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Low-Dose Aerosol Infection Model for Testing Drugs for Efficacy against *Mycobacterium tuberculosis*

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As a paradigm for chronic infectious diseases, tuberculosis exhibits a variety of clinical presentations, ranging from primary pulmonary tuberculosis to reactivation tuberculosis and cavitary disease. To date, the animal models used in evaluating chemotherapy of tuberculosis have been high-dose intravenous models that mimic the disseminated forms of the disease. In the present study, we have used a low-dose aerosol exposure model which we feel better reflects newly diagnosed tuberculosis in patients converting to tuberculin positivity. As appropriate examples of chemotherapy, four rifamycins (rifampin, rifabutin, rifapentine, and KRM-1648) were tested, first in an in vitro murine macrophage model and then in the low-dose aerosol infection model, for their activity against *Mycobacterium tuberculosis*. In both models, KRM-1648 had the highest level of activity of the four compounds. In the infected-lung model, rifabutin, rifapentine, and KRM-1648 all had sterilizing activity when given orally at 5 mg/kg of body weight per day. When given at 2.5 mg/kg/day, KRM-1648 had the highest level of activity of the four drugs, reducing the bacterial load by 2.7 logs over 35 days of therapy.

Disease caused by *Mycobacterium tuberculosis* continues to present a major global problem, with approximately 10 million new cases and close to 3 million deaths occurring each year (9, 30). This problem is further compounded by the increasing incidence of drug-resistant strains of *M. tuberculosis*, thus putting an increasing demand on the development of new compounds with which to treat the disease caused by this organism (1, 19).

Such compounds are first tested in animal models, predominantly in the mouse model. To date, most models have consisted of giving high (or even lethal) intravenous inocula of the H37Rv or Erdman strains of *M. tuberculosis* (50% lethal dose by this route, approximately 5×10^6 for both strains). If delivered properly as a single-cell suspension, over 99% of the inoculum is taken up by macrophages in the spleen and liver, thus mimicking clinical situations of immunodeficiency in which the infection has been allowed to widely disseminate from the lungs. While this is a reasonable model, it can be argued that it differs from primary pulmonary tuberculosis which begins with the inhalation of very small numbers of bacilli, the progressive growth of which is at least partially contained in the lungs by acquired immunity, concomitant with the conversion of the patient to tuberculin positivity (10). Under these conditions, reactivation tuberculosis can occur, sometimes leading to cavitation of the infectious lesions, if the patient is left untreated. Reactivation tuberculosis can be modelled in the mouse (21), whereas cavitation cannot. In fact, rabbits are the only rodents known to rapidly develop cavities following aerosol exposure, but this model is of course prohibitively expensive for routine chemotherapy evaluation.

To mimic newly diagnosed tuberculosis in which the patient has recently converted to tuberculin positivity, we present here a new model in which the lungs of mice are directly exposed to a low-dose aerosol of *M. tuberculosis*. The resulting infection grows progressively for approximately 3 weeks while the animals are in the process of generating protective immunity and a positive delayed-type hypersensitivity reaction. Since the latter event would prompt the initiation of chemotherapy, we took as examples rifamycins known to be active against *M. tuberculosis* in vitro and in other animal models. In addition to testing rifampin, we tested rifabutin (11, 14, 17, 22, 24–26, 28), rifapentine (2, 5–8, 12, 15), and a newly described compound, KRM-1648, which has recently been shown to have excellent activity against *M. tuberculosis* (13, 16, 18). In addition, we tested the three "newer" rifamycins with rifampin in a murine infection macrophage model, in which we arbitrarily compared the compounds in terms of the concentration of the drug needed to eliminate 99% of the bacterial load from the host cells (i.e., the 99% bactericidal concentration [BC_{99}]) (28). The data obtained further confirm previous observations of the high-level activity of compound KRM-1648.

MATERIALS AND METHODS

Mice. For the experiments, we used 6- to 8-week-old female specific-pathogen-free C57BL/6 mice purchased from the Charles River Laboratory (Wilmington, Mass.).

Bacteria. The virulent *M. tuberculosis* strains Erdman (TMCC 107) and CSU22 were grown to mid-log phase in Proskauer-Beck medium containing 0.01% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) and stored in ampoules frozen at −70°C until use. CSU22 is a multidrug-resistant strain resistant to rifampin.

Test compounds. KRM-1648 was provided by Kaneka Corp., Osaka, Japan; rifampin was provided by Sigma Chemical Co.; rifapentine was provided by Hoechst Marion Roussel, Inc., Cincinnati, Ohio; and rifabutin was provided by Pharmacia Adria, Columbus, Ohio.

Each drug was initially dissolved in a small volume of ethanol and then further dissolved in 0.05% methyl cellulose and 0.04% Tween 80 prior to oral gavage or was dissolved in tissue culture medium lacking 2-mercaptoethanol, antibiotics, and antimycotics for macrophage infection studies.

MIC determinations. MICs were determined by serially diluting each test compound in 7H9 broth in 96-well plates. An inoculum of 10^5 bacteria was added to each well. The plates were incubated for a minimum of 21 days and then examined for bacterial growth. The MIC was defined as the lowest concentration of drug at which no visible bacterial growth could be seen. The MICs determined for the Erdman strain were 0.3 μg of rifampin per ml, 3 μg of rifabutin per ml, 0.6 μg of rifapentine per ml, and 0.15 μg of KRM-1648 per ml. For strain CSU 22, MICs of all four compounds were >5.0 μg/ml.

Media and reagents. Bone marrow-derived macrophages were cultured in Dulbecco's minimal essential medium containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μg of streptomycin per ml,

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250 ng of amphotericin B per ml, and minimal essential medium nonessential amino acids supplemented with 10% heat-inactivated, low-endotoxin fetal calf serum (Summit Biotechnologies, Inc., Fort Collins, Colo.) and 10% L-929 fibroblast-conditioned medium. Macrophage monolayers were grown and infected with *M. tuberculosis* in the presence of decreasing concentrations of drugs as previously described (28). Bacterial colony formation was assessed 15 to 20 days following lysis and plating of the monolayers (28). The data were expressed as the log 10 value of the mean number of bacteria recovered for each macrophage lysate ($n = 3$). These data were then plotted against drug concentrations to determine the level needed to reduce the bacterial numbers by 2 logs (the BC_{99}, as previously described [28]).

In vivo infection. Mice were aerogenically infected with *M. tuberculosis* Erdman prior to drug treatments. The animals were placed in the exposure chamber of a Middlebrook Aerosol Generation Device (Glas-col Inc., Terre Haute, Ind.). With this device, compressed air is pumped into the chamber through a venturi nebulizer, in which we placed 10 ml of a suspension of bacteria at a concentration of 10^5/ml. It takes approximately 30 min to drain the nebulizer, during which time the mice within the chamber inhale approximately 50 to 100 bacilli into their bronchial tree and alveolar spaces. In our experience with this model over the past 10 years, we have found the inoculum size/tissue uptake algorithm to be highly reproducible, with a uniform uptake by the exposed animals. The course of the infection is then monitored by plating serial dilutions of individual whole-organ homogenates on nutrient 7H11 agar and assessing bacterial colony formation 14 to 21 days later, after incubation at 37°C in humidified air.

RESULTS

Efficacy of compounds in a macrophage model. The efficacy of the four compounds to inhibit the growth of *M. tuberculosis* Erdman and CSU22 within mouse bone marrow-derived macrophages was assessed. As shown in Fig. 1, all four compounds inhibited the growth of *M. tuberculosis* Erdman. Calculated BC_{99}s for the compounds were as follows: rifampin, 0.44 μg/ml; rifapentine, 0.43 μg/ml; rifabutin, 0.16 μg/ml; and KRM-1648, 0.08 μg/ml. None of the compounds had any activity against the rifampin-resistant strain CSU22 (Fig. 1).

Activity against low-dose aerogenic infection. The results in Fig. 2 illustrate the course of the infection in control mice, in which the initial inoculum increases 3 to 4 log before being contained in the lungs by the emergence of acquired immunity. By day 10 mice start to show evidence of delayed-type hypersensitivity to tuberculin (>0.2 mm), and they are strongly positive (0.4 to 0.7mm) by about day 15 or 20 (3). On the basis of these observations, which we contend model early diagnosed tuberculosis in humans, we began oral gavage of these mice with moderate doses of the four compounds on day 10. It was found that all four compounds reduced bacterial numbers to below the limit of detection when given at doses of 10 mg/kg of body weight per day (data not shown). When given at 5 mg/kg, rifampin was ineffective but the other compounds all reduced bacterial numbers to below the limit of detection after 14 days of treatment (Fig. 2). Finally, at 2.5 mg/kg, differences in effectiveness between the drugs began to become apparent, with KRM-1648 producing the greatest reduction in bacterial numbers (2.7 logs compared with peak numbers seen in controls) after 35 days of treatment.

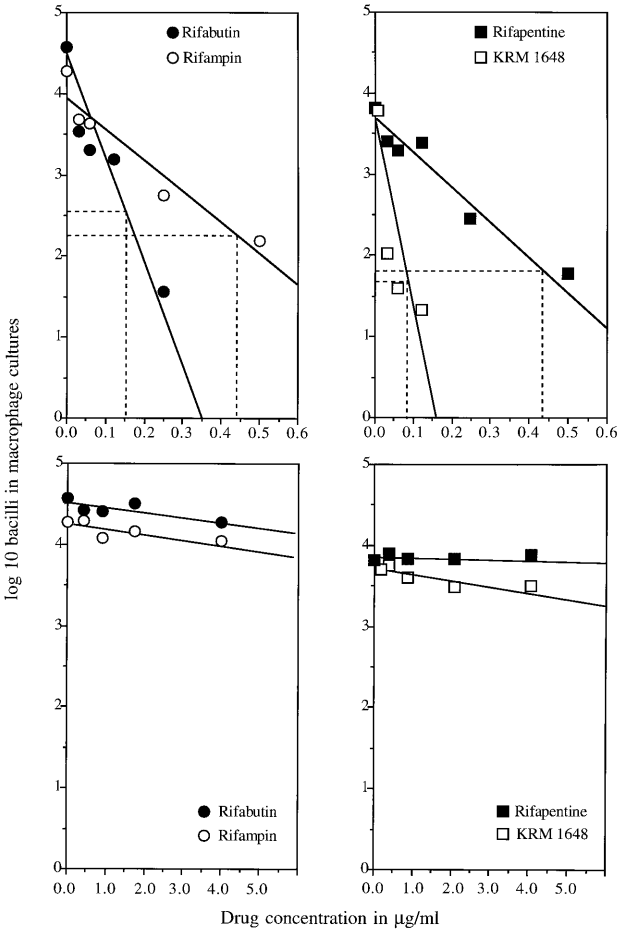

FIG. 1. The capacity of each compound to inhibit the growth of *M. tuberculosis* within monolayers of bone marrow-derived macrophages, arbitarily defined as the BC_{99}, or concentration of drug needed in the external medium to reduce the bacterial load by 2 logs, was determined by titration. Each datum point represents the mean number of viable bacilli recovered from triplicate cultures. A best-fit line was used to calculate the BC_{99}. The Erdman strain (top panels) and strain CSU22 (bottom panels) were used for the infections.

DISCUSSION

The mouse model has various limitations with regard to known events in the immunopathogenesis of tuberculosis in humans, but nevertheless it has proved a useful model in general for the evaluation of new chemotherapies. In most developed countries with established tuberculosis control programs, the diagnosis of disease and the onset of therapy often occur relatively early during the primary course of the infection when symptoms warrant the performance of a tuberculin skin test. Under these conditions, the disease is usually confined to the lungs and generally only presents as a disseminated disease if the patient is immunocompromised (31) or is very elderly (29). If the disease is left untreated, there is a risk that lesions that escape sterilization by T-cell-mediated immunity can become necrotic, potentially developing into liquefied cavities that erode into airways or major blood vessels. Unfortunately, in terms of animal models, only the rabbit consistently develops such cavities.

In this study, we present a low-dose aerosol infection model of primary pulmonary disease detected early by the emergence of delayed-type hypersensitivity to tuberculin. The results of the study show that three of the newer rifamycins, rifabutin, rifapentine, and KRM-1648, all had measurable levels of activity, even when given at relatively low dosages to mice infected by the aerogenic route with *M. tuberculosis*. Rifampin was effective at 10 mg/kg but not at lower doses, reflecting its lesser potency compared with those of the newer compounds. These data thus compare favorably with the results of earlier studies that have indicated the potential usefulness of these compounds in the therapy of tuberculosis (2, 5, 11, 14–16, 24, 26, 27).

The model presented in this work is very flexible. For instance, drug testing can be delayed until after the 30-day point, when the infection becomes chronic (20, 21); thus, the activities of compounds against bacteria that are in a latent rather

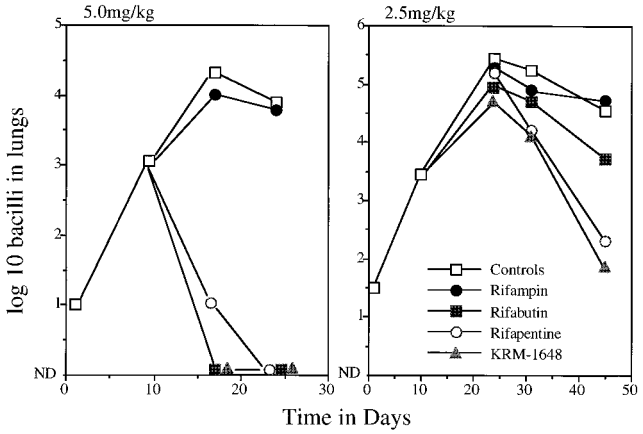

FIG. 2. Capacities of the test compounds, given at two doses, to reduce bacterial load in the lungs of mice infected by low-dose aerosol exposure to *M. tuberculosis*. The indicated compounds were given by gavage, starting on day 10 of the infection. Each datum point represents the mean number of bacilli recovered from four mice; the standard errors of the means are omitted for clarity (they did not exceed 0.35 logs). ND, not detected.

than an actively proliferating state can be tested. The inoculum size can be increased to nearly lethal levels (i.e., an uptake of 3×10^3 to 1×10^4 bacteria) mimicking acute pulmonary tuberculosis. Similarly, some clinical isolates are highly virulent and grow extremely well in this model (20). If mice with gene disruptions, such as interferon gamma gene knockout mice (4), are used, the infection grows progressively following aerosol exposure, giving rise to substantial caseous necrosis in the lungs. To date, however, such models have not been exploited for drug testing, despite the fact that they perhaps model the disease process somewhat better than the conventional high-dose intravenous-challenge model.

Since the first observation (22) of its activity in an in vivo model of tuberculosis, rifabutin has perhaps received the most attention of the newer compounds, and clinical observations of its activity have generally been highly favorable (11, 24–27). Rifapentine has been tested to a lesser extent, but clinical trials are now under way. KRM-1648 has not at the time of writing reached the clinical-trial stage, but the observed high-level activity of this compound indicates that clinical testing should be pursued. Indeed, all three compounds may be of use, particularly in terms of (i) their potential to reduce the length of short-course therapy, hence overcoming patient compliance problems and related problems, and (ii) their potential to fully sterilize infected lesions, thus reducing the possible incidence of recrudescent disease.

Of the compounds tested in this study KRM-1648 had the highest level of activity, both in terms of activity within the macrophage, where the infectious agent normally resides, and in the lung model, in which a 2.5-mg/kg dose of the compound still resulted in a significant reduction in bacterial numbers. In confirmation of an earlier study looking at the MICs of KRM-1648 (18), the drug was not active against a rifampin-resistant isolate of *M. tuberculosis* when tested in the macrophage model in the present study, although activity in vivo against a rifampin-resistant strain has been reported elsewhere (13). Furthermore, in earlier studies the MICs of this compound were often extremely low (0.0005 to 0.0125 μg/ml) whereas a recent screening of clinical isolates from our own collection has yielded MICs of KRM-1648 that are somewhat higher (0.15 to 1.25 μg/ml). The reason for this discrepancy is unclear.

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