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ATP Release by Infected Bovine Monocytes Increases the Intracellular Survival of *Mycobacterium avium* Subsp. *paratuberculosis*

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ATP release by infected bovine monocytes increases the intracellular survival of *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Mycobacterium avium subsp. *paratuberculosis* is the etiologic agent of Johne's disease, a chronic intestinal infection in ruminants. Adenosine 5′-Triphosphate (ATP) has been reported to induce killing of several *Mycobacterium* species in human and murine macrophages. We investigated whether ATP secreted from *M. avium* subsp. *paratuberculosis*-infected bovine monocytes affects intracellular survival of the bacilli. Bovine monocytes constitutively secreted ATP during an 8-day incubation period *in vitro*; however, *M. avium* subsp. *paratuberculosis* infection did not enhance ATP release. Removal of extracellular ATP by the addition of apyrase increased the viability of infected monocytes, but surprisingly decreased the number of viable intracellular bacilli. In contrast to previous reports, addition of extracellular ATP (1 mM) increased intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine monocytes. Neither apyrase nor ATP altered production of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI) by bovine monocytes. These results suggest that ATP release from infected bovine monocytes improves, rather than decreases, the intracellular survival of *M. avium* subsp. *paratuberculosis*.

Keywords: *Mycobacterium* subsp. *paratuberculosis,* bovine, monocytes, ATP, purinergic

Résumé

Mycobacterium avium sous-espèce paratuberculosis est l'agent étiologique de la maladie de Johne, une infection intestinale chronique des ruminants. L'adénosine 5′-Triphosphate (ATP) est connue pour induire la mort de plusieurs espèces de mycobactéries dans les macrophages humains et murins. Nous avons recherché si l'ATP secrétée par *Mycobacterium avium paratuberculosis* infectant les monocytes bovins affecte la survie intracellulaire des bacilles. Les monocytes bovins ont secrété de l'ATP pendant huit jours *in vitro*. Cependant, l'infection par *Mycobacterium avium paratuberculosis* n'augmente pas le taux d'ATP. Le déplacement de l'ATP extracellulaire par l'addition d'apyrase a augmenté la viabilité des monocytes infectés mais, étonnamment a diminué le nombre de bacilles intracellulaires viables. Contrairement au rapport précédent, l'addition d'ATP extracellulaire (1 mM) a augmenté la survie intracellulaire de *Mycobacterium paratuberculosis avium* dans les monocytes de bovins. Ni l'apyrase ni l'ATP n'ont modifié la production des réactions oxygènes intermédiaires (ROI) ou des réactions intermédiaires d'azote (RNI) dans les monocytes de bovins. Ces résultats suggèrent que le dégagement d'ATP à partir des monocytes de bovins infectés augmente plutôt que diminue la survie intracellulaire de *Mycobacterium avium paratuberculosis*.

Mots clés: paratuberculose, maladie de Johne, *Mycobacterium avium paratuberculosis,* ATP

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* is the etiologic agent of paratuberculosis (Johne's disease), a chronic granulomatous enteritis of ruminants [1]. There is reason to believe that some infected cows control *M. avium* subsp. *paratuberculosis* infection and do not develop clinical disease [2]. We know relatively little about the innate immune mechanisms that control the survival of *M. avium* subsp. *paratuberculosis* in bovine mononuclear phagocytes.

Adenosine 5′-Triphosphate (ATP) is secreted by lytic and nonlytic mechanisms from many types of cells, including monocytes and macrophages [3]. Once released, ATP binds to purinergic receptors (P2X) on the cell surface and modulates various cellular functions [4]. There have been several reports that addition of ATP induces apoptosis of macrophages and kills intracellular *Mycobacterium* species in human and murine macrophages [5–10]. For example, ATP binding to $P2X_7$ receptors increased intracellular calcium and phospholipase D (PLD) activity, and subsequently increased phagosome–lysosome fusion in *Mycobacterium tuberculosis*-infected human macrophages [8]. There is one report that ATP binding to $P2X_7$ receptors on bovine macrophages stimulated intracellular killing of *M. bovis* BCG [11].

Some cell types constitutively secrete ATP in culture at pM to nM concentrations [3]. ATP secreted from murine macrophages-induced spontaneous cell death; addition of apyrase, an ectoenzyme that hydrolyses ATP, enhanced macrophage viability [12]. As described above, addition of ATP (3–5 mM)-induced killing of intracellular *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG in human and murine macrophages, respectively [5–10]. However, there have been

no published reports on whether endogenously secreted ATP affects the intracellular survival of *Mycobacterium* species in mononuclear phagocytes.

In this study, we hypothesized that ATP secreted from *M. avium* subsp. *paratuberculosis*-infected monocytes would be cytotoxic to bovine monocytes and kill the intracellular bacilli. As expected, we observed that removal of extracellular ATP by addition of apyrase increased the viability of infected monocytes. However, to our surprise apyrase also decreased the intracellular survival of *M. avium* subsp. *paratuberculosis*. Conversely, short-term addition of ATP to the culture medium increased the number of viable *M. avium* subsp. *paratuberculosis* in infected monocytes. These data suggest that ATP release from infected bovine monocytes enhances, rather than diminishes, the intracellular survival of *M. avium* subsp. *paratuberculosis*.

2. Materials and methods

2.1. Bacteria culture

M. avium subsp. *paratuberculosis* strain K-10 was grown to a final concentration of 10^8 CFU/ml in Middlebrook 7H9 broth (Difco laboratories, Detroit, MI) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson Microbiology System, Sparks, MD), 0.05% Tween 80 and 2 μg/ml of mycobactin J (Allied Laboratories, Ames, Iowa). After harvesting and washing the bacteria with phosphate-buffered saline, single-cell suspensions of bacteria were made by using a motor-driven overhead stirrer and glass-Teflon homogenizer in a biosafety cabinet. To further remove clumped bacteria, the bacterial cells were allowed to settle for 30 min and the supernatant was centrifuged for 10 min at 200 × *g*. The bacteria in the supernatant were resuspended in PBS plus 10% glycerol, aliquoted and stored at −70 °C. The number of viable bacteria in the stock was determined by a radiometric (BACTEC) method, or by plate counts on 7H10 agar (CFU). For microscopic counting and differentiation of viable bacterial cells, the Live/Dead Baclight bacterial viability kit (Molecular Probes Inc., Eugene, Oregon) was used with the aid of a Petroff-Hauser chamber. Before each assay, an aliquot was thawed, diluted in RPMI-1640 medium without antibiotics and used for infection of cells *in vitro*.

2.2. Bovine monocytes

For isolation of bovine monocytes, blood was collected from the tail vein of healthy donor cows in the UW-Madison Dairy Science herd, using sodium citrate (0.38% (v/v) final concentration) as anticoagulant. Although this herd does not have a history of clinical Johne's disease, one cow tested positive (by ELISA) for Johne's each of the past 2 years and was culled. Neither of these cows were used as a blood donor in this study. The blood was centrifuged for 30 min at 400 × *g* and the plasma was removed. The buffy coat cells were resuspended in 35 ml of Hanks balanced salts solution (HBSS; Mediatech Inc., Herndon, VA) containing 4 mM EDTA, layered over 15 ml of Ficoll-Histopaque 1083 (Sigma Diagnostic Inc., St. Louis, MO) and centrifuged at room temperature for 40 min at 600 × *g*. The mononuclear cells were collected from the interface, the red blood cells were

lysed with RBC lysis buffer (150 mM NH_4Cl and 10 mM Tris, pH 7.5) and washed three times with HBSS. The isolated mononuclear cells were resuspended in RPMI-1640 medium (Mediatech Inc., Herndon, VA) supplemented with 1% FBS and adjusted to a concentration of 3×10^6 cells/ml. The cells were distributed (1 ml per well) into wells in a 24-well tissue culture plate (FALCON BD labware, Franklin Lakes, NJ). The monocytes were allowed to adhere for 2 h at 37 °C in 5% CO_2 and nonadherent cells were removed by washing with warm RPMI 1640. Adherent monocytes were used for infection assays and cultured in RPMI-1640 with 10% FBS (ATLANTA Biologicals, Lawrenceville, GA) without antibiotics. At the time of infection, the estimated number of adherent cells was approximately 2×10^5/well.

2.3. Measurement of ATP release from bovine monocytes

Monocytes were infected with *M. avium* subsp. *paratuberculosis* at a multiplicity of infection (MOI) of 10:1 (bacteria:monocyte) in the presence of 10% autologous serum for 3 h at 37 °C in 5% CO_2. The uningested bacilli were removed by washing three times with warm RPMI, and the monocytes were incubated further in RPMI with 10% FBS without antibiotics. Half of the medium was replaced with fresh RPMI supplemented with 10% FBS without antibiotics at day 4. At the indicated time points, we collected conditioned media, which were centrifuged at 200 × *g* for 5 min at 4 °C. The supernatants were distributed into wells (100 µl each well) of an opaque 96-well plate and an equal volume of CellTiter-Glo Luminescent Cell Viability reagent (Promega, Madison, WI) was added to the wells and incubated for 10 min at room temperature. The luminescent signal was measured using a luminometer (The Reporter; Turner Biosystems, Sunnyvale, CA). A series of dilutions of a known concentration of ATP was used to generate a standard curve from which ATP concentrations in the samples were estimated by extrapolation. In some experiments, we added 5 U of apyrase (grade VI, Sigma, St. Louis, MO) into the wells of *M. avium* subsp. *paratuberculosis*-infected monocytes at day 0 and day 4 after infection, and the ATP concentration in the conditioned media was measured as described above.

2.4. Quantitation of viable intracellular M. avium subsp. paratuberculosis

Infected monocytes were cultured in RPMI media supplemented with 10% FBS without antibiotics for up to 8 days at 37 °C in 5% CO_2. In some experiments, 5 U or 10 U of apyrase were added to the infected monocytes at the indicated time points. In other experiments, 0.1 mM or 1 mM Adenosine 5′-Triphosphate (ATP, Calbiochem, La Jolla, CA) was added to infected monocytes immediately, and 2 and 4 days after infection. In all experiments, we harvested conditioned media from infected monocytes at days 4 and 8. The conditioned media were centrifuged at 2000 × *g* for 30 min and lysed with 0.05% SDS to release any bacilli within the detached cells. The adherent monocytes were similarly lysed with 0.05% SDS and the lysate inoculated into BACTEC 12B vials along with the conditioned media lysate from the same well. The numbers of viable bacilli were estimated by a radiometric method as described previously [13].

2.5. Viability of infected monocytes

The viability of infected monocytes was assessed using the CellTiter-Blue Cell Viability Assay (Promega, Madison, WI). Monocytes were infected with *M. avium* subsp. *paratuberculosis* as described above. The media was removed, replaced with 360 µl of RPMI media supplemented with 10% FBS, and 40 µl of CellTiter-Blue reagent was added to each well. The plate was incubated at 37 °C for 2 h and the fluorescent intensity of each well was measured using a microplate reader (Synergy HT, Bio-Tek, Winooski, VT). The viability of apyrase treated monocytes was expressed as a percentage of the signal for untreated *M. avium* subsp. *paratuberculosis*-infected monocytes.We also differentiated live vs. dead monocytes using the LIVE/DEAD Viability/Cytotoxicity Kit (Product L3224, Invitrogen, Carlsbad, CA). The basis for this assay is that nonfluorescent Calcein AM is cleaved, by cytoplasmic esterases in intact cells, to calcein which gives an intense green fluorescence. In cells with a damaged cell membrane, ethidium homodimer-1 enters and binds to nucleic acids, resulting in an intense red stain. Infected monocytes were cultured in the presence or absence of apyrase, and Calcein AM (1 µM as final concentration) and Ethidium homodimer-1 (2 µM as final concentration) were added to infected monocytes at the indicated time points. After incubation at 37 °C for 10 min, the stained cells were examined using an inverted fluorescence microscope (Olympus IX70, Leeds Precision Instrument, Minneapolis, MN) with appropriate filter sets (excitiaion wavelength 488 nm, emission wavelength of 530 nm and 585 nm for calcein (green) and ethidium (red), respectively. Five different 400× magnification fields per well were examined and the numbers of live cells (green color) and dead cells (red color) were enumerated.

2.6. Reactive oxygen intermediate (ROI) and nitrogen intermediate (RNI) production by monocytes

ROI production by bovine monocytes was measured using Luminol (Calbiochem, La Jolla, CA). Bovine monocytes (10^5 cells/well) were cultured in wells of a white opaque 96-well plate (Nunc, Denmark) to which Luminol (20 µM) was added. After a 10 min incubation at 37 °C, *M. avium* subsp. *paratuberculosis* at MOI 10:1 (bacteria:monocyte), apyrase (5 U) and ATP (1 mM) were added to the wells. Unstimulated cells were used as a negative control, and cells incubated with 500 nM phorbol 12-myristate 13-acetate (PMA) (FisherBiotech, Fair Lawn, NJ) was used as a positive control. The plate was then incubated at 37 °C and photoemission was measured every 20 min for 120 min using a luminometer (The Reporter; Turner Biosystems, Sunnyvale, CA). To measure release of nitric oxide, conditioned media were collected, centrifuged at 200 × *g* for 5 min and 50 µl of each supernatant placed in separate wells of a 96-well plate. An equal amount of Griess reaction solution (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid) was added and the plate was incubated for 10 min at room temperature. The absorbance at 550 nm was measured with a plate reader (µQuant; Biotek instruments, Winooski, VT). A sodium nitrite solution was used to prepare a standard curve and the amount of NO_2^- was calculated by extrapolation.

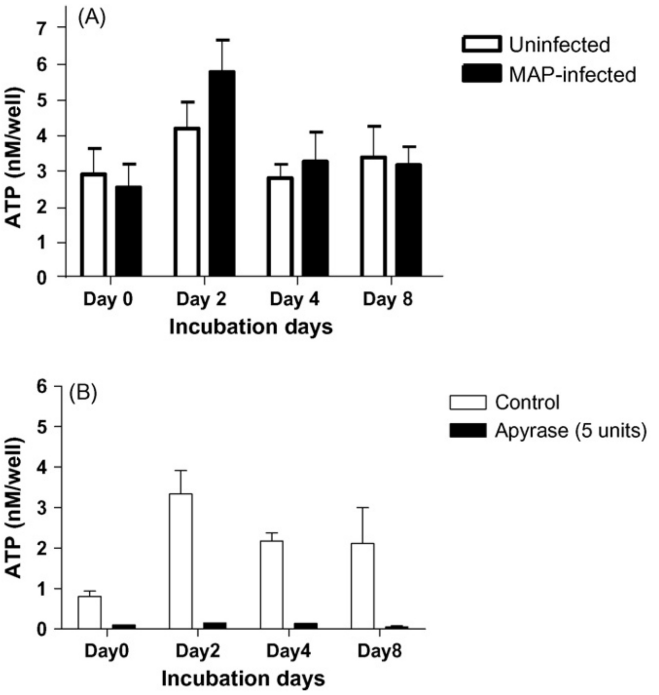

Figure 1. ATP release from uninfected or infected bovine monocytes. In (A), bovine monocytes were infected with *M. avium* subsp. *paratuberculosis* (MAP), as described in Section 2, and then incubated for up to 8 days at 37 °C in RPMI 1640 medium supplemented with 10% FBS. Conditioned media from uninfected and infected monocytes were collected at the indicated time points, centrifuged at 200 × *g* for 5 min at 4 °C, and the supernatants analyzed for ATP using a luminescence assay (Promega, Madison, WI) as described in Section 2. In (B), 5 U of apyrase (grade VI) was added to infected monocytes at day 0 and day 4 after infection and ATP release measured by the same procedure described above. Data presented are the mean ± S.E.M. of two independent experiments.

3. Statistical analysis

Data are presented as the mean ± the S.E.M. and were analyzed by ANOVA followed by a Tukey post-test for significance using the Prism 3 Statistical Software package (GraphPad, San Diego, CA).

4. Results

4.1. ATP release from bovine monocytes

Bovine monocytes constitutively released nM concentrations of ATP into the conditioned media during an 8-day incubation period. Prior ingestion of *M. avium* subsp. *paratuberculosis* did not significantly increase the amounts of ATP detected in monocyte conditioned medium (Figure 1A). Addition of apyrase, an ATP hydrolysis enzyme, completely removed ATP from conditioned media (Figure 1B).

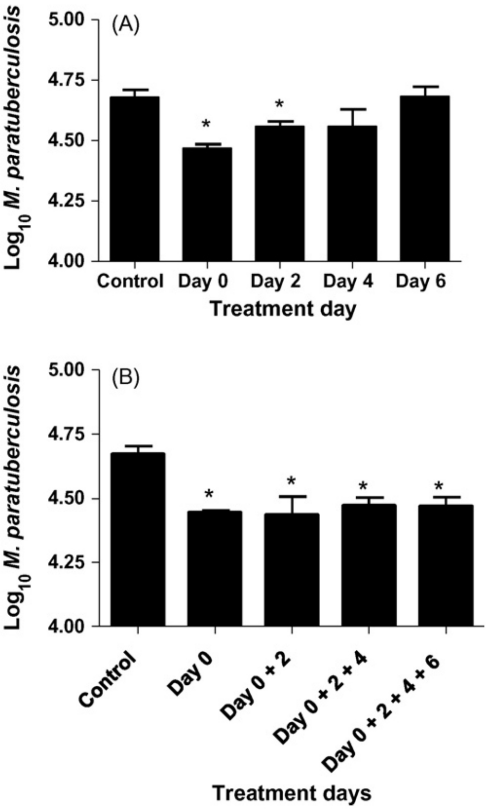

Figure 2. Intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine monocytes after a single (A) or multiple (B) treatments with apyrase. Bovine monocytes were incubated with *M. paratuberculosis* at a multiplicity of infection (MOI) of 10:1 (bacilli:monocyte) as described in Section 2. At the indicated time points, 5 U of apyrase was added and the infected cells further incubated until 8 days. At that point the cells were lysed, and the lysates and conditioned media from individual wells combined and inoculated into BACTEC 12B vials. The growth index was recorded every 24 h and the number of viable bacilli was calculated as described in Section 2. The data are the mean ± S.E.M. of two independent experiments (*$P < 0.05$).

4.2. Addition of apyrase decreases intracellular survival of M. avium subsp. paratuberculosis in bovine monocytes

To examine the effect of ATP release on intracellular survival of *M. avium* subsp. *paratuberculosis*, we treated infected monocytes with apyrase (an ATP diphosphohydrolase) at several time points. A single addition of apyrase reduced the number of viable *M. paratuberculosis* at 8 days after infection. The effect of apyrase was greater when added at day 0, rather than day 2 or day 4, and disappeared when addition of apyrase was delayed until 6 days after infection (Figure 2A). There was no greater reduction in CFU of *M. avium* subsp. *paratuberculosis* when apyrase was added more than once, as compared to a single treatment at day 0 (Figure 2B). When apyrase was added to infected monocytes at day 0 and day 4 after infection, we observed decreased numbers of viable intracellular *M.*

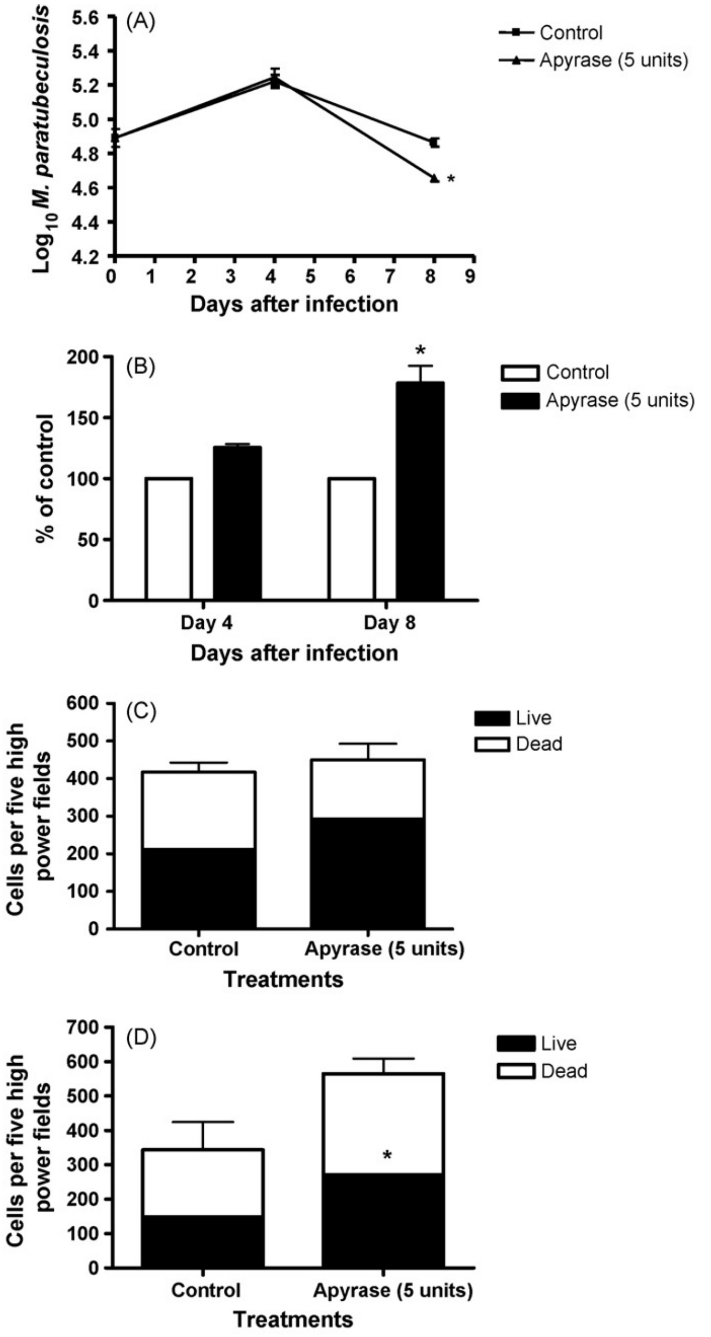

Figure 3. Effect of apyrase treatment on intracellular survival of *M. avium* subsp. *paratuberculosis* and viability of infected monocytes. In (A), bovine monocytes were infected with *M. avium* subsp. *paratuberculosis* as described in Section 2. Apyrase (5 U) was added to infected monocytes immediately after and 4 days after infection. At the indicated time points, monocytes were lysed and the lysates combined with the conditioned media from the same wells and inoculated into BACTEC 12B vials. The growth index was recorded every 24 h and the number of viable bacilli was calculated as described in Section 2. In (B),

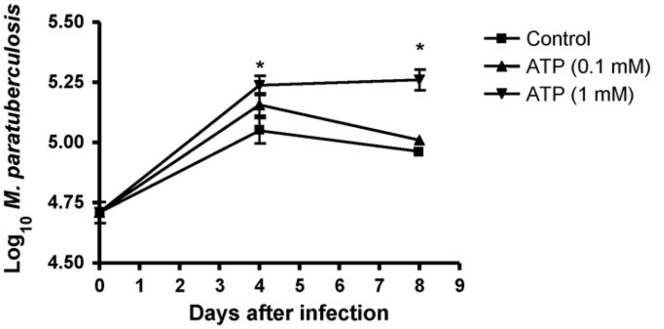

Figure 4. Intracellular survival of *M. avium* subsp. *paratuberculosis* is increased by addition of ATP. Bovine monocytes were infected with *M. avium* subsp. *paratuberculosis* as described earlier and ATP (0.1 mM or 1 mM) was added at 0, 2 and 4 days after infection. At the indicated time points, monocytes were lysed, and the lysates and conditioned media from the same wells combined and inoculated into BACTEC 12B vials. The growth index was recorded every 24 h and the number of viable bacilli calculated as described in Section 2. The results are the mean ± S.E.M. for two independent experiments (*$P < 0.05$).

avium subsp. *paratuberculosis* at day 8 (Figure 3A), but there was no significant effect of apyrase on intracellular bacilli at day 4.

Adding apyrase significantly increased monocyte viability by approximately 80% at day 8, as measured by an AlamarBlue assay (Figure 3B). We also performed Live/Dead staining (Invitrogen Product L3224) of monocytes at the same time points, and counted numbers of live and dead cells using inverted fluorescent microscopy. These data confirmed there were greater numbers of viable monocytes, and fewer viable bacilli, following apyrase treatment at 4 and 8 days after infection (Figure 3C and D). Taken as a whole, these data suggest that ATP released from infected monocytes supports survival of *M. avium* subsp. *paratuberculosis*, but reduces the viability of infected monocytes.

4.3. Adding exogenous ATP increases intracellular survival of M. avium subsp. paratuberculosis in bovine monocytes

We next added 0.1 mM or 1 mM ATP to *M. avium* subsp. *paratuberculosis*-infected monocytes at day 0, day 2 and day 4 after infection, and measured the number of viable bacilli by a radiometric method. Consistent with our apyrase data, adding 1 mM ATP significantly increased intracellular survival of *M. avium*

infected monocytes were treated with 5 U of apyrase immediately after and 4 days after infection). At the indicated time points, the conditioned media were removed, and monocyte viability assessed by staining with resazurin (Promega, Madison, WI) as described in Section 2. After a 2 h incubation, the fluorescent intensity of each well was measured with a fluorescent plate reader and the viability of apyrase treated monocytes expressed as the percentage increase compared to untreated monocytes. As a second measure of monocyte viability, apyrase-treated and untreated *M. avium* subsp. *paratuberculosis*-infected monocytes at 4 days (C) and 8 days (D) of infection were stained with the Live/Dead kit (Invitrogen) as described in Section 2. The monocytes were examined with an inverted fluorescent microscope and the numbers of live (green) and dead (red) monocytes in five different 400× fields were counted. Results are presented as the mean ± S.E.M. of three independent experiments (*$P < 0.05$).

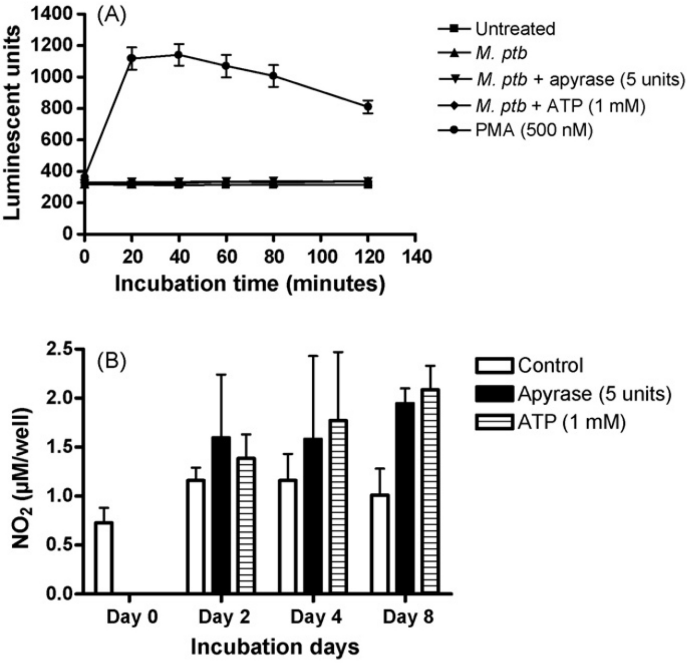

Figure 5. Addition of apyrase (5 U) or ATP (1 mM) does not alter production of ROI (A) or RNI (B) by bovine monocytes. To measure ROI (A) bovine monocytes were cultured in a white opaque 96-well plate (Nunc, Denmark) and Luminol (20 µM) and stimuli were added in RPMI media (Mediatech Inc., Herndon, VA). The cells were incubated for 120 min and photoemission was measured every 20 min with a Luminometer. Monocytes incubated with PMA (500 nM) were used as a positive control. To measure nitric oxide production (B), apyrase (5 U) was added to infected monocytes immediately after and 4 days after infection, or ATP (1 mM) was added at 0, 2 and 4 days after infection. The conditioned media were collected from each sample, centrifuged and RNI production measured with the Griess reagent as described in Section 2. The results are the mean ± S.E.M. of two independent experiments.

subsp. *paratuberculosis* (Figure 4). We also observed decreased viability of infected monocytes at the same time points (data not shown).

4.4. Addition of apyrase or ATP does not alter production of ROI and RNI by M. avium subsp. paratuberculosis-infected bovine monocytes

To investigate possible mycobactericidal mechanisms, we measured ROI and RNI production from *M. avium* subsp. *paratuberculosis*-infected monocytes after addition of apyrase or ATP. *M. avium* subsp. *paratuberculosis* infection did not stimulate production of ROI by bovine monocytes. Nor did addition of apyrase (5 U) or ATP (1 mM) alter production of ROI by bovine monocytes (Figure 5A). Likewise, bovine monocytes infected with *M. avium* subsp. *paratuberculosis* produced low levels (µM) of nitric oxide that were not significantly altered by addition of apyrase or ATP (Figure 5B).

5. Discussion

M. avium subsp. *paratuberculosis* infection in the intestinal tract of cattle results in granulomatous lesions that contain mononuclear phagocytes with intracellular bacilli [12]. Some of these infected cells may undergo apoptosis or necrosis, and could serve as a source of extracellular ATP. It is also reported that many different types of cells, including mononuclear phagocytes, secrete ATP by a nonlytic mechanism [3]. Once released, ATP can bind to purinergic receptors on the surface of nearby cells and evoke a variety of cellular responses [4].

Bovine monocytes constitutively secreted ATP (1–6 nM) during an 8-day incubation period. However, *M. avium* subsp. *paratuberculosis* infection did not enhance ATP secretion. These observations are similar to a previous report that murine macrophages constitutively release nM concentrations of ATP, that was not enhanced by addition of bacterial endotoxin (LPS) [14]. However, other investigators reported that LPS enhanced release of ATP from bovine endothelial cells [15]. It has been reported that thrombin, bradykinin, ADP, and oxidative stress increase ATP release from various cells [16] and [17]. *Chlamydia pneumoniae* was reported to increase intracellular ATP in murine macrophages by a Toll-like receptor 2-dependent manner, but these authors did not describe release of extracellular ATP in the same experiments [18]. We observed that *M. avium* subsp. *paratuberculosis* increased intracellular ATP in bovine monocytes (data not shown), but saw no significant change in extracellular ATP secreted from infected monocytes.

Addition of apyrase, which hydrolyzes ATP, completely removed extracellular ATP from the conditioned media. A single apyrase treatment decreased survival of *M. avium* subsp. *paratuberculosis* at 8 days after infection. The effect of apyrase was not enhanced by multiple treatments. Previously, other researchers reported that addition of extracellular ATP (3–5 mM)-induced killing of intracellular *Mycobacterium* species in human and murine macrophages [5–10]. In contrast to our observation, those earlier reports suggest that ATP suppressed intracellular survival of *Mycobacterium* species in human and murine macrophages. However, these experiments differed from our own in that we used apyrase to remove nM levels of endogenously secreted ATP, whereas the previous reports examined mycobacterial killing when exogenous ATP (3–5 mM) was added to the media [6]. To confirm that endogenous ATP supports the intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine monocytes, we added relatively low concentrations of ATP (0.1 mM or 1 mM) to infected monocytes. Addition of extracellular ATP increased the survival of *M. avium* subsp. *paratuberculosis*, but decreased the viability of infected monocytes at day 4 and day 8 after ATP treatment (data not shown). These observations are similar to other data we have reported in which short term (24 h) incubation with higher concentrations (5 mM) of ATP or benzyl-ATP was cytotoxic to bovine monocytes, but did not reduce the viability of *M. avium* subsp. *paratuberculosis* [19]. Nor did we observe in that study an effect of a 24 h incubation with 5 mM ATP on the numbers of viable *Mycobacterium bovis* BCG recovered from bovine monocytes.

Our data suggest that endogenous ATP is cytotoxic to bovine monocytes and slightly increased intracellular survival of *M. avium* subsp. *paratuberculosis*. Our observations are similar to a previous report that the J774 murine macrophage cell line spontaneously died during a 6-day incubation, and this cell death was

dependent on expression of the P2z receptor [12]. The same report also showed that apyrase rescued J774 cells from cell death [12]. Similarly, a separate report showed that apyrase decreased apoptosis of bovine endothelial cells in response to constitutive release of ATP [15].

How does removal of endogenous ATP enhance the killing of intracellular *M. avium* subsp. *paratuberculosis* in bovine monocytes? We considered the possibility that production of reactive oxygen or nitrogen was important. However, addition of neither apyrase nor ATP (1 mM) altered production of ROI and RNI by *M. avium* subsp. *paratuberculosis*-infected bovine monocytes. Bovine monocytes in general have a poor ability to produce reactive oxygen and nitrogen intermediates [20–22]. Our observations are consistent with a previous report that apyrase did not affect LPS stimulated production of nitric oxide by murine macrophages [14]. We also considered the possible role of calcium influx and phospholipase D in our experimental system. In preliminary experiments we did not observe an increase in intracellular Ca^{2+} in ATP-treated monocytes; whereas an increase in intracellular Ca^{2+} was observed in ionomycin (1 μM) treated monocytes, without any change in recovery of viable bacilli (data not shown). Nor did addition of ethanol (1%, v/v), which is reported to prevent phospholipase D activation and increase survival of *Mycobacterium tuberculosis* in the ATP-treated THP-1 human macrophage cell line [8], affect survival of *M. avium* subsp. *paratuberculosis* in bovine monocytes (data not shown).

In summary, we observed that removal of ATP from the culture medium of *M. avium* subsp. *paratuberculosis*-infected bovine monocytes increased monocyte viability and decreased intracellular survival of *M. avium* subsp. *paratuberculosis*. These data suggest that endogenous production of ATP might play a role in regulating the intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine monocytes.

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