**Review**

**Tactics of Mycobacterium avium subsp. paratuberculosis for intracellular survival in mononuclear phagocytes**

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Johnes’s disease is a condition that refers to chronic granulomatous enteritis in ruminants. It is believed that survival and replication of *Mycobacterium (M.) paratuberculosis* in mononuclear phagocytes plays an important role in the pathogenesis of Johnes’s disease. However, it is not clear how *M. paratuberculosis* survives for long time periods in mononuclear phagocytes, nor is it clear which factors trigger multiplication of these bacilli and result in the development of Johnes’s disease. Investigating the intracellular fate of *M. paratuberculosis* is challenging because of its very slow growth (more than two months to form visible colonies on media). Existing animal models also have limitations. Despite those obstacles, there has been progress in understanding the intracellular survival tactics of *M. paratuberculosis* and the host response against them. In this review, we compare known aspects of the intracellular survival tactics of *M. paratuberculosis* with those of other mycobacterial species, and consider possible mycobactericidal mechanisms of mononuclear phagocytes.

**Keywords:** intracellular, Johnes’s disease, Mycobacterium avium, Mycobacterium paratuberculosis

**Introduction**

*Mycobacterium (M.) paratuberculosis* is the etiologic agent of chronic enteritis of ruminants, known as paratuberculosis or Johnes’s disease [14]. *M. paratuberculosis* is a Gram-positive, acid-fast bacillus that belongs to the *M. avium* complex [10]. It grows very slowly and requires mycobactin J, an iron-chelating cell wall component produced by most other mycobacteria, for growth in vitro. As a result, visible colony formation takes 8 to 12 weeks or longer. Identification of *M. paratuberculosis* depends on mycobactin-dependent growth and detection of the species-specific IS 900 insertion sequence by polymerase chain reaction (PCR) [20]. Like other mycobacteria, the cell wall of *M. paratuberculosis* is lipid-rich and consists of several layers. The main components of the cell wall are lipoarabinomannan (LAM) and arabinomannan (AM) [90]. It has been reported that LAM is highly immunogenic and reacts with sera from infected cattle [47,89]. Several proteins that induce a humoral immune response in infected cows are produced by *M. paratuberculosis*, and the antigen 85 complex, which consists of four proteins [71]. These protein antigens have been investigated in an effort to find antigens that react specifically with sera from animals infected with *M. paratuberculosis*, and not those infected with other mycobacterial species. However, these antigens share epitopes with *M. avium* [71].

It is believed that young calves are infected by *M. paratuberculosis* via the oral route, through contaminated feces, colostrum, or milk. Most infected animals do not develop clinical symptoms. Only 10-15% of infected cows develop clinical disease, usually after two or more years of infection [72]. However, subclinically infected animals may shed bacilli intermittently in their feces, spreading infection in the herd. Cattle with clinical Johnes’s disease exhibit decreased production, diarrhea, and weight loss [19,71,90]. These animals usually shed bacilli in their feces and have detectable antibodies in their serum. The host response to *M. paratuberculosis* infection results in granulomatous lesions in the small intestine. The intestinal wall subsequently undergoes progressive thickening, which is caused by hypoproteinemia and edema due to decreased intravascular osmotic pressure [19]. Pathological changes in the small intestine result in malabsorption of nutrients and weight loss. It is not clear which bacterial factors trigger multiplication of *M. paratuberculosis*, or which host responses control *M. paratuberculosis* infection [14,18,21,22,71,83,94].

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Entry of *M. paratuberculosis* into mononuclear phagocytes

After ingestion by young calves, it is thought that the bacilli enter intestinal tissue through M cells in the Peyer's patches of the small intestine [67]. *M. paratuberculosis* expresses fibronectin attachment proteins (FAPs) [77]. Fibronectin bound to these receptors can, in turn, bind to integrins on M cells and mediate the uptake of *M. paratuberculosis* [78,79,95]. After crossing the intestinal epithelial layer, subepithelial macrophages phagocytose bacilli, presumably using several different surface receptors.

Mononuclear phagocytes serve as the intracellular niche for *M. paratuberculosis* survival and multiplication. Macrophages are known to have several receptors that are involved in the uptake of mycobacteria [7,73,75,85-87]. These receptors include complement receptors (CR1, CR3, and CR4), immunoglobulin receptors (FcR), the mannose receptor, and scavenger receptors [2,29,30,39,92]. Human macrophages exhibit decreased uptake of *M. avium* following the addition of anti-CR3 antibodies [11]. Serum opsonization of *M. tuberculosis* increases the uptake of bacilli by human monocytes, while blocking CR3 with specific antibody decreased the uptake of bacilli by about 87% [76]. Likewise, the uptake of *M. paratuberculosis* by murine macrophages was inhibited by preincubation with anti-CR3 monoclonal antibody [17]. Similarly, opsonization of *M. paratuberculosis* with serum from normal adult cows or from cows with clinical paratuberculosis enhanced the uptake of bacilli by bovine mononuclear phagocytes [43,106,112]. These observations suggest that complement opsonization is important to the uptake of *M. paratuberculosis* by bovine mononuclear phagocytes. It has also been reported that mononuclear phagocytes can synthesize and secrete complement proteins that opsonize particles for phagocytosis [62].

Possible mechanisms of intracellular survival or death of *M. paratuberculosis*

It is very important to understand the survival mechanisms of *M. paratuberculosis* in bovine mononuclear phagocytes if we are to develop more effective ways to control Johne's disease. Because infected animals develop clinical symptoms of Johne's disease relatively slowly, infected cows presumably can suppress multiplication of *M. paratuberculosis* and delay or prevent the development of Johne's disease.

Different routes of entry can alter the intracellular fate of ingested bacilli [6,7,23,28,45]. For example, complement receptor CR1-mediated uptake of particles does not stimulate the production of superoxide anion [23,45]. Mannose receptor-mediated uptake of pathogenic or nonpathogenic mycobacteria does not activate NADPH oxidase in human macrophages [7], and selective receptor blockade did not alter the intracellular survival of *M. tuberculosis* in human macrophages [111]. CR3-mediated binding and uptake of *M. tuberculosis* by macrophages does not seem to affect the intracellular fate of bacilli [96]. There was no difference in bacterial burden or granulomatous response between wild-type and complement component C3-deficient mice following *M. avium* infection [12]. Opsonization of *M. paratuberculosis* with normal serum from adult cows, or serum containing specific antibodies against *M. paratuberculosis*, increased the uptake of bacilli by bovine mononuclear phagocytes. However, intracellular survival of *M. paratuberculosis* in the bovine mononuclear phagocytes was not affected [43].

Toll-like receptors (TLR) are pattern-recognition receptors that detect microbes or microbial components and initiate inflammatory responses [63,93]. Antigen-presenting cells (APCs), which include dendritic cells and macrophages, express TLR receptors and initiate an immune response, and then bind to pathogen-associated molecular patterns (PAMPs) of microbes [109]. The 19 kDa lipoprotein of *M. tuberculosis* activates murine and human macrophages through TLR2, and this, in turn, activates a signaling pathway that kills intracellular bacilli [84,91]. This TLR2-mediated mycobactericidal effect is dependent on the production of nitric oxide in murine macrophages and enhanced expression of vitamin D receptor in human macrophages [58,91]. Bovine monocytes and macrophages express mRNA for TLR2 and TLR4 [105]. TLR ligands such as lipopolysaccharide (LPS), *Salmonella dublin*, and *Listeria monocytogenes* activate bovine mononuclear phagocytes and induce the production of reactive oxygen intermediates (ROIs) [104]. Although there have been no reports of how TLR activation alters the intracellular survival of *M. paratuberculosis*, pretreatment of J774 cells and bovine monocytes with LPS and IFN-γ slightly decreased the number of viable intracellular *M. paratuberculosis* bacteria [44,110].

During the process of phagocytosis by macrophages, the NADPH oxidase complex on the plasma membrane produces a series of ROIs, including superoxide anion, hydrogen peroxide, and hydroxyl radical [27,40,80]. These ROIs have been implicated in killing mycobacteria [46,50]. However, there have been conflicting reports on the mycobactericidal effect of ROIs against *M. tuberculosis* [16,34,55,56,61]. Mycobacteria have some ability to evade being killed by ROIs [41,61]. Bovine macrophages produce superoxide anion following stimulation with phorbol 12-myristate 13-acetate (PMA), zymosan, or LPS [104]. However, bovine monocytes and macrophages do not stimulate much ROI production after *M. paratuberculosis* infection, and IFN-γ activation of bovine monocytes and macrophages did little to increase the release of ROIs [103,113]. *M. paratuberculosis* secretes superoxide dismutase,
which is a possible protective mechanism for intracellular bacilli [59]. However, we need more evidence to clarify the role of ROIs on the intracellular fate of M. paratuberculosis in bovine mononuclear phagocytes.

Nitric oxide and other reactive nitrogen intermediates (RNI) are known to be major mycobactericidal molecules, especially in mice [15,16,32]. After activation with IFN-γ and TNF-α, murine macrophages produce significantly increased amounts of RNI [60]. Mycobacteria have the ability to inhibit recruitment of nitric oxide synthase to mycobacteria-containing phagosomes [65]. IFN-γ activation of bovine macrophages did not result in increased nitric oxide production, whereas IFN-γ-activated murine macrophages produced significant amounts of nitric oxide (45-83 μM) at 72 and 96 h after treatment [3]. Bovine macrophages can produce increased nitric oxide in response to other stimuli. LPS, Listeria monocytogenes, and Salmonella dublin all enhanced the production of nitric oxide (20 to 70 μM) by bovine macrophages [3,104]. In contrast, M. paratuberculosis-infected bovine macrophages and monocytes produced small amounts of nitric oxide (2-3 μM), and IFN-γ treatment increased nitric oxide production only to 6-8 μM [103]. Stimulation of monocytes with IFN-γ or IFN-γ and LPS increased the production of nitric oxide, but the amount produced was inadequate to kill intracellular M. paratuberculosis [110]. In the granulomatous lesions of bovine Johne's disease, the immunoreactivity of inducible nitric oxide synthase (iNOS) is weak and localized at or near the intestinal crypts [42]. Chemically-generated nitric oxide kills extracellular M. paratuberculosis, but bovine mononuclear phagocytes do not produce enough nitric oxide to kill intracellular M. paratuberculosis [110]. Although the inhibition of nitric oxide production resulting from the addition of N\textsuperscript{\textcircled{3}}-monomethyl-L-arginine (NMMA) increases the intracellular survival of M. tuberculosis in IFN-γ-pretreated murine macrophages [4], NMMA treatment does not promote intracellular survival of M. paratuberculosis in bovine monocytes [109]. These data suggest that nitric oxide might not be a major mycobactericidal mechanism with which to control M. paratuberculosis in infected cattle.

One of the potential microbicidal mechanisms of phagocytes is the phagosome-lysosome fusion [24,25,98]. The lysosomal vacuoles contain potent hydrolytic enzymes that kill and degrade ingested microbes [8]. These enzymes require an acidic environment for their optimal activity. This acidic condition is maintained by a membrane ATP-dependent proton pump, the vacuolar H\textsuperscript{+}-ATPase [64]. Mycobacterial phagosomes inhibit the recruitment of vacuolar H\textsuperscript{+} ATPase and phagosomal acidification [88]. Phagosomal maturation is also inhibited by retention of the small GTP-binding protein, Rab5, and by reduced recruitment of early endosomal autoantigen 1 (EEA1) to mycobacterial phagosomes [36,99]. M. tuberculosis LAM inhibits phagosome maturation [37,38,97]. Live M. paratuberculosis cells inhibit phagosome-lysosome fusion and phagosomal acidification in the J774 murine macrophage cell line, but killed M. paratuberculosis cells do not have this effect [51]. Similar results were seen in bovine mononuclear phagocytes, in which killed M. paratuberculosis cells were associated with greater phagosome-lysosome fusion than live M. paratuberculosis cells [107]. Pretreatment with IFN-γ and LPS enhanced phagosome-lysosome fusion in murine macrophages infected with M. avium or M. bovis BCG [74,100]. Treatment of M. paratuberculosis-infected J774 cells with IFN-γ and LPS also enhanced phagosome-lysosome fusion and the killing of intracellular bacilli [44]. However, the effect of IFN-γ and LPS treatment on the maturation of phagosomes containing M. paratuberculosis in bovine mononuclear phagocytes is unknown. There have been few investigations of the molecular mechanisms of phagosomal maturation and of the mycobacterial molecules that inhibit phagosomal maturation in bovine mononuclear phagocytes.

After infection with M. paratuberculosis, bovine monocytes produce TNF-α [1]. Although gene expression of TNF-α was identified in ileal tissues of cattle infected with M. paratuberculosis, no difference was seen between uninfected and infected cattle [57]. TNF-α treatment of murine macrophages infected with M. paratuberculosis resulted in either enhanced or decreased viability of intracellular bacilli, depending on the TNF-α concentrations and the lengths of incubation [82]. No report has yet been published on the effect of TNF-α on intracellular survival of M. paratuberculosis in bovine mononuclear phagocytes.

Although M. avium is antigenically and genetically very similar to M. paratuberculosis, it is generally considered to be relatively nonpathogenic in cattle. Bovine macrophages expressed greater amounts of IL-10 mRNA following infection with M. paratuberculosis than with M. avium [103]. IL-10 is an anti-inflammatory cytokine that suppresses the activation of macrophages [33]. The IL-10 gene is expressed to a greater extent in intestinal tissues and lymph nodes from cows clinically infected with M. paratuberculosis than in subclinically infected or healthy cows [49]. Bovine macrophages infected with M. paratuberculosis produce IL-10, and neutralization of IL-10 enhances the killing of intracellular bacilli [101]. Another report showed greater production of IL-10 from peripheral blood mononuclear cells (PBMC) isolated from cows with clinical Johne's disease than from healthy cows [49]. Addition of IL-10 increased intracellular survival of M. paratuberculosis in PBMCs isolated from healthy cows [48]. Neutralization of IL-10 also increased the production of IFN-γ by bovine peripheral blood mononuclear cells after infection with M. paratuberculosis, and enhanced phagosomal acidification and apoptosis of bovine macrophages [13,101]. However, the general role of IL-10 in resistance to mycobacterial infection is not clear. IL-10/ mice did not show greater resistance to acute M. tuberculosis.
infection than did wild-type mice [69].

Apoptosis is a process of programmed cell death that is characterized by DNA fragmentation, nuclear chromatin condensation, compacting of cellular organelles, and membrane blebbing [35]. It has been suggested that apoptosis of mycobacteria-infected macrophages induces the intracellular killing of bacilli, but that necrotic macrophage death does not induce the killing of mycobacteria [66]. There have been reports that apoptotic stimuli like Fas ligand, TNF-α, picolinic acid, ATP, and the mycobacterial 19 kDa lipoprotein can kill intracellular mycobacteria in mononuclear phagocytes [66,70,72,91]. However, it is not clear whether apoptosis of infected macrophages is required for intracellular killing of mycobacteria, nor is it clear whether stimuli that induce intracellular killing of bacilli are distinct from those that trigger apoptosis. M. tuberculosis induced 30-50% apoptosis of infected human alveolar macrophages at 2 and 4 days after infection, and this occurred via a TNF-α-dependent mechanism. Interestingly, pathogenic M. tuberculosis evades apoptosis of macrophages by inducing the secretion of TNF-R2 from infected macrophages that, in turn, is dependent on the production of IL-10 [9]. M. paratuberculosis induced 18 to 27% apoptosis of infected bovine monocytes at 6 and 48 h after infection, with live bacilli causing greater apoptosis than heat-killed M. paratuberculosis [5]. In that paper, however, the authors did not quantify changes in the intracellular survival of M. paratuberculosis. Interestingly, M. paratuberculosis caused less apoptosis in bovine macrophages than did M. avium [102]. More recently, evidence has indicated that the longer survival of M. paratuberculosis within bovine monocytes in vitro (4 to 8 days) results in morphological changes that may reflect reduced differentiation or survival of the infected monocytes [107].

Extracellular ATP can kill intracellular mycobacteria, presumably through the activation of purinergic receptors on the surface of infected mononuclear phagocytes. ATP is released by nonlytic and lytic mechanisms from both resting macrophages. ATP released from lymphocytes or mononuclear phagocytes in foci of mycobacterial infection might activate intracellular killing of bacilli are distinct from those that trigger apoptosis. M. tuberculosis induced 30-50% apoptosis of infected human alveolar macrophages at 2 and 4 days after infection, and this occurred via a TNF-α-dependent mechanism. Interestingly, pathogenic M. tuberculosis evades apoptosis of macrophages by inducing the secretion of TNF-R2 from infected macrophages that, in turn, is dependent on the production of IL-10 [9]. M. paratuberculosis induced 18 to 27% apoptosis of infected bovine monocytes at 6 and 48 h after infection, with live bacilli causing greater apoptosis than heat-killed M. paratuberculosis [5]. In that paper, however, the authors did not quantify changes in the intracellular survival of M. paratuberculosis. Interestingly, M. paratuberculosis caused less apoptosis in bovine macrophages than did M. avium [102]. More recently, evidence has indicated that the longer survival of M. paratuberculosis within bovine monocytes in vitro (4 to 8 days) results in morphological changes that may reflect reduced differentiation or survival of the infected monocytes [107].

Extracellular ATP can kill intracellular mycobacteria, presumably through the activation of purinergic receptors on the surface of infected mononuclear phagocytes. ATP is released by nonlytic and lytic mechanisms from both resting macrophages, as well as by cells undergoing apoptotic or necrotic cell death. ATP released from lymphocytes or mononuclear phagocytes in foci of mycobacterial infection might activate purinergic receptors on infected mononuclear phagocytes. Extracellular ATP is known to be cytotoxic to macrophages, and triggers the killing of mycobacteria in murine and human macrophages [26,31, 52-54,68]. This response reflects increased intracellular calcium and phospholipase D (PLD) activity following ATP activation of P2X7 receptors. This subsequently results in increased phagosome-lysosome fusion of mycobacterium-containing phagosomes [52,53]. It is not clear whether macrophage cytotoxicity is required for the mycobactericidal effect of ATP to occur. Bovine macrophages express mRNA for the P2X7 receptor, and the addition of ATP to bovine macrophages infected with M. bovis BCG induced the killing of intracellular bacilli [81]. Elimination of extracellular ATP by the addition of apyrase increased the survival of M. paratuberculosis-infected bovine monocytes, but unexpectedly reduced the survival of bacilli. Similarly, the addition of ATP or benzyl-ATP reduced the survival of M. paratuberculosis-infected monocytes, but not the survival of the bacilli themselves [108]. Thus, M. paratuberculosis may differ from other mycobacterial species in terms of how the presence of ATP affects the intracellular survival of bacilli within bovine mononuclear phagocytes.

In this review, we have described possible intracellular survival mechanisms of M. paratuberculosis in infected macrophages, mainly in comparison to M. tuberculosis and M. avium. M. paratuberculosis seemed to have shared strategies with other closely-related mycobacteria for intracellular survival, but it also has unique mechanisms. It is important to clarify the intracellular survival tactics of M. paratuberculosis in order to understand the pathogenesis of Johne's disease and develop means by which to prevent this disease. However, it is difficult to investigate the survival mechanisms of M. paratuberculosis in infected macrophages because of the long incubation period required to count visible colonies in media and the limited availability of bovine reagents to use for this purpose. With the development of new technologies and vigorous efforts, these survival mechanisms will be better elucidated in the future, and will allow us to understand the pathogenesis of this, one of the most challenging bacteria to study.

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