Extracellular ATP Is Cytotoxic to Mononuclear Phagocytes but Does Not Induce Killing of Intracellular \textit{Mycobacterium avium} subsp. \textit{paratuberculosis}

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\textit{Mycobacterium avium} subsp. \textit{paratuberculosis} is the etiologic agent of Johne’s disease, a chronic granulomatous enteritis in ruminants. ATP has been reported to induce cell death of macrophages and killing of \textit{Mycobacterium} species in human and murine macrophages. In this study we investigated the short-term effect of ATP on the viability of \textit{M. avium} subsp. \textit{paratuberculosis}-infected bovine mononuclear phagocytes and the bacilli within them. Addition of 5 mM ATP to \textit{M. avium} subsp. \textit{paratuberculosis}-infected bovine monocytes resulted in 50% cytotoxicity of bovine monocytes at 24 h. Addition of 2’(3’)-O-(4-benzoylbenzoyl) ATP tri-ethylammonium salt (Bz-ATP), which is a longer-lived ATP homologue and purinergic receptor agonist, significantly increased the uptake of YO-PRO, which is a marker for membrane pore activation by P2X receptors. Addition of Bz-ATP also stimulated lactate dehydrogenase release and caspase-3 activity in infected bovine monocytes. Neither ATP nor Bz-ATP reduced the survival of \textit{M. avium} subsp. \textit{paratuberculosis} in bovine mononuclear phagocytes. Likewise, addition of ATP or Bz-ATP was cytotoxic to murine macrophage cell lines (RAW 264.7 and J774A.1 cells) but did not affect the intracellular survival of \textit{M. avium} subsp. \textit{paratuberculosis}, nor were the numbers of viable \textit{Mycobacterium avium} subsp. \textit{avium} or \textit{Mycobacterium bovis} BCG cells altered in bovine mononuclear phagocytes or J774A.1 cells following ATP or Bz-ATP treatment. These data suggest that extracellular ATP does not induce the killing of intracellular \textit{M. avium} subsp. \textit{paratuberculosis} in bovine mononuclear phagocytes.

ATP can bind to purinergic receptors on the cell surface. Two types of ATP receptors (P2X and P2Y receptors) have been identified. P2Y receptors are metabotropic G-protein-coupled receptors, and P2X receptors are ligand-gated ion channel receptors (9, 27). P2Y receptors do not play a role in ATP-mediated cell death but instead preferentially respond to ATP and macrophage death are independent events (10, 33). In keeping with the latter scenario, the death of human macrophages following addition of anti-CD95 antibody, anti-CD69 antibody, or anti-major histocompatibility complex class II antibody plus complement or by addition of H$_2$O$_2$ did not result in the demise of intracellular \textit{M. bovis} BCG (21, 25).

In this study, we investigated the short-term effect of ATP addition on the viability of \textit{Mycobacterium avium} subsp. \textit{paratuberculosis}-infected bovine mononuclear phagocytes and the bacilli within them. In contrast to previous reports with human mononuclear phagocytes, ATP did not induce the killing of intracellular \textit{Mycobacterium} species in human and murine macrophages (10, 31).

\textbf{MATERIALS AND METHODS}

\textbf{Bacterial culture.} \textit{Mycobacterium avium} subsp. \textit{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 (Becton Dickinson Microbiology System, Sparks, MD), 0.05% Tween 80, and 2 µg/ml of mycobactin J (Allied Laboratories, Ames, IA). After the bacteria were harvested and washed with phosphate-buffered saline (PBS), single-cell suspensions of the bacteria were made by using a motor-driven overhead stirrer and a glass-Teflon homogenizer in a biosafety cabinet. To further remove bacterial clumps, the bacterial suspension was allowed to settle for 30 min and the supernatant was centrifuged at 200 $\times$ g for 10 min. The supernatant was resuspended in PBS plus 10% glycerol, aliquoted, and stored at $-70^\circ$C. The number of viable bacteria in the stock suspension was determined by a radiometric (BACTEC) method or by determination of plate counts (CFU) on 7H10 agar. To differentiate viable and dead bacterial cells microscopically, a Live/Dead...
Backlight bacterial viability kit (Molecular Probes, Inc., Eugene, OR) 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 to infect monocytes or macrophages in vitro. *Mycobacterium avium* subsp. *avium* (ATCC 35712; American Type Culture Collection, Manassas, VA) was prepared as described above. Single-cell suspensions of *Mycobacterium bovis* BCG (Pasteur strain; Statens Serum Institute) were obtained from M. Sandor (Madison, WI).

**Cell culture.** To isolate bovine monocytes, blood was collected from the tail veins of healthy donor cows, with sodium citrate (final concentration, 0.38% [vol/vol]) used as the anticoagulant (38). The blood was centrifuged at 400 × g for 30 min, and the plasma was removed. The buffy coat cells were resuspended in 35 mL of Hanks balanced salt solution (HBSS; Mediatech, Inc., Herndon, VA) containing 4 mM EDTA, layered over 15 mL of Ficol-Histopaque 1083 (Sigma Diagnostic, Inc., St. Louis, MO), and centrifuged at room temperature at 600 × g for 40 min. The mononuclear cells were collected from the interface, and the residual red cells were lysed with lysis buffer (150 mM NH₄Cl 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.) supplemented with 1% fetal bovine serum (FBS) and adjusted to a concentration of 3 × 10⁶ cells/mL. The cells were distributed (1 ml per well) into individual wells of a 24-well tissue culture plate or a 96-well tissue culture plate (Falcon; BD Labware, Franklin Lakes, NJ). The monocytes were allowed to adhere for 2 h at 37°C in 5% CO₂ and the nonadherent cells were removed by washing the plates with warm RPMI 1640 medium. The monocytes were cultured in RPMI 1640 medium with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) without antibiotics. At the time of infection, the estimated number of adherent cells was approximately 2 × 10⁵ cells/well. In some experiments, bovine monocyte-derived macrophages were obtained by culturing monocytes for 7 days in RPMI 1640 medium supplemented with 10% autologous serum for 3 days at 37°C with 5% CO₂. The medium was then removed and replaced with 360 μL of CellTiter-Blue cell viability assay (Promega, Madison, WI). Monocyte-derived macrophages were obtained by culturing the monocytes for 7 days at 37°C. Murine macrophage cell lines RAW 264.7 (ATCC TIB-71) and J774A.1 (ATCC TIB-67) were cultured in RPMI 1640 medium supplemented with 10% FBS and 10 mM ATP, respectively, compared to the numbers of dead cells (red color) were enumerated. The viability of ATP-treated monocytes was assessed by the CellTiter-Blue cell viability assay (Promega, Madison, WI). Monocyte-derived macrophages were obtained by culturing the monocytes for 7 days at 37°C. Murine macrophage cell lines RAW 264.7 (ATCC TIB-71) and J774A.1 (ATCC TIB-67) were cultured in RPMI 1640 medium supplemented with 10% FBS and 10 mM ATP, respectively, compared to the numbers of dead cells (red color) were enumerated. The viability of ATP-treated monocytes was assessed by the CellTiter-Blue cell viability assay (Promega, Madison, WI). Monocyte-derived macrophages were obtained by culturing the monocytes for 7 days at 37°C. 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YO-PRO uptake, LDH release, and caspase-3 activity of ATP or Bz-ATP treated bovine monocytes. High concentrations of ATP (mM) are reported to induce pore formation in cells via activation of the P2X7 receptors on the cell membrane (1, 5, 9, 14–16, 26, 30, 35). We investigated pore formation after ATP treatment of bovine monocytes, using a YO-PRO uptake assay. Addition of 1 to 5 mM ATP decreased YO-PRO uptake by bovine monocytes, whereas addition of Bz-ATP, which is a more potent agonist for purinergic receptors, increased YO-PRO uptake by bovine monocytes (Fig. 3A). Similar to these results, addition of Bz-ATP, but not ATP, induced LDH release from bovine monocytes (Fig. 3B). We also investigated whether ATP or Bz-ATP might induce apoptosis via activation of caspase-3. Only 5 mM Bz-ATP significantly increased caspase-3 activation in bovine monocytes (an approximately 60% increase in activity; P < 0.05); addition of ATP had no effect (data not shown).

ATP is cytotoxic to but does not affect survival of M. avium subsp. paratuberculosis in murine macrophage cell lines RAW 264.7 and J774A.1. For comparative purposes, we next investigated the effect of ATP on the viability of RAW 264.7 and J774A.1 murine macrophage cell lines using the CellTiter-Blue cell viability assay. RAW 264.7 cells and J774A.1 cells were more susceptible to ATP-induced cytotoxicity than bovine monocytes after a 4-h or a 24-h incubation with 5 mM ATP.
Because monocytes undergo morphological and functional changes into macrophage-like cells (i.e., monocyte-derived macrophages) after 4 to 7 days of culture in vitro (39), we also compared monocyte-derived macrophages with monocytes. ATP had similar cytotoxic effects on bovine monocytes and monocyte-derived macrophages at 4 and 24 h in culture (Fig. 4). Likewise, neither ATP nor Bz-ATP reduced the number of viable M. avium subsp. paratuberculosis in RAW 264.7 (Fig. 5) or J774A.1 (data not shown) murine macrophage cell lines. In subsequent experiments, we observed that neither ATP nor Bz-ATP induced the killing of M. avium subsp. avium in bovine monocytes (Fig. 6A), nor did ATP or Bz-ATP affect the survival of M. bovis BCG in bovine monocytes or monocyte-derived macrophages or M. bovis BCG in RAW 264.7 cells (Fig. 6B).

**DISCUSSION**

Johne’s disease is a chronic granulomatous enteritis of ruminants (4). Acid-fast bacilli can be prominent within mononuclear phagocytes in granulomatous lesions in the intestinal wall of cattle with clinical Johne’s disease (7, 29). It is possible that some of the cells in these foci of granulomatous inflammation are undergoing apoptosis or necrosis and, consequently, release ATP (22). Extracellular ATP is cytotoxic to human and murine macrophages and is reported to induce the...
P2X7 receptor plays a major role in the cytotoxic effect of ATP uptake of YO-PRO by bovine monocytes, addition of Bz-ATP, (9). Although ATP treatment did not result in the increased such as lucifer yellow and YO-PRO, after the addition of ATP the characteristics of the P2X7 receptor is its ability to create a of ATP for human and murine macrophages (10, 31). One of also reported to play a major role in the mycobactericidal effect for several cell types (12, 30, 34–36). The P2X7 receptor was intracellular killing of Mycobacterium tuberculosis and M. bovis BCG in human and murine macrophages (10, 18, 19, 21, 25, 33). We found that ATP exerted a dose-dependent (1 to 5 mM) cytotoxic effect on bovine monocytes and that M. avium subsp. paratuberculosis infection did not significantly increase the cytotoxicity of ATP. This finding is similar to that described in a previous report that ATP is no more cytotoxic to M. bovis BCG-infected human monocytes than uninfected monocytes (25). Based on prior reports, we expected the ATP-mediated cytotoxicity for bovine monocytes to be accompanied by the decreased survival of M. avium subsp. paratuberculosis. However, after ATP treatment the number of viable intracellular M. avium subsp. paratuberculosis cells was not reduced compared to the number of untreated bovine monocytes.

ATP can bind to several different purinergic receptors, including the P2X7, P2X1, and adenosine P1 receptors (3, 6). The P2X7 receptor plays a major role in the cytotoxic effect of ATP for several cell types (12, 30, 34–36). The P2X7 receptor was also reported to play a major role in the mycobactericidal effect of ATP for human and murine macrophages (10, 31). One of the characteristics of the P2X7 receptor is its ability to create a membrane pore that allows the entrance of large molecules, such as lucifer yellow and YO-PRO, after the addition of ATP (9). Although ATP treatment did not result in the increased uptake of YO-PRO by bovine monocytes, addition of Bz-ATP, which is a more potent P2X7 agonist (28), enhanced the uptake of YO-PRO in bovine monocytes. Likewise, Bz-ATP stimulated LDH release and caspase-3 activation in bovine monocytes in a dose-dependent manner, whereas ATP did not. Caspase activation was previously reported to be partially involved in ATP-induced cell death (8, 13, 23, 24). Overall, our data suggest that the ATP-mediated cytotoxicity might be P2X7 independent and that the Bz-ATP-mediated cytotoxicity might be P2X7 dependent in bovine monocytes.

Although Bz-ATP was cytotoxic for bovine monocytes, it did not induce the killing of M. avium subsp. paratuberculosis. Because macrophages express more P2X7 receptors than monocytes (11, 14, 15), we compared bovine monocytes and monocyte-derived macrophages for their responses to ATP and Bz-ATP. Neither ATP nor Bz-ATP induced the killing of intracellular M. avium subsp. paratuberculosis by bovine mono- cyte-derived macrophages. IFN-γ is reported to enhance the expression of P2X7 during the differentiation of monocytes into macrophages (14). However, prior activation of bovine monocyte-derived macrophages with IFN-γ followed by incubation with ATP or Bz-ATP did not increase the killing of intracellular M. avium subsp. paratuberculosis (data not shown). We found that 5 mM ATP was cytotoxic to RAW 264.7 and J774A.1 cells but did not cause the intracellular killing of M. avium subsp. paratuberculosis, nor did ATP or Bz-ATP induce the killing of M. avium subsp. avium or M. bovis BCG in bovine monocytes, monocyte-derived macrophages, or RAW 264.7 cells. These findings are in contrast to those of a previous report that incubation of the J774A.1 murine macrophage cell line with ATP increased the killing of intracellular M. bovis BCG (10).

It was previously suggested that the ability of ATP to increase the killing of intracellular mycobacteria in human macrophages (10, 18, 19, 33) may have been due to increased intracellular calcium concentrations and phospholipase D ac- tivity that in turn enhanced phagosome-lysosome fusion (18, 19). In our hands, ATP treatment did not increase intracellular calcium levels in bovine monocytes, nor did addition of calcium ionophore after the intracellular fate of M. avium subsp. paratuberculosis in bovine monocytes (data not shown). These find- ings are somewhat at odds with those described in previous reports that apoptosis, but not necrosis, of infected human macrophages is associated with the killing of intracellular M. bovis BCG (25) and that ATP-dependent killing of M. bovis BCG is P2X7 dependent (21). These findings highlight the differences and similarities in the responses of bovine mononuclear phagocytes compared with those of human or murine mononuclear phagocytes to purinergic stimulation, which might account for the differences in their responses to mycobacteria. One possible explanation for the discrepancy between monocyte death and mycobacterial survival might include differences in cell signaling via the P2X7 receptor in human and bovine mononuclear phagocytes. For example, KN-62, which is an antagonist of the P2X7 receptor, blocks the cation current and the uptake of ethidium after ATP treatment in human cells but not rat cells (17). It has also been shown that a polymorphism in the human P2X7 receptor reduces the killing of intracellular M. bovis BCG in human macrophages (31). However, it is also possible that the differences in the methods used in our study compared to those used in earlier studies.
though ATP was cytotoxic to bovine mononuclear phagocytes, reported for human and murine mononuclear phagocytes. Alphaphagocytes respond differently to ATP than was previously

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