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NOTES

Stimulation with Cytokines Enhances Penetration of Azithromycin into Human Macrophages

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An effective intracellular concentration of an antimicrobial agent is essential for therapy of infections caused by organisms of the *Mycobacterium avium* complex. We previously reported on the effect of the combination of azithromycin and tumor necrosis factor (TNF) against *M. avium* infection in macrophages. We now report that stimulation of macrophages either with recombinant human gamma interferon (IFN-γ, 10^2 U/ml) or with recombinant human TNF-α (10^2 U/ml) resulted in an increase in the intracellular concentration of azithromycin by approximately 200% within 3 h, compared with the concentration in unstimulated macrophages. Infection of macrophages with *M. avium* complex led to a decrease in the uptake of [^{14}C]azithromycin by infected cells, compared with that by uninfected controls. Stimulation of infected macrophages with recombinant IFN-γ or TNF-α overcame the inhibitory effect associated with infection. These results suggest that the increased bactericidal activity of the TNF-α-azithromycin or IFN-γ-azithromycin combination against *M. avium* is related to enhanced uptake of the antibiotic by the stimulated phagocyte.

Azithromycin is a new acid-stable macrolide in the class of azalides (16). It has been shown to have pharmacokinetic properties different from those of erythromycin, achieving rapid and sustained high concentrations in tissue after oral dosing (7, 17).

Bacteria of the *Mycobacterium avium* complex are facultative intracellular organisms which invade and survive within macrophages. In disseminated infection, a larger number of microorganisms is found within macrophages of the reticuloendothelial system (1). *M. avium* is a frequently isolated organism which causes pulmonary infections in patients with chronic lung diseases (6, 19). More recently, infections caused by organisms of the *M. avium* complex have been documented in a large percentage of patients with AIDS (10, 18). In AIDS patients, *M. avium* is associated with bacteremia and disseminated disease (15, 20). It is important to note that *M. avium* is commonly resistant to most antituberculosis antimicrobial agents (11, 13). A previous in vitro and in vivo study reported by our laboratories demonstrated the efficacy of azithromycin against bacteria of the *M. avium* complex (12).

Although azithromycin was shown to be active against intracellular *M. avium*, its bacteriostatic-bactericidal activities were significantly enhanced when macrophages were simultaneously stimulated with recombinant tumor necrosis factor alpha (TNF-α) (4). Such results suggested that exposure of infected macrophages to TNF-α induced intracellular bactericidal-bacteriostatic mechanisms that add to or synergize with azithromycin, since TNF-α by itself has no antimicrobial activity against *M. avium* (2). Such an effect might be the result of induction of the synthesis of a macrophage bactericidal protein(s). An alternative explanation is that stimulation by TNF-α may increase azithromycin uptake by macrophages, with a consequently greater intracellular concentration of the antibiotic.

To investigate the latter possibility we examined the effects of macrophage activation by cytokines on the intracellular concentration of [^{14}C]azithromycin.

[^{14}C]azithromycin was kindly provided by Pfizer Laboratories, Inc., New York, N.Y., and dissolved in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) to yield the desired concentration. The radiolabeled drug was determined to have a radiopurity of >97% and a specific activity of 15.0 mCi/mmol. Recombinant human TNF-α and human gamma interferon (IFN-γ) were gifts from Genentech, Inc., San Francisco, Calif., and had specific activities of 2×10^7 and 3×10^7 U/mg of protein, respectively. The recombinant cytokines were prepared in small aliquots and frozen at −70°C. Desired dilutions were prepared immediately before the experiments. Cytokine preparations were tested for endotoxin contamination by using the *Limulus* amebocyte lysate assay (Sigma, St. Louis, Mo.), and no contamination with endotoxin was observed. To further rule out the possible influence of endotoxin on the results, cytokine preparations were boiled and used as controls in the experiments. Boiling inactivates proteins but not endotoxin. Boiling cytokine preparations did not have any effect on [^{14}C]azithromycin uptake by macrophages.

Monocyte-derived macrophages were obtained from heparinized blood samples of healthy donors (three donors were used for all experiments) and processed as previously described (2, 4). Adherent monolayers containing 10^6 macrophages were maintained in culture with 1.5 ml of RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine, for 7 days. Although about 70 to 80% of the azithromycin in serum is bound to protein, only free drug is incorporated by phagocytic cells. There-

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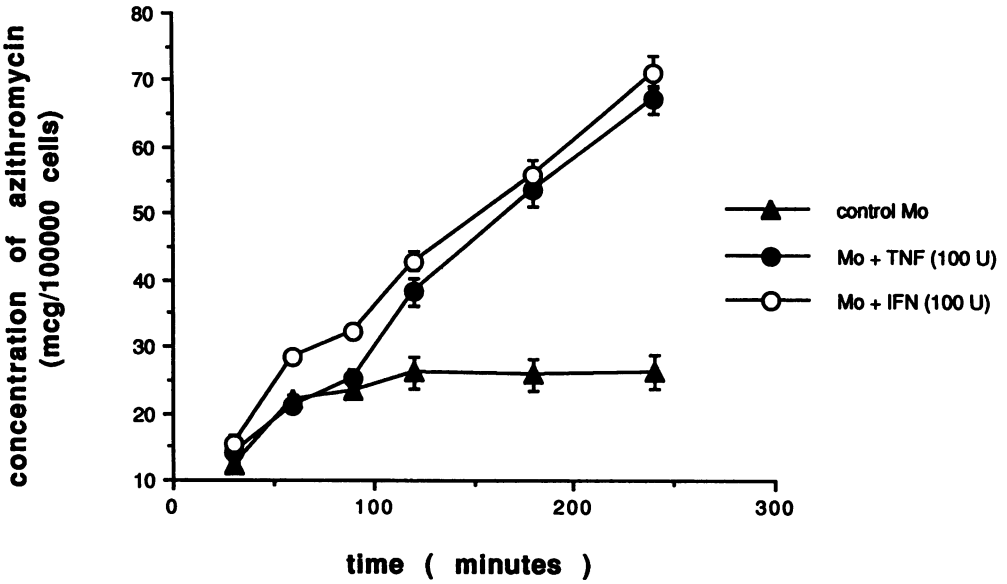

FIG. 1. Penetration of [^{14}C]azithromycin into human macrophages (Mo).

fore, the concentration of FBS in the medium should not interfere with the results. However, in order to examine the effect of the concentration in serum on azithromycin uptake, we carried out experiments using 5% FBS. The results did not differ from those obtained using 10% FBS (data not shown).

M. avium 101, serotype 1, was isolated from a patient with AIDS. The bacteria were grown in Middlebrook agar 7H10 medium (Difco Laboratories, Detroit, Mich.) for 10 days at 37°C. For the experiments, pure transparent colonies were obtained, resuspended in Hanks balanced salt solution, and washed twice. After resuspension in phosphate buffer, the suspension was adjusted for 10^7 bacteria per ml by using the McFarland standard. The bacterial suspension was vortex agitated for 2 min to disperse clumping, and a sample from the suspension was cultured onto 7H10 agar plates for quantitation of bacteria.

Monolayers containing approximately 5×10^5 cells were infected with 10^4 to 10^8 bacteria. After 4 h, monolayers were washed three times with Hanks balanced salt solution to remove extracellular bacteria. The number of viable intracellular bacteria was determined by lysing monolayers and plating the lysate onto 7H10 agar plates as previously described (2, 4).

Macrophage monolayers containing approximately 5×10^5 cells were incubated in RPMI 1640 supplemented with 10% FBS and 100 μg of radiolabeled antibiotic per 1.5 ml. At each time point, the monolayers were washed five times to remove extracellular antibiotic. The cells were then lysed with 1% Triton X-100 (Sigma), and the amount of radioactivity in the lysate was determined with an LKB beta counter. To determine the amount of the radioactive background (nonspecific binding to the plastic) in each experiment, [^{14}C]azithromycin was added to an empty well and removed by washing, and the well was treated with 1 ml of 1% Triton X-100. The radioactivity in the preparation was subtracted from this background. The concentration of antibiotic within macrophages was calculated from a standard curve.

Since some of the macrophage monolayers were activated with recombinant cytokines, we did not take into consideration the concentration of protein in the cell lysate. Cytokine-stimulated cells produce much larger quantities of protein than unstimulated macrophages. Therefore, our results are expressed as the amount of intracellular antibiotic detected, factored for the number of macrophages. In order to determine whether treatment with TNF-α or IFN-γ induced macrophage detachment from the plastic, the number of adherent cells was followed carefully during the experiment by counting the number of cells in the supernatant and the number of cells attached to the plastic, as previously reported (2). No differential detachment was observed between control and experimental groups when the reported concentrations of TNF-α or IFN-γ were used (2).

Incubation of human monocyte-derived macrophages with azithromycin was associated with a rapid increase in intracellular concentration of the antibiotic. After 30 min of incubation, azithromycin reached an intracellular concentration of 12.2 ± 2.3 μg/10^5 cells. This concentration increased to 26.2 ± 4.4 μg/10^5 cells after 4 h. Intracellular azithromycin concentrations plateaued after 2 h of incubation (Fig. 1). The intracellular concentration of azithromycin increased by 113.04% during the first 2 h of incubation; the increase in intracellular concentration by 4 h was 113.08%.

Our previous experiments have indicated an enhanced bactericidal effect against intracellular organisms of the *M. avium* complex when TNF-α is combined with azithromycin (4). We then studied the effect of stimulation of macrophages by recombinant cytokines such as TNF-α and IFN-γ on uptake of azithromycin by the phagocytic cell. For these experiments, macrophage monolayers were treated with TNF-α (10^3 U/ml) or IFN-γ (10^3 U/ml) for 18 h at 37°C. Cytokines were then removed by washing, and monolayers were subsequently incubated with [^{14}C]azithromycin at 37°C. As shown in Fig. 1, incubation of macrophages with 10^2 U of TNF-α per ml for 18 h was associated with increased uptake of azithromycin, compared with that of the control cells. Between 30 min and 2 h there was a 170.5% increase in the uptake of azithromycin by macrophages, while between 30 min and 4 h there was a 375.5% increase in

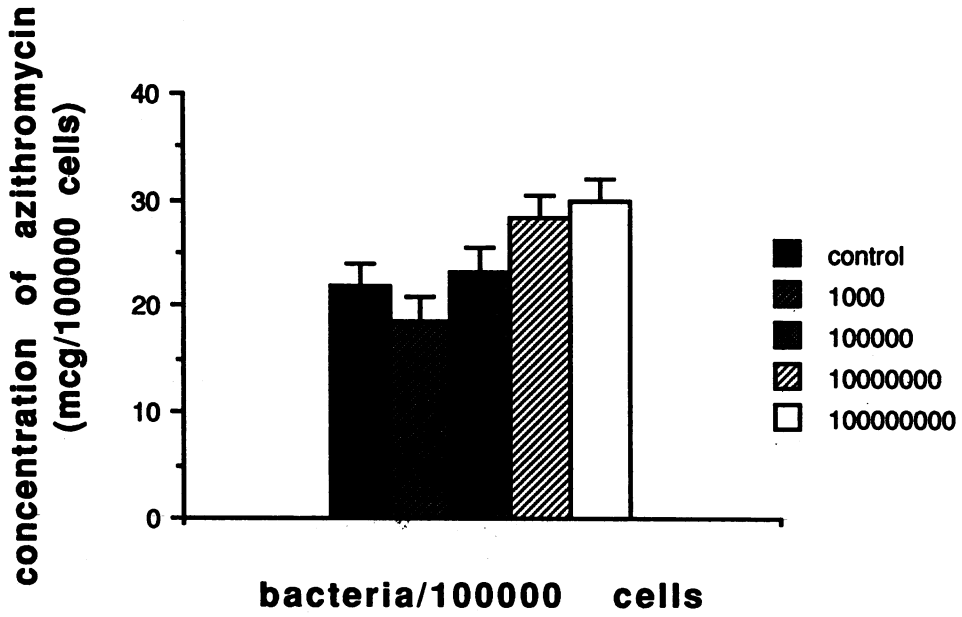

FIG. 2. Effect of *M. avium* infection for 24 h on the uptake of azithromycin by human macrophages. No statistically significant difference was observed among the experimental groups and the control group.

the incorporation of azithromycin by macrophages. Similar results were obtained when macrophages were stimulated with recombinant IFN-γ (10^2 U/ml). In the latter case, a 176% increase in uptake was observed between 30 min and 2 h, while a 359.2% increase was observed between 30 min and 4 h. These results suggest that stimulation of macrophages with either TNF-α or IFN-γ was associated with an increase in the uptake of azithromycin by macrophages of approximately 200%, compared with the uptake by unstimulated macrophages.

Since the uptake of some antibiotics has been reported to be mediated by active transport through cell membranes (14), we examined the effect of incubation of macrophages at 4°C on the uptake of azithromycin by unstimulated and stimulated (for 18 h at 37°C) macrophages. Incubation at 4°C prevented the uptake of azithromycin by both unstimulated and TNF-α- or IFN-γ-stimulated macrophages.

Previous studies have demonstrated that phagocytic cells also secrete azithromycin and can be responsible for the transport of the antibiotic to the site of infection (8). An alternative explanation for our findings is that cytokine-stimulated macrophages release smaller concentrations of incorporated antibiotic than unstimulated cells do. In order to examine this possibility, cytokine-treated and control macrophage monolayers were exposed to [^{14}C]azithromycin for 30 min, and any nonincorporated antibiotic was removed by washing. Monolayers were then monitored for 6 h to determine the concentration of azithromycin released in the supernatant. Both cytokine-stimulated and control macrophages secreted approximately 10% ± 4% of the intracellular antibiotic after 6 h.

Results reported by others as well as our results suggest an active transport of azithromycin through the cytoplasmic membrane (8, 9). Since our previous studies showed that infection of macrophages with large numbers of *M. avium* organisms could be associated with impairment of macrophage functions, we carried out experiments in which macrophage monolayers were infected with various amounts of *M. avium* 101, serotype 1, for 24 h and in which the uptake of azithromycin was evaluated both in control macrophages and in TNF-α- and IFN-γ-stimulated cells. Infection of monolayers containing 10^5 macrophages with approximately 10^3, 10^5, 10^7, and 10^8 bacteria for 24 h had no effect on the uptake of azithromycin by the phagocytes. However, when the cells were infected and the uptake of azithromycin was evaluated at 48 h, a 26% ± 4% decrease in uptake of the antibiotic was observed (Fig. 2). Incubation of infected cells with either TNF-α or IFN-γ increased the azithromycin uptake to normal levels (Fig. 3).

In order to determine whether a longer period of infection had any effect on azithromycin uptake, macrophages were infected with *M. avium* and uptake was measured after 5 days. Infection was associated with a 35% ± 4% decrease in the uptake of azithromycin by monolayers, while treatment of infected macrophages with either TNF-α or IFN-γ increased azithromycin uptake by macrophages to levels similar to those of uninfected cells (data not shown). We then examined the effect of filtrate of an *M. avium* 101 culture on the uptake of [^{14}C]azithromycin by macrophages. Supernatant of *M. avium* culture in 7H9 broth was concentrated by using a Centricon 3 microconcentrator (Amicon, Beverly, Mass.). The preparation containing 4.8 mg of protein per ml was diluted, and concentrations ranging from 0.1 to 10 μg/ml were added to macrophage monolayers for 5 days. Supernatant was then removed and [^{14}C]azithromycin was added for 2 h at 34°C. Concentrations greater than 1 μg/ml were associated with a dose-dependent decrease in the incorporation of [^{14}C]azithromycin by macrophages, up to 32.4% ± 6% (Table 1). These results suggest that the molecule(s) released by the bacteria is associated with the decreased uptake of [^{14}C]azithromycin by infected cells.

These results confirm previous studies by Gladue and colleagues (8), which show that azithromycin concentrates within macrophages. The increase in intracellular concentration of azithromycin was more evident during the first 2 h after treatment of macrophage monolayers and reached a

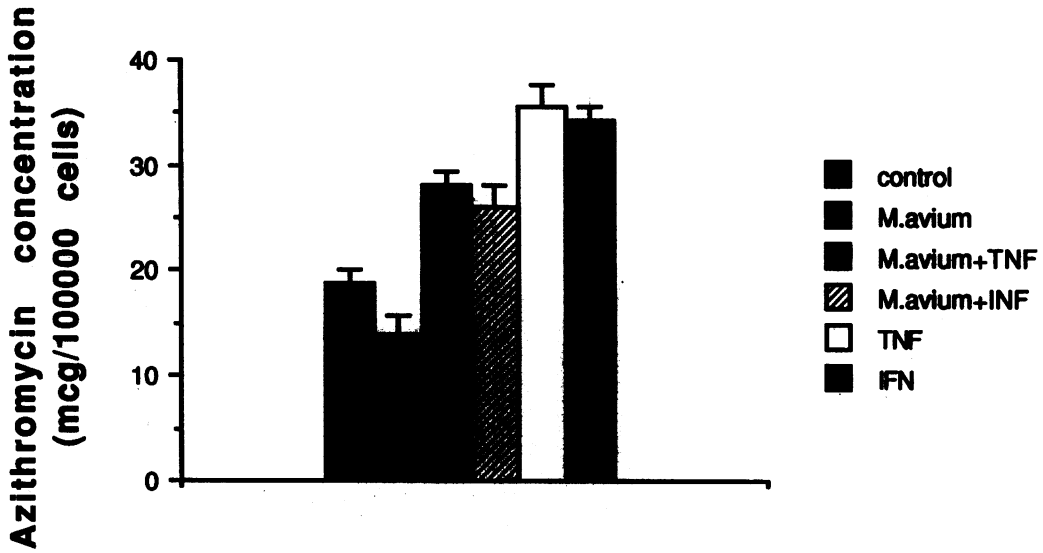

FIG. 3. Effect of *M. avium* infection (10^9 bacteria per 10^5 cells) for 48 h on the uptake of azithromycin by human macrophages. Influence of macrophage activation with TNF-α and IFN-γ on the uptake of [^{14}C]azithromycin by infected cells is also shown.

plateau thereafter. Incubation of macrophages at 4°C totally blocked drug uptake, suggesting that active transport through the membrane is the usual pathway for intracellular accumulation. Concentration of azithromycin within phagocytic cells explains the activity of the antibiotic against intracellular pathogens such as those of the *M. avium* complex (4).

Active transport mechanisms are clearly influenced by cell viability. Our results confirmed this hypothesis. Azithromycin uptake was influenced both by the degree of intracellular infection and by the time of infection. A decrease in uptake was observed only after 24 h of infection. Furthermore, only a 26% ± 4% decrease in uptake was observed after 48 h. However, if macrophages were maintained infected for 5 days, up to a 35% ± 4% decrease in uptake was observed. These findings may have small implications for the therapy of bacterial infections in which therapeutic drug concentrations greater than those obtained in serum are necessary to inhibit bacterial growth.

The active uptake of azithromycin by macrophages was also shown to be influenced by the degree of activation of the phagocyte. Our previous study demonstrated that treatment of *M. avium*-infected macrophages with a combination of TNF-α and azithromycin was associated with a significant increase in the intracellular killing of *M. avium*, compared with the killing seen in cells treated with either TNF-α or azithromycin alone (4). Our present findings suggest that increased uptake of azithromycin by cytokine-stimulated macrophages was responsible for the enhanced intracellular killing of *M. avium* observed in macrophages treated with TNF-α-azithromycin. However, the possibility that stimulation with TNF-α induced synthesis of intracellular bactericidal proteins (3), which led to a synergistic bactericidal effect with azithromycin, cannot be ruled out. Finally, macrophage activation prevents *M. avium*-mediated inhibition of phagolysosome fusion (5), which could potentiate the additive or synergistic effect of macrophage bactericidal proteins and azithromycin.

In summary, azithromycin was shown to achieve and maintain high intracellular concentrations within human macrophages. Antibiotic uptake is significantly increased by prior stimulation of macrophages with IFN-γ or TNF-α. Prolonged macrophage infection with *M. avium* impairs the uptake of antimicrobial agents; however, the decrease of drug uptake by infected macrophages can be overcome by stimulation with either IFN-γ or TNF-α after infection. Further investigations are necessary to determine the implication of these findings in vivo.

TABLE 1. Effect of *M. avium* supernatant filtrate on the uptake of [^{14}C]azithromycin by macrophages

Concn (μg/ml) of filtrate	Intracellular concn of azithromycin (μg/ml)[a]	% Decrease from control
No filtrate	31 ± 5	
0.1	30 ± 6	3.2
0.5	27 ± 4	12.9
1	24.4 ± 4	21.4
2	22.4 ± 3	27.7
5	21.7 ± 2	30.0
10	20.9 ± 4	32.4

[a] Incorporation was measured for 2 h after 5 days of incubation with *M. avium* culture filtrate. Values are means ± standard deviations.

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REFERENCES

1. **Bermudez, L. E., and L. S. Young.** 1987. Phagocytosis and intracellular killing of *Mycobacterium avium* complex by human and murine macrophages. Braz. J. Med. Biol. Res. **20**:191–200.
2. **Bermudez, L. E., and L. S. Young.** 1988. Tumor necrosis factor alone or in combination with IL-2, but not IFN-gamma, activates macrophages to kill *Mycobacterium avium* complex. J. Immunol. **140**:3006–3013.
3. **Bermudez, L. E., and L. S. Young.** 1989. Macrophage cytotoxic peptides (defensins) increase cell wall permeability and kill *Mycobacterium avium* complex, abstr. U-64, p. 165. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, D.C.
4. **Bermudez, L. E. M., and L. S. Young.** 1988. Activities of amikacin, roxithromycin, and azithromycin alone or in combi-

nation with tumor necrosis factor against *Mycobacterium avium* complex. Antimicrob. Agents Chemother. **32**:1149–1153.
5. **Black, C., L. E. Bermudez, L. S. Young, and J. S. Remington.** 1990. Co-infection of macrophages modulates IFNg and TNF-induced activation against intracellular pathogens. J. Exp. Med. **172**:977–980.
6. **Davidson, P. T., V. Khanijo, M. Goble, and T. S. Moulding.** 1981. Treatment of disease due to *Mycobacterium intracellulare*. Rev. Infect. Dis. **3**:1052–1059.
7. **Foulds, G., R. Shepard, and R. Johnson.** 1990. The pharmacokinetics of azithromycin in human serum and tissues. J. Antimicrob. Chemother. **25**(Suppl. A):73–82.
8. **Gladue, R. P., G. M. Bright, R. E. Isaacson, and M. F. Newborg.** 1989. In vitro and in vivo uptake of azithromycin (CP-62,993) by phagocytic cells: possible mechanism of delivery and release at sites of infection. Antimicrob. Agents Chemother. **33**:277–282.
9. **Gladue, R. P., and M. E. Snider.** 1990. Intracellular accumulation of azithromycin by cultured human fibroblasts. Antimicrob. Agents Chemother. **34**:1056–1060.
10. **Hawkins, C. C., J. W. M. Gold, E. Whimbley, E. Kiehn, P. Brannon, R. Cammarata, A. E. Brown, and D. Armstrong.** 1986. *Mycobacterium avium* complex infections in patients with the acquired immunodeficiency syndrome. Ann. Intern. Med. **105**: 184–188.
11. **Heifets, L. B., M. D. Iseman, A. J. Crowle, and P. J. Lindholm-Levy.** 1986. Pyrazinamide is not active in vitro against *Mycobacterium avium* complex. Am. Rev. Respir. Dis. **134**:1287–1288.
12. **Inderlied, C. B., P. T. Kolonoski, M. Wu, and L. S. Young.** 1989. In vitro and in vivo activity of azithromycin (CP 62,993) against the *Mycobacterium avium* complex. J. Infect. Dis. **159**:994–997.
13. **Inderlied, C. B., L. S. Young, and J. K. Yamada.** 1987. Determination of in vitro susceptibility of *Mycobacterium avium* complex isolates to antimycobacterial agents by various methods. Antimicrob. Agents Chemother. **31**:1697–1702.
14. **Johnson, J., W. Haud, J. Francis, N. Neva-Thompson, and R. Corwin.** 1980. Antibiotic uptake by alveolar macrophages. J. Lab. Clin. Med. **95**:429–439.
15. **Klatt, E. C., D. F. Jensen, and P. R. Meyer.** 1987. Pathology of *Mycobacterium avium-intracellulare* infection in acquired immunodeficiency syndrome. Hum. Pathol. **18**:709–717.
16. **Retsema, J., A. Girard, W. Schelkly, M. Manousos, M. Anderson, G. Bright, R. Borovoy, L. Brennan, and R. Mason.** 1987. Spectrum and mode of action of azithromycin (CP-62,993), a new 15-membered ring macrolide with improved potency against gram-negative organisms. Antimicrob. Agents Chemother. **31**:1939–1947.
17. **Shepard, R., and F. Falkner.** 1990. Pharmacokinetics of azithromycin in rats and dogs. J. Antimicrob. Chemother. **25**(Suppl. A):49–60.
18. **Wallace, J. M., and J. B. Hannah.** 1988. *Mycobacterium avium* complex infection in patients with acquired immunodeficiency syndrome. Chest **93**:926–934.
19. **Yeager, H., Jr., and J. W. Raleigh.** 1973. Pulmonary disease due to *Mycobacterium intracellulare*. Am. Rev. Respir. Dis. **108**: 547–552.
20. **Young, L. S., C. B. Inderlied, O. G. Berlin, and M. S. Gotlieb.** 1986. Mycobacterial infections in AIDS patients, with an emphasis on the *Mycobacterium avium* complex. Rev. Infect. Dis. **8**:1024–1033.