Activity and Penetration of Antituberculosis Drugs in Mouse Peritoneal Macrophages Infected with Mycobacterium microti OV254

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The activities of some commonly used antituberculosis drugs were investigated within unstimulated peritoneal macrophages and in 7H-9 medium without Tween 80 using Mycobacterium microti OV254 as the target organism. In macrophage cultures, serial concentrations of isoniazid, rifampin, pyrazinamide, or streptomycin were added after a 2.5-h phagocytosis period. Viable counts were carried out at daily intervals for 5 or 6 days. The patterns of susceptibility of the four drugs were similar for M. microti and Mycobacterium tuberculosis. To ensure comparability with daily drug replacements in the macrophage experiments, the period of exposure to drugs in 7H-9 medium was kept to only 3 days. With in vitro culture at pH 6.4, drug penetration, measured as the ratio of MICs in macrophages to MICs in 7H-9 medium, was approximately 5 for isoniazid, 5 for rifampin, and 10 for streptomycin. With in vitro culture at pH 7.4, drug penetration was 100 for streptomycin, and at pH 5.6 it was 1 for pyrazinamide. Pyrazinamide was only bacteriostatic in macrophages but weakly bactericidal during the first day of exposure in vitro.

MATERIALS AND METHODS

Media. All tissue culture media and supplements were obtained from Gibco-Europe Ltd. (Uxbridge, United Kingdom) unless specified otherwise. Dubos broth was prepared double strength (DSDB) from Dubos broth base (Diaflo Laboratories, West Molesey, Surrey, United Kingdom). Basic maintenance medium contained 5.9 ml of medium 199, 4 ml of heat-inactivated horse serum, 0.4 ml of bovine embryo extract (Flow Laboratories, Woodcock Hill, Hertfordshire, United Kingdom), and 0.1 ml of liver fraction L (1 mg/ml; United States Biochemical Corp., Cleveland, Ohio). Bacteria. M. microti OV254, obtained from R. J. W. Rees, National Institute for Medical Research, and then mouse passaged, was serially transferred at weekly intervals in DSDB.

Chemotherapeutic drugs. Isoniazid (Sigma Chemical Co., Poole, Dorset, United Kingdom), streptomycin (Evans Medical Ltd., Greenford, Middlesex, United Kingdom), and pyrazinamide (Merck Sharp & Dohme Ltd., Hoddesdon, Hertfordshire, United Kingdom) were dissolved in pyrogen-free water to obtain stock solutions of 100 μg/ml. Rifampin (CIBA Laboratories, Horsham, West Sussex, United Kingdom) was dissolved in 0.01 M HCl and immediately diluted in pyrogen-free water to make a stock solution of 100 μg/ml. Stock solutions were sterilized by filtration through membrane filters (0.22-μm pore size; Flow Laboratories).

Macrophage monolayers. (i) Preparation of monolayers. The peritoneal cavities of recently killed specific pathogen-free BALB/c mice weighing 18 to 20 g were washed out with medium 199 containing heparin (5 U/ml; Pines & Byrne Ltd., Greenford, United Kingdom). Pooled washings were centrifuged at 150 × g for 10 min at 4°C, and the cells were suspended in medium 199 containing 10% fetal calf serum. From this suspension, 5 × 10⁵ cells were put into Linbro multidishtes (16-mm diameter; Flow Laboratories) which were incubated at 37°C with 5% CO₂ for 2 h to allow adherence. Nonadherent cells were removed by washing four times with medium 199 containing 10% fetal calf serum.

Mycobacterium microti resembles Mycobacterium tuberculosis and has been used as an alternative to Mycobacterium bovis BCG for vaccinating children in Britain against tuberculosis (10). It is a convenient organism for laboratory studies because of its low virulence in humans, and M. microti OV254 has therefore been used by several workers, particularly in this laboratory, for studying phenomena relevant to the pathogenesis of tuberculosis in cultures of macrophages (9, 16, 17, 28). Recently, these studies have extended to the interaction between factors affecting immunity, including administration of lymphokines, and the activities of antituberculosis drugs (13). To interpret the results of such studies, we needed to know the activities of antituberculosis drugs within macrophages. Intracellular drug activity has been studied previously using radiolabeled drugs or with M. tuberculosis or M. bovis as the target organism (1, 4–8, 15, 18, 19, 26; N. Rastogi, M. C. Potar, and H. L. David, Letter, Antimicrob. Agents Chemother. 32:287, 1988), but there are no similar reports using M. microti. We have therefore examined the activities of the commonly used antituberculosis drugs, isoniazid, rifampin, pyrazinamide, and streptomycin, by doing serial CFU counts of M. microti OV254 in mouse peritoneal macrophages (PM) at different drug concentrations. The second aim of the study was to measure the penetration of the drugs into the macrophages by comparing MICs obtained in the macrophage with in vitro MICs obtained in a similar manner by serial CFU counts on cultures of M. microti containing a range of drug concentrations. Since the drugs were replaced daily in the macrophage cultures, the period of exposure to drugs in the culture medium was kept to only 3 days, a period as brief as possible consistent with measuring antibacterial activity. The MICs obtained in the macrophages and in culture medium are therefore comparable and could be used to measure more accurately than has previously been possible the effective penetration of the drugs into macrophages.

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incubation at 37°C, daily samples were ultrasonicated briefly, and viable counts were set up as above.

RESULTS
Each of the estimates of the MIC obtained in PM and in vitro was based on two or more experiments, except for the single experiment with rifampin in vitro. The MIC was taken as the lowest concentration that almost or completely inhibited growth and also yielded a curve distinctly lower than the curve for the drug-free control.

Isoniazid. The MICs of isoniazid were 0.05 μg/ml in PM and 0.01 μg/ml in vitro at pH 6.4 (Fig. 1). The endpoints of both titrations are sharp, lying between curves showing bactericidal activity and those no different from the controls.

Rifampin. The corresponding curves for rifampin (Fig. 2) indicate MICs of 0.05 μg/ml in PM and 0.01 μg/ml in vitro at pH 6.4. The endpoint in PM is gradual, as concentrations of 0.02 and 0.01 μg/ml slightly inhibited growth relative to the control, while the endpoint in vitro is sharp.

Pyrazinamide. The two experiments on pyrazinamide in PM yielded consistent results, but the corresponding in vitro experiments were less consistent. In PM, the MIC was taken as 20 μg/ml, but the curve for 10 μg/ml shows some inhibition. Although bacteriostasis is evident, none of the curves for 10 to 40 μg of pyrazinamide per ml in PM show any bactericidal activity. In experiments in vitro at initial pH values of 7.4 to 6.2, there was little evidence of any inhibition of growth by concentrations of up to 40 μg of pyrazinamide per ml. At pH 5.6, three of the four in vitro experiments, were consistent but the amount of antibacterial activity varied among them. Typical results of consistent experiments are shown in Fig. 3. The in vitro curves show slight bactericidal activity during the first day, which was also found in another of the three consistent experiments but not in the third experiment. The MIC was taken as 20 μg/ml,

The resulting monolayers of PM were cultured in basic maintenance medium.

(ii) Addition of M. microti and drug. A 6-day culture of M. microti in DSDB was washed twice by centrifugation at 1,000 x g for 15 min and suspended in Hanks balanced salt solution-HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) buffer and then ultrasonicated for 12 s (Rinco Vitrasonics UK Ltd., London, United Kingdom). The infective inoculum was prepared to give a bacteria to PM ratio of 1:1. After a phagocytosis period of 2.5 h, monolayers were washed four times to remove unattached mycobacteria and overlaid with fresh medium plus drug, diluted from stock in basic maintenance medium, at various concentrations (day 0). The medium and drug over the monolayers were replaced daily for 5 or 6 days.

(iii) Viable counts. PM were removed by scraping them from the Linbro wells with a polystyrene-fluorenyl-tipped rod and aspirating the cell suspensions, which were briefly ultrasonicated to disrupt cell clumps. Serial 10-fold dilutions were made first in digitonin (0.8 mg/ml) to lyse cells and then in distilled water, and 0.05-ml volumes were pipetted onto one-third segments of Dubos olic base agar medium plates (Difco). Plates were packed into polyethylene bags and incubated for 6 weeks at 37°C before CFU were counted. CFU counts were also set up on the well supernatants as a check on extracellular growth, which was always negligible.

In vitro titrations. After autoclaving, the pH of 7H-9 medium (Difco) without Tween 80 was adjusted with N/10 NaOH or HCl, and a 6-day culture of M. microti in DSDB was added (5 ml in 100 ml). After incubation for 3 days, the inoculated medium was dispensed into 30-ml screw-cap bottles and drugs were added. Over a 3-day period of
FIG. 3. Titrations of pyrazinamide in macrophages and in vitro. CFU counts of *M. microti* in macrophages growing in medium containing no drug (control) or 5, 10, 20, or 40 µg of pyrazinamide per ml and in vitro in medium at pH 5.6 containing no drug (control) or 5, 10, 20, or 40 µg of pyrazinamide per ml.

although there seemed to be slight activity at 5 and 10 µg/ml. Neither of the endpoints in PM or in vitro is sharp. No appreciable regrowth occurred in PM or in vitro at the ends of the experiments when the pH of the in vitro experiment was still 5.6.

**Streptomycin.** Titrations were done in the range of 30 to 0.05 µg of streptomycin per ml. The MIC in PM was 5 µg/ml (Fig. 4); the curve for this concentration and for 10 µg/ml showed bacteriostasis only, while 30 µg/ml was bactericidal. The MIC in vitro (Fig. 5) at pH 6.4 was 0.5 µg/ml, which was bactericidal, whereas at pH 7.4 the MIC was 0.05 µg/ml. These titrations show fairly sharp endpoints. In vitro, concentrations of 1 to 20 µg of streptomycin per ml were bactericidal at pH values of 5.8, 6.4, and 7.4, but lower concentrations were not tested at pH 5.8.

FIG. 4. Titration of streptomycin in macrophages. CFU counts of *M. microti* in macrophages growing in medium containing no drug (control) or 1, 2, or 5 µg of streptomycin per ml.

FIG. 5. Titrations of streptomycin in vitro at pH 6.4 and pH 7.4. CFU counts of *M. microti* in vitro in medium containing no drug (control) or 0.05, 0.1, 0.2, 0.5, or 1 µg of streptomycin per ml.

**DISCUSSION**

The results obtained in the present report are summarized in the left-hand section of Table 1, while in the right-hand section are (i) estimates of the MIC obtained in human macrophages by Crowle and his associates using a technique similar to ours but with *M. tuberculosis* Erdman as the target organism and (ii) estimates of in vitro MICs obtained in liquid medium without Tween 80 but with larger inoculum sizes and longer periods of incubation than in our tests or by the radiometric method. The MICs of isoniazid, pyrazinamide, and streptomycin obtained in human macrophages with *M. tuberculosis* are the same within the limits of experimental error as those we obtained in mouse PM using *M. microti* OV254, showing that the patterns of drug susceptibility in the two mycobacterial strains are very similar. The responses to isoniazid and pyrazinamide in macrophages and in vitro exemplify the close relationship between *M. microti* and *M. tuberculosis*, since these two species are unique among the mycobacteria in being susceptible to pyrazinamide and, together with the other mammalian tubercle bacilli, *M. bovis* and *M. africanum*, are almost the only species to be susceptible to isoniazid. The similarity of the drug susceptibility patterns of *M. microti* in mouse macrophages and of *M. tuberculosis* in human macrophages gives confidence that the results of chemotherapeutic experiments using the *M. microti* system are applicable to the treatment of human tuberculosis.

Previous attempts to measure the penetration of antibacterial drugs into macrophages are not entirely satisfactory. Techniques in which macrophages are cultured with radiolabeled drug and then separated by centrifugation from extracellular medium (1, 15) do not estimate the proportion of the intracellular drug available for antibacterial activity and may not be specific in the event of conversion to inactive metabolites. Alternatively, macrophages can be infected...
with a target organism and grown in medium containing serial concentrations of drug, as we have done. However, in previous studies (5-8, 18, 19, 26), the drug concentrations have not been sufficiently closely spaced to allow accurate estimations of the MICs, and of greater importance, a comparison has then been made between the intracellular MIC and an MIC obtained in vitro using a larger inoculum and a longer period of incubation, both of which may increase the MIC, and a medium containing Tween 80, which greatly decreases the MIC of most drugs. Even estimations of the MIC by the radiometric method in 7H-12 medium, which does not contain Tween 80 (Table 1), are not directly comparable with the macrophage results, mainly because drugs in the macrophage cultures were replaced daily while radiometric results are read after an incubation period of 6 to 9 days, which is sufficient long for deterioration of the drugs to occur, and also because the methods for determining the MICs are different. Our in vitro tests made it possible to make a more accurate comparison with the intracellular MICs because they were set up in medium without Tween 80, with a similar inoculum size and with an incubation period of only 3 days, longer than the 1-day intervals in the macrophage experiments but the minimum period necessary to demonstrate drug action. Nevertheless, there remains one potentially important difference between our intracellular and in vitro tests likely to affect the MICs. PM were cultured in medium containing 40% horse serum, reflecting the likely composition of inflammatory exudate, which might result in appreciable protein binding of the drug. However, the in vitro tests were in 7H-9 medium with only 0.5% bovine albumin, with little potential for protein binding. Thus, the ratio between the MIC in PM and the MIC in vitro should model not only the penetration and availability of the drug in PM but also the likely reduction of the MIC in PM by protein binding in their extracellular environment. We believe that our estimates of effective penetration are more accurate than previous measurements and are relevant to the choice of drug dosage in the treatment of tuberculosis.

The MIC of isoniazid against M. microtii in PM was five times higher than the MIC in vitro (Table 1). This finding, obtained with sharp endpoints and confirmed in duplicate experiments, is surprising since radiolabeled drug studies indicate similar intracellular and extracellular concentrations of isoniazid (1, 15) and its activity is not affected by pH (22) nor is it appreciably bound in plasma (3). Three possible explanations for this discrepancy might be advanced: intracellular activity may be reduced by intracellular binding or by antagonists or by metabolism within macrophages to an inactive compound, such as isonicotinic acid. Studies with radiolabeled rifampin suggest that it is concentrated two- to fivefold in alveolar macrophages (15), although this might be due to concentration in lipid cell structures distant to the microenvironment of mycobacteria. Our finding that the MIC is five times lower in PM might therefore be the result of the high degree (85%) of binding of rifampin in plasma (2).

The similarity of the MICs of pyrazinamide in PM and in vitro confirms earlier studies (1, 7, 18) that it penetrates PM well. Accurate comparisons of the MICs in PM and in vitro are difficult, however, because of differences in the shapes of the CFU curves. Pyrazinamide was only bacteriostatic in PM, as found previously in human macrophages (7, 18) and in a macrophagelike cell line (Rastogi et al., Letter), while it was bactericidal during the first day of exposure in vitro. No regrowth (7) occurred during the 6 days of the exposure in PM, probably because the drug was replaced every day. The greater bactericidal activity in vitro than in PM supports the evidence from other observations (12) that the main sterilizing activity of pyrazinamide in human lesions is due to its action on extracellular organisms in areas of acute inflammation where the pH is particularly low (23). This hypothesis explains the finding from clinical studies of pulmonary tuberculosis that pyrazinamide is an effective sterilizing drug during the early weeks of treatment, when areas of acute inflammation might be present, but loses this activity in the later months as inflammation dies down. It also suggests that pyrazinamide would not be useful for chemoprophylaxis of healed or dormant tuberculous lesions, since the bacilli in these would be in sites without acute inflammation. Our findings with streptomycin need little comment. They reflect the well-known decreased activity of streptomycin at lower pH values (20) and its much lower intracellular activity despite concentration in lysosomes (27).

ACKNOWLEDGMENT

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LITERATURE CITED

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