Comparative Effects of Sulfones and Rifampin on Growth of *Mycobacterium lepraemurium* in Macrophage Diffusion Chamber Cultures

W. A. RIGHTSEL, M. F. SAWYERS, AND J. H. PETERS

Department of Pathology, Baptist Memorial Hospital, and Department of Microbiology, University of Tennessee Center for the Health Sciences, Memphis, Tennessee 38146, and Life Sciences Division, SRI International, Menlo Park, California 94025

Received for publication 25 July 1977

A cell-impermeable diffusion chamber technique has been developed that lends itself to growth studies of *Mycobacterium lepraemurium*. This technique, in which the organism grows within macrophage cultures inside the chambers that are maintained on monolayer cultures of macrophages, provides a method for a strict in vitro evaluation of antileprosy drugs without the influence of a multiplicity of host factors. This system was used to compare the effect of three sulfone derivatives and rifampin on the growth of *M. lepraemurium* within these diffusion chamber cultures. Two sulfones, 4,4'-diaminodiphenyl sulfone and 4,4'-diacetamidodiphenyl sulfone, as well as rifampin, suppressed the growth of *M. lepraemurium*, but monoacetyl sulfone 4-amino-4'acetamidodiphenyl sulfone had no effect. The results indicate that the diffusion chamber technique can be used to evaluate the inhibitory effect of antileprosy drugs on the growth of *M. lepraemurium*. Also, the method provides for the first time a relatively rapid in vitro method for directly comparing the effects of drugs or their analogs when outside the metabolic influence of an animal host. This technique may be a useful tool for chemotherapy studies with other antileprosy compounds.

Successful growth of *Mycobacterium lepraemurium* has been achieved by use of a specialized diffusion chamber. Previous studies demonstrated that this acid-fast organism could be cultivated in these cell-impermeable porous chambers with a number of different host-chamber systems (10). Maximum growth of organisms consisting of a 31-fold increase was obtained in chambers containing macrophages that were maintained for 50 days by intraperitoneal implantation in mice. In the same studies, diffusion chambers inoculated with mouse macrophages and *M. lepraemurium* and then maintained for 40 days on monolayer petri plate cultures of mouse macrophages exhibited ninefold increases in numbers of acid-fast bacilli (AFB). This in vitro method seemed to provide a system for testing the inhibitory activity of antileprosy drugs against *M. lepraemurium* in the absence of host factors such as absorption, metabolism, and excretion of the compounds.

The most effective widely used antileprosy drugs today are dapsone (4,4'-diaminodiphenyl sulfone, DDS), the repository form of DDS, acedapsone (4,4'-diacetamidodiphenyl sulfone, DADDS), and rifampin (7, 8, 11, 12, 14). Studies in rats and humans have shown that monoacetyl DDS (4-amino-4'-acetamidodiphenyl sulfone, MADDS) is the principal circulatory metabolite of DDS and DADDS (3, 4, 7), but no direct tests of the possible antileprotic activity of MADDS or DADDS have been performed with *M. lepraemurium* or *M. leprae*. Levy et al. (5) found that MADDS was equal in activity to DDS in mice infected with *M. leprae*, but the observation that the mice deacetylated MADDS completely to DDS made it impossible to decide whether MADDS exhibited inherent antileprotic activity.

To determine the relative inhibitory activities of DDS, MADDS, and DADDS on *M. lepraemurium*, we used the diffusion chamber system containing mouse macrophages and the AFB maintained on monolayer petri plate cultures of macrophages. With this system we were able to evaluate the effects of these sulfones on the growth of the organism without the influence of an animal host. Measurements of the sulfones in the cultures were carried out to examine whether they were altered during the incubation periods. In addition, the inhibitory effect of rifampin on the growth of *M. lepraemurium* was
determined for comparison with those of the sulfones.

MATERIALS AND METHODS

*M. leprae*. The Hawaiian strain of *M. leprae* was maintained by continuous passage in CFW female mice by intraperitoneal injection of approximately 10⁶ AFB. Fresh suspensions of organisms for inoculation of diffusion chambers were prepared from fatty pads of mice infected 3 to 5 months previously. Approximately 1 g of fatty pad tissue was homogenized aseptically in a Ten Broeck tissue grinder with 30 ml of sterile Hanks balanced salt solution (BSS). Bacterial counts of the suspension were made by use of the technique described by Shepard and McRae (13).

Mouse peritoneal macrophages. Macrophages were obtained from CFW female mice that had been stimulated by intraperitoneal injection of 3.0 ml of thioglycolate broth (1). After 7 days, the animals were sacrificed to brood the macrophages were harvested by washing the peritoneal cavity with approximately 5.0 ml of Hanks BSS containing 10 U of heparin and 100 U of penicillin per ml. The macrophages were then centrifuged and resuspended in modified Chang’s 4:5:1 medium consisting of 40% fetal bovine serum, 50% NCTC 109 medium, and 10% beef embryo extract. The cell suspension was adjusted to a final concentration of 10⁷ macrophages per ml with this 4:5:1 medium.

Diffusion chambers. The assembly, sterilization, inoculation, and harvest of the specialized cell-impermeable diffusion membranes have been described in detail by Rightsel and Wygul (10).

Preparation of drugs. Stock solutions of the drugs were made in dimethylsulfoxide (Me₂SO). Each of the compounds was prepared at the following concentration: DDS at 1,900 μg/ml, MADDS at 1,300 μg/ml, MADD at 1,600 μg/ml, and rifampin at 1,900 μg/ml.

Chemotherapy trials. Petri plate cultures of mouse macrophages were prepared by inoculating petri plates (35 by 10 mm) with approximately 10⁶ macrophages in 3 ml of modified Chang’s 4:5:1 medium or in the same medium containing appropriate concentrations of the respective drugs. The stock solutions of each drug were diluted in medium to provide final concentrations of 0.0012 to 4.0 μg/ml in the petri plate cultures with Me₂SO present at levels ranging from 0.001 to 0.3%. Each experiment included control cultures with 0.3% Me₂SO in media without added drug as well as controls with media alone. These macrophage cultures, with or without drug, were incubated at 32°C in an atmosphere of 5% CO₂ in air for 3 days. After this initial incubation period, the petri plate cultures of macrophages were used to maintain diffusion chamber cultures inoculated with approximately 5 x 10⁶ AFB and 10⁷ macrophages, each contained in 0.1 ml of Hanks BBS. All petri plate monolayer cultures holding chambers, one chamber to each petri plate, were incubated at 32°C in a 5% CO₂ atmosphere, and the media, with the appropriate drug concentration or without drug, were changed twice weekly during the maintenance period. The effect of the drugs on the growth of *M. leprae* was evaluated after 42 to 48 days because Rightsel and Wygul (10) had demonstrated a ninefold increase of organisms during this time with this in vitro petri plate system. The chamber contents were harvested as previously described, and yields of AFB were determined from each replicate group for comparison of the total number and fold increase of *M. leprae* from both treated and untreated chambers with those containing 0.3% Me₂SO. In selected experiments, maintenance media from the petri plate cultures were analyzed for DDS and Madds by the chromatographic-fluorometric procedure of Murray et al. (6). Also, the viability and infectivity of the original AFB suspension used in each experiment were confirmed by inoculating 5 x 10⁸ bacilli into mouse footpads.

Statistical methods. The consistency and reproducibility of the bacillary counts among replicate chambers within the same group already have been demonstrated (10). In comparing results, statistical analyses were conducted by Student’s t test. The mean bacillary count and standard error of the mean were calculated for each group of four replicate chambers. The yields and fold increase were assessed using P values, with only P < 0.05 considered statistically significant.

RESULTS

Effect of drugs on the growth of *M. leprae* in diffusion chamber cultures of mouse peritoneal macrophages. The dose response of *M. leprae* to each of the three sulfones and rifampin was compared by adding maintenance media containing increasing half-log concentrations ranging from 0.0012 to 4.0 μg/ml of DDS, MADDS, DADDs, or rifampin to the macrophage petri plate cultures. Four replicate cultures were prepared for each dose of a given drug. Each chamber containing the standard inoculum of AFB and macrophages was placed on a 3-day monolayer culture containing 3 ml of media with the various drug concentrations. The cultures were refed by replacing 50% of the tissue culture fluid with fresh maintenance media containing the original drug concentration twice weekly during a 48-day incubation period. One set of controls consisted of medium with 0.3% Me₂SO and a second set of controls consisted of medium alone. The dose response to each of the three sulfone drugs and rifampin of *M. leprae* in diffusion chamber cultures held for 48 days is shown in Fig. 1. The average fold increase of AFB after treatment with increasing concentrations of each of the drugs showed that the effective concentrations of DDS occurred in the range from 0.04 to 4.0 μg/ml, those of DADDs were from 0.004 to 4.0 μg/ml, and those of rifampin were from 0.012 to 4.0 μg/ml. On the other hand, MADDS had no significant growth inhibitory effect. Also, the medium and Me₂SO solvent controls showed no growth inhibition of bacilli within the chamber.
Hence, DDS, DADDS, and rifampin all gave a good dose response, whereas MADDS was inactive.

Effect of diffusion on activity of drugs against *M. lepraemurium* within the diffusion chambers. Two of the sulfones, one inhibiting growth (DDS) and one causing no inhibition of growth (MADDS), as well as rifampin, were used to study the effect of diffusion on the activity of the drugs during growth of *M. lepraemurium* within the diffusion chamber cultures. Each of the drugs was placed both inside and outside the chamber at a concentration of 1.2 µg/ml. Other cultures contained the same concentration of a given drug in the petri plate medium only, thus permitting diffusion into the chamber. Another group of control cultures had no added drug in either the media or chambers. There were regular changes of the same type of culture media at 3-day intervals during a 48-day incubation period, after which the chamber contents were harvested and bacilli from replicate groups of four chambers containing each of the three drugs were counted. Results of this study on the effect of drug diffusion are summarized in Fig. 2, and the actual numbers of *M. lepraemurium* cells from the various diffusion chamber cultures after 48 days are given in Table 1. Both DDS and rifampin inhibited multiplication of *M. lepraemurium* at 1.2 µg/ml. Although fewer bacilli were found when the drugs were added both outside and inside the chamber than when they were added only to the petri plate media, the slight difference was not statistically significant. MADDS again showed no inhibitory effect no matter how the petri plate cultures with chambers were treated.

Comparative effects of the sulfones on

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**Fig. 1.** Dose response of *M. lepraemurium* in diffusion chamber cultures maintained for 48 days in petri plate cultures of macrophages to various concentrations of dapson (DDS), acedapsone (DADDS), monoacetyl DDS (MADDS), and rifampin (RMP). Media = controls with medium alone; DMSO = controls with medium plus 0.5% dimethylsulfoxide. The fold increase was established from the mean bacillary counts of each group of four replicate chambers at each drug concentration; also, the standard error (SE) of the mean was calculated for each group. As an example, the mean (± SE) bacillary count among four replicate chambers treated with 0.4 µg of DADDS/ml was 8.47 (± 0.19) × 10^6 and differed significantly (P < 0.001) from the media control chambers, which had a mean (± SE) bacillary count of 2.20 (± 0.06) × 10^6.

**Fig. 2.** Effect of diffusion of dapson (DDS), monoacetyl DDS (MADDS), and rifampin (RMP) on multiplication of *M. lepraemurium* in diffusion chamber macrophage cultures maintained for 48 days in petri plate cultures of macrophages. Examples of bacillary counts per group of four chambers are given in Table 1.

**Table 1.** Numbers of *M. lepraemurium* bacilli harvested from diffusion chamber cultures with drugs placed both inside and outside the chamber or only the inside chamber

<table>
<thead>
<tr>
<th>Drug</th>
<th>Location of drug</th>
<th>No. of bacilli</th>
<th>P^6</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDS</td>
<td>Out</td>
<td>16.7 (± 0.70) × 10^6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>In and out</td>
<td>13.4 (± 1.40) × 10^6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MADDS</td>
<td>Out</td>
<td>27.1 (± 1.86) × 10^6</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>In and out</td>
<td>28.3 (± 2.19) × 10^6</td>
<td>NS</td>
</tr>
<tr>
<td>Rifampin</td>
<td>Out</td>
<td>18.7 (± 1.01) × 10^6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>In and out</td>
<td>16.4 (± 1.32) × 10^6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>None</td>
<td>Media control</td>
<td>27.4 (± 0.74) × 10^6</td>
<td></td>
</tr>
</tbody>
</table>

^a Mean ± the standard error from four cultures for each drug after 48 days of incubation.
^b Compared with media control. NS, Not significant.
^c Not significantly different from group with same drug placed outside of chamber only (P > 0.5).
yields of *M. lepraemurium* from the diffusion chambers. To evaluate the comparative activity of the three sulfones against growth of *M. lepraemurium*, we used molar equivalents of the sulfones in the diffusion chamber system. In this study, DDS was used at 1.2 μg/ml, MADDS at 1.5 μg/ml, and DADDS at 1.6 μg/ml. There was a 50% medium change in the petri plate cultures twice a week for six weekly cycles, and the harvested tissue culture fluids were analyzed for DDS and MADDS at each interval. Also, duplicate chambers were harvested every 2 weeks and the total number of AFB from each group was determined. The comparative effect of molar equivalents of DDS, MADDS, and DADDS on yields of *M. lepraemurium* harvested from the chambers every 2 weeks is shown in Fig. 3. These results demonstrate that both DDS and DADDS significantly suppressed growth of the AFB, whereas molar equivalents of MADDS had no inhibitory effect. The chemical analyses for MADDS and DDS in the tissue culture media at the various sample times showed that no MADDS could be detected above the limit of sensitivity of the analytical method in the cultures containing added DDS (Table 2). Similarly, essentially no DDS was found in the cultures containing added MADDS or DADDS, indicating that hydrolysis of the acetylated drugs to DDS was negligible. Thus, the inhibitory effects of the added DDS and MADDS must be due to the unaltered compounds, and MADDS was inherently inactive.

**DISCUSSION**

This study shows that the diffusion chamber technique is useful and can be applied for the in vitro evaluation of antileprosy drugs against *M. lepraemurium*. As illustrated in Fig. 1, DDS, DADDS, and RMP consistently suppressed growth of the organism, permitting only a 2.5- to 3.5-fold increase of AFB in chambers at the end of 48 days; in contrast, both drug-free controls (Me₂SO and media) and cultures containing MADDS gave a 6.0- to 7.0-fold increase of AFB. Although this suppressive effect of DDS, DADDS, and rifampin appeared small, it was consistently reproduced in five different experiments.

Similar results were obtained in the diffusion experiments illustrated in Fig. 2. Both DDS and rifampin at concentrations of 1.2 μg/ml inhibited the growth of *M. lepraemurium*, whereas MADDS at the same level had no effect. Hence, the addition of drug inside the chamber did not significantly enhance its inhibitory effect.

In other comparative experiments, the growth of *M. lepraemurium* was inhibited by 1.2 μg of DDS or 1.6 μg of DADDS per ml, but MADDS showed no effect on the growth of the AFB at a molar equivalent concentration of 1.5 μg/ml. After 14 days, molar equivalent concentrations of each of the three sulfones permitted yields of bacilli comparable to that of the controls without

<table>
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<tr>
<th>Week</th>
<th>Sampling time (days)</th>
<th>MADDS found (μg/ml) after addition of DDS (1.2 μg/ml)</th>
<th>DDS found (μg/ml) after addition of MADDS (1.5 μg/ml)</th>
<th>Addition of DADDS (1.6 μg/ml)</th>
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</thead>
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<tr>
<td></td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<td>2</td>
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<td>0.012-0.023</td>
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<td>3</td>
<td>0, 4, 7</td>
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<td>&lt;0.01-0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>0, 4, 7</td>
<td>&lt;0.01</td>
<td>&lt;0.01-0.06</td>
<td>0.014-0.046</td>
</tr>
<tr>
<td>5</td>
<td>0, 3, 7</td>
<td>&lt;0.01</td>
<td>&lt;0.01-0.04</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6</td>
<td>0, 3, 7</td>
<td>&lt;0.01</td>
<td>&lt;0.01-0.08</td>
<td>0.018-0.046</td>
</tr>
</tbody>
</table>

* Controls (no drug added) at each interval: <0.01 μg/ml for all three drugs.
added drug. However, after 28 days the DDS and DADDS chambers yielded significantly fewer bacilli ($P < 0.001$), and at 42 days there was a small increase in growth with all groups but the inhibition with the DDS- and DADDS-treated chambers was still significant ($P < 0.001$). It has been possible to duplicate these small differences in growth rates of AFB in repeated trials with this system. As an example, both DDS and DADDS have consistently inhibited growth of the organism in a total of eight experiments, whereas MADS has never shown an inhibitory effect at a concentration equivalent to or higher than that of DDS. Chemical analyses of the tissue culture fluids for the sulfones at various intervals during the 48-day incubation showed that the suppressive effect was due to the unaltered compounds and that acetylation or deacetylation of the compounds did not occur in this system. Therefore, these metabolic alterations were not factors in the activity of the drugs, and the consistent inhibitory effect of DADDS on growth of \textit{M. lepraemurium} was surprising. This unexpected activity of DADDS could be due to the compound itself since no previous tests have been made wherein possible alteration of the drug by metabolism has been avoided. Another possibility is that DDS was formed from DADDS intracellularly even though tests indicated that DDS did not pass into the media. This system seems to provide a reliable in vitro method for determining the effect of drugs and their analogs outside the metabolic influence of an animal host. Rees and Wong (9) previously demonstrated that streptomycin and isoniazid inhibited in vitro growth of \textit{M. lepraemurium} in cultures of tissues initiated from infected animals. Also, Chang (2) reported on the suppressive effect of streptomycin on the growth of \textit{M. lepraemurium} in macrophage tissue cultures. However, this represents the first report on the use of a system for direct comparison of the efficacy of analogs of drugs and other antileprosy compounds.

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**LITERATURE CITED**