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Activities of Amikacin, Roxithromycin, and Azithromycin Alone or in Combination with Tumor Necrosis Factor against *Mycobacterium avium* Complex

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The effect of amikacin and two new macrolides (roxithromycin and azithromycin) used either alone or in combination with recombinant tumor necrosis factor (TNF) to inhibit or kill *Mycobacterium avium* complex in human macrophages was examined in vitro. Macrophage monolayers infected with *M. avium* complex (strain 101, serotype 1) were treated with antibiotics or TNF by using three different protocols: (i) antibiotics or TNF was added to the monolayers immediately after infection and washed out after 24 h, (ii) antibiotics or TNF was replenished daily for 4 days, or (iii) infected macrophage monolayers were treated with antibiotics plus TNF for 4 consecutive days. The number of viable intracellular bacteria was determined after 2 and 4 days of treatment by lysing cultured macrophages. Treatment for 24 h resulted in an inhibition of growth, as determined by macrophage lysis at day 4, for all three antimicrobial drugs and killing of 22% of intracellular bacteria after treatment with TNF. After treating the monolayers with amikacin, roxithromycin, or azithromycin for 4 consecutive days and replenishing the drug concentration daily, we observed 18 ± 6, 20 ± 4, and 22 ± 1% killing, respectively. TNF (100 U/ml) was added daily to the monolayers, which resulted in 54 ± 5% killing after 4 days. Combinations of antibiotics with TNF were associated with 62 ± 3% killing with TNF-azithromycin, 73 ± 6% killing with TNF-roxithromycin, and 56 ± 4% killing of intracellular *M. avium* complex with TNF-amikacin after 4 days. The mycobactericidal effect was enhanced (91 ± 4% killing) when TNF, amikacin, and roxithromycin were used together (compared with 68 ± 4% killing by roxithromycin-amikacin). Combinations of antimicrobial agents with immunomodulators like TNF may be useful for treatment of *M. avium* complex infection.

Organisms belonging to the *Mycobacterium avium* complex are the most common cause of bacteremia in patients with acquired immunodeficiency syndrome (12, 17). *M. avium* complex organisms are characteristically resistant to many antituberculous drugs, and alternative forms of therapy are necessary (1, 9, 13). As *M. avium* complex is an intracellular parasite, antimicrobial agents which can penetrate macrophages seem desirable. Amikacin has a predictable anti-*M. avium* complex activity in vitro, but previous studies have shown that amikacin, as well as other aminoglycosides, exerts primarily an inhibitory rather than a bactericidal effect on intracellular *M. avium* complex and *Mycobacterium tuberculosis* (3, 7). Roxithromycin and azithromycin are two new macrolide antibiotics with in vitro antimicrobial spectra similar to that of erythromycin but with a longer half-life and higher levels in serum (S. K. Puri, H. B. Lassman, I. Ho, R. Sabo, and A. Barry, Proc. 14th Int. Congr. Chemother., p. 128–137, 1986). Furthermore, both drugs, in contrast to erythromycin, are active in vitro against *M. avium* complex (M. Wu, P. Kolonoski, J. Yamada, C. Inderlied, and L. S. Young, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 564, p. 194, 1987) at levels which are achieved intracellularly and in tissues.

Classical antimicrobial agents may not be the only approach to treatment of mycobacterial infection in immunosuppressed patients. Mycobacteria are able to survive intracellularly within mononuclear phagocytes, despite treatment with bacteriostatic antibiotics (14). Thus, agents of ongoing interest such as cytokines, which can activate macrophages but are not mycobactericidal by themselves, could enhance intracellular killing of *M. avium* complex. Tumor necrosis factor (TNF) is a cytokine which we have found capable of activating macrophages to inhibit growth or kill intracellular *M. avium* complex (4). Thus, treatment with the combination of this cytokine with either mycobacteriostatic or mycobactericidal antibiotics might augment the ability of human macrophages to limit growth or kill intracellular *M. avium* complex. In this study, we examined this hypothesis in vitro by using an *M. avium* complex-infected human macrophage system.

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MATERIALS AND METHODS

Antimicrobial agents and TNF. Amikacin was kindly provided by Bristol Laboratories, Syracuse, N.Y. It was diluted in RPMI 1640 with 5% fetal calf serum (FCS) and stored in portions at a concentration of 500 μg/ml at −70°C. Roxithromycin was provided by Hoechst-Roussel Pharmaceuticals Inc., Somerville, N.J., as a powder. It was dissolved in RPMI 1640 with 5% FCS and stored at −70°C. Azithromycin was provided by Pfizer Laboratories, Inc., New York, N.Y., also dissolved in RPMI 1640 with 5% FCS, and stored at −70°C. Recombinant TNF was kindly provided by Genentech, Inc., South San Francisco, Calif., and had an activity of 3×10^7 U/mg of protein.

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Human macrophages. Heparinized human peripheral venous blood was obtained from healthy donors (three donors were used for all experiments), processed by centrifugation with Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo.), and cultured by the technique described by Crowle et al. (7). The solution was adjusted for a 10^7 suspension of mononuclear cells in RPMI 1640 supplemented with 10% heat-inactivated FCS (GIBCO Laboratories, Grand Island, N.Y.) and distributed in 1-ml samples in disposable 24-well tissue culture plates. The plates were incubated for 3 h at 37°C in 5% CO_2 in moist air for the adherence of monocytes. The medium and the nonadherent cells were then aspirated, and the monolayers, which contained approximately 10^6 monocytes, were counted by the method of Nakagawara and Nathan (16) and were washed twice with 1 ml of warmed Hanks balanced salt solution (37°C) to remove nonadherent cells. The adherent monocytes were maintained in culture with 1.5 ml of RPMI 1640 supplemented with 10% heat-inactivated (56°C, 30 min) FCS and 2 mM L-glutamine for 7 days. These cells developed the morphological criteria for macrophages in 4 days. About 20% of cultured infected macrophages detached from the plates (experiment and control) in the first 48 h. From then on, about 1 to 2% of the cells in both groups (activated and unactivated cells) were lost every 2 days. More than 97% of the cells in the monolayers were mononuclear phagocytes, as assessed by their ability to ingest neutral red.

***M. avium* complex.** *M. avium* complex strain 101 (serotype 1) was isolated from a patient with acquired immunodeficiency syndrome. Strain 101 is a virulent strain, as demonstrated by studies with animals (5). The mycobacteria were cultured in Middlebrook agar 7H10 medium (Difco Laboratories, Detroit, Mich.) for 10 days at 37°C. On the day of the experiment, pure transparent colonies were suspended in Hanks balanced salt solution and washed twice, and the suspension was adjusted for 10^7 bacteria per ml by using a McFarland standard. The bacterial suspension was vortex agitated for 2 min to avoid clumping, and a sample from the bacterial suspension was plated for confirmation of the number of bacteria.

Establishment of infection. Monocyte-derived macrophages (approximately 10^6 cells per ml) cultured in vitro for 7 days were incubated with a suspension of *M. avium* complex (10^7 bacteria per ml) in RPMI 1640 supplemented with 10% heat-inactivated FCS and 2 mM of L-glutamine. After 4 h, the extracellular bacilli were washed exhaustively with phosphate-buffered saline. The number of viable intracellular bacilli was determined after lysis of the macrophages; the bacilli were cultured onto 7H10 agar plates as described below to establish the number of phagocytosed bacteria per monolayer (implantation inoculum at time zero).

Antimycobacterial treatment. Infected macrophages were treated with amikacin (12 μg/ml), roxithromycin (2 μg/ml), azithromycin (10 μg/ml), or TNF (10^2 U/ml) alone or in combination. The concentrations utilized were based on the MIC of each drug for the bacteria and on an achievable concentration of the drug in serum or tissue according to pharmacokinetic studies, which made the working concentrations comparable for all three drugs (approximately six times the MIC). The MICs were obtained in broth (BACTEC system) by using a methodology previously described (15). The TNF concentration sufficient to stimulate macrophages to kill or inhibit growth of intracellular *M. avium* complex was based on results obtained in our previous experiments. Immediately after time zero was established, each drug was added and either washed out after 24 h (from then on the medium was replaced daily) or was added to the monolayers daily during the entire experimental period (replenished every day for 4 days). TNF was also added to the monolayers immediately after infection and either washed out after 24 h or replenished daily to maintain a constant concentration throughout the experiment. In the experiments in which drugs or TNF was replenished daily, the medium was replaced in all monolayers (including the control) to maintain similar conditions during the experiment. Macrophage rupture due to bacterial overgrowth was not observed in any experimental group. By observing any changes in the density of the monolayers and by performing cell counts on monolayer supernatants, we also determined that there is no preferential detachment of infected macrophages or of drug- or cytokine-treated macrophages from the surface of the plates.

Quantitation of acid-fast bacilli. Macrophages were cultured in Lab-Tek slides (Miles Scientific, Naperville, Ill.) with eight chambers for 7 days (6). The macrophage monolayers were infected with *M. avium* complex by following the protocol described above, and the intracellular bacteria were counted 24 h after infection. The slides were washed with Hanks balanced salt solution to remove extracellular bacteria, fixed with 4% glutaraldehyde, and stained with Kinyoun stain. From each chamber, 200 macrophages were counted microscopically at ×1,000 for acid-fast bacilli.

Quantitation of growth inhibition or killing of *M. avium* complex. Macrophage monolayers were infected with *M. avium* complex and cultured at 37°C in 5% CO_2 in moist air for several days in RPMI 1640 containing 10% heat-inactivated FCS. The number of bacteria per monolayer 4 h after infection (implantation inoculum at time zero) was obtained, and at 2 and 4 days after infection, the wells were washed with phosphate-buffered saline to remove any extracellular bacilli. To lyse macrophages, 0.5 ml of iced sterile water was added to each well and incubated for 15 min at room temperature. Then 0.5 ml of another lysing solution which contained 1.1 ml of 7H9 medium and 0.4 ml of 0.25% sodium dodecyl sulfate in phosphate buffer was added to each well for 10 min more. The wells were vigorously scraped with a rubber policeman, and the macrophage lysates were suspended in 0.5 ml of 20% bovine albumin in sterile water to neutralize the sodium dodecyl sulfate effect. The suspension was then vortex agitated for 2 min for complete lysis of macrophages. The macrophage lysate was sonicated for 10 s (power output, 2.5 W/s) to disperse the bacterial clumps and permit reliable pour plate quantitation. As a control for osmotic stability, mycobacteria without macrophages were subjected to the same procedure, and quantitative colony counts were determined by pour plate methods. The bacteria were 100% viable. To ensure that macrophages were totally disrupted, samples were examined by Giemsa staining.

The macrophage lysate suspension was diluted serially, and 0.1 ml of the final suspension was plated onto 7H10 agar. The plates were allowed to dry at room temperature for 15 min and were incubated at 37°C in 5% CO_2 in moist air for 2 weeks. The results are reported as mean CFU per milliliter of macrophage lysate suspension, obtained after 14 days in culture. Duplicate plates were prepared for each well. Inhibition of growth or killing of intracellular bacteria was concluded when the number of bacteria in treated monolayers was lower than that in control monolayers at the same time after infection or was lower than that in the implantation inoculum, respectively, using monolayers with a similar number of intracellular bacteria at time zero after infection.

Statistics. Each experiment was repeated at least 10 times.

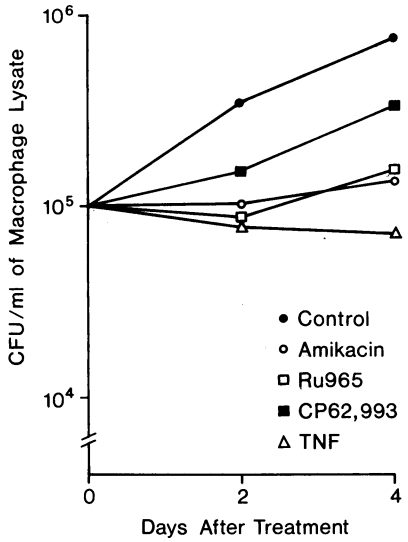

FIG. 1. Number of CFU of macrophage lysate per ml 2 and 4 days after treatment with a single dose of amikacin, roxithromycin (RU 965), azithromycin (CP62,993), and TNF. Each point represents a mean of 10 different experiments.

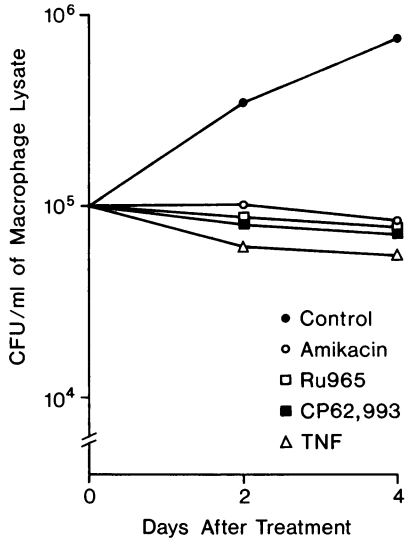

FIG. 2. Effect of amikacin, roxithromycin (RU 965), azithromycin (CP62,993), and TNF added onto macrophage monolayers daily for 4 days on *M. avium* complex survival within human macrophages, compared with control.

All data in each experiment were obtained on duplicate wells, and the means were calculated. The significance of differences in percent killing by treated and control macrophage cultures at identical times was tested by the Student *t* test.

RESULTS

Macrophages were infected similarly with *M. avium* complex in all performed experiments. At 24 h after infection, we observed that about 80 to 90% of the macrophages contained *M. avium* complex. The bacteria were able to survive and replicate intracellularly in normal macrophages (Fig. 1, control curve). The doubling time for the intracellular bacteria was about 26 h. To examine if treatment with mycobacteriostatic-mycobactericidal drugs such as amikacin and two macrolides, roxithromycin and azithromycin, could modify the intracellular survival of *M. avium* complex 101 when the drugs were used alone or in combination with recombinant TNF, we used three protocols. In protocol 1, infected macrophages were exposed to amikacin (12 μg/ml; 6 times the MIC), roxithromycin (2 μg/ml; 2 times the MIC), azithromycin (10 μg/ml; 0.3 times the MIC), or TNF (10^2 U/ml) shortly after infection and were removed by being washed after 24 h. The viable intracellular bacteria were quantitated after 2 and 4 days. To rule out a likely carry-over effect from drugs, sterile-filtered macrophage lysates obtained from antibiotics, TNF, and control monolayers were incubated with *M. avium* complex (10^4 and 10^6 bacteria per ml) and plated onto 7H9 agar plates. *M. avium* complex 101 grew equally well (as determined by the duplication time) in all tested groups (data not shown). Amikacin was inhibitory for *M. avium* complex for 48 h, with subsequent growth of the bacteria (Fig. 1). However, amikacin-treated monolayers had 81.3% fewer bacteria than controls had after 4 days. Both roxithromycin and azithromycin were inhibitory for *M. avium* complex, and the numbers of viable intracellular bacteria after 4 days were 80.0 and 56.0% fewer than that of controls. TNF was the only agent which induced predominant intracellular killing of *M. avium* complex (22%) after 4 days.

Because the regrowth of *M. avium* complex 101 observed 48 h after treatment with antimicrobial agents might be related to metabolism and reduction in effective intracellular concentration, drug concentration in the monolayers was replenished every day. All drugs were associated with intracellular killing of *M. avium* complex (Fig. 2). Thus, treatment with azithromycin was associated with 22 ± 1% killing of the initial inoculum after 4 days, and roxithromycin and amikacin were associated with 20 ± 4 and 18 ± 6% killing after 4 days. TNF (100 U), when added to the culture every day, was associated with 54 ± 5% killing after 4 days, compared with killing at time zero.

To determine if TNF-dependent activation of macrophages could amplify the mycobactericidal effect of antibiotics, infected macrophages were treated daily with TNF (10^2 U/ml) in combination with amikacin, roxithromycin, or azithromycin or with the combination of TNF-roxithromycin plus amikacin at the same concentrations used in the former experiments. The combinations were significantly more lethal than antibiotics alone (Fig. 3). Treatment with amikacin-TNF was associated with 56 ± 4% intracellular killing ($P < 0.05$ compared with the drug or TNF alone), treatment with azithromycin-TNF was associated with 62 ± 3% killing ($P < 0.05$ for drug and TNF alone), and treatment with roxithromycin-TNF was associated with 73 ± 6% killing ($P < 0.05$ for drug and TNF alone). The combination of amikacin-roxithromycin plus TNF was associated with 91 ± 4% intracellular killing, compared with 68 ± 4% killing by the amikacin-roxithromycin combination ($P < 0.05$ compared with TNF-amikacin and $P = 0.05$ compared with both the TNF-roxithromycin and roxithromycin-amikacin combinations).

DISCUSSION

M. avium complex has become the most common cause of bacteremia in patients with AIDS (12, 18). It is generally recognized that *M. avium* complex is resistant to most standard antituberculous drugs (9, 12). In this study, by using an in vitro model of *M. avium* complex infection (6, 8,

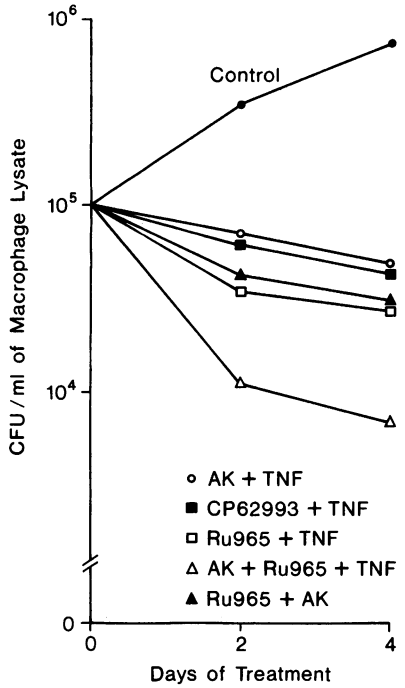

FIG. 3. Intracellular killing of *M. avium* complex after treatment with amikacin (AK)-TNF, azithromycin (CP62993)-TNF, roxithromycin (RU 965)-TNF, and amikacin-roxithromycin-TNF compared with control and roxithromycin-amikacin.

10), we demonstrated that the combination of antimicrobial agents active in vitro against *M. avium* complex and a cytokine that can activate mycobacteriostatic or mycobactericidal activity within human macrophages but does not have any mycobactericidal activity (3) can kill significantly greater numbers of intracellular *M. avium* complex than antimicrobial agents or cytokine alone.

The inability of drugs such as amikacin to achieve high intracellular concentrations may permit *M. avium* complex to survive within phagocytic cells. In contrast to amikacin, azithromycin and roxithromycin, two new macrolides, can be concentrated intracellularly within phagocytes. Studies by others (11; R. M. Shepard, D. J. Weidler, D. C. Garg, P. O. Madsen, C. E. Cox, K. H. Chan, and C. D. Bluestone, 27th ICAAC, abstr. no. 239, p. 138, 1987) have demonstrated that azithromycin and roxithromycin have a concentration in tissues up to 25 to 30 times greater than that in serum, which suggests an active mechanism of transport into cells (2). Although Hand and colleagues (11) have shown that the ingestion of microorganisms by the phagocytic cell decreases the ability of the cell to accumulate roxithromycin, the uptake of the drug by infected polymorphonuclear leukocytes is still impressive (11).

The particular interest in this study was the effectiveness obtained with concentrations of the antimicrobial agents in serum. Amikacin, azithromycin, and roxithromycin were bacteriostatic by themselves for the bacilli infecting human macrophages after a single dose. However, the efficacy of these drugs was improved when drug concentrations in tissue culture were replenished daily. Replenishing monolayers every day with azithromycin and roxithromycin, drugs which have a long half-life in tissues, may account for increased intracellular concentrations and greater efficacy in this model. Although the effect of each drug alone was most inhibitory or slightly bactericidal, we observed a significant bactericidal effect when the drugs were used in combination with TNF.

Why the combination of TNF and these antimicrobial agents was more effective than each drug by itself is a matter for speculation. All three antibiotics are transported into the cells by an active mechanism of the membrane. As a consequence, TNF-stimulated cells could transport a greater concentration of drugs intracellularly than unstimulated macrophages could. Also, TNF-stimulated cells synthesize cationic proteins not found in unstimulated cells, which are able to produce lysis in *M. avium* complex under determined conditions in vitro (unpublished observations). This effect could increase bacterial permeability to the antibiotics. Obviously, studies in vitro are essential for evaluation of this area of interest and are subjects of ongoing investigation.

The data obtained from these experiments in vitro demonstrated a significant mycobactericidal activity of amikacin, roxithromycin, and azithromycin combined with TNF and support further studies in vivo.

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