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Azithromycin Enhances Phagocytic Killing of *Aggregatibacter actinomycetemcomitans* Y4 by Human Neutrophils

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Abstract

Background—*Aggregatibacter actinomycetemcomitans* resists killing by neutrophils and is inhibited by azithromycin (AZM) and amoxicillin (AMX). AZM actively concentrates inside host cells, whereas AMX enters by diffusion. The present study is conducted to determine whether AZM is more effective than AMX at enhancing phagocytic killing of *A. actinomycetemcomitans* by neutrophils.

Methods—Killing assays were conducted in the presence of either 2 µg/mL AZM or 16 µg/mL AMX (equipotent against *A. actinomycetemcomitans*). Neutrophils were loaded by incubation with the appropriate antibiotic. Opsonized *A. actinomycetemcomitans* strain Y4 was incubated with the indicated antibiotic alone, with loaded neutrophils and antibiotic, or with control neutrophils (without antibiotic) at multiplicities of infection (MOIs) of 30 and 90 bacteria per neutrophil.

Results—Neutrophil incubation with 2 µg/mL AZM yielded an intracellular concentration of 10 µg/mL. At an MOI of 30, neutrophils loaded with AZM failed to kill significantly more bacteria than control neutrophils during the 60- and 90-minute assay periods. At an MOI of 90, neutrophils loaded with AZM killed significantly more bacteria than either AZM alone or control neutrophils during 60- and 90-minute incubations ($P < 0.05$), and killed significantly more bacteria after 90 minutes than the sum of the killing produced by AZM alone or neutrophils alone. Neutrophils incubated with AMX under identical conditions also killed significantly more bacteria than either AMX alone or control neutrophils, but there was no evidence of synergism between AMX and neutrophils.

Conclusions—Neutrophils possess a concentrative transport system for AZM that may enhance killing of *A. actinomycetemcomitans*. Its effects are most pronounced when neutrophils are greatly outnumbered by bacteria.

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The authors report no conflicts of interest related to this study.

Keywords

Aggregatibacter actinomycetemcomitans; anti-infective agents; immunity; innate; macrolides; neutrophil; periodontitis

Aggressive periodontitis (AgP) is an early-onset form of periodontitis characterized by periods of rapid periodontal breakdown and a tendency to occur in families.¹ The localized form of AgP is strongly associated with infections by the facultative species *Aggregatibacter actinomycetemcomitans*.^{2–4} This pathogen possesses several traits that enhance its virulence, including the ability to invade gingival epithelial cells, produce a leukotoxin that kills neutrophils, and resist phagocytic killing.^{5–10} Thus, it is difficult to completely eliminate *A. actinomycetemcomitans* from patients with AgP by mechanical removal of subgingival bacterial plaque alone.¹¹ A widely used protocol for treatment of AgP combines an adjunctive regimen of amoxicillin (AMX) and metronidazole with mechanical plaque debridement.¹² Although these two agents can enter cells by passive diffusion, neither is concentrated inside epithelium or neutrophils. This could limit this regimen's effectiveness in killing intracellular *A. actinomycetemcomitans*.

Azithromycin (AZM) is taken up by gingival epithelial cells and is known to accumulate inside neutrophils.^{13,14} It is active against *A. actinomycetemcomitans*, and its therapeutic levels in gingival crevicular fluid are sustained for at least 14 days after the last oral dose.^{15,16} Previous studies have shown that accumulation of AZM inside human gingival epithelial cells facilitates killing of intraepithelial *A. actinomycetemcomitans*.¹³ When active transport of AZM was inhibited by a competitive inhibitor, there was a significant decrease in the killing of intraepithelial bacteria. It is feasible that AZM accumulation by neutrophils could enhance phagocytic killing of *A. actinomycetemcomitans*. To address this hypothesis, the current authors characterized neutrophil AZM accumulation and examined its effects on killing of *A. actinomycetemcomitans*. The relationship between AZM accumulation and killing of intracellular bacteria was explored by determining whether AZM enhances phagocytic killing of *A. actinomycetemcomitans* to a greater extent than an equipotent concentration of AMX.

MATERIALS AND METHODS

Human neutrophils were isolated from blood collected from three healthy male volunteer donors (aged 24 to 57 years; mean age: 38 years). Written informed consent was obtained through a protocol approved by the Biomedical Sciences Institutional Review Board of The Ohio State University. The blood was subjected to Ficoll-Hypaque density gradient centrifugation and dextran sedimentation.¹⁷ Residual erythrocytes were eliminated by hypotonic lysis. Afterward, neutrophils were washed three times with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline and resuspended in Hanks balanced salt solution (HBSS). Isolated cells were >99% neutrophils and >99% viable as assessed by Trypan blue exclusion.

AZM transport was assayed by measuring changes in cell-associated radioactivity with time.¹³ Neutrophil suspensions were warmed to 37°C before incubation with [³H]-AZM[‡] at concentrations of 10 µg/mL in time course assays and 8 to 50 µg/mL in kinetic assays to

determine the Michaelis constant (K_m) and maximal velocity of transport (V_{max}). After the indicated time interval (3 minutes for kinetic assays and 1 to 20 minutes for assaying uptake time course), cell aliquots were withdrawn, layered over a mixture of canola oil/dibutylphthalate (3:10), and centrifuged for 35 seconds at $15,000 \times g$. Cell pellets were recovered and lysed for determination of AZM content by liquid scintillation counting. Intracellular AZM concentrations were calculated from measurements of intracellular AZM content and intracellular volume.¹³

Pure cultures of *A. actinomycetemcomitans* strain Y4 (43718)[§] were grown in brain–heart infusion broth^{||} at 37°C in humidified air with 10% CO₂. This strain is highly leukotoxic and belongs to the serotype most commonly isolated in patients with AgP.¹⁸ Bacteria were washed and opsonized at 37°C for 30 minutes in HBSS containing 20% human serum pooled from ≈ 200 donors.[¶] Neutrophils were loaded for 15 minutes at 37°C with either 2 µg/mL AZM[#] (the lowest concentration typically measured in gingival crevicular fluid during a 2-week period after systemic administration¹⁶) or 16 µg/mL AMX.[#] Broth dilution assays were used to confirm that these concentrations correspond to four times the minimum inhibitory concentration (MIC) for *A. actinomycetemcomitans* strain Y4.¹⁹ Control neutrophils were subjected to a similar incubation without antibiotic. Phagocytic killing assays were initiated by adding opsonized, prewarmed *A. actinomycetemcomitans* suspensions to vials containing one of the following: 20% human serum in HBSS, either 2 µg/mL AZM or 16 µg/mL AMX in 20% serum, control neutrophils in 20% serum, or antibiotic-loaded neutrophils in 20% serum containing either 2 µg/mL AZM or 16 µg/mL AMX. Assays were conducted at multiplicity of infection (MOI) ratios of 30 and 90 bacteria per neutrophil. The incubation vials were slowly rotated end over end for 90 minutes at 37°C to promote phagocytosis. Under these conditions, neutrophil viability was >98% after 90 minutes. At the beginning of the incubation and every 30 minutes thereafter, aliquots were removed and diluted in sterile water to lyse the neutrophils. After a second dilution, samples were spread on brain–heart infusion broth agar plates. After incubation for 48 hours at 37°C in 10% CO₂, surviving *A. actinomycetemcomitans* colonies were counted to assess killing.

Repeated-measures analysis of variance (ANOVA) was used to evaluate the concentration-dependent differences in the intracellular AZM concentrations and percentages of bacteria killed in the phagocytic killing assays. These data were normally distributed and exhibited equal variance. The Holm-Sidak test was used for post hoc comparisons. Differences in the cellular/extracellular concentration ratios were examined by Friedman repeated measures ANOVA on ranks because the data were not normally distributed.

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RESULTS

At concentrations similar to those found in gingival crevicular fluid (2 to 10 µg/mL), AZM was rapidly accumulated by neutrophils. Uptake exhibited Michaelis-Menten kinetics (Fig. 1, inset), with an observed Michaelis constant of 197 – 12.8 µg/mL and a maximum velocity of 59.9 – 5.52 ng/minute/10⁶ cells. AZM uptake saturated within 20 minutes (Fig. 1), yielding intracellular concentrations approximately five-fold higher than those in medium (Table 1). Through the range of 8°C to 37°C, AZM transport was highly temperature-dependent ($r = 0.975$, data not shown).

AZM-loaded neutrophils produced more effective bacterial killing than control neutrophils or AZM alone. The magnitude of this effect was dependent on incubation time and the MOI. At an MOI of 30 bacteria per neutrophil, AZM-loaded neutrophils exhibited a small, but statistically significant, enhancement of killing relative to control neutrophils at 30 minutes ($P < 0.05$, Holm-Sidak test), but failed to kill significantly more bacteria than controls at 60 and 90 minutes (Fig. 2). AZM-loaded neutrophils killed significantly more bacteria than AZM alone at 30 and 90 minutes ($P < 0.05$). At an MOI of 90, AZM-loaded neutrophils killed significantly more bacteria than control neutrophils or AZM alone at 60 and 90 minutes (Fig. 2, $P < 0.05$, Holm-Sidak test), but not at 30 minutes. At 60 minutes, the reduction in surviving colony-forming units (CFU) produced by AZM-loaded neutrophils (37.9%) was not significantly higher than the sum of the individual effects produced by AZM and neutrophils ($P = 0.22$, paired t test). At 90 minutes, however, the reduction by AZM-loaded neutrophils (58.5%) was significantly more pronounced than the sum of the individual effects of AZM and neutrophils ($P < 0.05$).

Under the same conditions (MOI = 90), AMX-loaded neutrophils also killed significantly more bacteria than control neutrophils or AMX alone at 60 and 90 minutes (Fig. 3, $P < 0.05$, Holm-Sidak test), but not at 30 minutes. At 60 minutes, the reduction in surviving CFU produced by AMX-loaded neutrophils (58.6%) was not significantly different from the sum of the individual effects produced by AMX and neutrophils. Similarly, at 90 minutes, the reduction by AMX-loaded neutrophils (65.6%) was not significantly different from the sum of the individual effects of AMX and neutrophils.

DISCUSSION

The findings confirm that neutrophils take up AZM through an active transport system. The observed Michaelis constant and maximum velocity values for AZM transport were consistent with a previous report.²⁰ Similar to clarithromycin (CLR), the interaction of AZM with the transporter is of relatively low affinity. However, the transport velocity and the degree of intracellular concentration observed with AZM are lower than those of CLR.²¹ In spite of this, the results suggest that neutrophils exposed to 2 µg/mL AZM while migrating through periodontal connective tissue toward the gingival crevice could accumulate an intracellular AZM concentration of ≈ 10 µg/mL. This is 20-fold higher than its MIC for *A. actinomycetemcomitans* strain Y4, at least five- to 40-fold higher than its MIC for most other strains of *A. actinomycetemcomitans* (0.25 to 2.0 µg/mL), and 10- to 80-fold higher than its MIC for most strains of *Porphyromonas gingivalis* (0.125 to 1.0 µg/mL).^{15,22} In

contrast, the intracellular activity of AMX and other β -lactam antibiotics is relatively modest because it is not actively concentrated.^{23,24} Although the 16- μ g/mL concentration of AMX used in this study is as effective as 2 μ g/mL AZM with respect to inhibition of *A. actinomycetemcomitans* strain Y4, this concentration is far above the normal therapeutic range. Treatment with AMX typically yields a concentration of 3 to 4 μ g/mL in gingival crevicular fluid.²⁵

The results suggest that AZM accumulation inside neutrophils enhances the phagocytic killing of *A. actinomycetemcomitans*. At equipotent concentrations, killing by AMX alone appeared to be more effective than by AZM alone. At the higher MOI, however, the magnitude of killing by AZM-loaded neutrophils was significantly greater than the sum of the individual effects of AZM and neutrophils alone, whereas killing by AMX-loaded neutrophils appeared to be additive in nature. To put these findings into context, AMX is a time-dependent antibiotic that produces optimal killing at concentrations that are two to four times the MIC,²⁶ whereas AZM is a concentration-dependent antibiotic with efficacy that is directly related to peak concentration and area under the concentration curve.²⁷ With concentration-dependent antibiotics, concentrations of 10 times the MIC are required to produce optimal bacterial killing.²⁸ Thus, the extracellular concentrations of AMX and AZM used in this study (which were both four times the MIC) were optimal for killing *A. actinomycetemcomitans* with AMX, but suboptimal for AZM. Intraneutrophil accumulation of AZM at concentrations 10 times the MIC presumably played a role in increasing its activity against *A. actinomycetemcomitans* and producing synergistic enhancement of killing by loaded neutrophils. Reinforcing the relationship between cell accumulation of AZM and its effect on killing of intracellular *A. actinomycetemcomitans*, the present authors' laboratory recently reported that intracellular AZM accumulation also facilitates killing invasive *A. actinomycetemcomitans* infections in cultured gingival epithelial cells.¹³ In that study, competitive inhibition of AZM transport into epithelial cells antagonized killing of intracellular *A. actinomycetemcomitans* laboratory strain Y4 and clinical strain SUNY 465 by AZM.

Because phagocytosis of *A. actinomycetemcomitans* by neutrophils is relatively inefficient, it is reasonable to doubt that intracellular accumulation of AZM could significantly enhance phagocytic killing. Along with resisting engulfment, however, *A. actinomycetemcomitans* also resists killing by neutrophils. Due in part to a weak oxidative burst response, the majority of ingested *A. actinomycetemcomitans* are still viable after 1 hour.²⁹ AZM is protonated and trapped in acidic subcellular environments in neutrophils, including lysosomes.³⁰ Under these conditions, prolonged exposure to high intracellular AZM concentrations has the potential to augment bacterial killing. It is important to note that AZM-loaded neutrophils were not consistently more effective at killing *A. actinomycetemcomitans* than control neutrophils in experiments that were run at an MOI of 30. Under these conditions, control neutrophils alone were capable of killing half of the bacteria during a period of 90 minutes. The beneficial effects of the system for intracellular AZM accumulation were mainly observed when neutrophils were presented with a greater number of bacteria than they could easily kill. It is possible that the serum used to opsonize the bacteria in this study could have influenced the degree of bacterial killing because it may

have contained antibodies that neutralized leukotoxicity. The serum was a commercial product that was pooled from many human donors, and a large proportion of individuals with periodontitis express antibodies against whole *A. actinomycetemcomitans* as well as *A. actinomycetemcomitans* leukotoxin.³¹ Although it is likely that these antibodies produced similar effects on bacterial killing by the two antibiotics used in this study, it would have been useful to characterize the levels of antibodies in the serum.

A similar study has been conducted with CLR,²¹ a macrolide that has a shorter half-life than AZM, is more active against *P. gingivalis* and *Prevotella intermedia*, and is comparably effective against *A. actinomycetemcomitans*.^{15,22,32} As with AZM, the most significant differences in killing *A. actinomycetemcomitans* by control and CLR-loaded neutrophils were observed at a relatively high MOI (100). One major difference was that the effects of CLR and neutrophils appeared to be additive rather than synergistic, despite the higher intracellular concentrations observed with CLR in neutrophils. In contrast to AZM, CLR is classified as a time-dependent antimicrobial agent, although it exerts postantibiotic effects that are enhanced at higher concentrations.^{26–28} This difference in the pharmacodynamic properties of AZM and CLR could be partially responsible for their somewhat divergent effects on phagocytic killing of *A. actinomycetemcomitans*. Clinically, systemic CLR appears to enhance reduction of probing depth and gain of clinical attachment resulting from non-surgical treatment of chronic periodontitis,³³ but, to our knowledge, only one randomized study has been conducted to date.

In addition to examining the killing of strain Y4, it would have been useful to include a clinical strain of *A. actinomycetemcomitans* in this study. Poly-*N*-acetylglucosamine, a matrix polysaccharide that helps *A. actinomycetemcomitans* resist phagocytic killing, is produced by most clinical strains, but not by Y4.³⁴ For this reason, it is possible that AZM accumulation inside neutrophils could have a more pronounced impact on phagocytic killing of a clinical strain than it does on Y4. While this omission must be considered a limitation, the assay conditions used in the present study were representative of conditions that occur in vivo. A recent pharmacokinetic study found that AZM concentrations in gingival crevicular fluid can approach 10 µg/mL and exceed 4 µg/mL for more than a week after the final oral dose.¹⁶ The study suggested that a standard regimen of AZM (initial dose of 500 mg, followed by 250 mg every 24 hours for the next 4 days) provides tissue levels in excess of the 2-µg/mL concentration used in the current study for at least 15 days. Counts of *A. actinomycetemcomitans*,^{35,36} *P. gingivalis*,³⁵ and neutrophils^{37,38} recovered from AgP sites suggest that MOI ranges from 14:1 at a typical site to 167:1 at sites with the highest levels of *A. actinomycetemcomitans*, and up to 150:1 at sites with the highest levels of *P. gingivalis*. Thus, it is feasible that neutrophil killing of *A. actinomycetemcomitans* could be enhanced in patients with AgP undergoing treatment with AZM, and conditions are also favorable for enhanced killing of *P. gingivalis*. Because of the low prevalence of the localized form of AgP, there have been no randomized clinical trials to assess the effectiveness of AZM as an adjunct to periodontal treatment. There is evidence that adjunctive use of AZM can enhance the clinical response to non-surgical treatment of the generalized form of AgP as well as non-surgical treatment of severe chronic periodontitis in smokers.^{39,40} AZM has also been shown to enhance clinical and microbiologic outcomes in the treatment of *P. gingivalis*–

associated chronic periodontitis.⁴¹ However, the improved clinical outcomes produced by adjunctive AZM have not always been associated with significant suppression of subgingival pathogens. Although some studies have shown that AZM suppresses periodontal pathogens,^{40,42} others either did not observe a significant adjunctive effect⁴³ or did not conduct a statistical analysis of microbiologic data.⁴⁴

CONCLUSIONS

Neutrophils express an active transport system for AZM. Intracellular accumulation of AZM may enhance killing of *A. actinomycetemcomitans*, especially under conditions in which neutrophils are greatly outnumbered by bacteria. The results of this study, in combination with a recent report that intracellular AZM accumulation can kill invasive *A. actinomycetemcomitans* in gingival epithelial cells,¹³ suggest that adjunctive AZM can potentially enhance the elimination of *A. actinomycetemcomitans* from patients with periodontitis.

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Figure 1.

Time course of accumulation of 10 µg/mL [³H]-AZM by suspended neutrophils at 37°C. The data represent the mean and SD. **Inset:** Representative Lineweaver-Burk plot of the initial phase of AZM transport by neutrophils.

Figure 2.

Effect of AZM on the killing of *A. actinomycetemcomitans* at MOI of 30 and 90. Opsonized bacteria were added to tubes containing 20% human serum (bacteria only), 2 $\mu\text{g/mL}$ AZM in 20% serum (bacteria + AZM), control neutrophils in 20% serum (bacteria + PMNs), or AZM-loaded neutrophils in 20% serum containing 2 $\mu\text{g/mL}$ AZM (bacteria, PMNs, and AZM). Data are presented as the mean and SD of five experiments. At each time point, pairwise comparisons that exhibited statistically significant differences (as determined by the Holm-Sidak post hoc test) are denoted by identical symbols.

Figure 3.

Effect of AMX on the killing of *A. actinomycetemcomitans* at an MOI of 90. Studies were conducted under the same conditions as the lower panel of Figure 2, except that AMX(16µg/mL) was substituted for AZM. Data are presented as the mean and SD of five experiments. At each time point, pairwise comparisons that exhibited statistically significant differences (as determined by the Holm-Sidak test) are denoted by identical symbols.

Table 1Intracellular Accumulation of AZM by Human Neutrophils (mean $\pm$ SD)

Extracellular AZM Concentration ($\mu\text{g/mL}$)	Intracellular AZM Concentration ($\mu\text{g/mL}$) [*]	Cellular/Extracellular Concentration Ratio [†]
2	9.82 $\pm$ 1.21	4.91 $\pm$ 0.60
5	23.8 $\pm$ 2.0	4.76 $\pm$ 0.40
10	49.1 $\pm$ 7.1	4.91 $\pm$ 0.70
20	94.8 $\pm$ 10.5	4.74 $\pm$ 0.52

^{*} Within the column, there is a significant treatment effect ($P < 0.001$, repeated measures ANOVA) and a significant difference in all pairwise comparisons ($P < 0.05$, Holm-Sidak).

[†] Within the column, there are no statistically significant differences ($P = 0.39$, Friedman repeated measures ANOVA on ranks).