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A Bone Marrow-Derived Murine Macrophage Model for Evaluating Efficacy of Antimycobacterial Drugs under Relevant Physiological Conditions

PAMELA S. SKINNER,[1]* SYNTHIA K. FURNEY,[1] MICHAEL R. JACOBS,[2] GILLES KLOPMAN,[3] JERROLD J. ELLNER,[4] AND IAN M. ORME[1]

Mycobacteria Research Laboratories, Department of Microbiology, Colorado State University, Fort Collins, Colorado 80523,[1] and Departments of Pathology,[2] Chemistry,[3] and Medicine,[4] Case Western Reserve University, Cleveland, Ohio 44106

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Even though the macrophage is the host cell for the intracellular bacterial parasite *Mycobacterium avium*, macrophages have undergone only limited evaluation as models for determining the capacities of antimycobacterial drugs to inhibit the growth of *M. avium* within this relevant intracellular environment. In the present study, we demonstrated that a panel of *M. avium* isolates could actively infect homogeneous monolayers of murine bone marrow-derived macrophages. A number of established and experimental antimycobacterial drugs were then added to these cultures at a range of concentrations, and their effects on the numbers of surviving bacilli were determined 8 days later. By plotting such numbers versus drug concentrations it was then possible to clearly distinguish between compounds with bactericidal activity (such as rifabutin and PD 125354) and those with bacteriostatic effects (such as clarithromycin), even though several of these compounds had very similar MICs. In addition, an estimate of the potential therapeutic efficiency of each drug could be made by determining the concentration needed to destroy an arbitrary percentage of the inoculum (in this case, the bactericidal concentration destroying 99% of the inoculum). Such values were considerably in excess of the MICs and may more realistically reflect the concentrations in serum required to effectively reduce the bacterial burden in vivo.

The incidence of infection and the extent of disease caused by *Mycobacterium avium* in AIDS patients are well documented (3, 19, 20, 26, 45). The most urgent problem associated with *M. avium* infections in these patients is the lack of definition of a standard optimal antimicrobial therapy. Despite this, however, it is clear that prolonged survival is associated with antimycobacterial treatment, with an average life extension of 8 months (6, 18).

Existing treatment regimens are primarily based on early results in the field in which multiple clinical isolates were tested for their in vitro susceptibilities to a wide variety of drugs, with the two most widely used methods being the broth microdilution method (43) and the radiometric BACTEC method (15, 21). In both of these methods, the antimicrobial agent is added directly to broth cultures, and hence is in direct contact with the bacilli. Under these conditions a minimal concentration of drug that inhibits the growth of the bacterial culture (the MIC) can be determined.

In this regard, however, some clinicians have voiced the concern that the MICs determined by these susceptibility tests may sometimes be of limited value because of their lack of correlation with the eventual clinical outcome in the patient (41). One possible explanation for this lack of correlation could be that tests based on direct exposure of the bacteria to drugs may not accurately reflect events in the host cell containing intracellular bacterial parasites. In fact, many potential factors could affect the activity of the drug during its interactions with the infected host cell and the intracellular bacteria that are not reflected in a broth culture assay. For instance, transport mechanisms in the cell may influence the intracellular drug concentration independently of the external fluid concentration; in this regard some drugs, such as macrolides and rifamycins, are known to concentrate well within macrophages (5, 35), while other drugs, such as aminoglycosides and β-lactams, (13) may be excluded. Second, the drug may be subject to chemical modification as it passes through the macrophage cytoplasm and into the phagosomal lumen. Finally, the local pH may affect the activity of the drug; for example, clarithromycin is more effective as the pH rises toward neutrality (39). This last observation is of interest, given the observation of Crowle and colleagues (8) that, contrary to earlier dogma, the pH of the mycobacterial phagosome is neutral rather than acidic, a finding that has recently been confirmed (38) and that has been shown to relate directly to the lack of ATPase-dependent proton pump formation on the phagosomal membrane harboring *M. avium*.

Given these considerations, we developed a screening assay for new antimycobacterial drugs based on the use of bone marrow-derived macrophages. In the study described here we compared the data obtained by use of this assay with data obtained by use of a conventional broth susceptibility test with five new experimental fluoroquinolones and three established agents, clarithromycin, ethambutol, and rifabutin. The results of the study showed that three distinct patterns of drug activity can be observed in the macrophage assay, enabling a clear distinction between compounds with bacteriostatic or bactericidal capacity, a distinction that was not possible on the basis of MICs alone.

MATERIALS AND METHODS

Bacterial isolates. *M. avium* LR114F and LR114R are transparent and smooth-domed variants, respectively, of a clinical isolate originally obtained from J. Crawford, Centers for Disease Control and Prevention, Atlanta, Ga. Strain 101, a clinical isolate that is highly virulent in animal models, was

* Corresponding author. Mailing address: Department of Microbiology, Colorado State University, Fort Collins, CO 80523. Phone: (303) 491-6587. Fax: (303) 491-1815.

kindly provided by Lowell Young, Kuzell Institute, San Francisco, Calif., *M. avium* serovar 2 strain 2-151 was kindly provided by Alfred Crowle, University of Colorado. Morphological variants of strain 2-151 were cloned by John Belisle, Mycobacteria Research Laboratories, Colorado State University; these included a variety of "rough" morphologic types that differed by visual inspection. All bacteria were grown to the mid-log phase in Proskauer Beck medium and were frozen at −70°C until they were needed.

Drugs. Five experimental fluoroquinolones (PD 135144, PD 143289, PD 125354, PD 119421, and PD 131575) were obtained from Parke Davis Pharmaceutical Research, Ann Arbor, Mich. (their structures are shown in references 22 and 23). Clarithromycin was obtained from Abbott Laboratories, Abbott Park, Ill.; ethambutol was from Lederle Laboratories, Pearl River, N.Y.; and rifabutin was from Pharmacia Adria, Dublin, Ohio.

MIC determinations. The MICs of each test compound were determined by a conventional method in which the compound was serially diluted in 7H9 broth in 96-well plates to which 10^5 bacteria were then added (43). Plates were incubated for 14 days and were examined for growth; the MIC was defined as the lowest concentration of drug at which no bacterial growth could be visually discerned.

Bone marrow-derived macrophage cultures. Bone marrow-derived macrophages were obtained from specific-pathogen-free C57BL/6 female mice purchased from Jackson Laboratories, Bar Harbor, Maine. Mice were euthanized by exposure to CO_2, and the 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 (note that this medium contains colony-stimulating factors, but at concentrations much lower than those needed to influence the growth of *M. avium* [1])–HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer–nonessential amino acids–L-glutamine–antibiotics by using a 26-gauge needle. Cell suspensions were then washed twice and were plated in 24-well tissue culture plates at a concentration of 10^6 cells per well in supplemented DMEM. The 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* 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 antibiotic-free DMEM containing the indicated concentrations (usually up to 8 μg/ml in the case of active compounds and 32 μg/ml in the case of less active compounds) of the compound being tested. Each concentration of drug was tested in triplicate wells. Control wells contained 1.0 ml of antibiotic-free DMEM. Wells were periodically observed under a microscope to check for cell viability or detachment (none of the study compounds except clarithromycin had any discernible toxic effects; clarithromycin caused a loss of cell viability when it was used at 32.0 μg/ml). After 8 days of incubation at 37°C in 5% CO_2, each well was gently washed and the monolayers were then lysed by using 0.1% saponin (Sigma, St. Louis, Mo.) dissolved in sterile water. The lysates were serially diluted in sterile saline and were plated onto 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 define antimicrobial activity. The equation of this line was used to calculate the bactericidal concentration of drug that gave a 2-log-unit (99%) reduction in bacterial numbers (BC99). In many cases in which the drug was only poorly effective, this value often fell well above the highest datum point, and hence is an extrapolation.

As we have observed previously (14), in general smooth-transparent (SmT) isolates grew 0.5 to 1.0 log units over the 8-day culture period (data not shown). In this situation, drugs could be categorized as bacteriostatic if they prevented growth over the initial inoculum value. In the case of smooth-domed (SmD) or rough (Rg) isolates, which grew only poorly or not at all in the cultures, it could not be discerned whether a drug was truly bacteriostatic or just ineffective.

RESULTS

Productive infection of macrophages with *M. avium*. As shown in Fig. 1, after 9 to 10 days of incubation cultures of macrophages consisted of a homogeneous monolayer of viable healthy cells (a few granulocyte-like cells could occasionally be seen; this contaminant was <1% of all cells). A few days after infection with virulent *M. avium* (the highly virulent SmT form of strain 2-151 is the example shown) the plasmalemmas of the macrophages showed characteristic spreading and extension. Note the absence of any extracellular bacteria (Fig. 1).

Growth of *M. avium* isolates in the presence of drugs. The capacity of each test compound to inhibit the growth of the *M. avium* isolates within macrophages over the 8-day culture period was then tested. When bacterial colony numbers were plotted against drug concentrations, three distinct patterns of activity were observed. These are illustrated with specific experimental examples in Fig. 2.

The first pattern of activity, designated pattern A, is representative of bactericidal drugs that gave rise to a dose-dependent reduction in bacterial numbers in a linear fashion, resulting in very few bacilli surviving in the macrophage culture monolayer. A second type of curve, designated pattern B, was also seen periodically and consisted of a linear reduction in bacterial numbers at low drug concentrations, which then gave rise to a plateau effect in which further bacterial clearance ceased. A third pattern, pattern C, was representative of drugs which were bacteriostatic (in the sense that they prevented the growth of virulent isolates [usually of the SmT type] in control cultures over the 8-day culture period) or were essentially ineffective (not influencing bacterial numbers when the isolate [usually of the SmD or Rg variety] did not grow appreciably over this time).

The types of patterns seen with each drug and each isolate are shown in Table 1, in which they are compared with MIC results. It can be seen that the activities of each of the experimental fluoroquinolones and rifabutin generally tended to be effective in reducing bacterial numbers, falling mostly into the A or B categories. The activity of the drug clarithromycin fell generally into the B or C category, while the activity of ethambutol was mostly of the C type.

Looking from the other perspective, test results for all except one of the isolates tested (isolate Rg2) recorded at least two compounds with A type patterns, indicating that a substantial reduction in bacterial numbers could be achieved if the correct compounds were chosen. Also note, however, that the types of graphical patterns recorded had no obvious correlations with the MIC for each isolate.

Comparison of MICs with effective drug concentrations observed in macrophage assay. The MIC data were then also

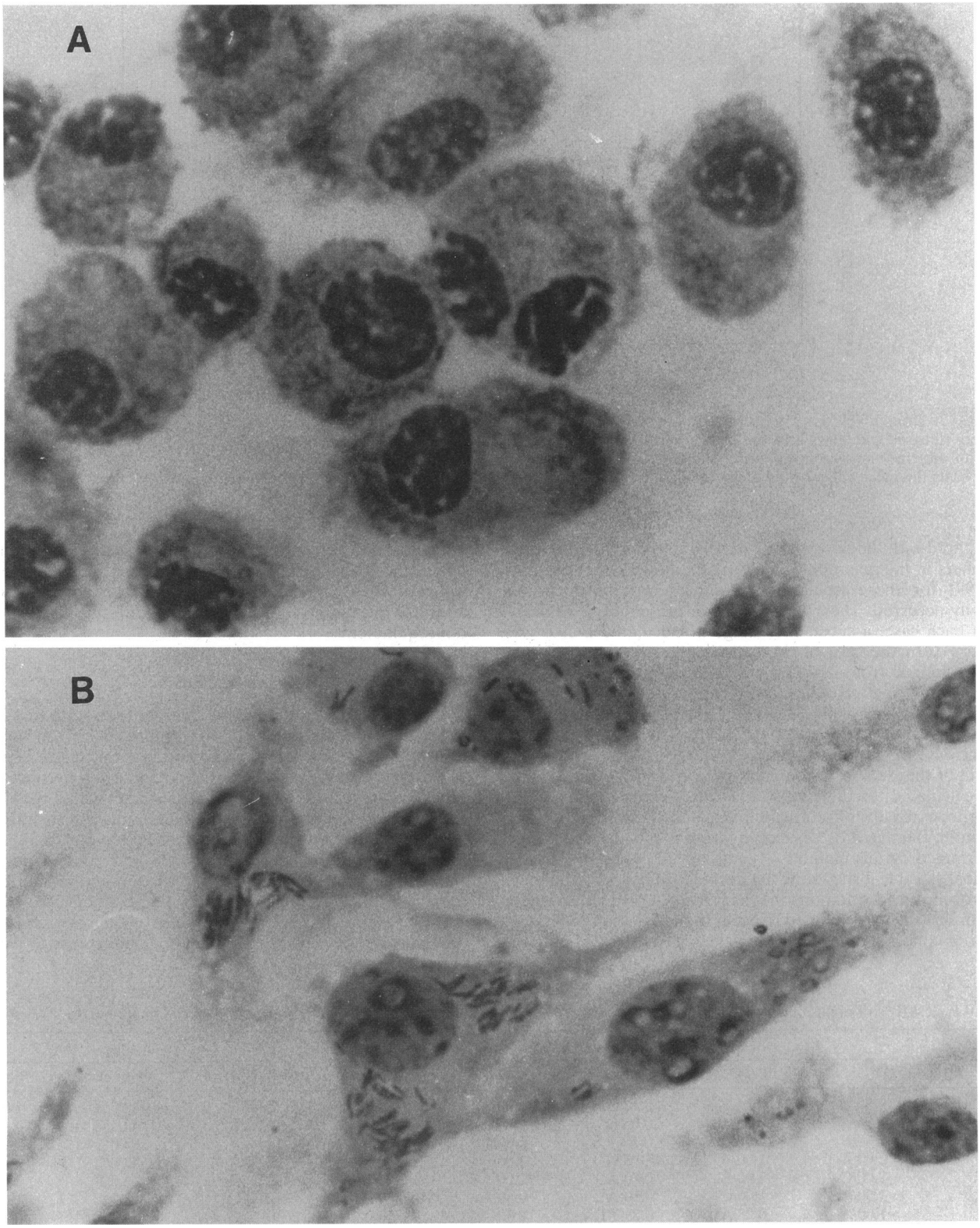

FIG. 1. Photomicrograph of a homogeneous monolayer of uninfected macrophages (A) and macrophages 8 days after infection with *M. avium* 2-151 SmT (B). Note the absence of any extracellular bacteria. Magnification, ×1,000.

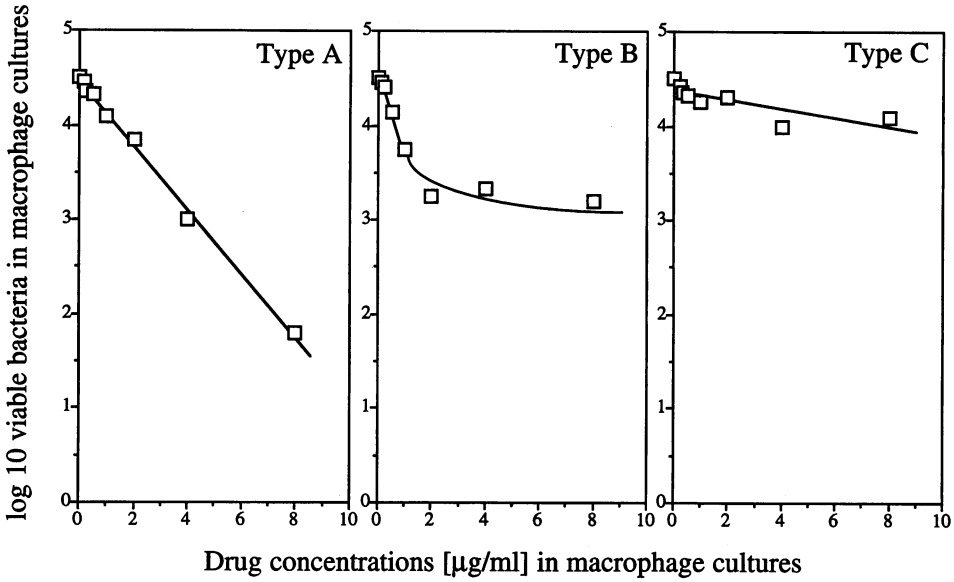

FIG. 2. Drug inhibition patterns observed in the macrophage model. Three types of curves were obtained; type A represents a bactericidal pattern (the example shown is inhibition of strain 2-151 SmT by PD 125354), type B was characterized by initial inhibition and then a plateau effect (clarithromycin versus strain LR114F), and type C represents a bacteriostatic pattern (ethambutol versus strain 2-151 Rg3). Each datum point represents the mean value of three determinations; standard errors of the mean are not shown, but they did not exceed 0.4.

compared with the dose of drug needed to reduce the bacterial numbers in the macrophage cultures by a factor of 2 log units (BC99) for those isolate-drug combinations in which this activity occurred. These latter data are presented in Table 2.

In each case, a substantial range in calculated BC99s was observed. In the case of the experimental fluoroquinolones, BC99s ranged from 4.5 μg/ml to 25.8 μg/ml on the basis of curve-fitting analysis. Of these compounds, PD 125354 had very good activity, with BC99s of 4 to 9 μg/ml inhibiting the three highly virulent isolates (LR114F, 101, and 2-151 SmT). In the case of clarithromycin, even though it had good MICs for all of the isolates tested, an estimated BC99 of 24.3 μg/ml was calculated for the single isolate in which some degree of inhibitory activity was observed (isolate 2-151 Rg2). As for rifabutin, estimated BC99s ranging from 5.4 to 12.4 μg/ml were determined by this method.

Finally, Table 3 shows the log decrease in bacterial numbers that occurred in situations in which the type B plateau effect was observed. These tended to occur in the 2- to 8-μg/ml range of drug concentrations; at these levels in most cases about a 1-log-unit decrease was seen; this represented a 90% destruction of the inoculum. In a few cases, however, the plateau effect was seen when larger reductions (1.7 to 2.8 log units) occurred; each of these reductions was seen with the experimental fluoroquinolones.

DISCUSSION

The results of the study described here illustrate that bone marrow-derived macrophages from inbred mice can be productively infected with *M. avium* and that this culture system can be used to test the capacities of potential antimycobacterial drugs to inhibit the growth of clinical isolates within these host cells under relevant physiological conditions. As such, therefore, this model may prove to be a useful method in aiding in the identification of new compounds and combinations of compounds that may potentially be used in antimycobacterial therapy prior to their evaluation in in vivo studies.

Despite their physiological relevance as the parasitized host cell, macrophage models have seen only limited use to date in

TABLE 1. MICs of eight antimycobacterial agents for 10 *M. avium* strains and drug activity patterns determined in macrophage assay system

M. avium strain	MIC (μg/ml)/drug activity pattern[a]							
	PD 135144	PD 143289	PD 125354	PD 119421	PD 131575	Clarithromycin	Ethambutol	Rifabutin
LR114F	0.50/B	0.25/C	0.12/A	1.00/A	0.25/A	2.00/B	4.00/B	4.00/A
LR114R	0.06/A	0.06/A	0.06/B	0.06/A	0.06/A	0.12/B	4.00/C	0.12/A
101	1.00/B	1.00/B	0.25/A	2.00/B	1.00/A	2.00/B	8.00/B	2.00/B
SmD	2.00/A	1.00/A	0.5/ND	1.00/B	1.00/C	0.12/A	4.00/C	0.06/A
SmT	2.00/C	2.00/C	0.50/A	2.00/A	2.00/B	2.00/B	16.0/B	2.00/A
Rg0	1.00/A	1.00/C	0.50/A	0.50/A	0.50/A	2.00/C	16.0/C	1.00/A
Rg1	1.00/A	0.25/A	0.25/ND	0.50/A	0.50/C	0.12/C	8.00/C	0.06/A
Rg2	2.00/B	1.00/B	0.5/ND	1.00/B	1.00/B	0.12/C	8.00/C	0.12/A
Rg3	0.50/B	0.25/A	0.25/ND	0.25/A	0.25/C	0.12/C	8.00/C	0.12/A
Rg4	0.50/A	0.50/A	0.25/ND	0.50/C	0.50/C	0.12/C	32.0/C	0.12/A

[a] A, bactericidal; B, plateau; C, bacteriostatic (see Fig. 2); ND, not determined.

TABLE 2. BC99s for those *M. avium* isolates in which bactericidal activity was observed

Drug	*M. avium* isolate	BC99 (μg/ml)
PD 135144	LR114R	9.20
	SmD	12.10
	Rg0	10.20
	Rg1	13.60
	Rg4	7.90
PD 143289	LR114R	10.70
	SmD	17.10
	Rg1	11.00
	Rg3	16.40
	Rg4	25.80
PD 125354	LR114F	4.50
	101	9.50
	SmT	6.40
	Rg0	7.80
PD 119421	LR114R	7.70
	LR114F	8.90
	SmT	8.80
	Rg0	12.20
	Rg1	9.10
	Rg3	16.10
PD 131575	LR114R	6.80
	LR114F	6.90
	101	11.30
	Rg0	7.70
Clarithromycin	SmD	14.30
Rifabutin	LR114R	9.70
	LR114F	12.40
	SmD	9.90
	SmT	7.60
	Rg0	7.70
	Rg1	5.40
	Rg2	6.00
	Rg3	8.70
	Rg4	5.40

TABLE 3. Log decrease in bacterial growth at onset of plateau effect and estimated drug concentration at plateau point for those *M. avium* isolates in which this effect was observed

Drug	*M. avium* isolate	Log decrease	Concn (μg/ml)
PD135144	LR114F	2.1	4.0
	101	0.8	2.0
	Rg2	0.9	2.0
	Rg3	1.3	4.0
PD143289	101	0.9	0.5
	Rg2	1.8	2.0
PD125354	LR114R	2.8	4.0
PD119421	101	0.9	1.0
	SmD	0.9	4.0
	Rg2	1.2	2.0
PD131575	SmT	1.7	4.0
	Rg2	0.9	2.0
Clarithromycin	LR114R	0.9	2.0
	L4114F	0.9	2.0
	101	1.2	1.0
	SmT	1.2	4.0
Ethambutol	LR114F	0.9	2.0
	101	1.1	2.0
	SmT	1.2	2.0
Rifabutin	101	0.8	2.0

studies designed to test the actions of drugs against intracellular infections with mycobacteria. A few studies with either human monocytes or the macrophage cell line J774 (7, 9, 30, 32, 41, 44) or peritoneal and alveolar macrophages (31) have been described; however, in general those studies tended to test only a single *M. avium* isolate or to test single concentrations of a compound for bacterial growth inhibition (usually either the broth MIC or the maximum concentration of drug in serum) rather than the range of doses used in the present study. For example, Mor and Heifets (27, 28) used *M. avium*-infected human monocytes and clarithromycin treatment and observed the activity of this drug at its calculated MICs and MBCs in a first study (27) and at a pulsed dose simulating the peak concentration in serum in a second study (28); in the latter study those workers observed a bacteriostatic effect, which was further confirmed by the results of the present study.

In support of the use of such models, a good correlation has previously been reported between the use of murine bone marrow-derived macrophages and the use of human monocytes for the evaluation of the bactericidal effects of therapeutic compounds (34). Moreover, studies that used different sources of macrophages (32, 44) observed that compounds which are very active in broth also tended to be active in the macrophage cultures.

In the present study, we found that by examining the capacities of serial dilutions of compounds to inhibit the growth of mycobacterial infections, the assay provided a clear distinction between agents that were bactericidal, partially active (plateau effect), or only bacteriostatic at best. Thus, although clarithromycin and rifabutin both had excellent MICs for the *M. avium* isolates tested, the bone marrow-derived macrophage assay provided evidence that the former agent usually tended to be bacteriostatic, while the latter was bactericidal. As described above, similar bacteriostatic effects of clarithromycin have been observed in both human monocytes and murine J774 cells (27).

Among the advantages of the macrophage assay is the fact that it can be used to identify the toxic effects of drugs. Thus, by examining the monolayers under a microscope (or, more stringently, by assaying for total protein in parallel cultures), one can quickly identify the agents (and their concentrations) that may have good activities in MIC assays but that have potential cytotoxic side effects. Another advantage is economy; one mouse provides approximately 10^8 bone marrow-derived macrophages, which in our hands is sufficient to test a range of concentrations of at least four compounds. In contrast, only about 10^7 macrophages can be elicited from the peritoneal cavity, and these cells are activated to some degree and tend to be of a heterogeneous nature.

As shown here, we occasionally observed a plateau effect in which only partial destruction of the inoculum occurred. Preliminary studies now strongly suggest that these residual bacilli had increased levels of resistance to the antimicrobial agent studied, and the resistance probably came about as a result of our usual practice of growing cultured isolates to

densities in excess of 10^8/ml, that is, to concentrations above the threshold frequency at which genetic mutations leading to drug resistance are probably likely to begin to occur. As can be seen in Table 3, the plateau effect was shown for several fluoroquinolone-isolate combinations, which is consistent with observations of bacterial resistance to this class of drugs (17, 25).

The data presented here show that cell-free in vitro tests tend to significantly underestimate the actual concentration that the compound must reach in tissue fluid in order to have a real therapeutic effect (arbitrarily assigned in this experimental model as the BC99). It was apparent in this regard that for some drug-isolate combinations the calculated BC99 was certainly within the range of concentrations that could be realistically achieved in a patient.

It is too early to attempt to extrapolate the data observed in the murine model described here to the clinical situation, however; and indeed, such observations must be regarded with caution given the very limited early results in clinical trials (11) that tended to indicate a more favorable response to clarithromycin opposed to that of rifabutin (the more active drug in the present study), and we hope that larger trials that include combinations of clarithromycin, rifabutin, and other compounds will provide more definitive answers (11). Despite this, however, there is already strong evidence that prophylaxic administration of rifabutin has a significant clinical benefit (29).

Hence, while we do not envision that the assay in the present study will compete at this time with other established in vitro assays, it may still prove to be a useful tool in the research laboratory as an adjunct to in vitro susceptibility testing in screening new compounds and thus allowing a distinction between bactericidal and inactive or bacteriostatic effects, as demonstrated above. When using experimental compounds, such information can then be applied to appropriate drug design computer programs so that investigators can make modifications to the structures of existing compound (24).

As for the use of the macrophage model for the identification of new compounds, the present study did identify some new fluoroquinolones, particularly PD 125354, that had bactericidal effects which were attained at BC99s that should be readily attainable in serum. In a previous study this compound was identified as having excellent MICs for a large panel of *M. avium* isolates (21), and the findings of the present study now confirm that the activity of this compound is indeed bactericidal within the infected host cell.

In terms of the inhibitory effects of the experimental fluoroquinolones, one interesting observation was the consistent trend that each of these agents tended to have better activity against the rough Rg3 and Rg4 variants of *M. avium* 2-151 than against the SmD variant. These organisms differ in that the SmD isolate possesses a copious, fully glycosylated glycopeptidolipid (GPL) coat which may potentially act as a drug barrier (12, 33); in contrast, the Rg3 and Rg4 morphotypes lack the GPL molecule (2). These data indicate, therefore, at least in a circumstantial manner, that the GPL layer may indeed act as a drug barrier by reducing the level of entry of the fluoroquinolones. We are examining the possibility that cycloserine, a drug that inhibits the racemase that converts L-alanine to the D-alanine present in the GPL peptide, may augment the activities of these fluoroquinolones by preventing GPL synthesis.

The data presented here also continue to illustrate the variability in drug activity not only from isolate to isolate but also within the different colony morphology variants that can be distinguished within the isolate. In the present study, as in other studies (36, 40), it was found that the SmT variant (2-151 SmT, LR114F, 101) generally tended to be more resistant to drugs in the broth assay, and this information is extended in the present study to show that the SmT isolates were often more resistant in the macrophage assay to drugs active against other morphological variants. Similar observations have been made with human monocytes (10, 37).

Finally, in addition to the above information, the macrophage model should also be applicable to testing combinations of drugs; for example, we have observed (37a) a predicted synergism between rifabutin and ethambutol in the macrophage model, as was seen previously in cell-free assays (16, 42, 44). Moreover, the model can be adapted to different physiological conditions that reflect the emergence of immunity in the infected host; for example, such macrophages are highly responsive to cytokine activation by tumor necrosis factor alpha or gamma interferon (1), and it will interesting to see if such macrophage activation (reflecting the influence of acquired immunity or, alternatively, immunotherapy in the immunocompromised and infected host) might enhance or interfere with the capacity of given drugs to inhibit mycobacterial survival. In this regard, similar experiments have been successfully conducted in human monocytes (4, 5).

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