemented with BHI and medium supplemented with bafilomycin A1, which is an inhibitor of H\textsuperscript{+}-ATPase, were 80.6, 84.9 % and 21.6 %, respectively. The fluorescence intensity of the cells exposed to the virulent supernatant was significantly lower than that of cells exposed to the avirulent
one (P < 0.05). The results indicate that the virulent organisms produced substance(s) to suppress acidification.

Recently, the complete DNA sequence of the virulence plasmids was reported (Takai et al., 2000). VapA, the product of the vapA gene, is located on the bacterial surface, and its expression is thermoregulated within the in vivo temperature range of 34–41°C (Takai et al., 1992). The bacterial virulence could hardly be explained by the expression of VapA alone (Giguere et al., 1999), and also the vapA gene was shown to be related to growth of the organisms in macrophages (Jain et al., 2003). Acidic pH and oxidative bursts in phagolysosomes are part of the bactericidal defence mechanism of phagocytic cells. However, the expressions of the vapA, -D, and -G genes is induced by acid pH or H₂O₂, conditions which correspond to situations encountered by R. equi inside macrophages (Benoit et al., 2001, 2002).

The plasmid in the virulent strain was shown to drop off after several passages (Takai et al., 1991). To avoid drop-off of the plasmid during the experiments, we always used each aliquot in one experiment. Each aliquot contained organisms with the plasmid that had been previously frozen, stocked and thawed before experiments. The possibility of the drop-off during each experiment seems to be low, although this was not confirmed.

We reported that VapA expression is below normal at pH 6 (Takai et al., 1996). As shown in Fig. 5, the acidity in phagolysosomes containing virulent R. equi did not increase much, whereas that in phagolysosomes having avirulent R. equi did not increase. Furthermore, we showed that the supernatant obtained from cultures of virulent R. equi contains molecules that hinder acidification in acidic compartments. The vapC, -D and -E genes, which are arranged in tandem downstream of vapA, are members of a gene family and encode secreted proteins that are approximately 50% homologous to VapA and each other (Byrne et al., 2001). These three genes are found only in R. equi strains that express VapA and are highly conserved in VapA-positive isolates from both horses and humans. VapC, -D and -E are secreted proteins that are regulated by temperature coordinately with VapA; the proteins are expressed when R. equi is cultured at 37°C but not at 30°C, a finding that is compatible with a role in virulence. As secreted proteins, VapC, -D and -E, produced by virulent R. equi, may regulate the acidity in phagolysosomes. It is known that both virulent and avirulent R. equi can withstand acidity to pH 4.0 (Benoit et al., 2000), but the killing effect of acid may depend not only on the pH but also the duration of exposure to it. Avirulent R. equi are exposed to acid for a longer time than virulent R. equi are exposed in the phagolysosome because they have no means to control acidification. The suppression of acidification may be an important mechanism for R. equi to establish a milieu in which to live and replicate. It may be due to substance(s) produced by the virulent organisms that block the function of H⁺-ATPase in the macrophages. Experiments are currently in progress in our laboratory. Phagolysosomes contain many antibacterial substances. These substances may also work strongly on avirulent R. equi in the acidic environment. A model depicting R. equi infection in the macrophage is shown in Fig. 7.

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REFERENCES


