Effect of Antipyretic Agents on Uptake, Transport, and Release of Antimicrobial Agents by Human Polymorphonuclear Leukocytes

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Polymorphonuclear leukocytes (PMNL) concentrate, transport, and release certain antimicrobial agents as they move in a chemotactic gradient. Antipyretic agents are frequently used in febrile patients receiving antimicrobial agents. Thus, the influence of ibuprofen, acetaminophen, and acetylsalicylic acid on uptake, transport, and release of azithromycin and moxifloxacin was studied. Uptake of the antimicrobial agents by human PMNL and the effect of the antipyretics were quantitated by bioassay of released antimicrobial agent. Transport and release were determined in chemotactic plates overlaid with sentinel bacteria that could detect transported and released antimicrobial agent. None of the antipyretics altered PMNL directed or nondirected movement. Uptake of azithromycin was significantly inhibited by acetylsalicylic acid but not by the other antipyretics. All of the antipyretic agents studied at therapeutic levels inhibited transport and release of both azithromycin and moxifloxacin. Administration of any of these antipyretic agents with antimicrobial agents that are transported and released by PMNL could compromise the efficacy of therapy.

Materials and Methods

Determination of MIC. We used the broth dilution method to determine the MIC of antimicrobial agents for the organism used in the assays [3].

Antimicrobial agents. Azithromycin was provided by Pfizer Pharmaceuticals, and moxifloxacin was provided by Bayer. A stock solution of azithromycin was made by initially dissolving azithromycin in ethanol and then diluting this with Hanks’s balanced salt solution (HBSS; Biowhittaker). Stock solutions of moxifloxacin were made in sterile water.

Antipyretic agents. Acetaminophen (Sigma Chemical; usual therapeutic plasma concentration, 20 μg/mL) [4], acetylsalicylic acid (Sigma; usual therapeutic plasma concentration, 20 μg/mL) [4], and ibuprofen (Sigma; usual therapeutic plasma concentration, 20 μg/mL) [5] were studied for effect on neutrophil function. Each agent was assessed at concentrations of 5, 10, 15, 20, 25, 30, and 35 μg/mL.

Bacterial strains. Streptococcus pyogenes ATCC 12344 was kept on chocolate agar plates and subcultured every other day. Overnight cultures of Micrococcus luteus ATCC 9341 (for azithromycin assays) or Staphylococcus aureus ATCC 27217 (for moxifloxacin assays) in TSB were used in bioassay plates. Bacteria used for the assays were selected on the basis of their susceptibility to the antimicrobial agent being studied.

Isolation of PMNL. Purified PMNL were obtained from normal heparinized (10 U/mL; Lymphomed, Fujisawa USA) venous blood by a ficoll-hypaque separation procedure adapted from Ferrante and Thong [6]. We layered 9 mL of fresh heparinized human blood onto a gradient consisting of 1 mL of ficoll-hypaque (ICN Biomedicals), 2 mL of One Step Polymorphs (Accurate Chemicals and Scientific), and 1 mL of neutrophil isolation medium (Cardinal Associates). The blood with the separation media was then centrifuged at 200 g for 25 min to produce a PMNL layer. PMNL were removed and washed 3 times with HBSS (Whittaker M.A. Bioproducts) with 10 U/mL heparin. Cells (95% PMNL) were resuspended in HBSS and counted by use of a hemocytometer.

Cell-associated antimicrobial agent concentration. In HBSS, 5 × 10⁶/mL PMNL were tumbled at 37°C for 4 h, with an antimicrobial agent with or without an antipyretic agent. Concentrations used were similar to published peak serum concentrations in humans after usual oral doses (0.1 μg/mL azithromycin or 4.5 μg/mL moxifloxacin and 20 μg/mL of the antipyretic agent). In addition, we...
studied 1.0 μg/mL azithromycin, a level achieved after intravenous administration. After the 4-h incubation period, the samples were centrifuged at 150 g, supernatants were decanted, and pellets were blotted to remove excess liquid. The pellets were then transferred to a 1.5-mL Eppendorf tube containing 150 μL of silicone oil (General Electric) and microfuged at 16,000 g for 3 min. This brought the PMNL pellet to the base of the tube and kept the fluid supernatant on top separated by the oil. Supernatant and silicone oil were removed by pipette. We used a sterile cotton swab to wipe carefully around the PMNL pellets to remove any remaining liquid.

Whereas some PMNL pellets were either freeze-thawed 3 times (by use of a dry ice–acetone slurry and a 37°C water bath) to lyse the cells, some pellets were not freeze-thawed to determine the effect of this procedure on antibiotic release. Microscopic examination indicated that all PMNL were lysed after freeze-thawing. Each pellet had 18 μL of sterile water added and was then triturated. Suspensions were placed into wells in tryptic soy agar (TSA) plates (Difco Laboratories) and seeded with either M. luteus or S. aureus. An overnight culture of bacteria in TSB (1 mL/50 mL of TSA for M. luteus or 1 mL/100 mL of TSA for S. aureus) plus 1 mL of 1 M HEPES/100 mL of TSA was mixed, and 30 mL of this mixture was poured into each 150 × 15–mm Petri dish (Becton Dickinson Labware) and allowed to solidify. We prepared a standard curve relating the size of zone of inhibition to the antimicrobial agent concentration by placing 20 μL of 4 known concentrations of each antimicrobial agent into 4-mm wells and incubating these on the same plate as the unknown concentrations.

M. luteus plates were used for azithromycin, and S. aureus plates were used for moxifloxacin. The plates were incubated at 37°C in 5% CO2 overnight. The diameters of the cleared zones were then measured and plotted along a line created from the standards, to determine the amount of antimicrobial agent released from the PMNL.

PMNL migration, transport, and release of antimicrobial agents. Double-layer agarose plates were made with a bottom layer of chemotaxis agarose and a top layer of TSA as previously described [7]. Three-mm-diameter wells were cut in the agarose 4 mm apart in a triplet pattern (figure 1). Each plate contained 1 control sample (PMNL incubated without any agent) in addition to samples of PMNL incubated with an antimicrobial agent and with or without antipyretic agents.

PMNL (5 × 10⁶/mL) were tumbled at 37°C for 1 h with concentrations of antimicrobial agents similar to peak serum levels reported in patients after usual oral doses (0.1 μg/mL azithromycin, 4.5 μg/mL moxifloxacin, or no antimicrobial agent added) [4, 5, 8] and with or without acetaminophen, acetylsalicylic acid, or ibuprofen. In addition, we studied 1.0 μg/mL azithromycin, a level achieved after intravenous administration. Neutrophils were washed once with HBSS and pellets were formed by centrifugation at 150 g for 5 min to remove extracellular antimicrobial agent. The supernatant was discarded, and pellets were blotted. Neutrophils were washed again with the respective concentration of antipyretic agent in HBSS and centrifuged at 150 g for 5 min. The supernatant was discarded, and pellets were blotted. PMNL were resuspended in the remaining supernate (~35 μL). We placed 8 μL of cell suspension of each condition (~2 × 10⁶ PMNL) in each of the middle wells of a triplet in the agar plates: 10⁻³ M FMLP (Sigma) or 10⁻⁷ M interleukin (IL)–8 (Sigma) were used as chemoattractants and placed in the outer wells. HBSS was placed in the inner well. Plates were incubated for 3 h at 37°C in 5% CO2 to allow migration of neutrophils. After incubation was complete, 1 set of plates was fixed with 100% methanol followed by phosphate-buffered formalin. After the agar was removed and plates were Giemsa stained, neutrophil migration distance toward chemoattractant and medium wells was measured microscopically [7].

Effect of antipyretic agents on transport and release of antimicrobial agents by PMNL. Human PMNL take up, transport, and release some antimicrobial agents effectively, as we showed elsewhere [2]. PMNL were tumbled for 1 h with azithromycin (0.1 μg/mL), azithromycin (1.0 μg/mL), or moxifloxacin (4.5 μg/mL) and with or without several concentrations of the antipyretic agents, and they were allowed to migrate for 3 h. Antimicrobial agents in the media were removed by washing with HBSS. Antipyretic agents were present during the 1 h of tumbling and the 3 h of migration.

The ability of neutrophils to transport and release intracellular antimicrobial agents was quantitated by measuring the zone of inhibition of bacterial growth present after migration. A 6-h broth culture of S. pyogenes was streaked directly onto the migration plate with a wire loop and incubated overnight at 37°C in a 5% CO2 incubator. The next morning, the zones of inhibition of bacterial growth toward chemoattractant wells and toward medium wells were measured under a microscope [2].

Results

MIC. The MIC of azithromycin for S. pyogenes and for M. luteus were 0.25 and 0.125 μg/mL, respectively. The MIC of moxifloxacin for S. pyogenes and for S. aureus were 0.16 and 0.078 μg/mL, respectively.

Figure 1. Illustration of plate for antimicrobial agent transport and release assay with triplet of wells punched in an assay plate. The 3 circles indicate 3-mm wells. Polymorphonuclear leukocytes (PMNL) are placed in center well and allowed to migrate toward chemoattractant (well containing FMLP), interleukin-8, or medium (Hanks’s balanced salt solution; HBSS). a, Directed migration; b, nondirected migration; c, inhibition of bacterial growth toward chemoattractant; d, inhibition of bacterial growth toward medium. Striped oval area indicates the pattern of PMNL migration after 3 h; dotted oval area indicates area of inhibition of bacterial growth.
Effect of antipyretic agents on uptake of antimicrobial agent by PMNL. Experiments were performed to compare the effect of freeze-thawing on release of azithromycin from PMNL incubated with 1.0 μg/mL azithromycin. We found that 65% more azithromycin was released from the freeze-thawed cells (12.6 ± 0.8 μg/5 x 10⁶ PMNL compared with 20.9 ± 0.5 μg/5 x 10⁶ PMNL, n = 3 and n = 18, respectively). The amount of antimicrobial agent released from freeze-thawed cells was considered to indicate uptake of antimicrobial agent. Figure 2 shows that there was no effect on uptake of moxifloxacin by the 3 antipyretic agents examined. In contrast, the uptake of 1.0 μg/mL azithromycin was decreased by acetylsalicylic acid in a dose-related fashion. The uptake of 0.1 μg/mL azithromycin also displayed a dose-related decrease; however, the decline was not statistically significant (P > .05). Ibuprofen decreased the uptake of 0.1 μg/mL azithromycin with statistical significance at 35 μg/mL ibuprofen. Ibuprofen did not have any effect on the uptake of 1.0 μg/mL azithromycin. There was no effect of acetaminophen on azithromycin uptake.

Effect of antipyretic agents on directed and nondirected PMNL migration. None of the 3 antipyretic agents had any effect on PMNL migration toward the chemoattractant FMLP (3.5 ± 0.02 mm, directed migration), the chemoattractant IL-8 (2.5 ± 0.06 mm, directed migration), or HBSS (1.9 ± 0.02 mm or 1.7 ± 0.04 mm, nondirected migration). Migration distances were equal for PMNL without antimicrobial agents, PMNL loaded with azithromycin, and PMNL loaded with moxifloxacin [2].

Effect of antipyretic agents on antimicrobial agent transport and release by PMNL. Figures 3 and 4 depict the results of the experiments performed to quantitate antibiotic transport and release. Experiments reported elsewhere [2] demonstrated that the mean zones of inhibition of bacterial growth produced by untreated PMNL were 0.89 ± 0.06 mm (n = 10) with directed migration inhibition toward FMLP and the zone produced during nondirected inhibition was 0.55 ± 0.09 mm (n = 10) with nondirected inhibition toward HBSS. There was no effect of the antipyretic agents on nondirected transport and release of antibacterial agents by PMNL except for experiments with moxi-
floxacin and ibuprofen at 20 \( \mu g/mL \), which showed inhibition of 87.7\% \pm 10\% compared with no ibuprofen (\( P = 0.02 \)).

Figure 3 shows that acetylsalicylic acid and ibuprofen significantly, and in a dose-dependent fashion, inhibited the directed (toward FMLP) transport and release of azithromycin and moxifloxacin. Acetaminophen significantly inhibited the directed (toward FMLP) transport and release of azithromycin and moxifloxacin at levels corresponding to therapeutic serum levels of acetaminophen. Figure 4 shows that acetaminophen also significantly inhibited the IL-8-directed transport and release of azithromycin.

**Discussion**

The results of the antimicrobial agent uptake experiments are similar to those previously reported. With no antipyretic agent, the ratio of cell-associated concentration of azithromycin divided by the external concentration (C:E) is 309 at 1.0 \( \mu g/mL \) extracellular concentration; for 0.1 \( \mu g/mL \), the C:E is 395. The C:E for moxifloxacin is 11.7. C:E ratios reported elsewhere are 338 for azithromycin [9] and 10.9 for moxifloxacin [10].

Acetylsalicylic acid had a small, but significant, effect on uptake of azithromycin but not moxifloxacin. The magnitude of this inhibition was only 12\% at 20 \( \mu g/mL \) acetylsalicylic acid and thus could not fully account for the 25\% decrease in transport and release of azithromycin by PMNL. No effect of acetylsalicylic acid was seen on uptake of moxifloxacin, but transport and release was inhibited. In addition, antimicrobial agent uptake was not inhibited by ibuprofen or acetaminophen, and these antipyretics significantly inhibited transport and release of antimicrobial agents. Thus, the inhibition of transport and release was not due to diminished uptake of antimicrobial agents by PMNL. It is not surprising that we found no effect (with the ex-
of their ability to inhibit the synthesis of prostaglandin E2 (PGE2) they are all potent antipyretics, and this is thought to be because inflammatory activity, less gastrointestinal toxicity, and less blockade of cell movement is inhibited with cytochalasin B [2].

Microbial agents by nondirected PMNL are minimal and not significantly different when compared with transport and release of microbial agents by PMNL. Some investigators have reported that antimicrobial agents are concentrated in PMNL granules [13], and thus inhibition of degranulation would be a reasonable hypothesis; however, salicylates do not inhibit degranulation [14].

The shape of the inhibitory curve depicting inhibition of antipyretic activity by therapeutic concentrations of acetylsalicylic acid or acetaminophen is inter-ception of 20 μg/mL ibuprofen with moxifloxacin of antipyretics on transport and release of antimicrobial agents by PMNL moving in a nondirected fashion. Transport and release of antimicrobial agents by nondirected PMNL are minimal and not significantly different when compared with transport and release where cell movement is inhibited with cytochalasin B [2].

Although acetylsalicylic acid, ibuprofen, and acetaminophen are all considered to be nonsteroidal anti-inflammatory drugs, there are significant differences. Acetaminophen has less anti-inflammatory activity, less gastrointestinal toxicity, and less blockade of platelet aggregation than the other 2 agents [11]. However, they are all potent antipyretics, and this is thought to be because of their ability to inhibit the synthesis of prostaglandin E2 (PGE2) [12].

The mechanisms of antipyretic inhibition of antimicrobial agent transport and release by PMNL are unknown. Because the effect is not related to decreased uptake or to decreased PMNL migration, it must be because of changes in release of antimicrobial agent from PMNL. Some investigators have reported that antimicrobial agents are concentrated in PMNL granules [13], and thus inhibition of degranulation would be a reasonable hypothesis; however, salicylates do not inhibit degranulation [14].

The shape of the inhibitory curve depicting inhibition of antimi-crobial agent transport and release by acetaminophen is interesting (figure 3). Significant inhibition is seen at 20 and 25 μg/mL but not at lower or higher concentrations. Simmons et al. [15] and Robak et al. [16] found a similar curve in a system that measured PGE2 release. Concentrations of ibuprofen 10 times greater than serum levels (200 μg/mL) were shown to inhibit PMNL chemotaxis by Nielsen and Webster [17]. We found no inhibition of chemotaxis at 20 μg/mL. Matzner et al. [18] measured PMNL migration toward zymosan-activated serum in a Boyden chamber and found no effect of acetaminophen but inhibition by acetylsali-cylic acid at therapeutic concentrations. We found no inhibition by use of FMLP as a chemoattractant in the agarose assay system that was utilized. Shalabi [19] found that therapeutic levels of acetaminophen decreased PMNL oxidative activity. Ruutu and Kosunen [20] did not find any inhibition of PMNL bacterial activity by therapeutic concentrations of acetylsalicylic acid or acetaminophen.

Although there is no firm proof that transport and release of antimicrobial agents by PMNL play a role in therapeutic success, evidence supports the importance of this phenomenon. Agents such as azithromycin are effective for a wide variety of infections, including those caused by "extracellular bacteria" such as Streptococcus pneumoniae and S. pyogenes, even though serum levels are often lower than the MIC of the microbes. For example, in a group of volunteers, after the administration of 500 mg of azithromycin on day 1 and 250 mg on days 2 and 3, the average peak serum level was 0.27 ± 0.1 μg/mL. PMNL levels were 57 ± 16 μg/mL [21]. Azithromycin is considered to be effective and eradicative therapy for S. pyogenes [22], and the MIC 90 is 0.12–4.0 μg/mL. Because the organism does not usually reside in PMNL [23], it is logical to assume that azithro-mycin released from cells including PMNL contribute to this effect. Reduction of transport and release by antipyretic agents thus could compromise therapy in situations where the microbes are not highly susceptible to the antimicrobials used.

References
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