Combined Effects of ATP on the Therapeutic Efficacy of Antimicrobial Drug Regimens against *Mycobacterium avium* Complex Infection in Mice and Roles of Cytosolic Phospholipase A<sub>2</sub>-Dependent Mechanisms in the ATP-Mediated Potentiation of Antimycobacterial Host Resistance

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Combined Effects of ATP on the Therapeutic Efficacy of Antimicrobial Drug Regimens against *Mycobacterium avium* Complex Infection in Mice and Roles of Cytosolic Phospholipase A₂-Dependent Mechanisms in the ATP-Mediated Potentiation of Antimycobacterial Host Resistance¹

Haruaki Tomioka²,* Chiaki Sano,* Katsumasa Sato,* Keiko Ogasawara,*† Tatsuya Akaki,*‡ Keisuke Sano,*† Shan Shan Cai,* and Toshiaki Shimizu*

ATP, which serves as a mediator of intramacrophage signaling pathways through purinoceptors, is known to potentiate macrophage antimycobacterial activity. In this study we examined the effects of ATP in potentiating host resistance to *Mycobacterium avium* complex (MAC) infection in mice undergoing treatment with a drug regimen using clarithromycin and rifamycin and obtained the following findings. First, the administration of ATP in combination with the clarithromycin and rifamycin regimen accelerated bacterial elimination in MAC-infected mice without causing changes in the histopathological features or the mRNA expression of pro- or anti-inflammatory cytokines from those in the mice not given ATP. Second, ATP potentiated the anti-MAC bactericidal activity of macrophages cultivated in the presence of clarithromycin and rifamycin. This effect of ATP was closely related to intracellular calcium mobilization and was specifically blocked by a cytosolic phospholipase A₂ (cPLA₂) inhibitor, arachidonyl trifluoromethylketone. Third, intramacrophage translocation of membranous arachidonic acid molecules to MAC-containing phagosomes was also specifically blocked by arachidonyl trifluoromethylketone. In the confocal microscopic observation of MAC-infected macrophages, ATP enhanced the intramacrophage translocation of cPLA₂ into MAC-containing phagosomes. These findings suggest that ATP increases the host anti-MAC resistance by potentiating the antimycobacterial activity of host macrophages and that the cPLA₂-dependent generation of arachidonic acid from the phagosomal membrane is essential for such a phenomenon. *The Journal of Immunology*, 2005, 175: 6741–6749.

Clinical management of *Mycobacterium avium* complex (MAC) infections is difficult, because MAC infections are frequently encountered in immunocompromised hosts, particularly AIDS patients (1–3), and MAC organisms are highly or moderately resistant to common antituberculosis drugs, such as isoniazid, ethambutol, pyrazinamide, and rifampin (4, 5). Although some new drugs, including clarithromycin, azithromycin, and rifabutin, are fairly effective in controlling MAC bacteria in AIDS patients (4–7), treatment of pulmonary MAC infections is still difficult even with the use of multidrug regimens containing these drugs (5, 8). Therefore, the development of new antimicrobials and administration protocols that are potently efficacious against MAC infections is urgently needed. Although some new antimicrobial agents active against MAC, such as new ketolides (telithromycin and ABT-773), pyrimidine derivatives (SoRI 8890), resorcinomycin A, and pyrrole derivatives (BM212), are being developed (9, 10), few have been subjected to clinical studies. Thus, at present it appears that attempts to devise potent anti-MAC administration protocols using ordinary antitymocobacterial drugs are more practical than awaiting the development of new anti-MAC drugs. In this context, it would be useful to devise regimens to treat MAC patients using ordinary anti-MAC agents in combination with immunomodulators.

Extracellular ATP is known to serve as a mediator of cell-to-cell communication by triggering a variety of biological responses in various cells, including hemopoietic cells, endothelial cells, and nerve cells, through ligation of plasma membrane purinergic receptors (11, 12). The biological activities of extracellular ATP are various and include mitogenic stimulation, gene expression, excitatory transmitter function, and induction of cell death. Two subfamilies of P2 purinoceptors have been described: ligand-gated ionotropic P2X receptors and G protein-coupled P2Y receptors (13, 14). Macrophages (Mφ) possess both P2X₇ (formerly P2Z) and P2Y₁ as major P2 receptors (15, 16). Ligation of P2X₇ receptors with low concentrations of ATP (~100 μM) causes the influx of extracellular calcium across the cell membrane (17, 18), whereas...
prolonged activation with high concentrations of ATP (3 mM) results in the formation of large nonselective membrane pores permeable to hydrophilic molecules of up to 900 Da (19). These events are thought to cause subsequent changes in intracellular signaling and metabolic pathways leading to the activation of NF-κB and stress-activated protein kinase (SAPK)/JNK, caspases, and cell apoptosis (16, 20–22). P2X2 receptors act via G proteins (Gq) to stimulate the phospholipase Cβ (PLCβ) signaling cascade, releasing Ca2+ from internal stores (16, 23).

It has recently been reported that treatment of human Moϕ with ATP (ATP4–) potentiates Moϕ activity in killing of Mycobacterium tuberculosis (MTB) complex mycobacteria, such as MTB and Mycobacterium bovis bacillus Calmette-Güerin (BCG) (24–28). ATP-induced killing of mycobacterial organisms within Moϕ is principally mediated by P2X7 receptors (24, 27), although it has also been reported that purinergic signaling regulates Moϕ activity in killing of BCG organisms via a P2X7-independent mechanism (29). In addition, ATP-mediated killing of mycobacterial organisms within Moϕ is mediated by phospholipase D, which is linked to leukocyte antimicrobial mechanisms dependent on the mobilization of intracellular Ca2+ and subsequent lysosomal fusion and acidification of mycobacteria-containing phagosomes (25–27).

These findings encouraged us to examine the effect of ATP administration to MAC-infected mice on the therapeutic efficacy of clarithromycin, a leading anti-MAC drug, in combination with rifampin or the new benzoxazinorifamycin rifalazil (30). We found that ATP significantly potentiated the therapeutic efficacy of clarithromycin/rifampin (CR) in treating MAC-infected mice during the early stage of infection, principally by potentiating the anti-MAC activity of rifamycin/rifampin (31). The organisms were cultured in Middlebrook 7H9 broth, and the MAC N-260 strain is much more virulent to mice than the MAC N-444 strain (31). The organisms were cultured in Middlebrook 7H9 broth, and bacterial suspension prepared with PBS containing 1% BSA was gently sonicated using a sonicator (model UR-20P; Tomy Seiko) for 5 s and centrifuged at 150 × g for 5 min to remove extracellular clumps, and the upper layer (~80% volume) was saved as an inoculum. In some experiments MTB H37Ra grown in 7H9 broth was used.

Materials and Methods

Organisms

MAC N-444 (serovar 8) and MAC N-260 (serovar 16) strains isolated from patients with MAC infection were used. These strains were identified as Mycobacterium avium (29). In addition, ATP-mediated killing of mycobacterial organisms within Moϕ is mediated by phospholipase D, which is linked to leukocyte antimicrobial mechanisms dependent on the mobilization of intracellular Ca2+ and subsequent lysosomal fusion and acidification of mycobacteria-containing phagosomes (25–27).

These findings encouraged us to examine the effect of ATP administration to MAC-infected mice on the therapeutic efficacy of clarithromycin, a leading anti-MAC drug, in combination with rifampin or the new benzoxazinorifamycin rifalazil (30). We found that ATP significantly potentiated the therapeutic efficacy of clarithromycin/rifampin (CR) in treating MAC-infected mice during the early stage of infection, principally by potentiating the anti-MAC activity of rifamycin/rifampin (31). The organisms were cultured in Middlebrook 7H9 broth, and the MAC N-260 strain is much more virulent to mice than the MAC N-444 strain (31). The organisms were cultured in Middlebrook 7H9 broth, and bacterial suspension prepared with PBS containing 1% BSA was gently sonicated using a sonicator (model UR-20P; Tomy Seiko) for 5 s and centrifuged at 150 × g for 5 min to remove extracellular clumps, and the upper layer (~80% volume) was saved as an inoculum. In some experiments MTB H37Ra grown in 7H9 broth was used.

Mice

Five-week-old BALB/c mice were purchased from Japan Clea.

Special agents

Special agents used in this study were as follows: clarithromycin (Taisho Pharmaceutical), rifalazil (Kaneka), rifampin (Sigma-Aldrich), ATP (ICN Biomedicals), benzoylbezoic ATP (BzATP; Sigma-Aldrich), calcium ionophore A23187 (Sigma-Aldrich), N6-monomethyl-l-arginine (NMMA; Dojindo), manoxilide (Wako Pure Chemical Industries), arachidonoyl trifluoromethylketone (a-TFMK; Sigma-Aldrich), nordihydroguaiaretic acid (NDGA; Sigma-Aldrich), indomethacin (Sigma-Aldrich), and colchicine (Sigma-Aldrich). IFN-γ (Genzyme Technne), superoxide dismutase (Wako Pure Chemical Industries), and catalase (Sigma-Aldrich). [3H]oleic acid were purchased from American Radio-Labeled Chemicals.

Experimental infection

Six-week-old BALB/c mice infected i.v. with 1 × 107 CFU of MAC were given, or not given, s.c. injections of clarithromycin (12 mg/kg) and rifampin (8 mg/kg) with or without simultaneous administration of ATP (40 mg/kg) s.c. once daily, five times per week, from day 1 after infection for up to 8 wk. Doses of test drugs were fixed to be nearly equivalent to their clinical dosages by weight. At intervals, mice were killed and examined for bacterial loads in the lungs and spleens by counting the number of CFU in the homogenates of individual organs using Middlebrook 7H11 agar plates.

Intracellular growth of MAC in Moϕ

Moϕ monolayer cultures prepared by seeding 1 × 104 mouse RAW264.7 Moϕ (RAW-Moϕ) or 5 × 104 J774.1 Moϕ (J774-Moϕ) on 96-well, round-bottom microculture wells were precultivated in 0.2 ml of RPMI 1640 medium containing 5% FBS and 25 mM HEPES at 37°C in a CO₂ incubator (5% CO₂-95% humidified air) for 18 h. After washing with HBSS containing 2% FBS, the Moϕ were incubated for 0.1 ml of 5% FBS-RPMI 1640 medium containing 2 × 106 CFU/ml MAC organisms (multiplicity of infection, 20) in a CO₂ incubator for 2 h. The MAC-infected Moϕ were then washed with 2% FBS-HBSS to remove extracellular organisms and thereafter cultivated in 0.2 ml of 5% FBS-RPMI 1640 medium with or without the addition of ATP, BzATP, A23187, or their combination in a CO₂ incubator for up to 6 days. In some experiments CR were added to the culture medium at the concentrations equivalent to their Cmax in the blood (clarithromycin, 2.3 µg/ml; rifampin, 6.2 µg/ml; rifalazil, 0.05 µg/ml) of humans who were given clinical dosages of these drugs. At intervals, the Moϕ were lysed with 0.07% SDS, followed by subsequent neutralization with 6% BSA. After collection of bacterial cells from the resultant Moϕ lysate by centrifugation at 2000 × g for 20 min and subsequent washing of recovered bacteria with distilled water by centrifugation, the number of CFU was counted on 7H11 agar plates.

Intracellular translocation of Moϕ membrane-associated AA and oleic acid to infected mycobacterial organisms

Profiles of intracellular translocation of membrane AA and oleic acid to mycobacterial organisms internalized in Moϕ phagosomes were examined as described previously (32). Briefly, zymosan A-induced mouse peritoneal exudate cells (2 × 104 cells) were cultured in 10 ml of 5% FBS-RPMI 1640 medium on a FBS-coated plastic culture dish (80-mm diameter; Falcon) at 37°C in a CO₂ incubator for 2 h. After rinsing with 2% FBS-RPMI 1640, adherent cells consisting of >90% Moϕ were gently scraped off into 20% FBS medium with a rubber policeman and collected by subsequent centrifugation at 250 × g for 5 min. Test Moϕ (6 × 106 cells) were incubated in 3.0 ml of 5% FBS-RPMI 1640 containing 4 nCi/ml [3H]AA or [3H]oleic acid (80 Ci/mmol) in polypropylene tubes (15 × 90 mm) at 37°C in a CO₂ incubator for 24 h. After rinsing with 2% FBS-HBSS, the resultant Moϕ (2.5 × 105 cells) loaded with [3H]AA or [3H]oleic acid were suspended in 200 ml of culture medium containing 1.0 × 106/ml MTB organisms and then incubated at 37°C for 2 h. After washing with 2% FBS-HBSS by centrifugation (120 × g, 5 min) to remove extracellular organisms, infected Moϕ were cultivated in a CO₂ incubator. After 12-h cultivation, Moϕ were collected, thoroughly washed with 2% FBS-HBSS, and lysed with 0.23% SDS solution for 10 min. Intramacrophage microorganisms were then collected by centrifugation (4200 × g, 10 min), and the radioactivity of recovered microorganisms was measured using toluene-based scintillator containing Triton X-100 using a Tri-Carb liquid scintillation spectrometer (Packard Instrument).

Intracellular translocation of cPLA2

Mouse peritoneal Moϕ (1 × 106 cells) were seeded onto a 17-mm cover glass and cultured in 5% FBS-RPMI 1640 medium overnight using six-well culture dishes (Corning). The resultant Moϕ monolayer was then infected with MAC N-260 by incubation in medium (5 ml) containing 1 × 106 MAC (multiplicity of infection, 100) at 37°C for 2 h, then incubated in medium with or without addition of either ATP (3 mM) or BzATP (0.3 mM) at 37°C for 10 min. After fixation with 4% paraformaldehyde and then methanol, the Moϕ specimen was permeabilized with sequential treatments with 50 mM ammonium chloride for 10 min and 0.2% Triton X-100 for 10 min, and thereafter blocked with 2% BSA in PBS for 30 min, followed by subsequent treatment with anti-Fc mAb for 30 min. Immunolabeling was performed using the following Abs at 37°C for 2 h: 1) MAC staining (mouse anti-MAC primary Ab) and Alexa Fluor 546-conjugated goat anti-mouse IgG1 mAb (secondary Ab), each used at a dilution of 1/500; and 2) cPLA2 staining: mouse anti-cPLA2 mAb (primary Ab) and FITC-conjugated rat anti-mouse IgG2b mAb (secondary Ab), each used at...
a dilution of 1/100. All specimens were viewed on an Olympus FV300 digital fluorescence confocal laser scanning microscope. The cPLA2 colocalization index, in terms of the relative intensity of cPLA2-fluorescence colocalizing with intracellular MAC organisms, was calculated as follows: cPLA2 colocalization index = (FITC’s cPLA2 fluorescence intensity of area surrounding individual bacterium)/(Alexa Fluor 546’s fluorescence intensity of area surrounding the same bacterium).

Expression of cytokine mRNA
RT-PCR analysis of cytokine mRNAs in lung tissues from mice infected with MAC was performed as follows. Total RNA was isolated from the lung tissues of MAC-infected mice with or without treatment harvested wk 4 after infection using the ISOGEN kit (Nippon Gene). After DNase I (Invitrogen Life Technologies) treatment (1 U of DNase/μg RNA sample) at room temperature for 15 min, the resultant RNA samples were reverse transcribed to the first chain of cDNA using random hexamer primers (Invitrogen Life Technologies) and 200 U of SuperScript II reverse transcriptase (Invitrogen Life Technologies) with the standard reaction mixture (20 μl): 1× reverse RT buffer (pH 8.3); 1 mM of each dNTP, 1 U of Taq polymerase (Takara Biomedicals), and 20 pmol of sense/antisense primers for test cytokines (synthesized by Greiner) and 2.0 U of RNase inhibitor (Invitrogen Life Technologies). After 1-h reaction at 42°C and subsequent heating at 72°C for 2 min, 1-μl aliquots of resultant cDNA were amplified specifically by PCR in the standard reaction mixture (50 μl) containing 1× PCR buffer (pH 8.3), 0.2 mM of each dNTP, 1 U of Taq polymerase (Takara Biomedicals), and 20 pmol of sense and antisense primers for test cytokines (synthesized by Greiner) as follows (sense/antisense): TNF-α, AGCCGACGCTG TACGACACACCAACA/ACACCCCCCTCCCTCAGGCACT; IFN-γ, GAAAGGCCTAGAAGCTGTAATAACT/ATCACGAGCGACTCCTTTCT CCGT; IL-10, TGACTGGCATGAGGATCAGCAG/ATCCTGAGGG TCTTCAGCTT; IL-13, ATGGCGCTCTGTGGACTCGAGTCC/ GAAGGGGGCCGTCGAAAACACAGTTGC; and TGF-β, AGCCCTTGAT ACCAATTTGTCCTGCTACAC/AAGGGGCGGGGCCCCGCG GGGCTTCACTGTC. Reactions were conducted in a DNA Thermal Cycler (ASTEC) for 30 cycles, including denaturing at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min for each cycle. PCR products were analyzed by electrophoresis on ethidium bromide-stained 2% agarose gels.

Statistical analysis
Statistical analysis was performed using a one-way ANOVA with a Bonferroni multiple comparisons post-test (StatView software; Hulinks).

Results

Effect of ATP administration on the therapeutic efficacy of CR against MAC infection
First, we examined the effects of ATP administration on MAC-infected mice, which were, or were not, given antimycobacterial CR drug regimens, on bacterial behavior at sites of infection (lungs and spleen). Fig. 1 shows the profiles of bacterial growth during the 4-wk period following infection in the lungs and spleens of MAC-infected mice, which were, or were not, treated with ATP, CR, or both. As shown in Fig. 1, A and B, in mice infected with the MAC N-444 strain (low virulence), persistent infection without bacterial growth was observed in the lungs, and gradual bacterial growth was noted in the spleen. ATP (50 mg/kg) alone did not affect the profiles of bacterial persistence in the lungs, whereas ATP in combination with CR (20 and 5 mg/kg, respectively) potentiated the bacterial elimination in the lungs due to the CR drug regimen (Fig. 1A). In the spleen, ATP alone slightly decreased the bacterial load at wk 4, whereas ATP in combination with CR enhanced CR-mediated bacterial elimination during wk 2–4 (Fig. 1B).

As shown in Fig. 1, A and B, in mice infected with the MAC N-260 strain (high virulence), significant levels of bacterial growth were observed in the lungs and spleen at wk 4 after infection. ATP alone did not affect bacterial growth in the lungs (Fig. 1C, □), but completely inhibited growth of MAC in the spleen (Fig. 1D, □). Administration of ATP in combination with CR caused more potent growth inhibition of the organisms in the lungs than that in mice given CR alone (Fig. 1C, ▪). As shown in Fig. 1D, bacterial elimination was clearly observed in the spleens of mice given ATP in combination with CR (□), whereas CR alone caused only growth inhibition of MAC (●).

Fig. 2 shows histopathological examination of the lungs of infected mice given, or not given, ATP. In mice without ATP treatment, a number of tubercular lesions consisting of epithelioid-like Mø surrounded by infiltrating lymphocytes were observed, but no caseous necrosis was noted (Fig. 2, A and B). As indicated in Fig. 2E, in this case, the number of granulomas per square millimeter of field was 0.71 ± 0.06 (average of 100 fields). Notably, ATP treatment did not affect the histopathological profiles of MAC-infected mice even after 8-wk administration of ATP (Fig. 2, C and D), and the number of granulomas was 0.73 ± 0.05/mm² field (Fig. 2E). In contrast, CR significantly decreased the number of tubercular lesions (photo not shown), and the number of granulomas was 0.16 ± 0.02/mm² field (Fig. 2E). These findings suggest that ATP treatment affects neither the formation nor the progression of granulomatous lesions in MAC-infected mice, unlike antimicrobial drugs.

We next examined profiles of the mRNA expression of cytokines that up-regulate Mø antimycobacterial activity (TNF-α, IFN-γ, and IL-2) and those that down-regulate it (IL-4, IL-10, IL-13, and TGF-β) (33) in the lungs of infected mice given, or not given, CR with or without ATP at wk 4 after infection. As shown in Fig. 3, TNF-α mRNA expression was not changed due to MAC infection, but was markedly decreased by administration of CR. In contrast, IFN-γ mRNA expression was up-regulated due to MAC infection, but was markedly decreased by administration of CR. We further examined profiles of those that down-regulate it (IL-4, IL-10, IL-13, and TGF-β) (33) in the lungs of infected mice given, or not given, CR with or without ATP at wk 4 after infection. As shown in Fig. 3, TNF-α mRNA expression was not changed due to MAC infection, but was markedly decreased by administration of CR. In contrast, IFN-γ mRNA expression was up-regulated due to MAC infection, but was markedly decreased by administration of CR.
infection, but was not affected by administration of CR. The expression of both IL-10 mRNA and IL-13 mRNA was markedly decreased by MAC infection. In these cases, administration of CR did not affect the mRNA expression of these cytokines. In contrast, TGF-β mRNA expression was slightly decreased in MAC-infected mice, but was not affected by administration of CR. Notably, ATP administration did not affect the profiles of mRNA expression of these cytokines in mice given CR alone. Neither IL-2 nor IL-4 mRNA expression was observed with any of the regimens tested (data not shown). These findings indicate that ATP administration did not modulate Th1 and Th2 cytokine gene expression in lungs of MAC-infected mice treated with CR.

Effects of ATP on antimicrobial activity of CR against intramacrophage MAC

To clarify the cellular mechanisms of the ATP-mediated increase in the in vivo efficacy of CR chemotherapy against MAC infection in mice, we examined the effects of high concentrations of ATP on the antimicrobial activity of Mφ (RAW-Mφ and J774-Mφ) against MAC organisms (N-444 and N-260 strains). As indicated in Fig. 4A, ATP at 10 mM, but not at 3 mM, weakly, but significantly, inhibited the growth of the low virulence MAC N-444 strain (31) in RAW-Mφ. Notably, both concentrations (3 and 10 mM) of ATP tested markedly potentiated the bactericidal activity of CR at the Cmax in blood against the MAC organisms residing within RAW-Mφ. As shown in Fig. 4B, in the case of RAW-Mφ infected with the high virulence MAC N-260 strain (31), ATP at 3 and 10 mM did not reduce, but, in fact, somewhat enhanced, the intramacrophage growth of organisms. These results, therefore, demonstrate that ATP exerts its antimicrobial activity against intramacrophage MAC mostly in the presence of antimycobacterial drugs. Nevertheless, both concentrations of ATP significantly augmented the bactericidal activity of CR against intramacrophage MAC. Fig. 4C shows the results of a similar experiment using J774-Mφ. In this case, ATP alone or in combination with CR exhibited similar effects on the behavior of intramacrophage MAC N-444 organisms, as in the case of RAW-Mφ shown in Fig. 4A. In this context, separate experiments showed that 10 mM ATP did not significantly augment the antimicrobial activity of CR against extracellular MAC when the organisms growing in 7HSF medium were treated with CR (1/2 Cmax each) in combination with ATP during 4-day culture. In a representative experiment, log-unit values of the bacterial CFU after 4-day culture (n = 3) were as follows: day 0, 4.41 ± 0.01; [1], clarithromycin/ rifampin, 2.08 ± 0.02; clarithromycin/rifampin plus ATP, 1.73 ± 0.03; and [2], clarithromycin/ rifalazil, 1.63 ± 0.23; clarithromycin/ rifalazil plus ATP, 1.80 ± 0.41. Therefore, it appears that ATP, even at 10 mM, does not affect the antimicrobial activity of CR against extracellular MAC.

Intracellular mechanisms of ATP-dependent potentiation of the antimicrobial activity of CR against intramacrophage MAC

Next we attempted to clarify the intracellular mechanisms of the ATP-mediated potentiation of antimicrobial activity of CR against MAC organisms residing inside Mφ. First, as shown in Fig. 5A, we found that the combination of ATP at a suboptimal concentration (0.3 mM) with calcium ionophore A23187 significantly potentiated Mφ anti-MAC activity, although ATP alone was ineffective in increasing this activity. Next, we examined the effects of ATP and BzATP (a potent P2X7 receptor agonist) on the anti-MAC antimicrobial activity of Mφ cultivated in medium in the presence of ionophore A23187 with or without the addition of CR. In this experiment, RAW-Mφ were treated with IFN-γ (500 U/ml) for 24 h, and the same concentration of IFN-γ was added to the culture medium of MAC-infected Mφ. In this case, ATP and BzATP at a suboptimal concentration (0.3 mM) failed to potentiate Mφ antimicrobial activity against intracellular MAC, even after stimulation of Mφ with IFN-γ, which is known to increase Mφ sensitivity to ATP-mediated induction of Mφ apoptosis and cause concomitant expression of Mφ antimycobacterial activity (24) (data not shown). However, as shown in Fig. 5B, when the Mφ were treated with the Ca2+ ionophore A23187 in combination with either ATP or BzATP at a suboptimal concentration (0.3 mM), significant bacterial killing of intramacrophage MAC was observed. This finding supports previous observations by Stober et al. (26) and Kusner and Barton (28) that ATP-mediated killing of mycobacteria within Mφ is dependent on intracellular Ca2+ mobilization. In this case, the bactericidal activity of CR against intramacrophage MAC was further potentiated by BzATP, but not by ATP. This finding indicates that extracellular ATP augments the antimicrobial activity of CR against intramacrophage MAC activity organisms, specifically through P2X7 receptors.

Next we examined the roles of ROI, RNI, and AA in the effector pathway(s) involved in the ATP-dependent increase in CR antimicrobial activity against intramacrophage MAC organisms, that is, ATP-mediated potentiation of Mφ anti-MAC activity with culture in the presence of CR. As shown in Fig. 5C (right columns), the potentiation by ATP at an optimal concentration (10 mM) of the antimicrobial activity of CR against intramacrophage MAC was markedly abrogated by a-TFMK (cPLA2 inhibitor), but not by...
indicate the following. First, it has been reported that the ED50 of secretory PLA2 inhibitor), NDGA (a lipoxygenase inhibitor), or colchicine (an inhibitor of phagocytosis), but not by manoalide (a mycobacteria was almost completely inhibited by a-TFMK and

bound to mycobacterial cell bodies (Fig. 5

AA directly

in ATP-mediated potentiation of M

were 3 and 7

FIGURE 3. A, Profiles of mRNA expression at wk 4 in the lungs of MAC N-260 (a high virulence isolate of M. intracellulare)-infected mice given, or not given, CR (clarithromycin, 12 mg/kg; rifampin, 8 mg/kg) with or without ATP (40 mg/kg). B, Averaged data of individual values of relative intensity are indicated.

superoxide dismutase/catalase (ROI scavengers) or NMMA (iNOS inhibitor). These findings suggest that cPLA2-mediated release of AA into MAC-containing phagosomes may play an important role in ATP-mediated potentiation of Mϕ anti-MAC activity in the presence of CR.

In this context, we previously found that in the case of [3H]AA-labeled Mϕ, membranous radioactive component(s) (presumably [3H]AA) translocated to the microorganisms internalized within Mϕ phagosomes (32). As shown in Fig. 5D, the translocation of the [3H]labeled membranous component(s) to intramacrophage mycobacteria was almost completely inhibited by a-TFMK and colchicine (an inhibitor of phagocytosis), but not by manoalide (a secretory PLA2 inhibitor), NDGA (a lipoxygenase inhibitor), or indomethacin (a cyclooxygenase inhibitor). Moreover, AA directly bound to mycobacterial cell bodies (Fig. 5E). These findings indicate the following. First, it has been reported that the ED50 of indomethacin for the inhibition of PG synthesis by zymosan A-stimulated Mϕ was only 0.01 μM (34, 35), and indomethacin at 10 μM caused 99% inhibition of PG production by LPS-stimulated Mϕ (36). In addition, the ED50 values of NDGA for the inhibition of PG and leukotriene synthesis by zymosan A-stimulated Mϕ were 3 and 7 μM, respectively (34, 35). Therefore, it is believed that both indomethacin and NDGA at the concentration (20 μM) used in the present study are able to effectively inhibit Mϕ PG and/or leukotriene synthesis. It thus appears that [3H]labeled AA, but neither [3H]labeled leukotrienes nor PGs/thromboxanes (lipoxygenase- and cyclooxygenase-catalyzed metabolites of AA, respectively), translocated to intracellular mycobacteria in host Mϕ. Second, the AA translocation was principally mediated by cPLA2 and was associated with Mϕ bacterial phagocytosis. It thus appears that ATP signaling causes cPLA2 activation via the intracellular Ca2+ mobilization pathway, thereby enhancing the cPLA2-mediated release of AA, one of the major antimycobacterial effectors of Mϕ (32, 37–39), into MAC-containing Mϕ phagosomes.

Fig. 6 shows profiles of the localization of cPLA2 in MAC-infected Mϕ that were cultured in the presence or the absence of ATP (3 mM) or BzATP (0.3 mM). First, in uninfected Mϕ cultured in the absence of ATP or BzATP, trace amounts of cPLA2 were diffusely expressed in the cytoplasm (Fig. 6b). Second, in MAC-infected Mϕ cultured in the absence of ATP or BzATP, moderately increased cPLA2 expression was observed in the cytoplasm (Fig. 6e). In this case, cPLA2 did not condense around intraphagosomal MAC organisms (Fig. 6, d and j). Third, in MAC-infected Mϕ cultured in the presence of ATP or BzATP, potently increased cPLA2 expression was noted (Fig. 6, h and k). In these cases, a part of cPLA2 was intensely condensed around intraphagosomal MAC organisms (Fig. 6, g, i and j, l, respectively). In this experiment, the cPLA2 colocalization indexes, in terms of the relative intensity of cPLA2 fluorescence colocalizing with intracellular MAC organisms, was estimated as follows (n = 30): MAC infection alone (control; Fig. 6, d-f), 0.57 ± 0.02; MAC infection plus ATP treatment (Fig. 6, g-i), 1.18 ± 0.02 (significantly greater than the control, p < 0.01); and MAC infection plus BzATP treatment (Fig. 6, j-l), 1.10 ± 0.03 (significantly greater than the control, p < 0.01). It thus appears that in MAC-infected Mϕ, ATP signaling via P2X7 receptors induces cPLA2 translocation to the phagosomal membranes surrounding internalized MAC organisms.

Discussion

The major findings of this study can be summarized as follows. 1) Administration of ATP in combination with the CR drug regimen accelerated bacterial elimination in MAC-infected mice without causing changes in the histopathological features or the expression of pro- and anti-inflammatory cytokine genes from those in mice not given ATP. 2) ATP potentiated the anti-MAC bactericidal activity of host Mϕ cultivated in the presence of clarithromycin and rifampicin. This effect of ATP was dependent on intracellular Ca2+ mobilization and cPLA2, which generates AA from phospholipids. 3) Intramacrophage translocation of membranous AA molecules to MAC-containing phagosomes was also mediated by cPLA2. Notably, ATP enhanced the intracellular translocation of cPLA2 into MAC-containing phagosomes. Concerning these findings, the following discussion can be made.
First, it is somewhat strange that ATP treatment potentiated the ability of MAC-infected mice to decrease bacterial loads, especially when infected mice were given the CR drug regimen, without affecting the formation/progression of granulomatous lesions and cytokine gene expression in the lungs of infected mice. Thus, it appears that ATP may principally enhance the host innate immunity that is much less dependent on Th1 cytokine-mediated granuloma formations than the case for acquired cellular immunity against mycobacterial Ags.

Second, the present findings also indicate that ATP potentiates Mφ antimicrobial activity against not only MTB complex mycobacterial organisms but also MAC-infected RAW-Mφ were cultured in the absence or the presence of ATP (10 mM) for 12 h. Solute control (0.1% DMSO), SOD/CAT, NMMA, or a-TFMK alone exerted no influence on the intramacrophage growth of the organisms. D, cPLA2- and phagocytosis-dependent translocation of Mφ membranous AA to mycobacterial organisms internalized in phagosomal vesicles. [3H]AA-labeled mouse peritoneal Mφ were infected with MTB and then cultured in the medium with or without addition of manoalide (20 μM), a-TF MK (100 μM), NDGA (20 μM), indomethacin (Indo; 20 μM), or colchicine (Colch; 2 μM) for 12 h. Radioactivity translocated to bacterial cells was measured as described in Materials and Methods. E, Profiles of time-dependent direct binding of [3H]AA to mycobacterial cells. Mφ organisms were incubated in the medium containing [3H]AA (12 μCi/ml) at 37°C for up to 12 h. Each bar or plot indicates the mean ± SEM (n = 4). The asterisks denote a statistically significant difference between two specified groups (p < 0.01). These data represent one of two or three experiments that were performed with similar results.
The interaction of MAC with the antimicrobial mechanisms of M. tuberculosis (MTB) has been generally accepted (42, 43). However, MTB-infected macrophages (Mφ) are not only important for the antimycobacterial function of host M. tuberculosis (MTB) but also play a critical role in the expression of anti-MAC activity against some intra-cellular pathogens (32, 39). In addition, the combination of RNI and ROI played a critical role in the expression of antimicrobial activity against BCG organisms (45). This implies that RNI is not involved in the antimycobacterial mechanisms of MAC-infected Mφ. 2) It has been shown that turpentine-lysosome fusion and acidification of mycobacteria-containing phagosomes (25–28). In addition, we previously found that release of free fatty acids (such as AA and linolenic acid) exhibited potent antimycobacterial activity against mycobacterial organisms, including MTB and MAC (32, 39). In addition, free fatty acids in combination with RNI played critical roles in manifestation of the activity of Mφ against mycobacterial pathogens, including MTB and MAC (32, 39, 47). The important roles of free fatty acids, such as AA and oleic acid, which are the major unsaturated fatty acyl residues of Mφ membrane phospholipids (48), in the antimycobacterial mechanisms of host Mφ are also supported by the following findings (32, 39). 1) MTB- or MAC-infected Mφ sequentially produced/released free fatty acid (AA) and subsequently RNI. 2) Sequential treatment of mycobacterial organisms with AA, then RNI, caused synergistic bactericidal activity against these pathogens. 3) In the case of [3H]AA-labeled Mφ, membrane radioactivity (presumably that of [3H]AA) was found to translocate to intraphagosomal microorganisms during chase incubation after MTB infection. 4) Mφ antimicrobial activity against MTB was strongly inhibited by a-TFMK (cPLA2 inhibitor). Moreover, as indicated in Fig. 5D, the present study also revealed that the intramacrophage translocation of membrane AA molecules to intraphagosomal MAC organisms was specifically blocked by a-TFMK. These findings strongly suggest that free fatty acids (especially AA) produced by the enzymatic action of cPLA2 play an important role as antimycobacterial effectors in the expression of Mφ antimicrobial activity against mycobacterial pathogens. This concept is in part supported by the finding of Duan et al. (49) that the apoptosis-mediated expression of anti-MTB antimicrobial activity of human monocytic-derived Mφ was dependent on cPLA2 and its product, AA. In this context, separate experiments indicated that also in the case of [3H]oleic acid-labeled Mφ, substantial amounts of [3H]oleic acid translocated to intraphagosomal MTB, although the translocation efficiency of [3H]oleic acid (1422 ± 100 cpm) was lower than that observed in the case of [3H]AA translocation (3069 ± 692 cpm). Notably, the [3H]oleic acid translocation was not affected by 100 μM a-TFMK (1392 ± 173 cpm), but was weakly inhibited by 20 μM manooladine (1277 ± 176 cpm), indicating that intracellular oleic acid translocation to intraphagosomal MTB is mediated by PLAs other than cPLA2, such as type IIA secretory PLAs and type V secretory PLAs. These findings indicate that intramacrophage AA mobilization is mediated by cPLA2 in a specific fashion, although not only AA, but also oleic acid, play important roles in the expression of Mφ antimicrobial activity against intracellular mycobacterial organisms.

In the present study it was found that ATP-induced potentiation of Mφ antimacrophage activity in the presence of CR was almost completely abolished by a cPLA2 inhibitor, a-TFMK, but not by ROI scavengers or iNOS inhibitor (Fig. 5C). This indicates that the anti-MAC bactericidal activity of ATP-stimulated Mφ is primarily mediated by a free fatty acid, AA, released by cPLA2 from phospholipids of the phagosomal membrane. In addition, we found that...
in MAC-infected Mφ, ATP signaling induced intracellular translocation/condensation of cPLA₂ molecules to phagosomes surrounding internalized MAC organisms (Fig. 6). To our knowledge, this is the first report that demonstrated intracellular translocation of cPLA₂ to intraphagosomal mycobacterial organisms and possible roles of cPLA₂ in intraphagosomal bacterial killing in ATP-treated Mφ during the course of mycobacterial infection. Because serine phosphorylation of cPLA₂ molecules (especially of Ser⁵⁰⁵) is known to promote membrane penetration of hydrophobic amino acid residues in their active site rim (50), the cPLA₂ condensation to phagosomal membranes in response to ATP signaling (Fig. 6) also appears to be related to the serine phosphorylation of cPLA₂.

Indeed, it was reported that ATP treatment of Mφ resulted in the activation of cPLA₂ due to MAPK/ERK1,2-mediated phosphorylation of serine residues (51–53). In separate experiments, ATP-induced expression of potentiated anti-MAC activity was found to be associated with apoptotic cell death of ATP-treated Mφ (H. Tomeoka, K. Sato, C. Sano, and T. Shimizu, unpublished observation), as previously reported for BCG-infected Mφ (24, 27).

This is consistent with the finding by Duan et al. (49) that MTB-induced Mφ apoptosis through at least two types of signaling pathways, TNF-α or cPLA₂-dependent cascades, each of which is also crucial for Mφ antimycobacterial defense mechanisms. In any case, these findings clearly indicate that ATP-mediated bacterial killing in MAC-infected Mφ is strictly dependent on cPLA₂ functions and its metabolic product, AA, but is substantially independent of RNI and ROI. This finding strongly supports that concept that AA generated by the enzymatic action of cPLA₂ plays a critical role in the antimycobacterial mechanisms of host Mφ.

Fourth, high concentrations of ATP (3–10 mM) were required to achieve significant levels of Mφ anti-MAC activity even in the case of the low virulence MAC (N-444 strain)-infected Mφ and to yield clear potentiation of Mφ anti-MAC activity in the presence of CR (Fig. 4). This implies that ATP acts via P₂X₇ receptors to induce/potentiate Mφ anti-MAC activity, because the active component of ATP interacting with P₂X₇ receptors is the fully ionized form of ATP (ATP⁺⁺), which is present as a small fraction of ATP at physiological pH. This conclusion is supported by the finding that these effects were mimicked by BzATP, a known agonist of P₂X₇ receptors (Fig. 5A). As shown in Fig. 5A, Ca²⁺ mobilization was required for Mφ to display significant levels of anti-MAC activity when ligation of P₁ receptors of MAC-infected Mφ was provided by a low concentration of ATP (0.3 mM). Under these conditions, P₂Y receptors (presumably P₂Y₂ and/or P₂Y₁₁ receptors) might participate in ATP-induced anti-MAC activity of Mφ by activating PLCβ, which is coupled with PKC activation, and subsequent activation of MAPK-mediated cPLA₂ activation (53).

Fifth, another important finding of the present study is that ATP strongly potentiated Mφ anti-MAC antimicrobial functions when Mφ were treated with CR, a combination of anti-MAC antimicrobial drugs (clarithromycin plus rifampicin). It thus appears that the use of ATP in combination with certain antimycobacterial drugs, including macrolides and rifamycins, may be beneficial in achieving efficacious control of patients with intractable MAC infections. ATP is an essential compound for all living things and can be administered to humans without severe adverse effects. At present, ATP is safely used as a vasodilator for clinical control of ischemia in the coronary arteries and paroxysmal tachycardia (54, 55). It thus appears that ATP can be safely administered to MAC patients.

There have been a number of attempts to establish efficacious regimens of adjunctive immunotherapy for management of patients with mycobacterial infections involving administration of certain immunomodulators in combination with antimycobacterial drugs (33). Adjunctive clinical trials using IL-2 or GM-CSF found these agents to be efficacious to some extent in improving patients with tuberculosis or disseminated MAC infections (33). However, these immunomodulating cytokines as well as IFN-γ and IL-12 do not appear promising as therapeutic agents for mycobacterial infections because of the possibility of induction of immunosuppressive cytokines, such as TGF-β, IL-10, and IL-13, during adjunct therapy and, in some cases, severe adverse effects (33). Thus, the development of new classes of immunomodulators other than cytokines, particularly those with no severe adverse effects, is needed. In this context, ATP may be one of the most promising agents for clinical immunotherapy of mycobacterial infections in combination with anti-MAC chemotherapy for the following reasons. First, ATP is safe for humans, as described above. Second, ATP acts directly on Mφ and rapidly causes not only potentiation of Mφ antimycobacterial activity, but also concomitant apoptosis of Mφ. It thus appears that ATP-stimulated Mφ do not serve as a cell source of immunosuppressing cytokines, which suppress Mφ antimycobacterial functions in an autocrine or paracrine fashion. In fact, in the present study prolonged ATP administration (4 wk) did not increase the mRNA expression of these immunosuppressing cytokines (Fig. 3). It will be of interest to examine the therapeutic effects of regimens involving ATP in combination with macrolides (clarithromycin and azithromycin), rifamycins (rifampin, rifabutin, and riflazil), and other drugs (ethambutol, streptomycin, and quinolones) against MAC infections over long observation periods at least 16 wk after infection. Additional studies are currently underway to elucidate the usefulness of ATP therapy of MAC infection in detail.

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Disclosures

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References


