# ACTIVITY OF ANTITUBERCULOSIS DRUGS AGAINST *MYCOBACTERIUM LEPRAE*

## STUDIES WITH EXPERIMENTAL INFECTION OF MOUSE FOOTPADS[1, 2]

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The search for drugs active against human leprosy has had to consist of trials in patients, because no experimental system providing multiplication of the etiologic agent has been available. Since improvement of patients is slow, and variable from patient to patient, the clinical trials have had to be very extensive to get an answer of any precision. Sometimes treatment of a few patients for a few months will give a preliminary notion that antileprosy activity is present. Unfortunately, however, the temporary improvement provided by many drugs has been followed by obvious deterioration, apparently because the drug is only partially active and ultimately permits the emergence of drug-resistant bacilli.

We have reported recently ([39]) that experimental infection of mouse footpads with *Mycobacterium leprae* ([36]) may be used to test drugs for antileprosy activity. The general principle is that a drug is studied to see if it will prevent multiplication of *M. leprae*; this is, of course, analogous to the search for other antibacterial drugs, in which test tubes containing known concentrations of drug are inoculated with small numbers of the bacterium in question to see if its multiplication will be prevented.

We have now tested a total of 11 drugs known to have antimycobacterial activity. In addition we have attempted to learn the least amount of DDS active against *M. leprae*.

### MATERIALS AND METHODS

The methods are described elsewhere in greater detail ([36, 37]). The inoculum was from a typical isolate in 6th passage, and the number of leprosy bacilli inoculated per mouse was $5 \times 10^3$. The mice were of the CFW strain of the Communicable Disease Center. They were inoculated in the right hind footpad and placed in cages with drug-containing or control diets. At monthly intervals a mouse from each control group was sacrificed for histologic sections. After the incubation period acid-fast bacteria (AFB) were detected in the sections, and mice were then sacrificed from each of the groups for counts of acid-fast bacteria in a suspension of the footpad tissue. Harvests were repeated

[1]Received for publication April 23, 1964.

[2]Symbols of the drugs are: B663, a rimino-compound of Barry *et al.* ([2]); CS, cycloserine; DDS, 4,4′-diaminodiphenyl sulfone (dapsone); DPT, 4-butoxy-4′dimethylaminodiphenyl thiourea, (SU-1906, CIBA-1906); EB, ethambutol ([41]); ETIP, diethyl dithiol*iso*phthalate (Etisul); INH, isoniazid; PAS, para-aminosalicylic acid; PZA, pyrazinamide; SM, streptomycin; TB1, 4-acetamidobenzaldehyde thiosemicarbazone (TB1/698) (Conteben) (amithiozone).

at approximately 2 month intervals. The method of counting AFB is a microscopic one, in which an equatorial strip is searched across a 2$\mu$l drop. In general, the microscopic search was more prolonged than in the previous work ([39]); 2 drops were first counted, and then up to 4 more if necessary, to bring the total bacilli counted to 25. If no AFB were found, the concentration was calculated on the basis of one bacillus seen in the same search, and the result expressed as less than this number. Portions of each suspension were inoculated on slants of Loewenstein-Jensen medium, 25 per cent blood agar, and 7H9 medium, and into 7H9 broth. The cultures were kept at 33°C, and examined regularly for 4 months. No acid-fast bacteria were isolated.

The drugs tested are listed in Table 1. Ethambutol was given as 0.5 per cent of the racemic mixture for the first 3 months, and thereafter as 0.25 per cent of the dextro (active) form. The streptomycin group received the control diet, and the drug was given by subcutaneous injection in 0.05 ml. of balanced salt solution 5 days a week. All other drugs were given as additions to the control diet (ground commercial chow).

Histopathologic studies were usually made on tissues of mice killed for counts of acid-fast bacilli. HAE (hematoxylin, azure, and eosin) and acid-fast stains were usually made on liver, spleen, kidney, and lung, and sometimes on lymph nodes and pancreas.

DDS was determined in tissues and serum by the method of Simpson ([40]). A variation was observed in the standard, due to precipitation of DDS from the stock solution at 5°C. So the stock solution was dispensed in 5 ml. amounts, which were kept at 5°C. For each test a fresh 5 ml. portion was heated and inspected carefully to ensure that solution was complete.

## RESULTS

Streptomycin, 2 mgm./day, B663, 0.01 per cent, and DDS, from 0.2 to 0.01 per cent, suppressed bacillary multiplication completely. There was no clear evidence of any bacillary increase. The occasional organisms encountered in the counts were probably remnants of the original inoculum of this very persistent mycobacterium. TB1 suppressed multiplication partially; there was some bacillary increase, but it was always less than that in any controls. PZA, ETIP, DPT, and ethambutol gave no evidence of suppression; the counts were not consistently different from those in the controls.

The incubation period (time before bacilli increased to detectable levels in sections or counts) was much longer in this experiment than in the previous one, and was near the upper limit of the variation that had been encountered with this experimental system ([36, 37]). It seems probable that the prolongation resulted in a more severe test of the drugs' efficacies. Certainly it was difficult to time the growth curve accurately enough to rule out a drug-induced delay in multiplication, such as that caused by CS in the first experiment ([39]).

As part of the same experiment, but not reported in Table 1, an attempt was made to learn if the result could be obtained earlier if more bacilli were inoculated. Doses of $1.6 \times 10^4$ and $5.0 \times 10^4$ leprosy bacilli were injected into mice given control diets and 0.1 per cent DDS. As expected, bacilli could be detected earlier, but the result was much less satisfactory because it was difficult to be certain whether there was true bacillary increase, or mere persistence of the bacilli inoculated. Fortunately it has been found in the meantime ([38]) that the

incubation period is a function of the proportion of solidly staining bacilli ([34]), and it seems probable that undesirably slow inocula can be avoided by selection on the basis of microscopic morphology.

*Drug toxicity.*—The very long duration of the experiment provided an unusual opportunity for the observation of chronic toxicity. Weight gain and behavior were normal throughout the group with the exception of those on 0.2 per cent DDS. This diet was not fully consumed and the growth rate was reduced. Histopathologic changes were not found except in mice receiving B663 and DDS.

The B663 mice, as expected, became increasingly red during the experiment and then became a deep reddish blue. Concentrations in the liver reached 5090 μg/gm. Grumbach ([26]) found 2300 μg/gm. in livers of mice with an intake of 1 mgm. B663/day for 60 days. Our mice received only about one-third of this amount per day, but they did so for a much longer period.

In frozen sections of the liver, red crystals were accumulated in foci from which parenchymal cells had disappeared (Fig. 1). The

TABLE 1.—*Effect of drugs on multiplication of* M. leprae *in foot-pads of mice.*[a]

| Drugs[c] | Harvest (AFB/mouse)[b] | | | Drug[d] activity |
|---|---|---|---|---|
| | 11 months | 13 months | 15½ months | |
| SM, 2 mgm./day | $2.1 \times 10^4$ (3)[e] | $<6.2 \times 10^3$ (0) | $<5.8 \times 10^3$ (0) | Complete |
| PZA, 0.5% | $1.3 \times 10^5$ (19) | $4.6 \times 10^5$ (53) | $7.5 \times 10^5$ (57) 3m[f] | None |
| ETIP, 0.5% | $7.9 \times 10^4$ (11) | $8.8 \times 10^5$ (76) | $4.4 \times 10^5$ (51) 5m | None |
| DPT, 0.1% | $7.0 \times 10^5$ (30) | $6.6 \times 10^5$ (62) | $1.3 \times 10^6$ (65) 1m | None |
| TB1, 0.1% | $8.4 \times 10^4$ (12) | $2.9 \times 10^4$ (5) | $7.9 \times 10^4$ (20) 3m | Partial |
| B663, 0.01% | $<7.3 \times 10^3$ (0) | $6.4 \times 10^3$ (1) | $<5.9 \times 10^3$ (0) | Complete |
| EB, 0.25% | $6.2 \times 10^5$ (27) | $1.0 \times 10^6$ (62) | $3.8 \times 10^5$ (64) 6m | None |
| Control | $1.7 \times 10^5$ (18) | $2.4 \times 10^5$ (42) | $2.8 \times 10^6$ (72) 1m | — |
| DDS, 0.01% | $<6.2 \times 10^3$ (0) | $<4.7 \times 10^3$ (0) | $1.3 \times 10^4$ (2) | Complete |
| DDS, 0.025% | $<6.8 \times 10^3$ (0) | $<6.2 \times 10^3$ (0) | $7.4 \times 10^3$ (2) 4m | Complete |
| DDS, 0.05% | $<7.9 \times 10^3$ (0) | $<5.5 \times 10^3$ (0) | $5.1 \times 10^3$ (2) 5m | Complete |
| DDS, 0.1% | $6.6 \times 10^3$ (1) | $<5.5 \times 10^3$ (0) | $3.6 \times 10^4$ (8) 3m | Complete |
| DDS, 0.2% | $<7.4 \times 10^3$ (0) | $<6.3 \times 10^3$ (0) | $<1.2 \times 10^4$ (0) 1m | Complete |
| Control | $1.5 \times 10^5$ (25) | $5.4 \times 10^5$ (65) | $4.2 \times 10^5$ (57) | — |
| DDS, 0.1% | $<7.1 \times 10^3$ (0) | $<5.9 \times 10^3$ (0) | $<2.3 \times 10^3$ (0) 5m | Complete |
| Control | $4.8 \times 10^5$ (55) | $2.4 \times 10^5$ (30) | $4.7 \times 10^5$ (54) 3m | — |

[a]Mice were inoculated with $5.0 \times 10^3$ leprosy bacilli and given diets containing the drugs shown or control diets (SM mice received control diet and SM by injection). Harvests of acid-fast bacteria (AFB) were begun after AFB appeared in sections of mice from control groups.

[b]Values preceded by "<" indicate that no AFB were found during counting procedure. The result was calculated on the basis of 1 organism and is recorded as less than this number.

[c]The percentage is the amount of drug mixed in diet.

[d]"Complete" indicates that multiplication of *M. leprae* was completely suppressed; the few AFB found during the counting procedure probably remained from the original inoculum. "Partial" indicates that multiplication of *M. leprae* occurred, but not so extensively as in the controls. "None" indicates that *M. leprae* multiplied to the same level as in the controls.

[e]The figure in parenthesis is the number of AFB observed during the counting procedure.

[f]Number of mice pooled in tissues for count; where no number is given, there were 2 mice per pool.

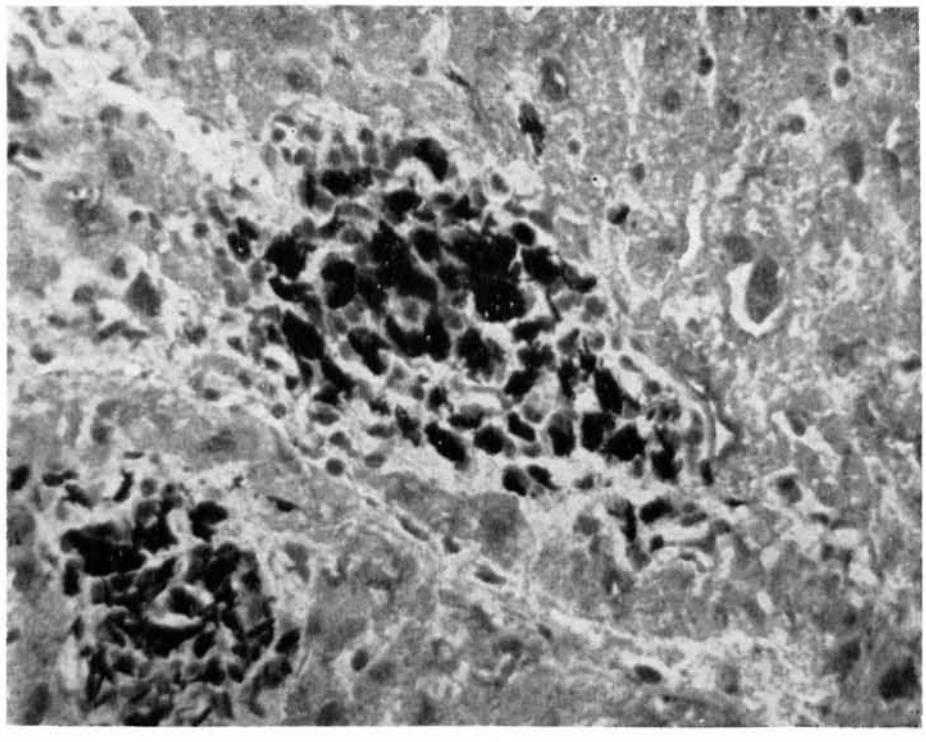

FIG. 1.—Liver of mouse receiving 0.01 per cent B663. Red crystals are in foci containing chiefly macrophages. Frozen section stained briefly with hematoxylin. X 500.

cellular reaction around the crystals was predominantly of macrophages. The foci were present at a frequency of 2 to 3 per low power (10x) field. The tissue changes were more severe than those described in experiments of shorter duration by Barry *et al.* ([2]) and by Grumbach ([26]). The tissue changes are apparently not lethal, since the life expectancy of mice (general purpose, NIH) receiving 0.01 per cent B663 in the diet is not reduced ([10]).

In the mice receiving higher concentrations of the DDS some Kupfer cells were distended with pigment colored green in HAE-stained sections (Fig. 2). In unstained sections the pigment was yellowish-brown, indistinguishable from that seen in spleens of most normal animals. Counts of the green cells in livers were made with the 20x objective. Averages of only 1 to 3 green cells per 20x field were observed with control mice, and mice receiving SM, PZA, and TB1. In the mice receiving DDS the averages were 193, 29, 9, 7, and 3 green cells for the diet concentrations of 0.2, 0.1, 0.05, 0.025, and 0.01 per cent, respectively. The ETIP and DPT mice also had moderate elevations to 14 and 10, respectively. The spleens of mice receiving higher dosages of DDS probably contained increased pigment, but it is not possible to be certain of this because normal mice contain

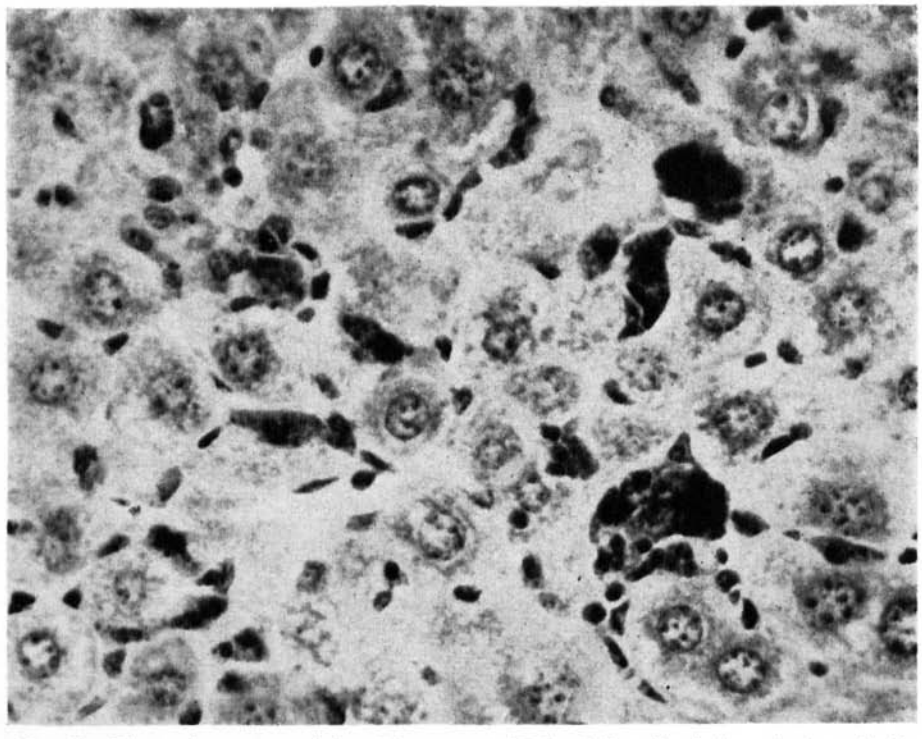

FIG. 2.—Liver of mouse receiving 0.2 per cent DDS. Cells with dark protoplasm in figure are green in HAE-stained sections. In unstained sections the pigment was brown. X 500.

variable amounts of what appeared to be the same pigment. The feet of DDS-treated mice were not studied histologically in the present experiment, but they were in the previous one ([39]). Review of those sections, representing monthly intervals, revealed that there was a distinct increase in number of green reticulum cells in the bone marrow beginning 3 months after the start of 0.1 per cent DDS.

*Levels of DDS in tissues.*—The concentration of DDS in various tissues is given in Table 2. Except at the 0.2 per cent level, where

TABLE 2.—*DDS concentrations in serum and tissues of mice.*

| Per cent of DDS in diet | Concentration of DDS (μg/gm.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Serum | | Liver | | Kidney | | Muscle | |
| | Free | Total | Free | Total | Free | Total | Free | Total |
| 0.2 | 22.1[a](3) | 43.8(3) | 45.8(3) | 94.4(3) | 36.9(3) | 81.4(3) | 20.6(3) | 33.6(3) |
| 0.1 | 19.0(3) | 31.0(4) | 38.8(4) | 71.7(5) | 24.2(4) | 52.1(5) | 13.8(4) | 21.4(5) |
| 0.05 | 2.4(2) | | 9.0(2) | | 3.5(2) | | 3.4(2) | |
| 0.025 | 5.2(2) | | 6.7(2) | | 6.3(2) | | 3.0(2) | |
| 0.01 | 2.6(4) | | 3.4(4) | | 2.6(4) | | 1.0(4) | |

[a]The concentration given is an average for the number of mice indicated in parenthesis.

food consumption was reduced, the tissue concentrations of DDS were directly proportional to the amount of DDS in the diet. Francis and Spinks ([25]) found a comparable value, 9 μg free DDS/ml. whole blood, for mice receiving 0.05 per cent DDS in diet. Our mice on 0.01 per cent DDS were receiving about 10 mgm. DDS/kgm. body weight; yet the concentration of free DDS in the tissue is similar to the value of 3.4 μg/ml. blood found by Lowe ([29]) for humans receiving about 1.4 mgm./kgm., i.e., 100 mgm./day, which is the usually recommended dose.

## DISCUSSION

*Comparative activities of the drugs in various antimycobacterial systems.*—These are reviewed in Table 3. All of the drugs are active against *Mycobacterium tuberculosis.* The apparent exception *in vitro,* ETIP, arises from the fact that this drug releases ethyl mercaptan by metabolic cleavage ([32]). Ethyl mercaptan is active at a level of 200 μg/ml. in the presence of albumin or serum ([17]). With the exception of DDS, the drugs causing complete suppression of the growth of *M. leprae* in mice have minimal inhibitory concentrations against *M. tuberculosis* of 1 μg/ml. or less.

The activities against *Mycobacterium tuberculosis* in mice could not be stated quantitatively since the technic used varied among reports. However, all the drugs have been reported active, with the exception of CS. The inactivity of CS against *M. tuberculosis* in mice arises from its rapid excretion after parenteral injections in that species ([12]).

*Mycobacterium lepraemurium* has been used as a test organism in studies of drugs that might be active against human leprosy. Like *M. leprae, M. lepraemurium* is found in an intracellular location, and it has not been cultivated on artificial medium. It does not appear that these similarities necessarily lead to parallelism in drug susceptibilities. DDS has only a low order of activity against *M. lepraemurium.* Of the four most effective drugs against *M. lepraemurium,* two, B663 and INH, were active against *M. leprae* in mice.

The effectiveness of drugs against *Mycobacterium leprae* in humans is difficult to state precisely. A drug is usually assessed by gross estimates of the number of acid-fast bacteria in skin smears, but the disease has a very long course even under treatment and only one study has followed many patients under adequate treatment to bacterial negativity. Lowe ([31]) was able to follow an unusually stable group of patients who had received full dosages of sulfones, chiefly DDS. He found that they all responded, although slowly. Thus at seven years 34 of 35 (97%) were smear-negative, at six years 31 of 35 (89%), and at five years 32 of 39 (83%). Those still positive had only a few bacilli and were continuing to improve. There was no evidence

of development of resistant forms, and although some patients remained stationary for long periods, none of them deteriorated. This finding would put DDS in a unique position in single-drug treatment of mycobacterial disease.

Studies with other drugs have not covered so long a period. The results selected for the last column of Table 3 are ones that covered a long period (to give an opportunity for deterioration to appear), or ones in which patients were assigned to treatment groups on a random basis. Besides DDS, all of the drugs for which such results have been reported have either given temporary improvement followed by deterioration after 4 months to 3 years, or they have been followed for periods less than 1 year. On the basis of the results with DPT in humans, one might have expected somewhat more activity in mice. It would have been helpful to know what DPT concentrations in blood and tissue were achieved in the mice, but the analytic methods are not sensitive enough ([24]).

It appears possible to rule out grossly inadequate blood levels as a cause for the failure of DPT and other drugs to achieve complete suppression of *M. leprae* in mice, because in each such case the drug had been shown to be active by the oral route against *M. tuberculosis* or *M. lepraemurium* in mice.

This review of comparative drug activities might be summarized by saying that the 5 drugs causing complete suppression of multiplication of *M. leprae* in mice were DDS, B663, and the three "first-line" drugs in human tuberculosis, INH, PAS, and SM. The most useful drug in the treatment of human leprosy, DDS, was not impressive in any experimental system except *M. leprae* in mouse footpads.

The disadvantages of DDS in treatment are the very long time apparently needed for cure, and the reactions to the drug that occur in some patients. As a substitute for DDS it would seem worthwhile to consider the use of combinations of INH, PAS, and SM, such as are used in human tuberculosis. In tuberculosis the combinations do not, on the average, achieve more rapid cures than the drugs used singly; instead they reduce the incidence of relapses, which are probably analogous to the late deterioration in treated leprosy.

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FOOTNOTES TO TABLE 3

[a]Except where otherwise noted the entries are for human tubercle bacilli and are taken from the review of Youmans and Youmans ([43]).

[b]Minimal concentration per ml. giving complete inhibition of bacillary growth.

[c]Index of chemotherapeutic effectiveness. The disease index in control group/disease index in treated group. A value of one means no chemotherapeutic activity. A value of ∞ means complete suppression of disease.

[d]The percentage is the amount of drug mixed in diet.

[e]Entries are from present paper except where noted.

[f]Disease index in treated group was 0.

[g]The amount of drug in the diet was incorrectly given in ([39]).

[h]Daily subcutaneous dose.

[i]Bovine tubercle bacilli.

TABLE 3.—*Comparative activities of the drugs in various systems.*

| Drugs | *M. tuberculosis*[a] | | | *M. lepraemurium* | *M. leprae* | |
|---|---|---|---|---|---|---|
| | *In vitro*[b] | In mice | In man | In mice[c d] | In mice[d e] | In man |
| DDS | 7μg (42) | Active (28) | Inactive | 1.8 (0.1%) (7) | Complete suppr. (0.01%) | Drug of choice. (see text) |
| B663 | 0.3μg (1) | Active (1) | | ∞[f] (0.01%) (10) | Complete suppr. (0.01%) | Temporary improvement. Deterioration at 12 months (4, 5). |
| INH | 0.015-0.25 μg | Active | Active | 13.8 (0.01%) (6) | Complete suppr. (0.01%)[g] (39) | Temporary improvement. Deterioration at 12 months (15). |
| PAS | 1μg | Active | Active | 1.0 (0.6%) (7) | Complete suppr. (0.6%) (39) | Results ambiguous at 48 wks (20). See (39). |
| SM | 1μg | Active | Active | 2.8 (3 mg)[h] (7) | Complete suppr. (2 mg)[h] | As active as DDS at 32 and 48 weeks (20). |
| CS | 5-20μg | Not active | Active | 5.6 (1.0%) (8) | Partial suppr. (0.5%) (39) | As active as DDS at 48 weeks (21). |
| TB1 | 5μg (18) | Active[i] (19) | Active | 0.9 (0.3%) (7) | Partial suppr. (0.1%) | Temporary improvement. Deterioration in third year (30). |
| DPT | 2-4μg | Active (23) | Active (33) Inactive (27) | 0.9 (0.2%) (7) | Inactive (0.1%) | Less active than DDS (22). Temporary improvement. Deteriora- after 3 years (14). |
| EB | 5μg (35) | Active (41) | Active (3) | 3.4 (0.5%) (11) | Inactive (0.25%) | No report. |
| ETIP | Inactive (17) | Active (16) | | 18.8 (0.5%) (9) | Inactive (0.5%) | Temporary improvement. Deterioration after 4 months (13). |
| PZA | 6.25μg | Active | Active | 40.0 (0.5%) (6) | Inactive (0.5%) | Trials limited. |

*Toxicity.*—Toxic reactions were encountered with B663. The focal hepatic lesions containing crystals were apparently more severe than those reported by others ([2, 26]). Our mice had received the drug for much longer periods. If accumulation of the drug in the tissues is to be avoided in long-continued administration of the drug in a chronic disease such as leprosy, it may be necessary to interrupt treatment occasionally, perhaps until the plasma concentration falls sufficiently.

In the mice receiving higher doses of DDS there was an increase in the amount of pigment in Kupfer cells. Presumably this is a product of hemoglobin that arises from the increased hemolysis that frequently accompanies DDS intake. With the lowest doses of DDS there was no increased pigment, but there was still complete suppression of multiplication of leprosy bacilli.

SUMMARY

Eleven drugs have now been tested against *Mycobacterium leprae* in mouse footpads. Multiplication of *M. leprae* was suppressed completely by DDS (4,4′-diaminodiphenyl sulfone), B663 (a rimino compound), isoniazid, para-aminosalicylic acid, and streptomycin. It was suppressed partially by cycloserine and amithiozone. Diphenyl thiourea, ethambutol, diethyl dithiol*iso*phthalate, and pyrazinamide were inactive. Various decreasing dosages of DDS were tried, and the least amount used, 0.01 per cent in the diet, was completely suppressive. This dosage resulted in tissue concentrations averaging 1.0 to 2.6 μg/gm.

B663 in the dosage used, 0.01 per cent in the diet, produced frequent focal accumulations of crystals in the liver. DDS in the higher dosages caused the deposition of pigment in the liver, presumably secondary to the increased hemolysis that it frequently causes. In the lower dosages (0.01%), which were still completely effective against the bacilli, there was no deposition.

A comparative review is made of the activities of the 11 drugs against *M. tuberculosis, M. lepraemurium,* and *M. leprae.*

RESUMEN

Se han ensayado hasta ahora 11 drogas contra el *Mycobacterium leprae* en las patas de ratones. La multiplicación del *M. leprae* fué completamente suprimida por el DDS (4,4-diaminodifenilsulfona), B663 (un compuesto rimino), isoniacida, acido para-aminosalicilico, y estreptomicina. Fué suprimido parcialmente por las cicloserina y amitiozona. Fueron inactivas la difenil tiourea, etambutol, dietil-ditiolisoftalato y la pirazinamida. Fueron ensayadas diversas dosis decrecientes de DDS, y la menor cantidad usada, 0.01 por ciento en la dieta, fué completamente supresiva. Estas dosis resultaron en los tejidos en concentraciones promediadas en 1.0 a 2.6 μg/gm.

B663 en las dosis usadas, 0.01 por ciento en la dieta, produjo frecuentes acumulaciones focales de cristales en el hígado. El DDS en altas dosis produjo la deposición de pigimento en el hígado, presumiblimente secundaria a la hemolisis aumentada que frecuentemente lo produce. En las dosis menores (0.01%) las cuales fueron todavía completamente efectivas contra el bacilo, no hubo deposición.

Se hace un estudio comparado de las actividades de las 11 drogas contra los *M. tuberculosis, M. lepraemurium* y *M. leprae.*

RÉSUMÉ

Onze produits ont été à présent mis à l'épreuve pour leur action à l'égard de *M.leprae* dans la sole plantaire de la souris. La multiplication de *M.leprae* a été complétement arrêtée par la DDS (4,4′-diaminodiphenyl sulfone), le B663 (un rimino-composé), l'isoniazide, l'acide para-aminosalicylique, et la streptomycine. Elle fut partiellement arrêtée par la cyclosérine et l'amithiozone. La diphenyl thiourée, l'ethambutol, le diethyl dithiolisophthalate et la pyrazinamide se sont révélés inactifs. Divers dosages de DDS ont été essayés en ordre descendant, et la quantité la plus petite utilisée, 0.01% dans la nourriture, a témoigné d'une action suppressive entière. Ce dosage a entraîné des concentrations tissulaires de l'ordre de 1.0 à 2.6 μg/g.

Aux doses utilisées, 0.01% dans la nourriture, le B663 a fréquemment entraîné des accumulations de cristaux localisés en foyers dans le foie, probablement à la suite de l'hémolyse accrue que cause souvent ce produit. Aux dosages les plus bas (0.01%), encore parfaitement efficaces contre les bacilles, il n'a pas été noté de telles dépositions.

Une revue est faite des activités comparées de ces 11 produits contre *M.tuberculosis, M.lepraemurium,* et *M.leprae.*

*Acknowledgements:* We would like to acknowledge our indebtedness to Dr. Guy P. Youmans and Dr. Anne S. Youmans for letting us make use of their valuable review ([43]) before its publication. We are indebted to Mrs. Dorothy McRae and Mrs. Mary Ann Edwards for their careful technical contribution.

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