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Activities of Rifabutin, Clarithromycin, and Ethambutol against Two Virulent Strains of *Mycobacterium avium* in a Mouse Model

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Rifabutin, clarithromycin, and ethambutol, which were administered at concentrations similar to those used in clinical trials that are now under way, were tested for their capacities to inhibit the growth of two highly virulent isolates of *Mycobacterium avium* in inbred C57BL/6 mice and in vitro in a bone marrow-derived murine macrophage model. In the latter model rifabutin and clarithromycin had modest activities against strain 101 and somewhat better activities against strain 2-151. When they were tested in vivo, however, the best results, against strain 101 were seen when the three drugs were given in combination, whereas against strain 2-151 the combination therapy showed no significant improvement over that of clarithromycin given alone. It will be of interest to note to what degree the eventual outcomes of the current trials correlate with the predictions of these animal model systems.

Disseminated infection caused by members of the *Mycobacterium avium* complex is relatively common in AIDS patients with low CD4 T-cell counts (2, 7, 13, 33). Although therapy of such infections was initially difficult, given the resistance of the organism to most conventional antimycobacterial drugs (7, 18), some considerable recent advances have been made with the availability of a variety of new active compounds including macrolides, rifamycins, and fluoroquinolones.

Of these, perhaps the most promising compound has been clarithromycin, which has been shown to be active in a variety of in vitro and in vivo models (4, 8, 15, 17, 19, 20, 23, 25, 30) and which has been demonstrated in recent clinical trials to significantly reduce the levels of bacteremia in patients with disseminated disease (5, 6). Similarly, the rifamycin rifabutin also has considerable activity against both *M. avium* (3, 9, 10–12, 16, 22, 24, 27, 31, 32) and *Mycobacterium tuberculosis* (22) in rodent models and has recently been shown to be of clinical benefit when it was given as a prophylactic therapy (21) and when it was used in a recent prospective trial (29).

Use of the combination of clarithromycin and rifabutin with a third drug, ethambutol, is now being evaluated in the clinical setting. Despite this, however, adequate data on this combination from animal models are still lacking, as is a consensus on appropriate experimental protocols. For example, most experimental analyses performed in the mouse model to date have been carried out with *M. avium* isolates that, with very few exceptions, appear to be mostly of low to moderate virulence (4, 15, 16, 22, 23). Moreover, the range of doses of drugs applied in such models are sometimes higher than those that can be realistically applied in the clinical setting.

In view of this, we compared the activity of the three-drug combination, given at dosages within the range of those currently being applied in clinical trials, against the in vivo growth of two *M. avium* isolates deliberately chosen for their known high levels of virulence in the mouse model. At these dosages each agent had only modest activities, if any. However, when the drugs were given as a triple combination improved reductions in the bacterial load of one of the two virulent strains were observed in some tissues. As such, it will therefore be interesting to see how closely the results obtained with this animal model predict the eventual outcomes of current clinical trials.

We used two *M. avium* isolates that are of high virulence for C57BL/6 mice (in that a modest inoculum of 10^5 to 10^6 bacteria given intravenously will grow progressively in target organs and kill the mouse in 3 to 4 months). These isolates were strain 101, which was kindly provided by Lowell Young, Kuzell Institute, San Francisco, Calif., and a smooth transparent variant (1) cloned from strain 2-151, an isolate that was originally kindly provided by Alfred Crowle, University of Colorado. All bacteria were grown to the mid-log phase in Proskauer Beck medium and were frozen at −70°C until they were needed.

Bone marrow-derived macrophages were obtained from specific-pathogen-free female C57BL/6 mice purchased from the Jackson Laboratories, Bar Harbor, Maine, by a previously described procedure (28). Briefly, mice were euthanized by exposure to CO_2, and femur bones were dissected out. The bones were trimmed at each end, and the marrow was flushed out with 5.0 ml of Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 10% L-929 fibroblast-conditioned supernatant, HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, nonessential amino acids, L-glutamine, and antibiotics by using a 26-gauge needle. Cell suspensions were then washed twice and plated in 24-well tissue culture plates at a concentration of 10^6 cells per well in supplemented DMEM. Monolayers were then incubated at 37°C in 5% CO_2, with the medium changed every 3 days. Macrophages were used 8 to 9 days later; they were infected with a 1.0-ml suspension containing 10^6 *M. avium* organisms suspended in antibiotic-free DMEM and were incubated as described above for 4 to 5 h. The wells were then thoroughly washed to remove the extracellular bacteria and were replaced with 1.0 ml of antibiotic-free DMEM containing the indicated concentrations (up to 8 μg/ml) of the compound being tested. Each concentration of drug was tested in triplicate wells. Control wells contained 1.0 ml of antibiotic-free DMEM without any drug. Wells were periodically observed under a microscope to check for cell viability or detachment (none of the compounds used in the study had any discernible

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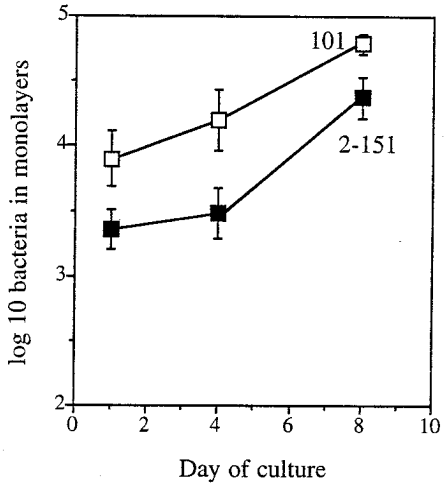

FIG. 1. Growth of *M. avium* 101 (□) and 2-151 (■) in bone marrow macrophage monolayers over the 8-day culture period. Data are expressed as means ± standard errors of the means for triplicate cultures.

toxic effects). After 8 days of incubation at 37°C in 5% CO_2, each well was gently washed and monolayers were then lysed with 0.1% saponin (Sigma, St. Louis, Mo.) dissolved in sterile water. Lysates were serially diluted in sterile saline and were plated on nutrient 7H11 agar (Difco, Detroit, Mich.). Bacterial colony formation was enumerated after incubation of the plates for 10 to 14 days at 37°C in humidified air. Data were expressed as the $\log_{10}$ mean numbers of bacteria in the triplicate cultures for each drug concentration; this information was entered into a simple computer graphics program (Cricket Graph; Cricket Software, Malvern, Pa.) in which a curve-fit line was established to determine the slope of bacterial killing.

Clarithromycin was obtained from Abbott Laboratories, Abbott Park, Ill., ethambutol was obtained from Lederle Laboratories, Pearl River, N.Y., and rifabutin was obtained from Pharmacia Adria, Dublin, Ohio. Stock solutions were prepared by using instructions from the manufacturers and were then diluted in sterile water prior to oral administration by gavage. All drugs were given at 15 mg/kg of body weight per day. MICs for each compound for the strains tested were as follows; for strain 101 the rifabutin MIC was 2.0 μg/ml, the clarithromycin MIC was 2.0 μg/ml, and the ethambutol MIC was 8.0 μg/ml; for strain 2-151 the rifabutin MIC was 2.0 μg/ml, the clarithromycin MIC was 2.0 μg/ml, and the ethambutol MIC was 16.0 μg/ml.

Mice were infected intravenously with 10^5 bacilli and were given drugs for 40 days from day 20 postinoculation. The bacterial numbers in the target organs were then determined by plating serial dilutions of individual whole-organ homogenates on nutrient 7H11 agar (Difco) and counting the bacterial colony formation after 10 to 20 days of incubation at 37°C in humidified air. Differences in bacterial load were tested by analysis of variance.

Figure 1 shows the growth of the two isolates in the bone marrow macrophage assay system over the 8-day culture period. For evaluation of drug activity, the compound was added after infection of the macrophages, potential effects on bacterial numbers were assayed on the eighth day, and bacterial numbers were compared with those in control (no drug) wells; these data are given in Fig. 2. Strain 101 was relatively resistant to all three compounds; hence, given the fact that this strain grew about 0.8 log unit over the 8-day culture period in the control wells, the actions of the three drugs can be regarded as bacteriostatic at best. Better activity was observed against strain 2-151, with significant reductions in bacterial numbers in cultures containing rifabutin or clarithromycin. Despite this, however, the drug concentrations needed to reduce bacterial loads by 2 log units (28) were still relatively high (6 μg for rifabutin and 5.5 μg for clarithromycin).

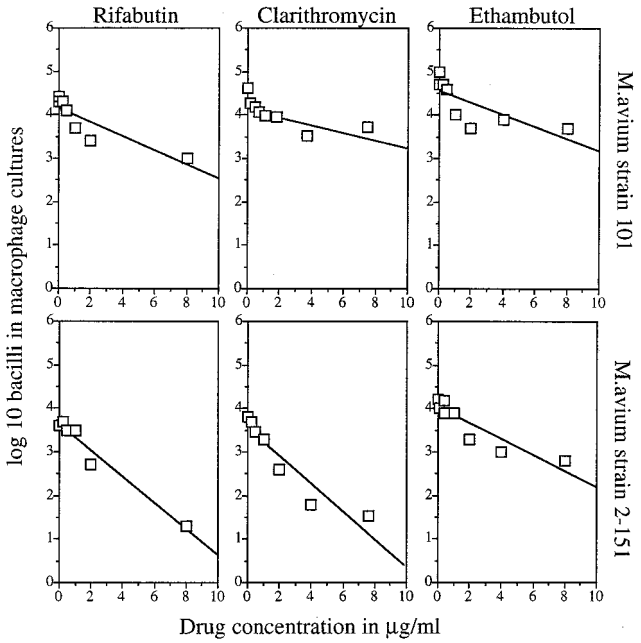

FIG. 2. Capacities of drugs to inhibit the growth of *M. avium* inside cultures of bone marrow-derived macrophages. Cultures were exposed to a single bolus of increasing concentrations of drugs at the time of infection (day zero), and the potential effects on bacterial numbers were assessed 8 days later. Data are expressed as mean (n = 3). Standard errors of the means are omitted; they did not exceed 0.35.

The results of the in vivo experiments are given in Fig. 3 and 4. Both strains grew well in infected mice, especially in the spleens and lungs (Fig. 3). Dissemination to the bone marrow

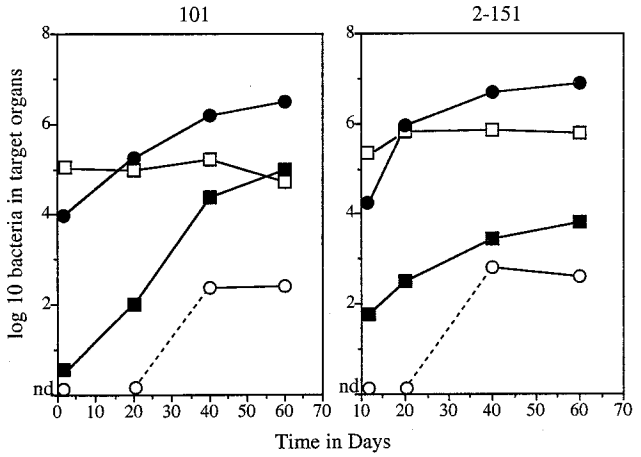

FIG. 3. Growth of the two isolates in target organs of untreated mice over the course of the experiment. Data are means (n = 4). Standard errors of the means are omitted; they did not exceed 0.3. The effects of drug therapies from day 20 to day 60 are shown in Fig. 4. Symbols: □, livers; ●, spleens; ■, lungs; ○, bone marrow.

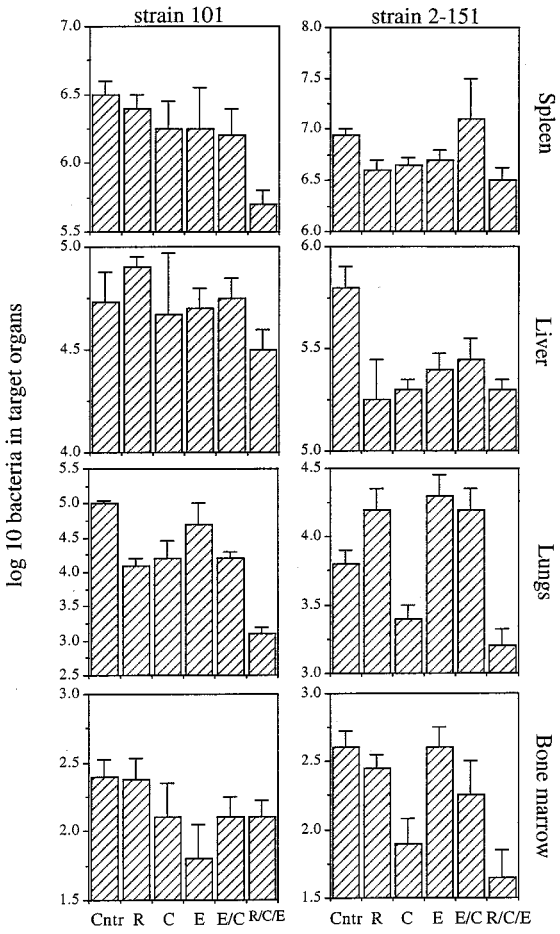

FIG. 4. Capacities of drug combinations to inhibit the growth in vivo of the two *M. avium* strains. Drug treatment was given from day 20 of the infection for 40 days, after which time the bacterial numbers in the target organs of test and control (untreated) mice were determined. Data expressed as means ± standard errors of the means. ($n = 4$). Cntr, untreated mice; R, rifabutin; C, clarithromycin; E, ethambutol (all drugs were given at 15 mg/kg/day).

was noted in both groups of mice. For evaluation of drug effects, mice were administered compounds by gavage starting on day 20 of the infection, and then the resulting bacterial numbers were assessed on day 60 in test and control (untreated) mice; these latter data are given in Fig. 4. In animals infected with strain 101, the drug treatments were mostly ineffective, with the only good activity being observed when the three compounds were used in combination. This activity was seen in the lungs ($P < 0.01$) and spleen ($P < 0.02$) but not in the other tissues. When strain 2-151 was tested, a very modest but significant reduction in the numbers of bacilli in the bone marrow ($P < 0.02$) and liver ($P < 0.04$) was seen with clarithromycin.

The results of the present study therefore further confirm the potential usefulness of clarithromycin as a major therapy for *M. avium* infections. In the model described here, clarithromycin was active against two highly virulent strains of *M. avium*, even though the dose given was modest and even though the achievable level in mouse serum at this dose is somewhat lower than that which can be achieved in humans (0.8 compared with 2 μg/ml [26]). The model also predicts, however, that, perhaps at least in some cases, a combination of rifabutin and ethambutol given with clarithromycin will be more effective. Such a regimen has a considerable attraction versus the use of monotherapy, given the dangers of emerging drug resistance.

Both rifabutin and clarithromycin had modest effects against the two virulent strains in the macrophage model, which is similar to the results described in an earlier report (28), and this is consistent with models that use human monocytes as the host infected cell, in which very high doses of clarithromycin were needed to significantly reduce bacterial loads (19, 20). In this regard, it is noteworthy that the modest drug activities predicted by the bone marrow macrophage assay were in fact also borne out by the results of the subsequent in vivo experiments. All three agents given alone had little effect on the growth of strain 101 in vivo, whereas clarithromycin had the best effect against strain 2-151 in both assays.

These data therefore support the results presented in earlier reports indicating the clinical use of clarithromycin (5, 6). The third drug, ethambutol, is ineffective alone but may potentiate the effects of these other compounds (12, 14, 25), probably by disrupting the arabinogalactan layer of the mycobacterial cell wall, allowing the other compounds to better penetrate the organism. In view of this and taking into account current problems with emerging drug resistance in mycobacteria, the use of such combination therapy seems highly appropriate.

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