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Clarithromycin Accumulation by Phagocytes and Its Effect on Killing of *Aggregatibacter actinomycetemcomitans*

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Abstract

Background—Clarithromycin inhibits several periodontal pathogens and is concentrated inside gingival fibroblasts and epithelial cells by an active transporter. We hypothesized that polymorphonuclear leukocytes (PMNs) and less mature myeloid cells possess a similar transporter for clarithromycin. It is feasible that clarithromycin accumulation inside PMNs could enhance their ability to kill *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 examined with PMNs loaded by incubation with clarithromycin (5 µg/ml). Opsonized bacteria were incubated at 37°C with control and clarithromycin-loaded PMNs.

Results—Mature human PMNs, HL-60 cells differentiated into granulocytes and undifferentiated HL-60 cells all took up clarithromycin in a saturable manner. The kinetics of uptake by all yielded linear Lineweaver-Burk plots. HL-60 granulocytes transported clarithromycin with a K_m of approximately 250 µg/ml and a V_{max} of 473 ng/min/10⁶ cells, which were not significantly different from the values obtained with PMNs. At steady state, clarithromycin levels inside HL-60 granulocytes and PMNs were 28- to 71-fold higher than extracellular levels. Clarithromycin-loaded PMNs killed significantly more *A. actinomycetemcomitans* and achieved shorter half times for killing than control PMNs when assayed at a bacteria-to-PMN ratio of 100:1 ($P < 0.04$). 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 help PMNs cope with an overwhelming infection by *A. actinomycetemcomitans*.

Keywords

macrolides; neutrophils; granulocyte precursor cells; periodontitis

INTRODUCTION

Periodontitis, the major cause of adult tooth loss, is the result of infection by specific bacteria. Most species of subgingival bacteria associated with periodontitis can be

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Summary: Polymorphonuclear leukocytes possess a transport system for accumulating clarithromycin, which could help them cope with an overwhelming infection by *A. actinomycetemcomitans*.

eliminated by mechanical debridement (root planing). However, *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 early-onset periodontitis and recurrent adult 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 possess virulence factors that frustrate the host response and thwart conventional efforts to eliminate them. In particular, both are capable of invading the epithelial lining of the periodontal pocket.⁴ *A. actinomycetemcomitans* can invade epithelial cells and pass into the underlying connective tissue,⁵ while *P. gingivalis* can invade epithelial cells and linger inside.⁶ *A. actinomycetemcomitans* also resists phagocytic killing^{7, 8} and produces a leukotoxin that kills PMNs, the first line of defense against bacteria in the gingival crevice.^{9, 10} While only a few bacterial pathogens are capable of invading and surviving within epithelial cells and PMNs, these infections are among the most difficult to eradicate. Persistent infections by *A. actinomycetemcomitans* or *P. gingivalis* are frequently associated with continued periodontal breakdown.¹¹

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.¹² Beta-lactam antibiotics are poorly suited for treating intracellular infections because they do not penetrate mammalian cells. Our previous work has suggested that clarithromycin is a good candidate for treating invasive pathogens because it is accumulated inside gingival fibroblasts and epithelial cells.¹³ Clarithromycin is a macrolide that inhibits protein synthesis by binding to the 50S subunit of the bacterial ribosome.¹⁴ This agent reaches higher levels in gingiva than in serum and yields higher levels in inflamed gingiva than in healthy gingiva.¹⁵ Azithromycin and clarithromycin are effective against *A. actinomycetemcomitans* and *P. gingivalis*.¹⁶⁻¹⁹ Both exhibit good activity against spirochetes, *Eikenella corrodens*, *Prevotella* species, fusobacteria, and other anaerobic and facultative oral pathogens.¹⁹⁻²¹ We hypothesized that PMNs and HL-60 cells (a human promyelocytic leukemia cell line that can be induced into immature PMN-like cells) possess similar active transport systems that facilitate intracellular accumulation of clarithromycin. A transporter of this type could benefit the host by enhancing the phagocytic killing of *A. actinomycetemcomitans*. These two hypotheses were tested in the present laboratory study.

MATERIALS AND METHODS

HL-60 cell culture

Human promyelocytic leukemia (HL-60) cells* were cultured at 37°C in 5% CO₂ in RPMI 1640 medium[†] supplemented with 15% heat-inactivated fetal bovine serum[‡]. Immature HL-60 cells were differentiated into granulocytic HL-60 cells by culturing with 1.3% DMSO for seven days.²²

PMN isolation

Blood was obtained by venipuncture from volunteers who were in good general health with no radiographic signs of periodontal bone loss and no pocket probing depths greater than 4 mm. The donors were recruited and consented under an IRB-approved protocol and had not taken antibiotics or anti-inflammatory agents in the 30 day period prior to the blood draw.

* American Type Culture Collection, Manassas, VA, USA

[†] Invitrogen Corporation, Carlsbad, CA, USA

[‡] Sigma Chemical Company, St Louis, MO, USA

PMNs were isolated from citrated whole blood, using Ficoll/Hypaque density gradient centrifugation and dextran sedimentation.²³ 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 cyto-spin preparations stained with Wright-Giemsa) and >99% viable (based on trypan blue exclusion).²⁴ PMNs were suspended in Hank's balanced salt solution (HBSS)[§] at densities of 5×10^6 cells/ml and 10^7 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.¹³ PMN suspensions were warmed to 37°C prior to incubation with [³H]-clarithromycin[¶] at concentrations of 10 µg/ml for time course assays and 8-50 µg/ml in kinetic assays to determine the Michaelis constant (K_m) and maximal velocity of transport (V_{max}). After the indicated interval (3 minutes for kinetic assays and 2-60 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 $15,000 \times g$ in a microcentrifuge.²⁵ 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 ml of water for 12 hrs. Lineweaver-Burk analysis was used to determine K_m and V_{max} .

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 37°C with [³H]-water[#] (5 µCi/ml). To correct for the extracellular water trapped in the pellet, cells were equilibrated with [¹⁴C]-inulin^{**} (2 µCi/ml) 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)^{††} were grown in brain heart infusion broth (BHI)^{‡‡} at 37°C in humidified air with 10% CO₂. Bacteria were harvested from broth cultures, washed, and opsonized for 30 minutes at 37°C in HBSS containing 20% pooled human serum^{§§}.

[§]Invitrogen Corporation, Carlsbad, CA, USA

[¶]American Radiolabeled Chemicals, St Louis, MO, USA

[#]NEN Life Science Products, Boston, MA, USA

^{**}NEN Life Science Products, Boston, MA, USA

^{††}American Type Culture Collection, Manassas, VA, USA

^{‡‡}Becton, Dickinson and Company, Sparks, MD, USA

^{§§}Sigma Chemical Company, St Louis, MO, USA

PMNs were loaded with 5 µg/ml clarithromycin for 15 minutes at 37°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.²⁶

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 their clarithromycin 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 4°C (Fig. 1 inset). The kinetics of uptake by HL-60 granulocytes yielded linear plots when analyzed by the Lineweaver-Burk method (Fig. 2A). This analysis suggested that HL-60 granulocytes possess a transporter that can internalize clarithromycin with an estimated K_m of 250 ± 7.7 µg/ml and a maximum velocity (V_{max}) of 473 ± 24.9 ng/min/10⁶ cells. Kinetic analysis of clarithromycin transport by human PMNs and undifferentiated HL-60 cells yielded linear Lineweaver-Burk plots (not shown). In comparison to HL-60 granulocytes, PMNs transported clarithromycin with a similar K_m and a V_{max} that was lower, but 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, the steady-state concentration inside HL-60 granulocytes was 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 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 a 1:20 dilution of the clarithromycin concentration in the extracellular medium (Fig. 2B). 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

^{¶¶}Becton, Dickinson and Company, Sparks, MD, USA

pH ($P < 0.001$, ANOVA). Transport activity was similar at pH 7.8 and 8.3, but there were significant differences in all other pair-wise comparisons ($P < 0.05$, Holm-Sidak test).

When PMNs were incubated for 15 minutes in HBSS containing 5 $\mu\text{g/ml}$ clarithromycin prior to their addition to a bacterial killing assay, the intracellular clarithromycin concentration reached 290 $\mu\text{g/ml}$. 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. 4A), 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 both killed *A. actinomycetemcomitans* rapidly, while clarithromycin alone produced a more gradual pattern of inhibition (Fig. 4B). 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 CFU additions that corresponded to a bacteria-to-PMN ratio 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 also significantly shorter than for clarithromycin alone ($P < 0.05$).

DISCUSSION

The present study characterized the uptake of clarithromycin by human cells derived from three different stages of myeloid maturation. HL-60 cells are a human promyelocytic cell line used as a model for studying cell differentiation, and can be differentiated into cells that possess some of the functional features of mature granulocytes.^{22, 27} Undifferentiated (promyelocytic) and differentiated (granulocytic) forms of HL-60 cells were compared to purified human PMNs to determine whether clarithromycin transport activity is similar at different stages of myeloid maturation. Our results support the hypothesis that undifferentiated HL-60 cells, differentiated HL-60 cells, and mature peripheral blood PMNs 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. 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} . However, the efficiency of transport (as assessed by V_{max}/K_m) was somewhat similar at all three stages of maturation. The expression of transport activity by myeloid cells from different stages of maturation suggests that the transporter is not highly specialized and may play an important and conserved role in cell survival (i.e. scavenging nutrients and biosynthetic precursors).^{28, 29} In addition to clarithromycin, PMNs also actively transport ciprofloxacin, doxycycline, minocycline and clindamycin.^{25, 30, 31}

Although the observed affinity of the transporter was relatively low, its other characteristics suggest that it can actively accumulate clarithromycin inside PMNs that have infiltrated gingival connective tissue and migrated into periodontal pockets. In the presence of 2 $\mu\text{g/ml}$ clarithromycin, a concentration that is clinically attainable in the gingiva,¹⁵ the transporter accumulated intracellular levels that exceeded 140 $\mu\text{g/ml}$. 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 appeared to be most efficient under neutral to mildly alkaline conditions. This finding is pertinent because the environment in inflamed periodontal pockets is slightly alkaline, while the pH

of healthy gingival crevices is nearly neutral.^{32, 33} Thus, conditions for PMN clarithromycin uptake are more favorable in a periodontal pocket than in a healthy gingival crevice.

Transporters can 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 sustain effective levels of clarithromycin at these locations.

While the results support the hypothesis that clarithromycin accumulation by PMNs is associated with enhanced killing of *A. actinomycetemcomitans*, the effects of clarithromycin were dependent on the multiplicity of infection. At a bacteria-to-PMN ratio of 30:1, control and clarithromycin-loaded PMNs had nearly the same ability to kill most of the *A. actinomycetemcomitans*, and both were more effective than clarithromycin alone. In these experiments, the half-times for killing by control and clarithromycin-loaded PMNs were not significantly different. At a bacteria-to-PMN ratio of 100:1, it was more difficult for PMNs to kill all of the 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. Previous studies of patients with localized aggressive periodontitis have recovered approximately 2.8×10^4 viable PMNs from periodontally involved sites.^{34, 35} These deep sites typically harbor $>10^7$ bacteria, although counts of $>1.2 \times 10^8$ have been recovered from individual sites.³⁶ Assuming that *A. actinomycetemcomitans* constitutes approximately 3.9% of the total bacterial count at these sites,³⁷ the ratio of *A. actinomycetemcomitans*-to-PMN is typically around 14:1, but can exceed 167:1 at some individual sites. Thus, the finding that clarithromycin can enhance bacterial killing at an *A. actinomycetemcomitans*-to-PMN ratio of 100:1 is clinically relevant.

Previous studies have suggested that other antibiotics may enhance PMN killing of *A. actinomycetemcomitans*. 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 50:1³⁸. However, a more recent study of *A. actinomycetemcomitans* phagocytosis by crevicular PMNs from healthy subjects demonstrated that clindamycin elevates the percentage of phagocytosing PMNs and enhances the intracellular killing of *P. gingivalis* and *A. actinomycetemcomitans*. It is unclear whether these effects are related to the known ability of clindamycin to enhance PMN Fc receptor expression or whether clindamycin accumulation inside PMNs is a critical factor.³⁹ With regard to ciprofloxacin, a previous study demonstrated that PMNs loaded with ciprofloxacin killed significantly more *A. actinomycetemcomitans* strain Y4 and achieved shorter half times for killing than control PMNs alone when assayed at bacteria-to-PMN ratios of 30:1 and 90:1.²⁴ At bacteria-to-PMN ratios of 10:1 or lower, the effect of ciprofloxacin was not significant. Under comparable experimental conditions, ciprofloxacin produced a more dramatic enhancement of bacterial killing by PMNs than we observed with clarithromycin in the present study. While both agents are accumulated by PMNs, there are differences in their antimicrobial activity. Ciprofloxacin produces potent bacteriocidal effects against *A. actinomycetemcomitans*, whereas clarithromycin is bacteriostatic. It is likely that these differences in activity against *A. actinomycetemcomitans* were reinforced by the relatively short (30 to 90 minute) duration of the bacterial killing assay.

To put the findings and limitations of the current study into context, the bacterial killing assays were conducted with only one strain of *A. actinomycetemcomitans*. The Y4 strain belongs to the serotype *b* classification of *A. actinomycetemcomitans*, which is highly leukotoxic and is the most commonly isolated serotype of aggressive periodontitis patients.⁴⁰ The Y4 strain used in this study was commercially obtained and was not freshly cultivated from the oral cavity. Laboratory strains of *A. actinomycetemcomitans* can be more susceptible to killing by PMNs than fresh isolates.⁸ Thus, clarithromycin could have a greater impact on the killing of *A. actinomycetemcomitans* in vivo than it did in this in vitro study.

A. actinomycetemcomitans is a challenging therapeutic target that invades epithelial cells, where they can multiply and spread into adjacent tissues. Recent studies have found high levels of *A. actinomycetemcomitans* inside epithelial cells associated with periodontal pockets, gingival crevices and buccal mucosa in healthy individuals and patients with chronic periodontitis.^{41, 42} *A. actinomycetemcomitans* releases a leukotoxin that forms pores in the plasma membrane of PMNs, causing osmotic instability and cell death.^{43, 44} It is also capable of inhibiting PMN chemotaxis and impairing production of antibacterial agents such as hydrogen peroxide.^{45, 46} For these reasons, it is difficult for PMNs to eradicate this pathogen. The present study demonstrates that HL-60 cells and mature human PMNs both possess an active transporter that takes up clarithromycin. It is well suited for accumulating high intracellular concentrations of this agent under conditions found in inflamed gingiva. PMNs loaded with clarithromycin appear to have an enhanced ability to cope with an overwhelming infection by the leukotoxic *A. actinomycetemcomitans* strain Y4, although this benefit is less pronounced at lower multiplicities of infection. While clarithromycin has not been widely used in periodontal therapy, it is effective against *A. actinomycetemcomitans* and several other periodontal pathogens, can reach bacteria that have invaded oral epithelium¹¹ and yields higher local tissue levels in inflamed gingiva than at healthy sites.¹³ In combination with results from previous studies, the current findings suggest that clarithromycin could be useful for treating periodontitis associated with infections by *A. actinomycetemcomitans*.

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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 $\mu\text{g/ml}$ 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. **A:** Regression line from Lineweaver-Burk analysis of clarithromycin transport kinetics ($r = 0.992$). **B:** 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 $\pm$ 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. actinomycetemcomitans* by PMNs. Data are presented as the mean of at least four experiments $\pm$ SEM. **A:** The experiments portrayed in this panel were conducted with a ratio of 100:1 bacteria-to-PMN. 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). **B:** The experiments portrayed in this panel were conducted at a bacteria-to-PMN ratio of 30:1. 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).

Table 1

Kinetics of Clarithromycin Transport by Myeloid Cells*

Cell Type	K_m ($\mu\text{g/ml}$)	$V_{\max}$ (ng/min/ 10^6)	$V_{\max}/K_m$
Undifferentiated HL-60 Cells	$149 \pm 12.7^{\# \&}$	$202 \pm 22.5^{\dagger}$	$1.36 \pm 0.09^{\ddagger}$
HL-60 Granulocytes	$250 \pm 7.67^{\#}$	$473 \pm 24.9^{\dagger}$	$1.89 \pm 0.07^{\ddagger!!}$
Human PMNs	$249 \pm 7.63^{\&}$	315 ± 28.3	$1.26 \pm 0.08^{!!}$

* Resting cells were suspended in HBSS and incubated with several different concentrations of [^3H]-clarithromycin for 3 minutes. Transport assays were terminated by centrifuging the cells through an oil cushion. K_m and $V_{\max}$ values were determined by Lineweaver-Burk analysis. Results are expressed as mean $\pm$ 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 denote comparisons that were significantly different ($P < 0.05$, Holm-Sidak test).

Table 2

Intracellular Accumulation of Clarithromycin by Resting HL-60 Granulocytes and Human PMNs

Extracellular Concentration ($\mu\text{g/ml}$)	Cellular/Extracellular Concentration Ratio, HL-60 Cells	Cellular/Extracellular Concentration Ratio, PMNs
2	30.0 ± 1.5	71.3 ± 2.6
5	30.8 ± 2.1	58.0 ± 1.3
10	27.8 ± 2.1	57.5 ± 2.9

Results are expressed as the mean of 6 determinations $\pm$ SEM.

Table 3

Half-times for Killing of *Aggregatibacter actinomycetemcomitans* Strain Y4 by PMNs and Clarithromycin (in minutes)

Incubation conditions	9.0×10^7 <i>A. a.</i>	3.0×10^8 <i>A. a.</i>
<i>A. a.</i> and PMNs	$20.6 \pm 2.16^{\#}$	$71.4 \pm 6.8^*$
<i>A. a.</i> and clarithromycin	$47.7 \pm 5.2^{\#}$	$104.0 \pm 11.0^*$
<i>A. a.</i> and PMNs + clarithromycin	$19.7 \pm 0.9^*$	$42.7 \pm 3.6^*$

In these experiments, the indicated number of *A. a.* colony forming units were added to tubes containing PMNs (3×10^6), clarithromycin (5 μ g/ml), or PMNs loaded with clarithromycin (5 μ g/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 $\pm$ 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 ($P < 0.05$, Holm-Sidak Test).