Assessment of New Therapies for Infection Due to the *Mycobacterium avium* Complex: Appropriate Use of In Vitro and In Vivo Testing

Jacques H. Grosset

Laboratory testing is a prerequisite to predictions about the potential value of human clinical trials—the gold standard for the assessment of new therapies for infection with the *Mycobacterium avium* complex (MAC). These laboratory assessments must be made in the proper sequence, with appropriate models and methodology used to obtain data valid in determining whether clinical trials are warranted. In vitro testing permits measurements of minimal inhibitory and minimal bactericidal concentrations, identification of the synergism or antagonism of various agents, definition of an agent’s pharmacokinetic properties (e.g., hydrophilicity or lipophilicity), and evaluation of a drug’s intracellular penetration and activity against intracellular organisms. The most appropriate animal model for in vivo testing of activity against MAC is the beige mouse. Experiments in this model provide important data on an agent’s minimal effective dose and on its optimal dose, dosing frequency, and route(s) of administration. Evaluations in the beige mouse also document whether the agent is bactericidal or bacteriostatic, whether it selects drug-resistant mutants, and whether its use in combination with other agents is beneficial.

A rational approach to the development of effective therapy for infection with the *Mycobacterium avium* complex (MAC) involves a broad range of laboratory investigations whose results predict which agents are most likely to be clinically effective—and therefore to be worth testing in humans. Because of the natural resistance of MAC in vitro to almost all drugs active against the *Mycobacterium tuberculosis* complex, no possible approach should be neglected in the search for antimicrobial agents effective in the treatment of MAC infection; the identification of such drugs is especially important with regard to patients with AIDS [1].

A variety of in vitro and in vivo laboratory methods are available for testing the efficacy of new antimicrobial agents against MAC. As recently recommended by the World Health Organization Working Group on Tuberculosis Research and Development [2], these techniques should be applied in a logical sequence before any clinical evaluations are undertaken.

**In Vitro Laboratory Testing**

Determinations of MIC and MBC and evaluation of synergism. Laboratory testing of a new antimicrobial agent begins with determination of its MIC and MBC [3]. The results of these measurements should be compared with those for related compounds to determine whether the newly tested agent is more active. For example, the MICs of rifabutin (a semisynthetic ansamycin antibiotic derived from rifamidein S) and clarithromycin (a 6-O-methyl erythromycin derivative) for MAC are 5 log₂ dilutions and 3 or 4 log₂ dilutions lower than the MICs of the respective parent compounds [4] (table 1). Data on synergism and antagonism of the tested agent and other drugs with established activity should also be considered. These assessments may indicate whether it will be beneficial to combine these agents [5-7]. For example, at concentrations comparable to those obtained with the recommended therapeutic doses, ethambutol and rifampin exhibit synergism against a great majority of MAC strains [8].

Investigators conducting in vitro studies should be wary of the possibility of inappropriately discarding potentially useful agents. For example, in light of some in vitro results, clarithromycin might have been considered only marginally useful for the treatment of MAC infection. At pH 6.6 on 7H11 agar (Difco, Detroit), its MIC for MAC was 8–16 μg/mL, and pharmacokinetic studies in humans indicated that the administration of 1,000 mg twice daily produced a serum concentration of only 7.5 μg/mL [9]. However, macrolides are more effective at basic pH than at acidic pH. Thus the MIC of clarithromycin for MAC at physiological pH is 2–4 μg/mL—well below the range of achievable levels in human serum [10].

In addition to pH, the nature of the medium used for the cultivation of MAC should be taken into account. Egg-containing media are not recommended for the screening of new agents against MAC because of their high protein content and their inspissation at 85°C, which may inactivate the drug. As shown in table 2, the MIC of rifabutin for 90% of the tested strains of MAC is 2 μg/mL on 7H11 agar medium and 16 μg/mL on egg medium [11]. Likewise, Tween-containing broth is not recommended for the initial screening of agents.
agents clarithromycin and rifabutin are more active against compartment effectively. By extension, the more lipophilic against intracellular organisms. Liposome-encapsulated are strongly hydrophilic compounds, do not penetrate the cell membrane and are relatively or completely inactive agent’s pharmacokinetics in humans and its MIC for MAC gism, efficacy testing is not warranted when the MIC is against MAC is to use an in vitro culture of human mono­
cytoid macrophages [12-14]. In this cell model, macro­phages are infected with MAC on day 6 of culture. Intracel­ular bacteria are counted 60 minutes after inoculation by enumeration of acid-fast bacteria and colony-forming units (cfu). The antimicrobial drug is then added, and extracellu­lar and intracellular bacteria are counted on days 4 and 7 after inoculation. In controlled experiments [15], clarithro­mycin (4 μg/mL) and rifabutin (0.5 μg/mL) significantly slowed the intracellular replication of MAC \((P < .01)\). In contrast, a drug like amikacin, which does not reach high intracellular concentrations because of its strong hydrophilic­ity, cannot kill or inhibit MAC in this system [13].

Relation of pharmacokinetics to MIC. If in vitro laboratory data are promising, then the relation between the agent’s pharmacokinetics in humans and its MIC for MAC should be evaluated. Unless there is reason to suspect syner­gism, efficacy testing is not warranted when the MIC is higher than the agent’s peak serum level, especially if the drug exhibits poor intracellular penetration. If the MIC for MAC is below the peak level in human serum and the agent penetrates the intracellular compartment effectively, in vivo assessment of its efficacy—both alone and in combination with other agents active against MAC—is warranted.

### In Vivo Experimental Testing

Because of its standardization and the ease with which it can be handled, the mouse is the model of choice for studies of MAC infection in vivo. The different strains used have included immunocompetent inbred mice [16], immunodeficient beige mice [17], thymectomized mice [18], and nude mice [19]. Although the beige mouse is deficient only in natural killer cells [20] and does not mimic AIDS (in terms of specific CD4 cell–mediated immunodepression) as closely as does the thymectomized mouse or the nude \((nu/nu)\) mouse, it is most often recommended for two reasons. First, the experimental disease that follows intravenous infection of the beige mouse with MAC is multibacillary and often lethal when not actively treated [17]. Second, the beige mouse may be maintained under conventional conditions. In fact, its only disadvantage is that it is costly.

**Comparative pharmacokinetics in humans and in mice.** Data on the comparative pharmacokinetics of the study drug in humans and in the mouse model should be collected as a prerequisite to tests in mice. Comparison of the blood level curves for humans and mice forms the basis for determinations of the dosages to be studied. Among the many pharmaco­kinetic parameters that should be considered, the area under the concentration-time curve (AUC) is probably the most important in the selection of comparable doses. Because drug pharmacokinetics are related more closely to body surface area than to body weight, appropriate doses for testing in the mouse model are usually as much as 12 times higher per kilogram than those administered to humans. As shown in table 3, the AUC after oral administration of clarithromycin (50 mg/kg) to mice is comparable to the AUC after oral administration of a single 400-mg dose of clarithromycin \((i.e., 5-7\text{ mg/kg})\) to humans.

**Determination of the minimal effective dose (MED).** After appropriate doses, routes, and frequencies of drug administra-

**Table 1.** MIC for MAC: comparison of derivatives with their parent compounds.

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC range (MIC(_{90})) of indicated drug for MAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifabutin* (derivative)</td>
<td>0.5–2 (2)</td>
</tr>
<tr>
<td>Rifampin* (parent)</td>
<td>16–64 (64)</td>
</tr>
<tr>
<td>Clarithromycin(^\dagger) (derivative)</td>
<td>2–8 (4)</td>
</tr>
<tr>
<td>Erythromycin(^\dagger) (parent)</td>
<td>16–64 (64)</td>
</tr>
</tbody>
</table>

* As measured in Difco 7H11 agar [11].
† As measured in Difco 7H9 broth with Tween 80 [4].

because it has been shown to enhance antimicrobial activity. Agar media with standardized protein contents are recom­mended for screening whenever possible [2].

**Intracellular activity.** Another important consideration is the relative hydrophilicity and lipophilicity (drug partition coefficient) of the new agent. Lipophilic drugs dissolve easily in the phospholipids of the cell membrane and penetrate the macrophage, which is the predominant site of MAC multiplic­ation in patients with AIDS. The macrolides and rifamy­cins, which are lipophilic agents, are active against intracel­lular organisms because they penetrate the intracellular compartment effectively. By extension, the more lipophilic agents clarithromycin and rifabutin are more active against MAC in patients with AIDS than are erythromycin and rifampin, respectively. Conversely, aminoglycosides, which are strongly hydrophilic compounds, do not penetrate the cell membrane and are relatively or completely inactive against intracellular organisms. Liposome-encapsulated aminoglycosides may be able to cross the cell wall barrier, however, because of their lipophilicity.

The simplest way to test the intracellular activity of a drug against MAC is to use an in vitro culture of human monocyte-derived macrophages [12-14]. In this cell model, macro­phages are infected with MAC on day 6 of culture. Intracel­lular bacteria are counted 60 minutes after inoculation by enumeration of acid-fast bacteria and colony-forming units (cfu). The antimicrobial drug is then added, and extracellu­lar and intracellular bacteria are counted on days 4 and 7 after inoculation. In controlled experiments [15], clarithro­mycin (4 μg/mL) and rifabutin (0.5 μg/mL) significantly slowed the intracellular replication of MAC \((P < .01)\). In contrast, a drug like amikacin, which does not reach high intracellular concentrations because of its strong hydrophilic­ity, cannot kill or inhibit MAC in this system [13].

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### Table 2. Effect of pH and inspissation of egg medium on the MICs of drugs active against MAC.

<table>
<thead>
<tr>
<th>Medium (pH)</th>
<th>Clarithromycin</th>
<th>Rifabutin</th>
</tr>
</thead>
<tbody>
<tr>
<td>7H11 agar (6.6)</td>
<td>2–32 (16)</td>
<td>0.5–2 (2)</td>
</tr>
<tr>
<td>Egg medium (6.6)</td>
<td>4–8 (8)</td>
<td>4–16 (16)</td>
</tr>
<tr>
<td>OADC*-supplemented</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mueller-Hinton agar (7.3)</td>
<td>0.5–8 (2)</td>
<td>2 (2)</td>
</tr>
</tbody>
</table>

* Oleate-albumin-dextrase-catalase.
Table 3. Comparative pharmacokinetics of oral clarithromycin in humans and mice.

<table>
<thead>
<tr>
<th>Species, dose</th>
<th>Peak level (μg/mL)</th>
<th>AUC (mg·min/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400 mg, single dose</td>
<td>1.13</td>
<td>333</td>
</tr>
<tr>
<td>600 mg, single dose</td>
<td>2.03</td>
<td>926</td>
</tr>
<tr>
<td>500 mg bid</td>
<td>3.1</td>
<td>1,230</td>
</tr>
<tr>
<td>1,000 mg bid</td>
<td>7.5</td>
<td>3,570</td>
</tr>
<tr>
<td>2,000 mg bid</td>
<td>16.0</td>
<td>7,038</td>
</tr>
<tr>
<td>Mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 mg/kg, single dose</td>
<td>1.5</td>
<td>130</td>
</tr>
<tr>
<td>50 mg/kg, single dose</td>
<td>4.3</td>
<td>372</td>
</tr>
</tbody>
</table>

NOTE. Data are from [4, 9].

Assessment of therapy have been determined, in vivo testing of the drug can begin. The first step is to determine the MED—i.e., the minimal dose necessary to prevent death and the development of lesions in the lungs, spleen, and liver after intravenous infection with a standard MAC inoculum. For example, daily treatment for 12 weeks with clofazimine (20 mg/kg) or kanamycin (20 mg/kg), beginning 24 hours after MAC infection, prevents the multiplication of organisms and the development of lung lesions in C57Bl/6 mice. In contrast, such a course of treatment with rifabutin (10 mg/kg) or ethambutol (20 mg/kg) is only marginally active in this experimental model [16]. As shown in table 4, lung lesion scores and cfu counts are almost the same in untreated controls as in mice treated with rifabutin or ethambutol.

Assessment of bactericidal activity. If the outcome of MED determination is favorable, the efficacy of the study drug should be evaluated in mice. Therapy should be started when infection—initiated by the intravenous injection of a standard inoculum—is well established. Through enumeration of cfu in organs (primarily the spleen and the lungs), it is possible to determine whether the drug exhibits bactericidal or bacteriostatic activity, whether it selects resistant mutants when used alone, whether it prevents the selection of resistant mutants when given in combination with one or more other drugs, and whether it eradicates MAC when given for a sufficient length of time.

For example, as shown in figure 1, mice were treated six times per week with clarithromycin alone (200 mg/kg) from 2 weeks after intravenous infection with 7.9 log10 cfu of MAC organisms (by which time the total MAC population in the spleen had reached 7 log10 cfu) up to 18 weeks. The total number of cfu in the spleen decreased by ~2 log10 during the 16 weeks of treatment [21]. While drug-susceptible bacteria were killed, however, clarithromycin-resistant mutants were progressively selected. Thus, in beige mice as in humans, the key to successful treatment of disseminated MAC infection does not lie in the use of a single drug, even a drug with documented bactericidal activity against MAC.

A mouse model can also be used for comparison of the antibacterial activity of drug combinations with that of a drug used alone. As shown in figure 2, for example, cfu counts after 4 weeks of treatment were significantly lower in beige mice treated daily with a combination of clarithromycin (200 mg/kg), ethambutol (125 mg/kg), and rifabutin (10 mg/kg) than in those treated with clarithromycin alone (P < .05; author's unpublished data).

If carefully used, this animal model is a reliable predictor of clinical efficacy, although its degree of accuracy is <100%. Although studies with animals are an obligatory prerequisite, they obviously cannot replace clinical evaluations.

**Summary**

Many laboratory procedures must be employed in the development of effective new therapies for MAC infection. Relevant in vitro laboratory methods include determination of
the MIC and the MBC, evaluation of synergism, and assessment of activity against intracellular MAC organisms in macrophage culture. The beige mouse appears to be the best animal model for testing of the in vivo activity of new drugs used alone and in combination with other agents. If used appropriately, this animal model can reliably predict clinical efficacy. Studies with animals should precede—but cannot replace—clinical evaluations, which remain the gold standard for drug testing.

References