CLARITHROMYCIN ACCUMULATION BY PHAGOCYTES AND ITS EFFECT ON KILLING OF AGGREGATIBACTER ACTINOMYCETEMCOMITANS

THESIS

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ABSTRACT

Background: Previous studies have shown that clarithromycin inhibits several periodontal pathogens and is concentrated inside gingival fibroblasts and epithelial cells by an active transporter. We hypothesized that polymorphonuclear cells (PMNs) and other cells of myeloid lineage possess a similar transport system for taking up clarithromycin. In addition, we predicted that clarithromycin accumulation inside PMNs would enhance killing of *Aggregatibacter actinomycetemcomitans*.

Methods: To test the first hypothesis, purified PMNs and cultured HL-60 cells were incubated with [³H]-clarithromycin. Clarithromycin transport was assayed by measuring changes in cell-associated radioactivity over time. The second hypothesis was evaluated with PMNs loaded by brief incubation with clarithromycin (5 µg/ml). Opsonized bacteria were incubated at 37° C with control and clarithromycin-loaded PMNs and in the presence and absence of clarithromycin (5 µg/ml).

Results: Undifferentiated HL-60 cells, HL-60 cells that had been differentiated into PMN-like granulocytes, and mature human PMNs all took up clarithromycin in a saturable manner. The kinetics of uptake by all three types of cells yielded linear Lineweaver-Burk plots. PMNs and HL-60 granulocytes transported clarithromycin with a $K_m$ of approximately 250µg/ml and a $V_{max}$ that was not significantly different. When
extracellular concentrations were diluted, HL-60 granulocytes loaded with clarithromycin lost approximately 20% of their antibiotic content within 3 minutes and approximately 45% of their original content within 60 minutes. HL-60 granulocytes and PMNs accumulated intracellular levels of clarithromycin that were 28- to 71-fold higher than their extracellular concentration. When assayed at a bacteria-to-PMN ratio of 100:1, clarithromycin-loaded PMNs killed significantly more bacteria and achieved shorter half times for killing than control PMNs (P < 0.04). At a ratio of 30:1, these differences were not consistently significant.

**Conclusion:** PMNs and less mature myeloid cells possess a transporter that takes up and concentrates clarithromycin. This system could enhance the ability of PMNs to cope with an overwhelming infection by *A. actinomycetemcomitans.*
DEDICATION

This document is dedicated to my mother, Lily,
for all her love and support throughout my life.
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CHAPTER 1:
INTRODUCTION

Periodontitis is a bacterially induced chronic inflammatory disease which leads to the destruction of the underlying structures of the teeth, such as the periodontal ligament, cementum, and bone. Most species of subgingival bacteria associated with periodontitis can be eliminated by mechanical debridement (root planing). However, some bacterial species, such as Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis, are exceptionally difficult to eliminate by mechanical therapy alone.\(^1\) A. actinomycetemcomitans is a Gram-negative microaerophilic coccobacillus that is strongly associated with aggressive and refractory forms of periodontitis, while P. gingivalis is a Gram-negative anaerobic rod that is associated with severe adult periodontitis, failing guided tissue regeneration, and acute periodontal abscesses.\(^2,3\) Both species possess virulence factors that frustrate the host response and resist conventional efforts to eliminate them.

One such virulence factor that is prevalent in both bacterial species is the ability to invade the epithelial lining of the periodontal pocket.\(^4\) A. actinomycetemcomitans can invade epithelial cells and pass into the underlying connective tissue, while P. gingivalis can invade epithelial cells and linger inside the cell.\(^5,6\) Invasion by A. actinomycetemcomitans occurs through the binding of bacterial fimbriae or extracellular
vesicles onto the cell surface. This causes ruffling and invaginations on the epithelial cell membrane, which allows the bacteria to be internalized.\(^7\) Once inside, A. *actinomycetemcomitans* induces membrane protrusions, which allow for bacterial migration into adjacent epithelial cells and connective tissue while escaping detection from host immune cells.\(^8,\,9\) Hypothetically, invisibility from immune cells may partly explain why individuals with aggressive periodontitis typically do not exhibit massive inflammation clinically.\(^9\) While only a few bacterial pathogens are capable of invading and surviving within epithelial cells, these infections are among the most difficult to eradicate. Within this context, it is not difficult to envisage how inter- and intraepithelial invasion may lead to disease occurrence and resistance to treatment.

In addition, A. *actinomycetemcomitans* resists phagocytic killing by secreting low molecular-weight compounds that inhibit neutrophil chemotaxis, producing heat-stable proteins which inhibit the neutrophil’s ability to produce hydrogen sulfide, and resisting defensins formed by neutrophils.\(^10\)-\(^12\) The bacteria also produces leukotoxin, a protein which induces apoptosis in polymorphonuclear cells (PMNs). PMNs are the first line of defense against bacteria near the gingival margin, and their inhibition allows for pathogenic bacteria to remain in the periodontium.\(^13\)-\(^15\) Persistent infections by A. *actinomycetemcomitans* or *P. gingivalis* are frequently associated with continued periodontal breakdown.\(^16\) One study has found that the continual presence of A. *actinomycetemcomitans* in periodontally treated patients increased the occurrence of recurrent periodontitis despite following a 3 month maintenance schedule.\(^17\) Thus, the
presence of the bacteria is notable not only for its ability to evade and inhibit the immune response, but also for its ability to produce further breakdown when the bacteria is sustained in the oral cavity.

Invasive pathogens can be eliminated by introducing an appropriate antimicrobial agent into invaded host cells. In cases where there is continued periodontal breakdown after scaling and root planing, antimicrobial chemotherapy is often used as an adjunct to mechanical therapy.\\(^{18}\) Several studies have noted the beneficial effects of systemic antibiotics in the treatment of aggressive periodontitis, such as reduced pocket depth and additional clinical attachment gains of approximately 0.45 mm.\\(^{19,20}\) The combination of metronidazole and amoxicillin has been widely prescribed for aggressive periodontitis due to its consistent ability to reduce pocket depths, reduce the amount of periodontal pathogens, and increase clinical attachment gain.\\(^{21}\) The combination of both antibiotics is also more effective than either antibiotic alone.\\(^{22}\) However, beta-lactam antibiotics are poorly suited for treating intracellular infections because of their inability to penetrate mammalian cells. Amoxicillin also cannot be used for patients allergic to penicillin, with a history of cross-reactions occurring between 1-10% of all patients.\\(^{23-26}\) Compliance may also be an issue, as the antibiotics must be taken together three times a day for at least seven days.

Although not widely used in periodontal therapy, macrolide antibiotics may be suitable for incorporation due to several advantageous features. First, macrolides such as
azithromycin and clarithromycin may accumulate with high efficiency inside epithelial cells and immune cells such as PMNs and macrophages.\textsuperscript{27, 28} Macrolides are able to inhibit bacterial growth by binding to the 50S subunit of the prokaryotic 70S ribosome and disrupting protein synthesis.\textsuperscript{29} Furthermore, both azithromycin and clarithromycin exhibit good activity against spirochetes, \textit{Eikenella corrodens}, \textit{Prevotella} species, fusobacteria, and other anaerobic and facultative oral pathogens such as \textit{A. actinomycetemcomitans} and \textit{P. gingivalis}.\textsuperscript{30-35}

Clarithromycin is a semi-synthetic macrolide which is similar to erythromycin but demonstrates a broader spectrum of activity. It is effective towards both most gram-positive bacteria and also a number of gram-negative bacteria in the oral environment. Previous work has suggested that clarithromycin is a good candidate for treating invasive pathogens because it is accumulated inside gingival fibroblasts and epithelial cells by active transport.\textsuperscript{36} This agent reaches higher levels in gingiva than in serum and yields higher levels in inflamed gingiva than in healthy gingiva.\textsuperscript{37}

As of now, little is known about the mechanism by which macrolide antibiotics are taken up by granulocytic cells. We hypothesized that PMNs and HL-60 cells (a human promyelocytic leukemia cell line that can be differentiated into immature PMN-like cells) possess similar active transport systems that facilitate intracellular accumulation of clarithromycin. We further hypothesized that a transporter of this type could be expected
to benefit the host by enhancing the phagocytic killing of \textit{A. actinomycetemcomitans}.

These two hypotheses were tested in the present laboratory study.
CHAPTER 2:
MATERIALS AND METHODS

**HL-60 cell culture:** Human promyelocytic leukemia cells (HL-60, American Type Culture Collection, Manassas, VA) were cultured at 37° C in 5% CO₂ in RPMI 1640 medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 15% heat-inactivated fetal bovine serum (Sigma Chemical Company, St Louis, MO). Immature HL-60 cells were differentiated into granulocytic HL-60 cells by culturing with 1.3% DMSO for seven days.\(^{38}\)

**PMN isolation:** Human PMNs were isolated from citrated whole blood obtained from healthy volunteers, using Ficoll/Hypaque density gradient centrifugation and dextran sedimentation.\(^{39}\) Residual erythrocytes were eliminated by hypotonic lysis. The remaining cells were washed three times with phosphate buffered saline. Cells isolated in this manner are generally >99% PMNs (based on cytospin preparations stained with Wright-Giemsa) and >99% viable (based on trypan blue exclusion). PMNs were suspended in Hank's balanced salt solution (HBSS, Invitrogen Corporation, Carlsbad, CA) at densities of 5 x 10⁶ cells/ml and 10⁷ cells/ml, respectively, for the transport assays and phagocytic killing assays described below.

**Clarithromycin transport:** Clarithromycin transport was assayed by measuring cell-associated radioactivity over time as previously described.\(^{36}\) PMN suspensions were
warmed to 37° C prior to incubation with [³H]-clarithromycin (American Radiolabeled Chemicals, Saint Louis, MO) at concentrations of 10 µg/ml for time course assays and 8-50 µg/ml in kinetic assays to determine the Michaelis constant (Kₘ) and maximal velocity of transport (Vₘₐₓ). Unlabeled clarithromycin was obtained from United States Pharmacopeia (Rockville, MD). After the indicated interval (3 minutes for kinetic assays and 2 to 60 minutes for uptake or efflux time courses), 0.5 ml aliquots of cell suspension were rapidly withdrawn, layered over 0.3 ml of a mixture of canola oil/dibutylphthalate (3:10) and centrifuged for 35 seconds at 15,000 x g in a microcentrifuge. After removal of aqueous and oil layers, cell pellets were recovered by cutting off the ends of the microcentrifuge tubes. The pellets were lysed for liquid scintillation counting by agitating in 1.0 ml of water for 12 hrs. Lineweaver-Burk analysis was used to determine Kₘ and Vₘₐₓ.

**Clarithromycin efflux:** Suspended HL-60 granulocytes were loaded to a steady-state intracellular clarithromycin concentration by incubation for 20 minutes at 37° C in HBSS containing 10 µg/ml [³H]-clarithromycin. To trigger efflux of intracellular clarithromycin stores, the concentration of clarithromycin in the extracellular medium was abruptly diluted 1:20 with 37° C HBSS. The decrease in intracellular clarithromycin concentration was monitored for 60 minutes.

**Intracellular clarithromycin concentration:** Aliquots of suspended cells were loaded to steady-state with [³H]-clarithromycin, and intracellular clarithromycin content was
assayed as described above. The intracellular volume of identical cell aliquots was measured by incubation for 20 minutes at 37º C with [³H]-water (5 µCi/ml, NEN Life Science Products, Boston, MA). To correct for the extracellular water trapped in the pellet, cells were equilibrated with [14C]-inulin (2 µCi/ml, NEN Life Science Products) and processed similarly. Intracellular clarithromycin concentrations were calculated by dividing cell content by cell volume.

**Bacterial killing:** Pure cultures of *A. actinomycetemcomitans* strain Y4 (ATCC 43718, American Type Culture Collection, Manassas, VA) were grown in brain heart infusion broth (BHI, Becton Dickinson and Company, Cockeysville, MD) at 37º C in humidified air with 10% CO₂. Bacteria were harvested from broth cultures, washed, and opsonized for 30 minutes at 37º C in HBSS containing 20% pooled human serum (Sigma Chemical Company, St Louis, MO).

PMNs were loaded with 5 µg/ml clarithromycin for 15 minutes at 37º C. Control PMNs were subjected to a similar incubation without the agent. The phagocytic killing assay was initiated by adding opsonized, prewarmed *A. actinomycetemcomitans* suspensions to polypropylene microcentrifuge tubes containing one of the following: 20% human serum (in HBSS), 5 µg/ml clarithromycin in 20% human serum, control PMNs in 20% human serum, or clarithromycin-loaded PMNs in 20% human serum containing 5 µg/ml clarithromycin. Clarithromycin was maintained in the medium of the loaded PMNs throughout the assay. The incubation tubes were slowly rotated end-over-end for 90
minutes at 37° C to facilitate phagocytosis. At the beginning of the incubation and every 30 minutes thereafter, aliquots were removed and diluted in sterile water to lyse the PMNs. After further dilution, the samples were spread on BHI agar plates and incubated for 48 hours at 37° C in 10% CO₂. Surviving A. actinomycetemcomitans colonies were counted to assess bacterial killing. Assays were conducted at bacteria-to-PMN ratios of 30:1 and 100:1. Half-times for killing were calculated as previously described.⁴¹
CHAPTER 3:  
RESULTS

Undifferentiated HL-60 cells took up clarithromycin from the extracellular medium in a saturable manner, reaching steady state levels within 15 minutes. Neutrophil-like HL-60 granulocytes also took up clarithromycin, although the content per cell was slightly lower and they required less time to reach steady-state than with undifferentiated cells (Fig. 1).

Clarithromycin uptake by HL-60 granulocytes was strongly temperature dependent and was completely inhibited at temperatures below 4º C (Fig. 1 inset). The kinetics of uptake by HL-60 granulocytes yielded linear plots when analyzed by the Lineweaver-Burk method (Fig. 2, upper panel). This analysis suggested that HL-60 granulocytes possess a transporter that can internalize clarithromycin with an estimated $K_m$ of $250 \pm 7.67 \, \mu g/ml$ and a maximum velocity ($V_{max}$) of $473 \pm 24.9 \, ng/min/10^6$ cells.

Clarithromycin transport by human PMNs and undifferentiated HL-60 cells was also associated with linear Lineweaver-Burk plots (not shown).

In comparison to HL-60 granulocytes, PMNs transported clarithromycin with a similar $K_m$ value, and a $V_{max}$ value that was lower, but it was not significantly different (Table 1). With undifferentiated HL-60 cells, the $K_m$ and $V_{max}$ values for clarithromycin transport were significantly lower than with HL-60 granulocytes ($P < 0.05$).
HL-60 granulocytes and PMNs accumulated relatively high intracellular clarithromycin concentrations. When incubated at 37° C in medium containing 2 µg/ml clarithromycin, steady-state intracellular concentrations in HL-60 granulocytes were approximately 60 µg/ml (Table 2). Incubation with 10 µg/ml clarithromycin yielded an intracellular concentration of 278 µg/ml. Incubation of PMNs in the presence of 2 µg/ml clarithromycin yielded an intracellular concentration of 142.6 µg/ml, while incubation with 10 µg/ml was associated with an intracellular concentration of 575 µg/ml.

HL-60 granulocytes loaded with clarithromycin lost approximately 20% of their antibiotic content within 3 minutes after dilution of the clarithromycin concentrations in the extracellular medium (Fig. 2, lower panel). Under these conditions, approximately 35% of the clarithromycin content was lost within 20 minutes. After 60 minutes, granulocytes retained more than 50% of their original steady-state clarithromycin content.

To determine the effect of pH on granulocyte clarithromycin transport, the kinetics of transport were analyzed over the range of pH 6.3 to pH 8.3 (Fig. 3). Within this range, the efficiency of transport (as assessed by V_{max}/K_m ratio) exhibited a positive relationship with pH (P < 0.001, ANOVA). Transport activity was similar at pH 7.8 and 8.3, but there were significant differences in all other pairwise comparisons (P < 0.05, Holm-Sidak test).
When PMNs were incubated for 15 minutes in HBSS containing 5 µg/ml clarithromycin prior to their addition to a bacterial killing assay, the intracellular concentration of clarithromycin reached 290 µg/ml. The effect of clarithromycin on killing of *A. actinomycetemcomitans* was dependent on the ratio of bacteria to PMNs in the assay. At a bacteria-to-PMN ratio of 100:1 (Fig. 4, upper panel), clarithromycin-loaded PMNs killed significantly more bacteria than control PMNs at 30, 60 and 90 minutes (P < 0.05, paired t-test).

When the bacteria-to-PMN ratio was decreased to 30:1, control and clarithromycin-loaded PMNs rapidly killed *A. actinomycetemcomitans*, while clarithromycin alone produced a more gradual pattern of inhibition (Fig. 4, lower panel). PMNs loaded with clarithromycin killed a slightly greater number of bacteria than control PMNs, but the difference was significant only at 90 minutes (P < 0.05, paired t-test). The half-times for bacterial killing were consistent with these findings (Table 3). Using colony-forming units (CFU) additions that yielded bacteria-to-PMN ratios of 30:1, the half-times for killing by control or clarithromycin-loaded PMNs were similar, but both were significantly shorter than with clarithromycin alone (P < 0.05, ANOVA with Holm-Sidak post test). At a bacteria-to-PMN ratio of 100:1, the half-time for killing by clarithromycin-loaded PMNs was significantly shorter than that of control PMNs (P < 0.05, ANOVA with Holm-Sidak post test), which was, in turn, significantly shorter than for clarithromycin alone (P < 0.05).
CHAPTER 4:
DISCUSSION

The present study characterized the uptake of clarithromycin by human cells derived from three different stages of myeloid maturation. Both human PMNs and the HL60 cell line were used in this study. HL-60 cells are a human promyelocytic cell line used as a model for studying cell differentiation and proliferation. The HL-60 cell line can be differentiated into cells having many of the morphological features of mature granulocytes. By using undifferentiated (promyelocytic) and differentiated (granulocytic) forms of HL-60 cells, we can compare these cells to human PMNs, and observe whether transporter activity was present during the different stages of myeloid maturation.

Our results support the hypothesis that undifferentiated HL-60 cells, differentiated HL-60 cells, and peripheral blood PMNs all possess a similar active transport system for clarithromycin. The observed transport activity was saturable, concentrative, sensitive to changes in temperature, and consistent with Michaelis-Menten kinetics. HL-60 cells were tested under these conditions because they offer a simple, convenient model in which to observe transporter activity, as compared to PMNs, which must first undergo a lengthy purification process from extracted blood. Furthermore, since HL-60 cells are an
immature precursor to PMNs, one can surmise that they both may share a similar transport system, which is evident in the kinetic assay results. Clarithromycin transport by differentiated HL-60 cells and PMNs exhibited similar $K_m$ and $V_{max}$ values, whereas undifferentiated HL-60 cells exhibited a lower $K_m$ and $V_{max}$. The differences in mean $K_m$ and $V_{max}$ between cell lines were statistically significant. Despite different kinetic values, it can be evident from these results that all three stages of myeloid maturation possess a transport system, and that the efficiency of the transport system is similar at several different stages of myeloid maturation. This suggests that the transporter is not highly specialized and may play an important and conserved role in cell survival. Clarithromycin, as a weak base, could potentially be taken up by transport systems whose primary role is to scavenge nutrients and biosynthetic precursors$^{42, 43}$. Aside from clarithromycin, other studies have found that PMNs also actively transport ciprofloxacin, doxycycline, minocycline and clindamycin$^{27, 40, 44}$.

Although the observed affinity of the transporter is relatively low, our results suggest that transporters accumulate clarithromycin in PMNs that have migrated into periodontal pockets. In the presence of 2 $\mu$g/ml clarithromycin, a level that is easily attainable in the gingiva$^{37}$, the transporter accumulates intracellular levels of clarithromycin that exceed 140 $\mu$g/ml. This corresponds to a level that is approximately 40-fold higher than peak serum concentrations of clarithromycin and 70-fold higher than its MIC$_{90}$ for A. actinomycetemcomitans.$^{16}$ Clarithromycin transport also appears to be most efficient under neutral to mildly alkaline conditions. This finding is pertinent because the
environment in inflamed periodontal pockets is slightly alkaline in comparison to the nearly neutral pH of healthy gingival crevices.\textsuperscript{45,46} Thus, conditions for clarithromycin uptake by PMNs are more favorable in a periodontal pocket than a healthy gingival crevice. In a clinical setting, this characteristic could potentially enhance the effectiveness of clarithromycin for periodontally diseased sites in both basic and acidic conditions. In a basic periodontal pocket, PMNs could readily take up antibiotics and phagocytose the bacteria at the same time, leading to efficient bacterial killing within the neutrophil. PMN killing of bacteria could potentially be enhanced in an acidic environment as well. In a periodontal abscess, where low pH is often encountered, the uptake of clarithromycin may not be very efficient, but the acidic environment could potentially trigger PMN lysis, which would release the contents of the antibiotics into the periodontal abscess and promote disease resolution.

Transporters can also move substrates in a forward or reverse direction across the plasma membrane to maintain equilibrium between the substrate concentrations in the intracellular and extracellular environments. Reverse transport allowed clarithromycin-loaded HL-60 cells to release a portion of their intracellular clarithromycin pool when the concentration of clarithromycin was abruptly decreased in the extracellular medium (Fig. 2). It is reasonable to speculate that reverse transport could provide a mechanism by which PMNs that have migrated to inflammatory sites can help maintain effective levels of clarithromycin at these locations.
The results of the study further suggest that clarithromycin accumulation by PMNs is associated with enhanced killing of \emph{A. actinomycetemcomitans} strain Y4, but this is strongly dependent on the multiplicity of infection. At a bacteria-to-PMN ratio of 30:1, control and clarithromycin-loaded PMNs have nearly the same ability to kill most of the \emph{A. actinomycetemcomitans}, and both are more effective than clarithromycin alone. Under these conditions, the half-time for killing by control and clarithromycin-loaded PMNs is not significantly different. At a bacteria-to-PMN ratio of 100:1, however, it is more difficult for PMNs to kill all of the available bacteria. Under these conditions, clarithromycin-loaded PMNs killed significantly more bacteria than control PMNs at all time points, and the half-time for killing by clarithromycin-loaded PMNs was significantly shorter than with control PMNs. In a biofilm-infected periodontal pocket, pathogenic bacterial counts range from 10 million to 1 billion, and PMN counts range from $3.1 \times 10^4$ to $3.7 \times 10^4$ cells/site.\textsuperscript{47,48} Thus, there is at least a 300:1 bacteria-to-PMN ratio evident in a periodontally diseased site, which may overwhelm the host immune defense. This study suggests that the high concentrations of clarithromycin found in loaded PMNs will enhance the phagocytic killing of \emph{A. actinomycetemcomitans}, especially when bacteria-to-PMN ratios are high.

Previous studies have suggested that antibiotics other than clarithromycin may enhance PMN killing of \emph{A. actinomycetemcomitans}. PMNs loaded with ciprofloxacin killed significantly more bacteria and achieved shorter half times for killing than control PMNs alone when assayed at bacteria-to-PMN ratios of 30:1 and 90:1.\textsuperscript{49} At bacteria-to-PMN
ratios of 10:1 or lower, the effect of ciprofloxacin was not significant. With respect to clindamycin, one study concluded that it had no significant effect on bacterial killing by freshly isolated PMNs at bacteria-to-PMN ratios of up to 50:1. However, a later study of *A. actinomycetemcomitans* phagocytosis by crevicular PMNs from healthy individuals demonstrated that clindamycin elevated the percentage of phagocytosing PMNs and enhanced the intracellular killing of *P. gingivalis* and *A. actinomycetemcomitans*. It is unclear whether these results are due to the known ability for clindamycin to enhance Fc receptor expression, or whether clindamycin accumulation inside PMNs itself is the critical factor.

*A. actinomycetemcomitans* is a challenging therapeutic target because it can invade epithelial cells, multiply, and spread into adjacent tissues. Recent studies have found high levels of *A. actinomycetemcomitans* inside epithelial cells associated with periodontal pockets, and also in the gingival crevices and buccal mucosa of patients with and without chronic periodontitis. Furthermore, these intracellular pathogens can show signs of resistance to antibiotic therapy. In an *in vitro* study, oral epithelial cells infected with *A. actinomycetemcomitans* and *P. gingivalis* were incubated with up to 100-fold minimum inhibitory concentration (MIC) of several antibiotics (clindamycin, doxycyline, metronidazole, and moxifloxacin). Antibiotic treatment could not completely eradicate the intracellular bacteria, therefore viable pathogenic colonies remained. In a clinical study, aggressive periodontitis patients were treated with root planing, systemic antibiotics, and chlorhexidine rinses. Most bacterial species (*A. *
*Actinobacillus actinomycetemcomitans, P. intermedia, T. forsythus*) were significantly reduced but repopulated within 3-6 months. Furthermore, at post-treatment all species (including *P. gingivalis*) were detected intracellularly and the percentage of infected cells did not decrease after therapy. This study suggests that while scaling and root planing is effective in reducing bacterial populations of *A. actinomycetemcomitans* and *P. gingivalis* for 3-6 months, intracellular reservoirs of these bacteria may possibly contribute to refractory or progressive periodontitis. Thus, intracellular bacteria may prove to be resistant to treatment and contribute to disease progression in certain individuals.

Conventional antibiotic therapy for the elimination of *A. actinomycetemcomitans* involves a regimen of amoxicillin and metronidazole. Studies have found that this regimen reduced pocket depth and improved clinical attachment gain. Systemic antibiotics can suppress *A. actinomycetemcomitans* in the oral cavity up to 12 months or more. Yet a small subset of patients still tested positive for the bacteria and may develop progressive attachment loss despite mechanical debridement and antibiotic therapy. In a 5-year follow-up study of patients treated for aggressive periodontitis and maintained, 2-5% of these patients experienced recurrent episodes of loss of attachment. Up to now, there are very few studies on the ability of amoxicillin and metronidazole to penetrate tissues – however, the combination may not be effective in treating intracellular infections. Compared to other antibiotics, beta-lactam antibiotics demonstrate poor penetration into epithelial cells, and do not easily cross the plasma membrane. Furthermore, both beta-lactams and metronidazole have a limited ability
to concentrate within immune defense cells such as phagocytes.\textsuperscript{63-65} Because periodontal pathogens are not completely eliminated intracellularly and a small subset of individuals continue to break down despite treatment, intracellular penetration of an antibiotic could play an important role in periodontal therapy.

Human PMNs migrate from the bloodstream to a site of infection following signals from chemoattractants. Ideally, PMNs could serve as vehicles by which antibiotics could be delivered in high doses to the sites of infection. For this to occur, the PMNs must concentrate a high dose of the antibiotic, sustain appropriate doses of the antibiotic over time, and penetrate epithelial cells where intracellular bacteria reside. Several \textit{in vitro} studies have demonstrated that PMNs not only take up and concentrate macrolides, but that they can also transport these antibiotics toward a chemoattractant gradient, while delaying the release of their contents until bacterial sites were reached.\textsuperscript{28,66} Furthermore, the concentration of clarithromycin is low in serum but can be highly concentrated within gingival tissues.\textsuperscript{37} Our study has found that PMN transporters actively take up clarithromycin in large concentrations from the surrounding extracellular environment. Theoretically, if clarithromycin were used by an individual with active periodontal disease, the individual’s circulating PMNs may pick up low levels of clarithromycin in the circulation (serum), but then may rapidly concentrate the antibiotic as the PMNs travel through the connective tissue and into sites of inflammation in the gingiva. The concentrative characteristics of PMNs and the delayed release of antibiotic until the site
of infection is reached underscore the ability of PMNs to act as an effective vehicle for delivering high doses of antibiotics in infected sites.

There are a few limitations to the present study. First of all, only one strain of *A. actinomycetemcomitans* was tested. The Y4 strain was commercially obtained and was not freshly cultivated from the oral cavity. Although the Y4 strain is a highly leukotoxic strain, there is a potential for mutation over time during storage of the bacteria. Furthermore, although the killing half-times achieved for clarithromycin-loaded PMNs were significantly shorter compared to regular PMNs, the effect does not seem substantial. This contrasts with a similar study that tested ciprofloxacin for phagocytic killing of *A. actinomycetemcomitans* and found significant bacterial killing with as low as a 30:1 bacteria-to-PMN ratio. The enhanced killing of bacteria by ciprofloxacin compared to clarithromycin at corresponding ratios can be partly explained by the fact that ciprofloxacin is bacteriocidal, whereas clarithromycin is mainly a bacteriostatic agent. For bacteriocidal effects of *A. actinomycetemcomitans* to be noted with clarithromycin, more observation time than the 30-90 minute intervals used in the present study is required. Thus, the limited time in which clarithromycin-loaded PMNs was incubated with the bacteria could partly explain why the phagocytic killing of these PMNs seems modest in comparison to unloaded PMNs. Future studies could introduce the antibiotic clinically to patients with localized aggressive periodontitis, and the effects of intracellular killing could be determined by obtaining both epithelial cells and subgingival samples of *A. actinomycetemcomitans* prior to and post antibiotic therapy.
Clarithromycin was approved for use by the FDA in October 1991, and is traditionally used for the treatment of pneumonia, sinusitis, bronchitis, and *H. pylori*-associated duodenal ulcers. Clarithromycin is generally administered at an oral dose of 500 mg twice a day, with a peak serum level of 2-3 µg/ml and an elimination half-life is 5-7 hours. While clarithromycin has not been widely used in periodontal therapy, it is effective against *A. actinomycetemcomitans* and other periodontal pathogens, can reach bacteria that have invaded oral epithelium, and can yield higher levels in inflamed gingiva than at healthy sites. There are few side effects associated with clarithromycin, the most common being diarrhea (3%), nausea/vomiting (3%/6%), dysgeusia (3%), dyspepsia (2%), abdominal pain (2%), and headache (2 %). These symptoms are relatively mild and rarely require discontinuation of drug therapy. Overall, clarithromycin seems to have promising potential for periodontal applications.

In conclusion, both HL-60 cells and mature human PMNs possess an active transporter that takes up clarithromycin and is concentrative. The transporter appears to be expressed in the early stages of myeloid maturation. The transporter also appears to function most efficiently in an alkaline environment, which is characteristic of periodontally diseased sites. Furthermore, clarithromycin-loaded PMNs exhibit an enhanced ability to kill *A. actinomycetemcomitans Y4*, and its effects are most pronounced when the bacteria-to-PMN ratio is high. In combination with results from previous studies, the current findings suggest that clarithromycin could be a viable
therapeutic agent for treating periodontitis cases associated with infections by *A. actinomycetemcomitans*.
REFERENCES


Table 1: Kinetics of Clarithromycin Transport by Myeloid Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>$K_m$ (µg/ml)</th>
<th>$V_{max}$ (ng/min/10^6)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undifferentiated</td>
<td>149 ± 12.7$^#&amp;$</td>
<td>202 ± 22.5$^\dagger$</td>
<td>1.36 ± 0.09$^\ddagger$</td>
</tr>
<tr>
<td>HL-60 Cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL-60 Granulocytes</td>
<td>250 ± 7.67$^#$</td>
<td>473 ± 24.9$^\dagger$</td>
<td>1.89 ± 0.07$^\ddagger‼$</td>
</tr>
<tr>
<td>Human PMNs</td>
<td>249 ± 7.63$^&amp;$</td>
<td>315 ± 28.3</td>
<td>1.26 ± 0.08$^\ddagger‼$</td>
</tr>
</tbody>
</table>

Resting cells were suspended in HBSS and incubated with several different concentrations of $[^3]$H-clarithromycin for 3 minutes. Transport assays were terminated by pelleting the cells through an oil cushion. $K_m$ and $V_{max}$ values were determined by Lineweaver-Burk analysis. Results are expressed as mean ± SEM. Differences in mean $K_m$ and $V_{max}$ values were greater than would be expected by chance ($P < 0.001$, ANOVA). Identical superscripts within a column ($^\#$, $^\&$, $^\dagger$, $^\ddagger$, or $^\ddagger‼$) denote comparisons that were significantly different ($P < 0.05$, Holm-Sidak test).
Table 2: Intracellular Accumulation of Clarithromycin (µg/ml) by Resting HL-60 Granulocytes and Human PMNs

<table>
<thead>
<tr>
<th>Extracellular Concentration (µg/ml)</th>
<th>Cellular/Extracellular Concentration Ratio, HL-60 Cells</th>
<th>Cellular/Extracellular Concentration Ratio, PMNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>30.0 ± 1.5</td>
<td>71.3 ± 2.6</td>
</tr>
<tr>
<td>5</td>
<td>30.8 ± 2.1</td>
<td>58.0 ± 1.3</td>
</tr>
<tr>
<td>10</td>
<td>27.8 ± 2.1</td>
<td>57.5 ± 2.9</td>
</tr>
</tbody>
</table>

Results are expressed as the mean of 6 determinations ± SEM.
Table 3: Half-times for Killing of *A. actinomycetemcomitans* Strain Y4 by PMNs and Clarithromycin (in minutes)

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>9.0 x 10⁷ A. a. (30:1)</th>
<th>3.0 x 10⁸ A. a. (100:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. a. and PMNs</td>
<td>20.6 ± 2.16#</td>
<td>71.4 ± 6.8*</td>
</tr>
<tr>
<td>A. a. and clarithromycin</td>
<td>47.7 ± 5.2#*</td>
<td>104.0 ± 11.0*</td>
</tr>
<tr>
<td>A. a. and PMNs + clarithromycin</td>
<td>19.7 ± 0.9*</td>
<td>42.7 ± 3.6*</td>
</tr>
</tbody>
</table>

In these experiments, the indicated number of *A. a.* colony forming units were added to tubes containing PMNs (3 x 10⁶), clarithromycin (5µg/ml), or PMNs loaded with clarithromycin (5µg/ml). Bacterial survival was assayed at 30 min as previously described. Half-times were calculated as described by Van Furth et al.²³ and are presented as the mean ± SEM of four experiments. Within each data column, significant treatment effects were observed (P < 0.001, repeated measures ANOVA). Within columns, significant differences between pairs of incubation conditions are denoted by identical superscripts # and * (P < 0.05, Holm-Sidak Test).
APPENDIX B: FIGURES
Fig. 1. Time course of clarithromycin accumulation by undifferentiated HL-60 cells and HL-60 granulocytes. Inset: Temperature dependence of HL-60 granulocyte clarithromycin transport (r = 0.975). In the presence of 10 µg/ml clarithromycin, the transport activity observed in the 37°C control was 14.8 ± 1.43 ng/min/10^6 cells.
**Fig. 2.** Characterization of clarithromycin uptake by and efflux from HL-60 granulocytes.

*Upper panel:* Regression line from Lineweaver-Burk analysis of clarithromycin transport kinetics ($r = 0.992$). *Lower panel:* Efflux of clarithromycin from loaded cells. Cells were loaded to a steady-state intracellular concentration by incubation for 20 minutes at 37° C in HBSS containing 10 µg/ml clarithromycin. Efflux was triggered by abruptly diluting extracellular clarithromycin 1:20 with 37° C HBSS and monitored by the decrease in cell-associated radioactivity.
**Fig. 3.** The pH dependence of clarithromycin transport by resting HL-60 granulocytes.

Cells were suspended in HBSS adjusted to the indicated pH, and clarithromycin transport was assayed at 37°C. Data are presented as the mean of four experiments ± SEM. Transport was significantly influenced by pH (P < 0.001, ANOVA). At pH 7.8 and 8.3, the observed transport activity was similar. However, there were significant differences in all other pairwise comparisons (P < 0.05, Holm-Sidak test).
**Fig. 4.** The effect of clarithromycin on killing of *A. a.* by PMNs. Data are presented as the mean of at least four experiments ± SEM. The experiments summarized in the upper panel were conducted with a bacteria to PMN ratio of 100. Under these conditions, PMNs loaded with clarithromycin killed significantly more bacteria than control PMNs at 30, 60 and 90 minutes (P < 0.05, paired T test). The experiments portrayed in the lower panel were conducted with 30 bacteria per PMN. In these experiments, clarithromycin loaded PMNs killed significantly more bacteria than control PMNs at the 90 minute time point (P < 0.05, paired T test).