Activity of Amikacin against *Mycobacterium avium* Complex under Simulated In Vivo Conditions

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We studied the activity of amikacin against *Mycobacterium avium* complex strain 101 by using continuous-level, changing concentrations which simulated levels in serum in a patient, and pulsed exposures. Amikacin at a concentration of 5 or 15 µg/ml showed rapid bactericidal action following constant exposure of the organisms. With the in vitro model, a peak concentration of 10 or 20 µg/ml, complete sterilization was obtained by day 7. In pulsed-exposure studies, a minimum period of contact of 72 or 96 h at a concentration of 10 µg/ml was needed for complete sterilization.

*Mycobacterium avium* complex organisms cause severe pulmonary disease and, in immune-deficient patients, disseminated disease (10). They are common, serious opportunistic pathogens in patients with acquired immune deficiency syndrome (11, 13, 14). Since the organisms are highly resistant to most of the available antimycobacterial drugs (2), chemotherapeutic management poses serious problems. We and others are involved in concentrated efforts to uncover potentially active drugs against these infections.

Amikacin, an aminoglycoside antibiotic, has been shown in in vitro studies (C. B. Inderlied, D. A. Bruckner, and L. S. Young, Program Abst. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 395, 1984) to be highly active against several strains of *M. avium* complex. These findings naturally warranted more detailed in vitro and in vivo investigations of its chemotherapeutic efficacy against *M. avium* complex disease in order to clarify its potential clinical application. We have carried out various investigations with this drug alone or in combination under several dynamic in vitro conditions and assessed its efficacy in free and liposome-encapsulated forms in experimental *M. avium* complex disease in beige-mouse and macrophage models. The results with the animal and macrophage systems are presented in other reports (P. R. J. Gangadharam, V. K. Perumal, N. R. Podapati, L. Kesavalu, and M. D. Iseman, submitted for publication; N. Duzgunes, V. K. Perumal, R. J. Debs, and P. R. J. Gangadharam, submitted for publication; L. Kesavalu, V. K. Perumal, N. Duzgunes, R. Debs, and P. R. J. Gangadharam, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, U59, p. 142); this report discusses antimycobacterial activity of amikacin against *M. avium* complex studied by using continuous exposure in a roller tube, dynamic exposure in an in vitro model (4), and a pulsed-exposure system using the membrane filter technique (1). These experiments are important links between conventional in vitro and in vivo studies, with the ultimate aim of providing useful preclinical data on the drug.

**MATERIALS AND METHODS**

**Amikacin.** Amikacin sulfate (1 g/4 ml; Amikin) was purchased from Bristol-Myers Co. Aseptic dilutions of a stock solution were made in sterile distilled water to obtain the requisite concentrations and stored at room temperature.

**M. avium complex.** *M. avium* complex strain 101 (serotype 1) was used. This strain, originally obtained from the blood of an acquired-immune-deficiency-syndrome patient from California, is highly susceptible in vitro to several drugs, including amikacin. The organisms were grown in Middlebrook and Cohn 7H9 broth, and single-cell suspensions of the transparent colonies were obtained as described previously (5, 7). The suspension, consisting of >95% single and double cells, was stored at −70°C in individual vials and used after a single thawing. Counts of CFU were made on 7H11 agar medium.

**Continuous-exposure experiments.** In the continuous-exposure studies, *M. avium* complex strain 101 was exposed to 5 and 10 µg of amikacin per ml. A drug-free tube served as a control. Both tubes were mounted in a roller tube drum, and the CFU in samples were measured daily up to 11 days.

**Dynamic-exposure experiments.** The dynamic-exposure studies were done using the in vitro model (model man of Gangadharam and co-workers) (4), which simulates exposure of the organisms to various diminishing concentrations of the drug, a situation analogous to levels in serum in a patient receiving chemotherapy. This model has been used by us (4, 8, 12) and others (14, 16) in several studies. In brief, the model (Fig. 1) consists of a cellophane dialyzing tube tightly attached to an open end of a screw-cap glass tube fitted through a rubber stopper to a 1-liter flask. The other end of the 3-in (ca. 8 cm)-long cellophane tube was closed by tying it into a tight knot.

In the current study, *M. avium* complex strain 101 (10^4 CFU/ml) and arbitrarily chosen peak concentrations ranging from 10 to 40 µg of amikacin per ml were placed within the cellophane dialyzing tube in individual models in 7H9 broth while the outside compartment contained only 7H9 broth, so that the drug alone could diffuse out (Fig. 1). A drug-free control model was set up under similar conditions. The organisms were exposed to diminishing concentrations of the drug, similar to what occurs under in vivo conditions. A typical picture of the diminishing concentrations of a drug inside the bag is shown in Fig. 1. Samples of 7H9 broth from inside the cellophane bag, containing the organisms from the drug-treated and drug-free (control) models, were taken daily up to 13 days, and CFU counts were determined. With separate sets of models, using only the drugs and media, the
diffusion of the drug through the dialyzing tube was studied by taking samples from the tube at different intervals. The levels of amikacin were measured by using the fluorescence polarization immunoassay developed by Abbott Laboratories (Abbott TDX Manual 1987, p. 16.01-16.08).

**Pulsed-exposure experiments.** In the pulsed-exposure method (1) a young culture of *M. avium* complex strain 101 in 7H9 broth was exposed to amikacin (10 µg/ml) for 24, 48, 72, or 96 h. The drug was then removed rapidly by passage through a membrane filter (pore size, 0.22 µm; Millipore Corp.) and repeated washing with 7H9 broth. More than 90% of the drug was removed by each washing, and after four washings, insignificant amounts of the drug were detected in the wash (data untabulated). The bacteria on the filter after five washings were suspended in fresh drug-free 7H9 broth to the original volume and incubated at 37°C. A drug-free control was set up under the same conditions. CFU in samples of these reconstituted cultures were counted on 7H11 agar medium after various periods of exposure to the drug; CFU in samples of the drug-free controls were also counted.

**RESULTS**

**Continuous exposure.** A rapid decline of CFU counts in *M. avium* complex culture incubated with a concentration of 15 µg of amikacin per ml commenced by day 1, with complete elimination of recoverable organisms by day 5; the sterility of the drug-treated tube was maintained throughout the experimental period (Fig. 2). With 5 µg/ml, a similar decline of the CFU counts was seen; however, complete elimination of recoverable organisms was not seen until day 7. The drug-free control tubes showed logarithmic increases of CFU counts, as expected. The counts from the control and drug-treated (15 µg/ml) tubes were different significantly (*P* < 0.01) from day 1 onward. Between the two drug-treated tubes, statistically significant differences were seen only at 3 days.

**Dynamic-exposure experiments.** Preliminary experiments using a peak concentration of 40 µg/ml resulted in rapid elimination of CFU counts (data untabulated). Even with a concentration of 20, 15, or 10 µg/ml, there was a rapid decline of the CFU counts, with complete elimination of the counts by day 7 (Fig. 3). The elimination of the counts was slightly more rapid with 20 µg/ml in the earlier periods than with 10 and 15 µg/ml, although all three drug-containing models showed complete elimination of CFU counts by day 7. There was little difference between 10 and 15 µg/ml at any period of observation, although the differences between the 20-µg/ml model and the other two drug concentrations were significant at 3 and 5 days (*P* < 0.05). The control model showed an increase of the CFU counts similar to that in the continuous-exposure control tube, although the increase in growth with the in vitro model stabilized after day 9.

**Falloff concentrations of amikacin.** Disappearance of amikacin from the cellophane tube through diffusion was assessed by using models provided with arbitrary peak concentrations of about 10 and 20 µg/ml and measuring the amikacin levels at regular intervals thereafter. Typical time-concentration curves with 13 µg/ml are shown in Fig. 4. There was a gradual disappearance of amikacin up to 24 h, even though approximately 3 µg/ml was noted at 24 h. It is not clear whether complete elimination of the drug will result at periods beyond 24 h and whether considerable accumulation of the drug will occur following multiple doses. An essentially similar picture was seen with models receiving 20-µg/ml peak concentrations (data not shown).

**Pulsed-exposure experiments.** Exposure of *M. avium* complex strain 101 to 10 µg of amikacin per ml for 24 h resulted in a bacteriostatic type of action, arresting growth; after the drug was removed, resumption of growth promptly started in
the logarithmic phase up to 7 days, at which time it stabilized (Fig. 5). With a 48-h exposure, the CFU counts fell by 2 logs by the time the drug was removed, after which the growth resumed as with the 24-h exposure and a similar pattern was seen. In contrast with 72- and 96-h exposures, by the time the drug was washed out, rapid bactericidal action had occurred, with virtually no recoverable organisms seen at any time thereafter.

**DISCUSSION**

Discovery and development of antimycobacterial agents against the *M. avium* complex group of organisms are urgently needed because of the high prevalence of disseminated disease in acquired immune deficiency syndrome patients and an apparent increase in pulmonary cases due to opportunistic pathogens in the United States (11, 13, 15). After conventional in vitro screening, all promising agents should be subjected to rigorous in vivo testing in susceptible animals and macrophage systems. In addition, a wide array of other detailed pharmacological and toxicological studies must be performed before controlled clinical investigations.

Prior to the detailed animal studies, there are several valuable in vitro studies which can be used which partially simulate in vivo conditions. Some of these studies mimic pharmacodynamic features of the host, whereas others represent the anatomic location of the parasites inside the body. These are sometimes referred to as in vitro-in vivo tests (3). The experiments described in this report dealing with constant, dynamic, and pulsed exposures thus provide a bridge between the conventional in vitro screening tests and in vivo animal studies.

It is encouraging that amikacin showed considerable promise in the three types of dynamic experiments discussed in this report. In the constant-exposure experiments, bactericidal action was evident with 15 μg/ml as early as 5 days. If the concentration was slightly reduced, similar bactericidal action was seen, although at a slightly slower pace, the cultures becoming negative by day 7 (Fig. 2). Pulsed exposure for 72 or 96 h resulted in similar bactericidal action with 10 μg of amikacin per ml. Thus, in these systems, a minimum concentration of 10 μg/ml and a minimum period of 3 to 4 days of contact seem to be essential for complete bactericidal action.

Comparison of the results between the constant- and pulsed-exposure experiments (Fig. 2 and 5) reveals interesting differences. For instance, with constant exposure to a 15-μg/ml concentration, complete elimination of the counts was seen by day 5, whereas in the pulsed-exposure studies with a lower concentration of the drug (10 μg/ml), complete sterilization was obtained after 3 or 4 days of exposure. This discrepancy can be explained by the fact that in the pulsed-exposure studies, the organisms were washed several times.

FIG. 3. Log CFU counts of *M. avium* complex inside the dialyzing tube from amikacin-treated (10, 15, and 20 μg/ml) and drug-free-control models. Appropriate dilutions of samples taken from the drug-treated and drug-free (control) models were plated on 7H11 agar medium, and the CFU were enumerated after 3 weeks of incubation at 37°C.

FIG. 4. Time-concentration (conc.) curves of amikacin levels inside the cellophane bag, with an initial peak concentration of 13 μg/ml. Amikacin levels were measured by using the fluorescence polarization immunoassay of Abbott Laboratories (Abbott TDX Manual 1986, p. 16.01–16.08).

FIG. 5. Log CFU counts of *M. avium* complex from cultures in 7H9 broth that were exposed to amikacin for 24, 48, 72, and 96 h. After each period of exposure, the drug was washed out by at least five washings, using suction, with sterile 7H9 medium. The bacilli remaining after the fifth wash were suspended in 7H9 medium to the original volume and then reincubated. CFU were measured in samples from the reconstituted suspensions in drug-free and drug-containing tubes for several days.
to get rid of all the drug and the suspension was reconstituted to the original volume before the CFU counts were determined. These operations (centrifugation, multiple washings, and resuspension) naturally involve loss of the bacterial population. This presumably is reflected in lower CFU counts compared with those in the roller tube cultures, in which no such operations were involved. However, in either situation, 5 days of constant or pulsed exposures with a concentration of 10 or 15 μg/ml resulted in complete sterilization of the culture.

These results can be compared with the action of powerful drugs like isoniazid against Mycobacterium tuberculosis (4) and 6-cyclo-octylamino-5,8-quinoline quinone (CQQ), now called gan-gamicin (gangamicin is the patent name for CQQ; patent application filed by National Jewish Center for Immunology and Respiratory Medicine), against M. avium complex under similar conditions (9). These results can also be contrasted with those for a less effective compound, clofazimine, which showed a bacteriostatic type of action with exposure for 48 h at 1 μg/ml, followed by a resurgence of growth after day 4 (8), and with a slower bactericidal action shown by rifabutin with exposure for up to 3 to 4 days; complete elimination of the counts resulted at 21 and 13 days, respectively (12).

Dynamic studies with the in vitro model man (4) demonstrated bactericidal action with complete elimination of the counts by day 7, a time similar to that obtained with continuous exposure. Thus, in situations simulating pharmacodynamic drug disposition by humans and animals, amikacin causes rapid elimination of the CFU counts by 1 week.

These investigations, which are a natural extension of the conventional in vitro studies, confirm the antimonycobacterial potential of amikacin against M. avium complex. These findings are of value and form a bridge to the in vivo and clinical studies. However, the chemotherapeutic activity of amikacin has to be established in animal and macrophage models before it is subjected to clinical trials.

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LITERATURE CITED