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Azithromycin Kills Invasive *Aggregatibacter actinomycetemcomitans* in Gingival Epithelial Cells

Pin-Chuang Lai,[a,b] John D. Walters[b]

Divisions of Oral Biology[a] and Periodontology,[b] The Ohio State University Health Sciences Center, Columbus, Ohio, USA

Aggregatibacter actinomycetemcomitans **invades periodontal pocket epithelium and is therefore difficult to eliminate by periodontal scaling and root planing. It is susceptible to azithromycin, which is taken up by many types of mammalian cells. This led us to hypothesize that azithromycin accumulation by gingival epithelium could enhance the killing of intraepithelial *A. actinomycetemcomitans*. [^{3}H]azithromycin transport by Smulow-Glickman gingival epithelial cells and SCC-25 oral epithelial cells was characterized. To test our hypothesis, we infected cultured Smulow-Glickman cell monolayers with *A. actinomycetemcomitans* (Y4 or SUNY 465 strain) for 2 h, treated them with gentamicin to eliminate extracellular bacteria, and then incubated them with azithromycin for 1 to 4 h. Viable intracellular bacteria were released, plated, and enumerated. Azithromycin transport by both cell lines exhibited Michaelis-Menten kinetics and was competitively inhibited by L-carnitine and several other organic cations. Cell incubation in medium containing 5 μg/ml azithromycin yielded steady-state intracellular concentrations of 144 μg/ml in SCC-25 cells and 118 μg/ml in Smulow-Glickman cells. Azithromycin induced dose- and time-dependent intraepithelial killing of both *A. actinomycetemcomitans* strains. Treatment of infected Smulow-Glickman cells with 0.125 μg/ml azithromycin killed approximately 29% of the intraepithelial CFU of both strains within 4 h, while treatment with 8 μg/ml azithromycin killed ≥82% of the CFU of both strains ($P < 0.05$). Addition of carnitine inhibited the killing of intracellular bacteria by azithromycin ($P < 0.05$). Thus, human gingival epithelial cells actively accumulate azithromycin through a transport system that facilitates the killing of intraepithelial *A. actinomycetemcomitans* and is shared with organic cations.**

Periodontitis is the result of infection by a specific group of subgingival bacteria. These pathogens induce host immunological and inflammatory responses in periodontal tissues, leading to the destruction of connective tissues and alveolar bone (1). While nonsurgical periodontal treatment can usually eliminate most periodontal pathogens and arrest periodontal attachment loss, invasive pathogens like *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* are difficult to eliminate by conventional therapy (2, 3). If efforts to eliminate these bacteria are unsuccessful, they can multiply within gingival crevicular epithelial cells and recolonize adjacent periodontal pockets.

A. actinomycetemcomitans is a Gram-negative facultative rod that possesses several virulence factors that can overwhelm the host defense. In addition to its ability to invade epithelial cells (4), *A. actinomycetemcomitans* resists phagocytic killing (5) and produces a leukotoxin that kills polymorphonuclear lymphocytes (PMNs) (6). Previous studies have shown that extracrevicular reservoirs of *A. actinomycetemcomitans* exist and may contribute to recurrent or refractory diseases in some subjects (7, 8). Thus, it is rational to use a systemic antibiotic as an adjunct to nonsurgical periodontal treatment to facilitate the elimination of pathogens from subgingival and extracrevicular niches and enhance the response to therapy.

Azithromycin (AZM), a derivative of erythromycin, is effective against *A. actinomycetemcomitans* (9) and possesses a long half-life. In addition, AZM produces anti-inflammatory effects by inhibiting nuclear factor kappa B in oral epithelium (10) and by reducing levels of proinflammatory cytokines in gingival crevicular fluid (GCF) (11). Several clinical trials have demonstrated promising clinical and microbiological benefits of AZM in the treatment of periodontal diseases (12–14). Unlike beta-lactam antibiotics, AZM is concentrated inside human PMNs and fibroblasts (15, 16). Clarithromycin (CLR), a closely related macrolide, is also actively transported and accumulated by human oral epithelial cells, gingival fibroblasts, and PMNs (17, 18). PMNs that have taken up CLR exhibit enhanced phagocytic killing of intracellular *A. actinomycetemcomitans* (18).

Although the mechanism by which AZM is taken up by epithelium is unclear, it is feasible that intracellular accumulation of AZM could be useful in eradicating invasive bacteria from gingival epithelial cells. AZM and other weak organic bases can potentially interact with organic cation transporters or organic anion-transporting polypeptides, which have relatively broad substrate specificity. To the extent that this occurs, substrates of these transport systems could competitively inhibit AZM transport. In the present study, we characterized AZM transport by two different cultured oral epithelial cell lines and utilized an *in vitro* model of epithelial invasion to examine the effect of intracellular AZM accumulation on the elimination of two different strains of *A. actinomycetemcomitans* from cultured gingival epithelium.

MATERIALS AND METHODS

Epithelial cell culture. Smulow-Glickman (SG) gingival epithelial cells, originally derived from human attached gingiva (19), and SCC-25 epithelial cells (CRL-1628, ATCC, Manassas, VA), derived from oral epidermoid carcinoma (20), were used in this study. SG cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum

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Address correspondence to John D. Walters, walters.2@osu.edu.

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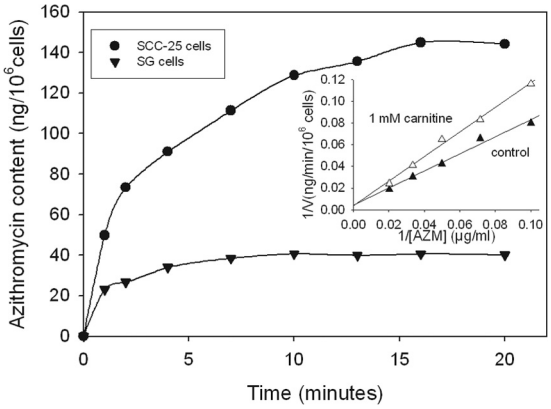

FIG 1 Time course of AZM accumulation in SCC-25 and SG epithelial cells cultured at 37°C. The assay was initiated by the addition of [^{3}H]AZM to suspended cells. The data represent the mean of three experiments. The inset is a representative Lineweaver-Burk plot of the initial phase of AZM transport by SG cells in the presence or absence of 1 mm L-carnitine. The intercepts are consistent with competitive inhibition.

(Atlanta Biologicals, Norcross, GA), while SCC-25 cells were grown in 50% DMEM–50% Ham's F-12 medium (Invitrogen Corp) containing 10% heat-inactivated fetal bovine serum and 0.4 μg/ml hydrocortisone. Both cell lines were fed every 3 days and cultured to confluent monolayers at 37°C in the presence of 5% CO_2 in separate 24-well tissue culture plates.

Bacterial culture. Pure cultures of *A. actinomycetemcomitans* strains SUNY 465 (clinical isolate) and Y4 (ATCC 43718) were grown in brain heart infusion (BHI) broth (Becton, Dickinson and Company, Sparks, MD) at 37°C in an environment containing 10% CO_2.

Assay of AZM transport. Confluent SG and SCC-25 cell monolayers were washed with Hank's balanced salt solution (HBSS; Invitrogen Corp.), harvested by brief treatment with 0.25% trypsin-EDTA (Invitrogen Corp.), and suspended in HBSS at a density of 10^6 cells/ml. AZM transport was assayed as previously described, by measuring changes in cell-associated radioactivity over time (17, 21). Aliquots of cell suspension were incubated at 37°C with [^{3}H]AZM (American Radiolabeled Chemicals, St. Louis, MO) at a concentration of 10 μg/ml for time course assays and at 10 to 50 μg/ml for kinetic assays to determine the Michaelis constant (K_m) and maximal velocity of transport (V_{max}). After the indicated interval (1 to 20 min for uptake time course assays and 3 min for kinetic assays), 0.5-ml aliquots of cell suspension were rapidly withdrawn, layered over 0.3 ml of a mixture of canola oil-dibutyl phthalate (3:10), and centrifuged for 30 s at 15,000 × *g* in a microcentrifuge (22). After removal of the aqueous and oil layers, the cell pellets were recovered, lysed by agitation in 1 ml of sterile water, and subjected to liquid scintillation counting.

The intracellular volume associated with identical cell suspensions was measured by incubation with [^{3}H]water (5 μCi/ml; NEN Life Science Products, Boston, MA) for 20 min at 37°C. Volume determinations were corrected for extracellular water trapped in the pellet, which was determined by incubation under the same conditions with [^{14}C]inulin (1 μCi/ml; PerkinElmer, Waltham, MA) (23).

Several organic cations and organic acids were examined for the potential to inhibit AZM transport. All were purchased from Sigma Chemical Company (St. Louis, MO). Individual agents were added to 0.5-ml cell suspension aliquots simultaneously with [^{3}H]AZM. Lineweaver-Burk analysis was used to determine the mechanisms of inhibition.

Effect of AZM on killing of intraepithelial *A. actinomycetemcomitans*. Epithelial invasion by *A. actinomycetemcomitans* was induced as described by Meyer et al. (24). In this model, *A. actinomycetemcomitans* enters the epithelial cells in a host-derived membrane-bound vacuole and lyses the vacuolar membrane soon after entry (25). Microtubules play a critical role in cell invasion by *A. actinomycetemcomitans*. Paclitaxel (originally named taxol), which stabilizes polymerized microtubules, enhances invasion and inhibits the subsequent exit of *A. actinomycetemcomitans* from infected epithelial cells. Thus, the assay was conducted with DMEM containing 10 μM paclitaxel (Sigma Chemical Company, St. Louis, MO) to help maintain the intracellular *A. actinomycetemcomitans* levels over the course of the assay. Confluent SG cell monolayers in 24-well culture plates were washed and pretreated with assay medium for 30 min prior to the addition of bacteria. Bacterial cultures were harvested, washed, resuspended in assay medium, and added to each culture plate well at a multiplicity of infection of 1,000. After infection for 2 h at 37°C, SG cell monolayers were washed five times with HBSS. Adherent extracellular bacteria were removed by treatment with 100 μg/ml gentamicin for 1 h. After the removal of gentamicin-containing medium, the infected monolayers were washed five times with HBSS. To confirm the absence of viable extracellular bacteria, an aliquot of the final wash was plated on BHI agar (Becton, Dickinson and Company, Sparks, MD). Infected SG cell monolayers were then cultured in the presence of AZM for periods of 1, 2, and 4 h. As a positive control, infected monolayers were cultured under identical condition in the absence of AZM. At the indicated intervals, monolayers were washed four times with HBSS and lysed in sterile water to release intracellular *A. actinomycetemcomitans*. Dilutions of the lysate were plated on BHI agar for the enumeration of surviving CFU. Data were expressed as a percentage of the colonies recovered from the positive controls. The effect of amoxicillin (AMX), which does not accumulate inside cells (26), was tested for comparison to AZM. Experiments were carefully monitored to rule out any cytotoxic effects of reagents on cultured epithelial cells or *A. actinomycetemcomitans*.

RESULTS

Epithelial AZM transport. AZM accumulation by SCC-25 and SG cells was saturated within 20 min (Fig. 1), resulting in steady-state intracellular concentrations that were more than 20-fold higher than the extracellular concentrations (Table 1). Transport activity exhibited Michaelis-Menten kinetics (Fig. 1, inset). The observed K_m values for AZM transport by SCC-25 and SG cells were similar, but SG cells transported AZM at approximately half the maximal velocity observed with SCC-25 cells (Table 1). At steady state, with an extracellular AZM concentration of 5 μg/ml, the cellular/extracellular concentration ratio of SCC-25 cells was slightly higher than that of SG cells. To examine the substrate specificity of the system that transports AZM, several organic cations and anions were tested as potential inhibitors. In kinetic studies with SCC-25 cells, the organic cations quinidine, pyrilamine, procainamide, and L-carnitine acted as competitive inhibitors of AZM transport (Table 2 and Fig. 1 inset). Probenecid, an organic acid, also produced competitive inhibition of AZM transport. At inhibitory concentrations, none of these agents altered the pH of the assay medium. The organic anions spironolactone, pravastatin, bromosulfophthalein, hydrocortisone, taurocholate, and estrone-3-sulfate produced little or no inhibition of AZM transport.

TABLE 1 Kinetic constants of AZM transport by SG and SCC-25 cells[a]

Cell type	K_m (μg/ml)	V_{max} (ng/min/10^6)	Cellular/extracellular concn ratio
SG	198 ± 23.8	249 ± 13[b]	23.7 ± 0.97[b]
SCC-25	176 ± 10.5	486 ± 20.1	28.8 ± 1.53

[a] K_m and V_{max} values were determined by Lineweaver-Burk analysis of transport activity during the rapid initial phase of uptake (first 3 min). The cellular/extracellular concentration ratio was determined after incubation for 20 min in medium containing 5 μg/ml AZM. All data are expressed as the mean ± the standard error of the mean of at least three experiments.

[b] The values in this column are significantly different ($P < 0.05$, *t* test).

TABLE 2 Inhibition of SCC-25 cell AZM transport by organic cations and probenecid[a]

Agent	K_m (μg/ml)	V_{max} (ng/min/10^6)	Mechanism of inhibition (K_i [mM])	Chemical classification
Control	176 ± 10.5	486 ± 20.1		Not applicable
Quinidine	382 ± 2.0	464 ± 8.5	Competitive (0.82 ± 0.09)	Organic cation
Pyrilamine	266 ± 12.6	503 ± 6.6	Competitive (0.4 ± 0.07)	Organic cation
Procainamide	269 ± 56.5	513 ± 112	Competitive (3.03 ± 0.46)	Organic cation
L-Carnitine	314 ± 32	483 ± 46.5	Competitive (0.46 ± 0.05)	Organic cation
Probenecid	236 ± 3.2	483 ± 30.9	Competitive (0.57 ± 0.08)	Organic acid

[a] The values shown were derived from Lineweaver-Burk analysis of transport activity observed in the presence or absence of the indicated agents. All data are expressed as the mean ± the standard error of the mean of at least three experiments.

Treatment with 10 μM paclitaxel, used to enhance epithelial infection by *A. actinomycetemcomitans*, also had no significant effect on AZM transport by SG cells.

Killing of intraepithelial *A. actinomycetemcomitans* by AZM. Since *A. actinomycetemcomitans* exhibits strain-dependent differences in susceptibility to AZM, the effects of AZM on two different invasive strains were examined. Infected SG epithelial cells were treated with AZM concentrations similar to those found in blood (0.125 to 0.5 μg/ml) and GCF (2 to 8 μg/ml) (27). AZM produced dose- and time-dependent intraepithelial killing of both strains (Fig. 2, $P < 0.001$, repeated-measures analysis of variance). Cells infected with SUNY 465 required 4 h of treatment with 0.125 μg/ml AZM to produce a significant degree of bacterial killing ($P < 0.01$, Holm-Sidak test). Treatment with 0.5 μg/ml AZM produced significant killing after 2 h, while treatment with ≥2 μg/ml produced significant killing after 1 h ($P < 0.01$). Treatment with 8 μg/ml AZM killed approximately 49% of the control SUNY 465 CFU after 1 h, 83% after 2 h, and 85% after 4 h ($P < 0.01$, Holm-Sidak test). Under similar experimental conditions, treatment for 2 h with 4 μg/ml AMX killed only 14% of the SUNY 465 CFU ($P > 0.05$; data not shown).

Although AZM produced dose- and time-dependent bacterial killing in cells infected with the Y4 strain, concentrations of ≥0.5 μg/ml required a longer treatment time to produce the degree of inhibition observed with SUNY 465 (Fig. 2, lower panel). Interestingly, treatment with 0.125 μg/ml AZM produced significant killing after 1 h ($P < 0.01$, Holm-Sidak test).

To determine whether inhibition of AZM transport impairs the killing of intraepithelial *A. actinomycetemcomitans*, 1 mM L-carnitine (alone or in combination with 2 μg/ml AZM) was added to the culture medium of invaded SG cells (Fig. 3). Under these conditions, carnitine reduced the steady-state intracellular AZM concentration from 43.5 μg/ml to approximately 33.3 μg/ml (data not shown). Treatment for 1 h with carnitine had no significant effect on the survival of SUNY 465, while treatment with AZM killed 30% of the bacteria ($P < 0.05$, Holm-Sidak test). In the presence of a combination of carnitine and AZM, killing of

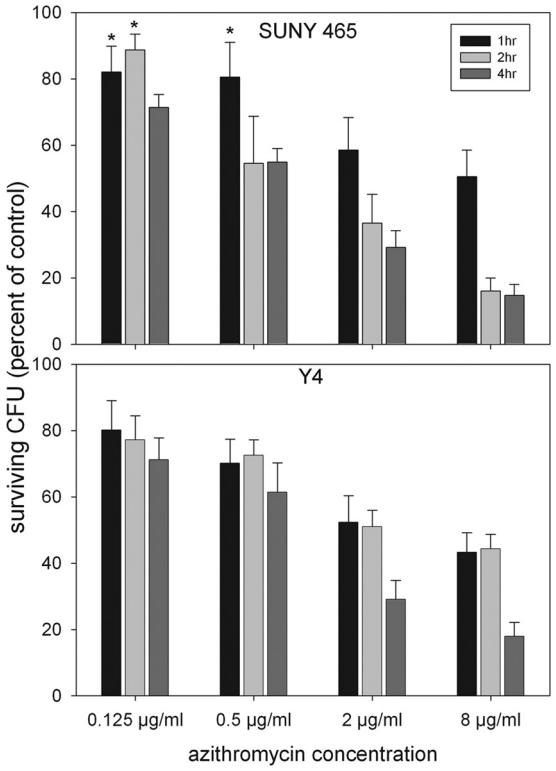

FIG 2 Effect of AZM on the killing of intracellular *A. actinomycetemcomitans*. SG cell monolayers infected with either the SUNY 465 or the Y4 strain of *A. actinomycetemcomitans* were incubated in AZM-containing (treatment) or AZM-free (control) medium. Intracellular *A. actinomycetemcomitans* bacteria were released by cell lysis, plated on BHI agar, and enumerated. The data presented are percentages of the colonies recovered from the controls. The data represent the mean ± the standard error of the mean of six to eight experiments. Treatments that failed to produce significant inhibition compared to the control are denoted by asterisks ($P > 0.05$, Holm-Sidak test).

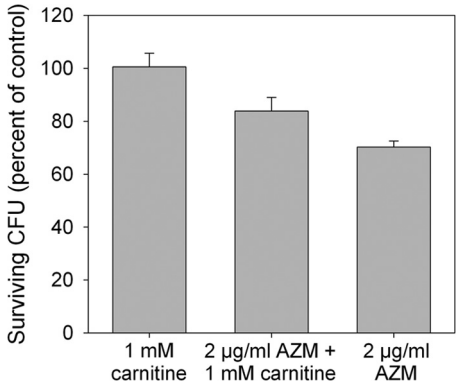

FIG 3 Impairment of AZM killing of intracellular SUNY 465 by L-carnitine. Infected SG cell monolayers were incubated with L-carnitine alone, AZM alone, or a combination of AZM and L-carnitine for 1 h. Cells cultured in the absence of treatment agents served as controls. The data presented are percentages of the colonies recovered from the controls. The data represent the mean ± the standard error of the mean of seven experiments. The results of all three treatments were significantly different from each other ($P < 0.05$, Holm-Sidak test).

SUNY 465 was reduced to approximately half of that produced by AZM alone ($P < 0.05$, Holm-Sidak test).

DISCUSSION

The results obtained in this study demonstrate that human oral epithelial cell lines possess an active transport system that concentrates AZM and facilitates the killing of *A. actinomycetemcomitans* inside infected gingival epithelium. AZM transport by both epithelial cell lines exhibited Michaelis-Menten kinetics and yielded steady-state intracellular concentrations that were substantially higher than those in the extracellular medium. In addition to human phagocytes and fibroblasts, in which active cellular uptake of AZM has been reported (16, 28), previous studies provide evidence of AZM concentration inside epithelium (21, 29). The cellular/extracellular AZM concentration ratios observed in the present study were approximately 3-fold higher than those reported for canine kidney, McCoy, and Hep-2 epithelial cells. The differences might be associated with the different origins of these epithelial cell lines and different levels of transporter gene expression. SCC-25 cells took up AZM at a V_{max} 2-fold higher than that of SG cells and exhibited a significantly greater degree of intracellular AZM accumulation.

As a weak organic base, AZM is a candidate for interaction with transporters that carry organic cations. In this study, the organic cations quinidine, pyrilamine, procainamide, and L-carnitine competitively inhibited epithelial AZM transport. Control experiments confirmed that none of these agents altered the pH of the assay medium. This suggests that AZM uptake by these oral cell lines is mediated by a transport system that accepts organic cations as substrates. Except for probenecid, none of the organic anions we examined inhibited AZM transport. Probenecid reportedly interacts with organic cation transporters, possibly through binding to both the hydrophobic and anionic binding sites of some organic cation transporters (30).

Antibiotics that can penetrate eukaryotic cell membranes and remain active in the intracellular environment are most suitable for treating infections by invasive bacteria (31). In the present study, AZM accumulation by SG cells was associated with time- and concentration-dependent killing of intracellular *A. actinomycetemcomitans*. While the more susceptible SUNY 465 strain was substantially inhibited after 2 h of treatment with AZM, longer treatment times were required to produce comparable inhibition of Y4. Despite the limitation posed by a relatively short experimental treatment time with a bacteriostatic antibiotic, almost 90% of the SUNY 465 strain cells were killed inside SG cells incubated with AZM at concentrations comparable to those found in GCF (8 μg/ml). Inhibition of AZM transport by carnitine significantly impaired this killing. AMX, which does not concentrate inside cells, was significantly less effective at killing intraepithelial *A. actinomycetemcomitans* at the concentrations found in GCF (4 μg/ml).

The connection between intracellular AZM accumulation and killing of invasive bacteria in this study is consistent with results obtained with *Listeria*- and *Staphylococcus*-infected macrophages (32). Inhibitors of the P-glycoprotein efflux pump increased the intracellular accumulation of AZM and enhanced the killing of intracellular bacteria. Similarly, PMNs that take up and accumulate CLR exhibit enhanced phagocytic killing of the leukotoxin-producing Y4 strain of *A. actinomycetemcomitans* (18). Our findings can also be related to a previous study of antibiotic killing of *A. actinomycetemcomitans* NCTC 9710 within cultured KB epithelial cells (33). In that study, the effects of moxifloxacin and doxycycline, which are actively transported by human oral epithelial cells (23), were examined. Intracellular *A. actinomycetemcomitans* was completely eliminated by treatment for 4 h with 0.115 μg/ml moxifloxacin or 6.25 μg/ml doxycycline. Thus, it appears that moxifloxacin, doxycycline, and AZM can kill intraepithelial *A. actinomycetemcomitans* at concentrations near those attainable in GCF. One advantage of AZM is that its therapeutic levels in GCF are sustained for an unusually long time (≥14 days after the last oral dose) (34).

In summary, two cell lines derived from oral epithelium possess active transport systems for AZM that exhibit Michaelis-Menten kinetics and accept other organic cations as substrates. The resultant concentration of AZM inside cultured gingival epithelial cells is effective at killing invasive infective *A. actinomycetemcomitans*. To date, there have been no clinical studies to examine the adjunctive effects of AZM in the treatment of localized aggressive periodontitis, the variant of periodontitis that is most strongly associated with infection by *A. actinomycetemcomitans* (35). Our findings provide a rationale for conducting these studies.

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