A Radiometric Method for Predicting Effectiveness of Chemotherapeutic Agents in Murine Leprosy [1, 2]

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Drug susceptibility of *M. lepraemurium* has been extensively used to screen substances which might be active against *M. leprae* ([2-9, 14-18]). This model is used because of the biologic similarities of *M. lepraemurium* and *M. leprae*, the causative agents of murine and human leprosy respectively ([18, 21]). Although neither organism can be cultured in cell-free media, *M. lepraemurium* is easier to study in the laboratory because it can be grown more conveniently in larger numbers in murine hosts.

Most of the models to evaluate *M. lepraemurium* susceptibility to drugs involve inoculation into mice, and since the organisms grow slowly, at least three months of observation are required before drug effect can be determined ([7]).

In 1969, a sensitive radiometric method for the detection of bacterial metabolism was introduced by DeLand and Wagner ([12, 13]). The technic measures $^{14}CO_2$ produced by the bacterial metabolism of ^{14}C-labeled substrates. Recently, it has been shown that pure suspensions of *M. lepraemurium* are metabolically active ([21]). Based on these observations, a radiometric method has been developed which permits rapid detection of the metabolism of *M. lepraemurium* by measuring the $^{14}CO_2$ produced by conversion of acetate-U-^{14}C or glycerol-U-^{14}C ([1]).

The current paper reports the application of this radiometric technic to the rapid evaluation of the susceptibility of *M. lepraemurium* to a series of drugs.

MATERIALS AND METHODS

Preparation of the bacilli. *M. lepraemurium* (Hawaiian strain) was harvested from infected livers of female CBA/J mice (Jackson Laboratories) which had been inoculated intraperitoneally and intravenously three to four months previous with 5×10^8 bacteria. The bacteria were separated from tissue components using the technic of Tepper and Varma ([21]). At the end of this procedure, the bacteria were suspended in sterile water at a final concentration of 1×10^9 bacteria/ml.

Buffer system. The simple K-36 buffer of Weiss ([22]) (0.1M KCl, 0.01M NaCl, 0.05M KH_2PO_4, pH 7.0) was used as a buffer system solution for the bacteria.

Drugs. The effect of the following drugs on the metabolism of *M. lepraemurium* was studied: bacitracin, cephaloridine, chloramphenicol, cycloserine, dactinomycin, dapsone (DDS), ethionamide, isoniazid (INH), kanamycin, methenamine mandelate (mandelamine), nitrofurantoin, oxacillin, polymyxin B, rifampin (rifampicin), streptomycin, sulfadimethoxine, and vancomycin (see Appendix). Except for dactinomycin (0.5 mg/vial), the dose for all of the drugs was 5 mg/vial.

In a subsequent experiment, a combination of nitrofurantoin and ethionamide (5 mg/vial, each) was inoculated into four flasks containing 1×10^9 bacteria, suspended in K-36 buffer and into three flasks containing the complex NC-5 medium ([1]); four extra controls, two for each suspending medium, without the drugs were also prepared. After 15 days of incubation, the contents of these vials were pooled and 0.1 ml was inoculated intraperitoneally into three CFW female mice. The same was done with the suspensions of the control vials.

Reaction system. In order to detect bacterial metabolism, 10 ml of K-36 buffer were placed in a 20 ml multidose vial, along with 5 μCi of acetate-U-^{14}C (New England Nuclear), 1 ml of the final suspension of bacteria (1×10^9 bacteria/vial) and the drug. All flasks were prepared in duplicate.

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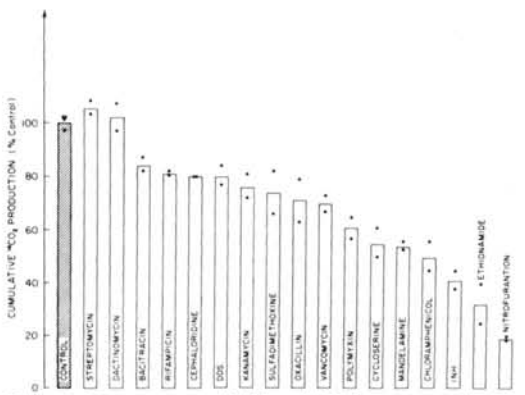

FIG. 1. Drug susceptibility of *M. lepraemurium*.

Controls without drugs were prepared in the same manner. In order to evaluate background levels and substrate stability, additional controls were prepared by adding autoclaved bacteria to vials which contained K-36 buffer and acetate-U-^{14}C.

Radiometric measurement. Vials were incubated at 30°C and the $^{14}CO_2$ produced by bacterial metabolism was measured radiometrically. The details of operation of the measurement device (Bactec R-301, Johnston Laboratories), have been published previously ([1]). Measurement of the $^{14}CO_2$ produced in each vial was begun at 24 hours and continued until 15 days after inoculation. In order to evaluate the effect of a particular drug on the metabolism of *M. lepraemurium*, the sum of the $^{14}CO_2$ produced in the presence of drug over the 15 day testing interval was compared to that produced in the control vials without drug. The data were expressed as percent control radioactivity or as "index unit" (where 100 = 0.025 μCi) representing the cumulative $^{14}CO_2$ production ([1]).

Sterility testing. Sterility tests were performed on all samples and consisted of subculture on chocolate-agar and radiometric sterility testing with glucose-U-^{14}C ([10-13]).

RESULTS

Several drugs inhibited the metabolism of *M. lepraemurium* as shown in Figure 1. The inhibitory effect of the drugs increased as follows: bacitracin, rifampin, cephaloridine, DDS, kanamycin, sulfadimethoxine, oxacillin, vancomycin, polymyxin B, cycloserine, mandelamine, chloramphenicol, INH, ethionamide and nitrofurantoin.

Dactinomycin and streptomycin seemed to stimulate the metabolism of *M. lepraemurium*. Ethionamide and nitrofurantoin had the most marked inhibitory effect on the metabolism of these bacteria (Fig. 1). INH and chloramphenicol also had a significant inhibitory action.

The time course curves for the most effective drugs, ethionamide and nitrofurantoin are shown in Figure 2. The combination of these drugs, tested in a separate experiment was effective both in the K-36 buffer (Fig. 3)

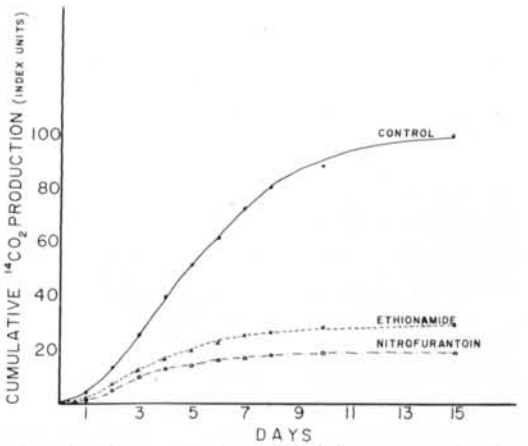

FIG. 2. Susceptibility of *M. lepraemurium* to nitrofurantoin or ethionamide alone.

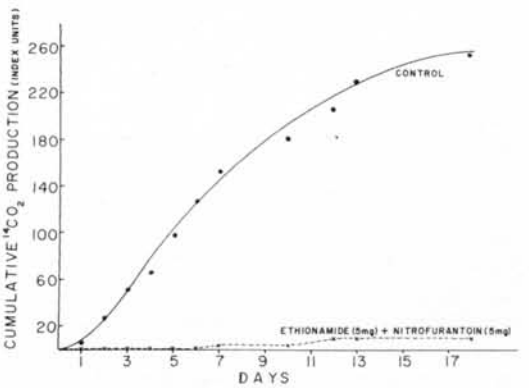

FIG. 3. Susceptibility of *M. lepraemurium* to a combination of nitrofurantoin and ethionamide in K-36 buffer.

and in the NC-5 medium (Fig. 4). In the K-36 buffer, after 15 days the activity in the vials which contained the combination of drugs was less than 5% of that of the controls (Fig. 3); in the NC-5 medium, the activity was about 14% of that of the controls after 13 days (Fig. 4).

Four months later, the mice injected with drug-treated bacteria were free of lepromas; the control mice showed a small leproma in

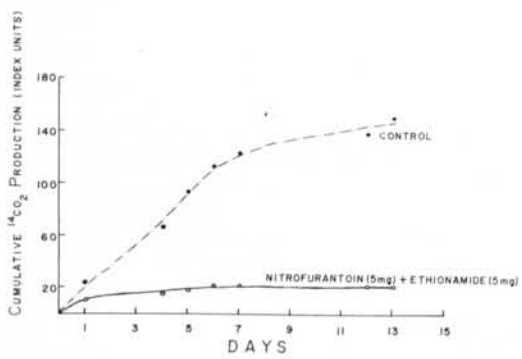

FIG. 4. Susceptibility of *M. lepraemurium* to a combination of nitrofurantoin and ethionamide in NC-5 medium.

the site of injection. However, no histologic assessment was done on the sites of injection either in the drug-treated or in the control mice.

As expected, $^{14}CO_2$ production was not observed in the vials inoculated with autoclaved bacteria. All sterility tests were negative.

DISCUSSION

The *in vivo* effect of a number of drugs on the growth of *M. lepraemurium* has been studied exhaustively in a mouse model by Chang ([2-9]), Hadler ([14]), Hadler and Mauri ([15, 16]) and by Mauri *et al* ([17]). The technics employed in these studies required observation of the animals for a minimum of three months for interpretation of the effect of the drug. In contrast, the radiometric technic which is described in this paper determined the effect of 17 drugs on the metabolism of *M. lepraemurium* within 15 days. The data obtained were used to rank the drugs from maximum to minimum inhibition of the metabolism of these organisms (Fig. 1).

It is presumed that a positive radiometric test is important information because it would indicate that at least one of the metabolic reactions of *M. lepraemurium* would be blocked. However, drugs not effected by the radiometric method should be further tested by methods involving active multiplication of these organisms. Therefore, it seems that this method has potential at least as a simple test for rapidly screening a large number of drugs or combination of drugs which might be effective against murine leprosy.

It is difficult to draw conclusions about the effect of a particular drug on the *in vivo* growth of *M. lepraemurium* based solely on metabolic inhibition. However, in a limited number of experiments, the combination of the drugs which showed the greatest inhibition, nitrofurantoin and ethionamide, proved to be bactericidal *in vitro*. This effect has not been previously reported, although ethionamide alone has been shown to be bactericidal against *M. leprae* in mice foot pads ([19]). Based on the observation of the additive effect of ethionamide and nitrofurantoin, a trial of the effect of this combination against *M. leprae* in the mouse foot pad may be warranted.

At the time of this writing, the authors have just begun to study *M. leprae* using the radiometric method described. Studies are planned using *M. leprae* from lepromas harvested from the armadillo ([20]). This method would seem to be ideal for the study of *M. leprae* and it is hoped that a screening test can be developed based on the inhibition of the metabolism of *M. leprae in vitro*. Such a test may bring us another step closer to the ideal screening method for drugs active against human leprosy.

SUMMARY

A simple radiometric method has been developed for evaluating the effect of drugs on the metabolism of *M. lepraemurium*. The method is based on the measurement of the $^{14}CO_2$ produced through bacterial metabolism of acetate-U-^{14}C. Seventeen drugs were tested: bacitracin, cephaloridine, chloramphenicol, cycloserine, dactinomycin, DDS, ethionamide, INH, kanamycin, methenamine mandelate, nitrofurantoin, oxacillin, polymyxin B, rifampicin, streptomycin, sulfadimethoxine and vancomycin. The drugs which caused most marked inhibition were chloramphenicol, INH, ethionamide and nitrofurantoin in order of increasing effectiveness.

The radiometric study which is completed in 15 days permits direct study of the drug effect on the metabolism of *M. lepraemurium* and a more rapid screening of antileprosy drugs than has previously been possible. Currently, these observations are being extended to studies of the structure-activity relationships of antileprosy drugs and the metabolism and drug susceptibility of *M. leprae in vitro*.

RESUMEN

Se ha desarrollado un método radiométrico simple para evaluar el efecto de las drogas en el metabolismo del *M. lepraemurium*. El método está basado en la medición del $^{14}CO_2$ producido a través del metabolismo bacteriano de acetato-U-^{14}C. Se estudiaron 17 drogas: bacitracina, cefaloridina, cloramfenicol, cicloserina, dactinomicina, DDS, etionamida, INH, kanamicina, mandelato de metenamina, nitrofurantoina, oxacilina, polimixina B, rigampicina, estreptomicina, sulfadimetoxina y vancomicina. Las drogas que causaron la inhibición más marcada fueron el cloramfenicol, INH, etionamida y nitrofurantoína, en orden de incremento de efectividad.

El estudio radiométrico, que se completa en 15 días, permite un estudio directo del efecto de la droga sobre el metabolismo del *M. lepraemurium* y un estudio selectivo de drogas contra la lepra, más rápido que lo que había sido posible hasta ahora. Actualmente, estas observaciones se están extendiendo a estudios sobre las relaciones estructura-actividad de drogas contra la lepra, y el metabolismo y la susceptibilidad a las drogas del *M. leprae in vitro*.

RÉSUMÉ

On a mis au point une méthode radiométrique simple pour évaluer l'action des médicaments sur le métabolisme de *M. lepraemurium*. La méthode est basée sur la mesure du $CO_2{}^{14}$ produit lors du métabolisme de l'acetate-U-C^{14} chez les bactéries. Dix-sept médicaments ont été étudiés, à savoir la bacitracine, la céphaloridine, le chloramphénicol, la cyclostérine, la dactinomycine, la DDS, l'éthionamide, l'INH, la Kanamycine, le mandélate de méthenamine, la nitrofurantoïne, l'oxacilline, la polymyxine B, la rifampicine, la streptomycine, la sulfadimethoxine et la vancomycine. Les médicaments ayant entraîné l'inhibition la plus prononcée étaient le chloramphénicol, l'INH, l'éthionamide et la nitrofurantoïne, ces médicaments étant énumérés dans l'ordre d'une efficacité croissante.

L'étude radiométrique, qui a été achevée en 15 jours, permet une étude directe de l'action d'un médicament sur le métabolisme de *M. lepraemurium*, de même qu'un triage plus rapide des médicaments anti-lépreux qu'il n'avait été jusqu'à présent possible. On procède actuellement à un élargissement de ces observations, en vue d'étudier les relations entre la structure et l'activité des médicaments anti-lépreux, de même que le métabolisme et la susceptibilité aux médicaments de *M. leprae in vitro*.

APPENDIX

Drug Suppliers

Bacitracin: The Upjohn Company, Kalamazoo, Michigan.

Cephaloridine (Loridine): Eli Lilly and Company, Indianapolis, Indiana.

Chloramphenicol: Mann Research Laboratories, New York, New York.

Cycloserine: Mann Research Laboratories, New York, New York.

Dactinomycin (Cosmegen): Merck, Sharp and Dohme, West Point, Virginia.

DDS: Parke, Davis and Co., Detroit, Michigan.

Ethionamide: Ives Laboratories, Inc., New York, New York.

Isoniazid (Nydrazid): E. R. Squibb and Sons, Inc., New York, New York.

Kanamycin: Mann Research Laboratories, New York, New York.

Methenamine mandelate (Mandelamine): Warner Chilcott Laboratories, Morris Plains, New Jersey.

Nitrofurantoin: Sigma Chemical Company, St. Louis, Missouri.

Oxacillin (Bactocill): Beecham-Massengill Pharmaceuticals, Bristol, Tennessee.

Polymyxin B: Pfizer, Inc., New York, New York.

Rifampin (Rifampicin): Mann Research Laboratories, New York, New York.

Streptomycin: Eli Lilly and Company, Indianapolis, Indiana.

Sulfadimethoxine: Hoffman-LaRoche, Inc., Nutley, New Jersey.

Vancomycin (Vancocin): Eli Lilly and Company, Indianapolis, Indiana.

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