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New Approach for the Evaluation of Antimycobacterial Drug Combinations In Vitro (the Laboratory Model Man)

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An attempt was made to study quantitatively the antimicrobial effect of combinations of commercially available antituberculosis drugs and antibiotics on the growth of multiple drug-resistant strains of *Mycobacterium intracellulare* under simulated in vivo conditions. Combinations of erythromycin, isoniazid, methenamine, or oxacillin eliminated populations of *M. intracellulare* when drug combinations in concentrations achievable in man were kept in contact with the organism for 10 hr daily. Although combinations of INH and rifampin failed to eliminate populations of *M. intracellulare*, this pair seemed to be the most effective two-drug combination available. The requirement for successful treatment of drug-resistant mycobacteriosis is the selection of an effective drug regimen and the maintenance of combined action of all drugs in the serum for approximately 10 hr daily. An in vitro model is described which enables the bacteriologist to design an effective combination of drugs and to measure its efficiency under simulated in vivo conditions.

The judicious use of effective multiple drug regimens, coupled with improved laboratory methods for monitoring the patients' response to chemotherapy, is resulting in the steady reduction of cases of drug-susceptible *Mycobacterium tuberculosis* in this country. On the other hand, routine drug susceptibility tests are of little value in the treatment of patients infected with multiple drug-resistant mycobacteria other than tubercle bacilli. In particular, strains of *M. intracellulare* (Battey bacilli) multiply in vitro and in vivo in the presence of single, commonly used antituberculosis drugs. The treatment of patients infected with such organisms often is empirical, and will continue to be so until laboratories investigate the antimycobacterial effect of various drug combinations in an effort to find a regimen which will effectively inhibit drug-resistant mycobacteria in vitro.

This study was undertaken (i) to evaluate quantitatively the bactericidal effect of certain drug combinations on *M. intracellulare* under simulated in vivo conditions, and (ii) to determine the synergistic effect of combinations of drugs which might enable a significant quantitative reduction in drug intake, thereby reducing undesirable side effects of these drugs in man.

MATERIALS AND METHODS

Investigations were performed on 30 strains of *M. intracellulare* isolated from patients in the United States. The cultures were identified and their drug susceptibility patterns were determined in this laboratory. All strains were considered clinically resistant to the commonly employed concentrations of isoniazid (INH), streptomycin, *p*-aminosalicylic acid, ethionamide, viomycin, cycloserine, kanamycin, ethambutol, and pyrazinamide (1-5).

The drugs used and the reasons for their inclusion in this study are as follows. Cycloserine, INH, and ethionamide were chosen because of their occasional antimicrobial activity against *M. intracellulare* in routine susceptibility tests performed in this laboratory. Methenamine was selected because of its unique mode of action, i.e., the release of bactericidal concentrations of formaldehyde from the molecule at pH levels below 7. The new drug rifampin was used following reports of its antituberculosis activity (10). Penicillinase-resistant oxacillin was investigated because of reported synergistic effects of INH-penicillin combinations on *M. tuberculosis* (9). Erythromycin was also studied based upon its reported synergistic effect with penicillin (3). The concentrations of each drug studied were selected in accordance with their achievable serum levels in man (6), and are listed in Table 1.

TABLE 1. *The drugs and their concentrations employed in the laboratory model man studies*

Drug	Concn investigated[a]
Cycloserine	20
Erythromycin	5, 10
Ethionamide	5
Isoniazid	1, 10
Methenamine	400
Oxacillin	1.2, 10
Rifampin	1, 5

[a] Values are expressed as microgram per milliliter.

Twenty strains of *M. intracellulare* were tested against 15 different combinations of INH, methenamine, erythromycin, and oxacillin incorporated in 7H-10 agar. Sixteen strains were tested in 7H-10 agar against (i) four quantitatively different combinations of INH and rifampin; (ii) 1 and 5 μg of rifampin per ml alone; (iii) twelve different combinations of INH with rifampin and either ethionamide, ethambutol, or cycloserine. The various drug-containing media and drug-free controls were dispensed in 5-ml amounts into the quadrants of sectioned, plastic, petri dishes. The percentages of drug-resistant colonies were determined according to the proportion method of Canetti and co-workers (1). The antimicrobial actions of 10 different two- and three-drug combinations of INH, methenamine, erythromycin, and oxacillin, as well as the effect of four different combinations of 1 to 5 μg/ml of both rifampin and INH were studied in a model apparatus ("Laboratory Model Man") similar to that of Gangadharam and co-workers (2). The lower fifth of a plastic screw-capped test tube was cut off and a cellophane dialysis bag (pore size, 48 A units) was fastened to the open end of the screw-top portion of the tube. The plastic tube and attached dialysis tubing were sterilized in ethylene oxide gas and then introduced into a 250-ml Erlenmeyer flask containing 195 ml of 7H-9 broth, with or without the drug combinations. The rate of dialysis of the drugs was determined by biological and chemical assay methods prior to the experiments with living cultures of *M. intracellulare*. The levels of INH and ethionamide in the Erlenmeyer flask and in the dialysis bag were quantitatively determined every other hour for a period of 24 hr by the Vertical Diffusion method using H37Rv as the test strain (8). The levels of cycloserine, erythromycin, oxacillin, and rifampin were determined by the cylinder method of the Food and Drug Administration; a drug-susceptible strain of *Staphylococcus aureus* was used as test strain on regular nutrient agar (6). The levels of methenamine were determined by the quantitative chemical test according to the method of Knight and co-workers (4). The simulated daily absorption and excretion of four drugs in the Laboratory Model Man is shown in Fig. 1. Isoniazid entered into the dialysis tubing within 4 hr, while erythromycin required 6 hr to equalize the concentration of the drug between the dialysis tubing and the surrounding broth reservoir (Fig. 1). The rate of dialysis of the other drugs ranged between 4 to 6 hr, depending on molecular size. The time required for dialysis approximated the absorption rates in vivo (6).

A 5 ml-amount of 2- to 5-day-old 7H-9 liquid culture of the strain of *M. intracellulare* being tested was pipetted into each dialysis bag. A sample of each culture suspension also was plated on 7H-10 agar to determine the initial bacterial population. The Laboratory Model Man cultures were incubated for a maximum of 10 days at 37 C without carbon dioxide. When the dialysis bags containing suspensions of *M. intracellulare* were placed alternately into drug-containing and drug-free 7H-9 broth, it was possible to simulate the absorption and excretion of drugs in man. On each of the 10 days of incubation, the growing organisms were exposed for a predetermined time to freshly prepared drug solutions. The passage of drugs from the Erlenmeyer flask "reservoir" inte the culture-containing dialysis sac simulated the in vivo absorption of drugs. Peak levels of drugs were maintained for 2 to 10 hr (depending upon the experiment) and then dialysis sacs were transferred to flasks of drug-free 7H-9 broth, thereby simulating excretion of drugs from patients' tissue and serum. In order to determine quantitatively the antimicrobial effect of each drug combination, samples of the cultures were plated onto 7H-10 medium every other day following the time of complete drug "excretion." After 2 weeks of incubation at 37 C in an atmosphere of 10% CO_2, the numbers of surviving organisms were calculated by multiplying the number of colonies on 7H-10 plates by the dilution factor. Samples from an untreated control Model Man were also taken to determine the logarithmic increase in the number of organisms.

Bacilli which survived the 10-day treatment with any two- or three-drug combinations were tested again on 7H-10 agar medium against the same drug concentrations. This was to determine the rate of drug resistance resulting from treatment of *M. intracellulare* under simulated in vivo conditions in the Model-Man experiment.

RESULTS AND DISCUSSION

Studies on the susceptibility of *M. intracellulare* to single drugs or multiple combinations of

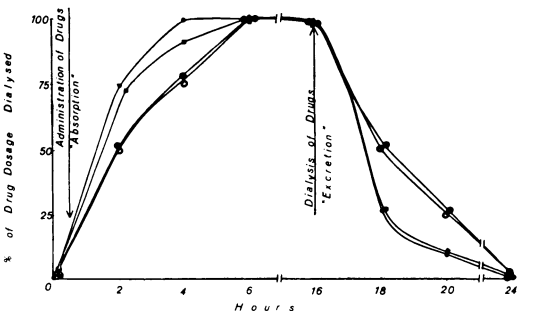

FIG. 1. *Simulated daily absorption and excretion of drugs in the Laboratory Model Man. Symbols: ●, INH; ■, methenamine; ○, erythromycin; ●, oxacillin.*

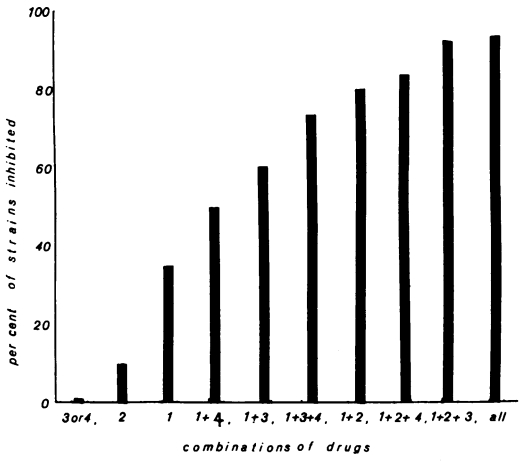

FIG. 2. *Susceptibility of M. intracellulare to combinations of methenamine, erythromycin, isoniazid, and oxacillin incorporated in 7H-10 agar. Symbols: (1) 400 μg of methenamine per ml; (2) 5 μg of erythromycin per ml; (3) 1 μg of isoniazid per ml; (4) 1.2 μg of oxacillin per ml.*

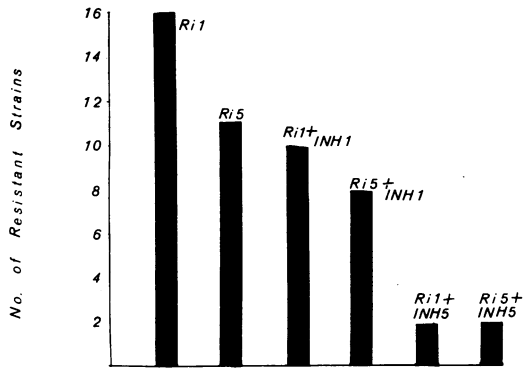

FIG. 3. *Susceptibilities of 16 strains of M. intracellulare to different combinations of rifampin and isoniazid. Symbols: Ri 1 and Ri 5, 1 and 5 μg of rifampin per ml, respectively; INH 1 and INH 5, 1 and 5 μg of INH per ml, respectively.*

INH, methenamine, erythromycin, and oxacillin in 7H-10 agar medium revealed methenamine to be the most effective single compound (Fig. 2). A methenamine concentration of 400 μg/ml in 7H-10 agar inhibited the growth of 35% (7 out of 20 strains) of the *M. intracellulare* strains studied. Addition of 1.0 μg of INH per ml to the methenamine concentration increased the percentage of strains inhibited to 60% (12 out of 20 strains). A three-drug combination of methenamine, INH, and erythromycin inhibited the growth of 95% (19 of 20) of the *M. intracellulare* strains tested. This study revealed a synergistic effect of six drug combinations out of a total of 15 tested in 7H-10 agar medium (Fig. 2). A combination of four drugs was not superior to three-drug combinations. Sixteen strains of *M. intracellulare* were tested in a similar manner against 1 and 5 μg/ml of rifampin and four different combinations of 1 or 5 μg of rifampin and INH per ml, respectively. (Fig. 3). No strains of *M. intracellulare* were inhibited by 1 μg of rifampin per ml, whereas five were totally inhibited by 5 mcg of rifampin per ml. The addition of 1 μg of INH per ml to either 1 or 5 μg of rifampin per ml decreased the number of resistant strains to 10 and 8, respectively. No differences were detectable when concentrations of either 1 or 5 μg of rifampin per ml were combined with 5 μg of INH per ml; only 2 strains of *M. intracellulare* grew in the presence of either combination. The addition of 5 μg/ml of either ethionamide or erythromycin or 20 μg of cycloserine per ml to the rifampin and INH regimen did not prevent these two strains from growing on 7H-10 agar medium.

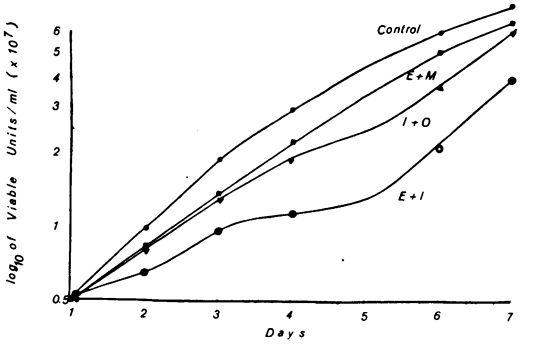

FIG. 4. *Antimicrobial effect of combinations of two drugs on the growth of M. intracellulare in the Laboratory Model Man. Symbols: I, INH; E, erythromycin; O, oxacillin; M, methenamine.*

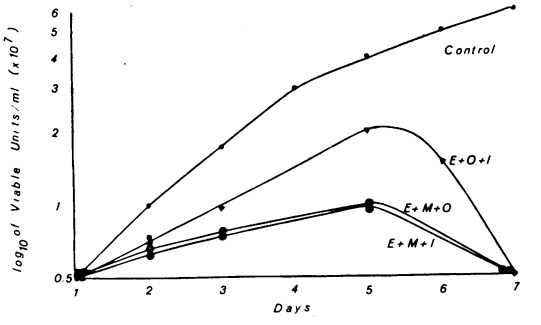

FIG. 5. *Antimicrobial effect of combinations of three drugs on the growth of M. intracellulare in the Laboratory Model Man. Symbols: I, INH; E, erythromycin; O, oxacillin; M, methenamine.*

10 hr and after increasing the INH, oxacillin, and erythromycin dosages each to 10 μg/ml. The methenamine concentration of 400 μg/ml was maintained throughout the experiments. Under these conditions, a population of 500,000 organisms was eliminated or reduced to 10^0 within 10 days. This was achieved by using high dosages of drugs for the first 5 days and reverting to the lower dosages of the previous experiments for the next 5 days. The total time of exposure to drugs during the 10-day period, whether high or low dosage was employed, was 10 hr per day. The treatment with antituberculous drugs was discontinued after 10 days in experiments using both bacteriostatic and bactericidal combinations of drugs. The experiment using bacteriostatic drug combinations demonstrated an increase of viable units during a 7-day follow-up (simulating the absconding of the positive patient from the hospital), whereas the experiment using bactericidal combinations only occasionally yielded surviving organisms during the 7-day period (Fig. 6). This follow-up simulated the discharge of the cured patient. Single surviving organisms of *M. intracellulare* were resistant to the same drug combination when tested again on 7H-10 agar medium. The mutation rate of *M. intracellulare* for the combination of INH, erythromycin, and either methenamine or oxacillin seems to be in the range of 1 in 10^6–10^7, which approximates the mutation rate of *M. tuberculosis* for a single major drug (7). Optimal results were obtained when a population of *M. intracellulare* was exposed for 24 hr per day to low concentrations of three drugs—a situation achieved by the usual drug susceptibility tests, but certainly not attained in tuberculous patients. A population of 10^5 organisms was reduced to one or two viable units following

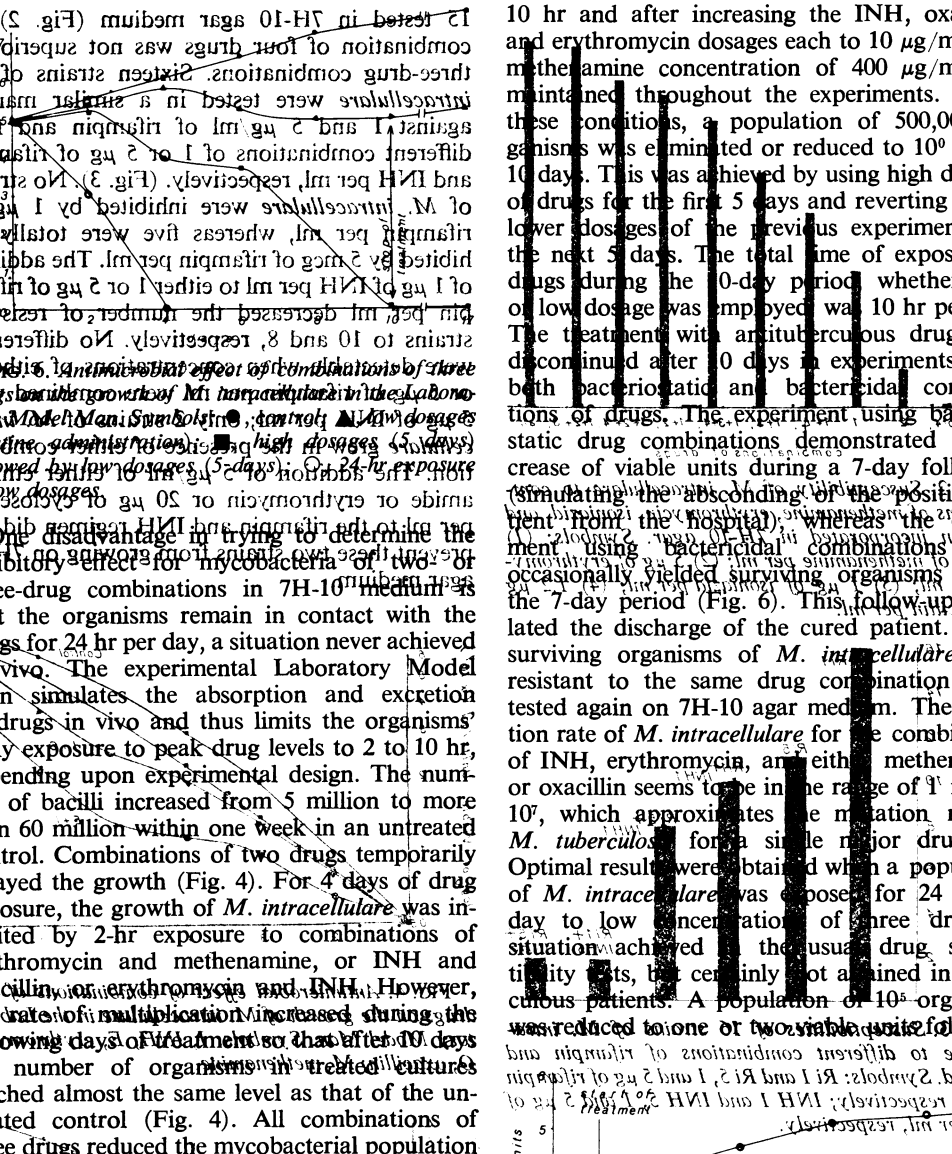

FIG. 6. *Antimicrobial effect of combinations of three drugs on the growth of M. intracellulare in the Laboratory Model Man System. ●, control; ▲, low dosages (routine administration); ■, high dosages (5 days) followed by low dosages (5 days); ○, 24-hr exposure to low dosages.*

One disadvantage in trying to determine the inhibitory effect for mycobacteria of two- or three-drug combinations in 7H-10 medium is that the organisms remain in contact with the drugs for 24 hr per day, a situation never achieved in vivo. The experimental Laboratory Model Man simulates the absorption and excretion of drugs in vivo and thus limits the organisms' daily exposure to peak drug levels to 2 to 10 hr, depending upon experimental design. The number of bacilli increased from 5 million to more than 60 million within one week in an untreated control. Combinations of two drugs temporarily delayed the growth (Fig. 4). For 4 days of drug exposure, the growth of *M. intracellulare* was inhibited by 2-hr exposure to combinations of erythromycin and methenamine, or INH and oxacillin, or erythromycin and INH. However, the rate of multiplication increased during the following days of treatment so that after 10 days the number of organisms in treated cultures reached almost the same level as that of the untreated control (Fig. 4). All combinations of three drugs reduced the mycobacterial population within one week to the original number in the starting inoculum, despite an initial logarithmic multiplication which occurred a few days after onset of "treatment." None of the drug combinations proved to be bactericidal, but all combinations containing 5 μg of erythromycin per ml proved to be bacteriostatic (Fig. 5).

Three-drug combinations containing erythromycin, INH, and either oxacillin or methenamine are shown in Fig.6. The number of viable units in the control increased from 10^5 to more than 10^7 within 10 days. A bactericidal effect of combinations of three drugs was obtained both after prolongation of the daily drug exposure from 2 to

4 days of constant drug exposure (Fig. 6). Experiments using INH, methenamine, erythromycin, and oxacillin in one combination, either incorporated in 7H-10 agar or in the Laboratory Model Man, did not enhance the antimycobacterial effect of the three-drug combinations (Fig. 2).

Figure 7 demonstrates the antimicrobial effect of combinations of rifampin and INH on *M. intracellulare* in the Laboratory Model Man. The number of organisms increased in an untreated "control" from less than 1,000 to more than 10^5 within 10 days of incubation. The combination of 5 μg of rifampin per ml plus 1 μg of INH per ml acted bacteriostatically since the number of organisms dropped to the initial inoculum size after a logarithmical multiplication occurred during the first 3 days of treatment (Fig. 7). Combinations of 1 to 5 μg of rifampin per ml plus 5 μg of INH per ml reduced the population of *M. intracellulare* to 10 to 100 organisms after an initial increase to 10^4 was observed. However, between the 8th and 10th days of treatment, increasing colony counts were again recorded, which indicated the emergence and multiplication of rifampin- and INH-resistant mutants. This was confirmed by drug susceptibility tests in 7H-10 agar. The antimycobacterial effect of the INH-rifampin combinations was not enhanced by adding either 20 μg of cycloserine or 5 μg of either ethionamide or erythromycin per ml to the combination.

This study attempted to cope more realistically with the problems of antimycobacterial chemotherapy by employing a laboratory model simulating more closely in vivo conditions. To date, the treatment of human mycobacterioses caused by *M. intracellulare* has been poor. Some attempt should be made to treat human infections with *M. intracellulare* in a more rational manner by employing drug combinations designed in the laboratory. The most important requirement for the elimination of multiple drug-resistant organisms is the combined action for a certain length of time each day of appropriate concentrations of previously determined effective drug combinations. The Laboratory Model Man represents an alternative to the stereotyped and empirical treatment of drug-resistant cases of human mycobacterioses by taking into account the particular drug susceptibility pattern of each acid-fast isolate, and by determining in the patient the achievable superimposed peak levels of all drugs employed in the regimen.

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