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Sensitivity of *Mycobacterium leprae* to Low Levels of 4,4'-Diaminodiphenyl Sulfone.* (31282)

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The most widely used drug for treatment of leprosy is 4,4'-diaminodiphenyl sulfone (DDS). Although it must be administered for years to achieve cure in lepromatous disease, it is eventually effective in nearly all cases(1). The usual dosage is 100 mg/day, and it produces 1-5 μg DDS/ml blood(2). DDS is also effective against *M. leprae* in mice(3,4). The lowest intake previously studied was 0.01% in the diet; it also was completely effective against the bacilli, and it produced about 3 μg DDS/ml serum(4).

In the present study in mice the range of DDS dosages tested has been extended to very low levels, without encountering the endpoint.

Materials and methods. In the experimental system employed(5,3,4), mice were injected in a rear foot pad with 5×10^3 *M. leprae*. The bacillary growth curve was then monitored by monthly harvests of foot pads from untreated mice and counts of the contained acid-fast bacteria (AFB). In the method for counting AFB the technique for applying the sample to the slide has been modified somewhat (unpublished). When the counts rose to a level near 1×10^6 AFB/mouse, harvests were made from each treated and control group. The strain of *M. leprae* used was in first mouse passage. Due to difficulties in the breeding colony, the line of mouse used previously, CFW, was not available in adequate numbers, and BALB/C mice were used instead.

DDS was administered in the diet starting on the day of infection. Since it was essential to ensure homogeneous distribution in the food, the drug was dissolved in 95% ethanol and mixed into the unpelleted diet with a liquid-solid twin-shell blender (Patterson-Kelly Co.). In this apparatus the liquid is sprayed into cavities formed in the rapidly mixing diet by a spinning feed bar so that aggregation of food particles and localized soaking does not occur. The same amount of ethanol was mixed into the control diet. Diets were always mixed in the order of increasing DDS concentration, and the apparatus was carefully cleaned after each day of use.

Sulfone (unconjugated) in blood was determined by the procedure of Simpson(6), modified for smaller volumes for measurement in the 3 ml cell of a Beckman DU spectrophotometer. To 1 ml of heparinized blood, 0.25 ml 0.2 M Na_2HPO_4 was added, and extraction was carried out with 6 ml ethyl acetate by mixing on a vortex mixer for 15 seconds. After centrifugation at 2000 RPM for 5 minutes, 5 ml of the organic phase was transferred by pipette (fitted with a safety bulb) to another tube, and extraction was carried out in the same way with 5 ml 1 N HCl. After the same centrifugation 4 ml of the aqueous phase was removed similarly to another tube. The efficiency of the extrac-

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TABLE I. Effect of DDS on Multiplication of *M. leprae* in Mice (Foot Pads). Mice were inoculated in a hind foot pad with 5×10^3 *M. leprae* (12% solidly staining(7)). The bacillary growth curve was then monitored with periodic harvests of 4 mice taken from 4 control groups; the harvest at 98 days was $<5 \times 10^4$ AFB/mouse, at 133 days 3.5×10^4, at 163 days 1.9×10^5, and at 198 days 5.1×10^5. Harvests were carried out from 4 mice from each group shortly thereafter and at 3 month intervals.

Mouse No.	% DDS in diet	Harvest (AFB/mouse) ($\times 10^4$) 220–221 days	320–336 days	447–449 days	DDS in blood (μg/ml) 320–336 days	447–449 days
1– 20	Nil	138	60	94	0 (2),* 0 (2)	0 (2),* 0 (2)
21– 40	.03	<2	<2	2	2.0 (1), 4.4 (2)	3.1 (2), 3.8 (2)
41– 60	.01	<2	<2	<2	.5 (1), .6 (3)	1.0 (1), 1.7 (1), 1.5 (1)
61– 80	Nil	8	29	29	0 (2), 0 (2)	0 (3), 0 (1)
81–100	.003	<2	<2	<2	0 (1), .9 (3)	0 (1), .4 (1), .4 (2)
101–120	.001	<2	<2	<2	.2 (1), .3 (3)	0 (1), 0 (3)
121–140	Nil	19	2	7	0 (3), 0 (1)	0 (3), 0 (1)
141–160	.0003	<2	<2	4	0 (1), 0 (3)	0 (1), 0 (3)
161–180	.0001	<2	<2	<4	0 (1), 0 (2), 0 (1)	0 (2)
181–200	Nil	131	29	24	0 (1), 0 (3)	0 (2), 0 (2)
201–220	.00003	<2	2	<2	0 (1), 0 (2), 0 (1)	0 (2), 0 (2)
221–240	.00001	<2	2	3	0 (1), 0 (3)	0 (1), 0 (3)
241–260	Nil	54	42	—	0 (3), 0 (1)	—

* In parentheses is given the number of mice in pool.

tions was demonstrated by comparison to the standard procedure. Color was developed by adding 2 drops of the necessary reagents. The 2 standards contained, respectively, 10 and 2 μg DDS in 1 ml. Each determination was done in duplicate. With these modifications the lower limit of detectability representing an optical density of about 0.010, is about 0.2 to 0.3 μg/ml sample.

Results. At all levels of DDS intake, including the smallest amount tried (0.00001%), the multiplication of *M. leprae* was completely suppressed (Table I). An intake of 0.00001% in the diet amounts to a daily intake of about 0.3 μg/mouse/day or 10 μg/kg/day. The concentrations of DDS in the blood corresponded to those observed previously(4). The lowest dosage producing detectable levels of sulfone was 0.001% in the diet, which gave 0.2 to 0.3 μg/ml blood.

Discussion. 1. *Reliability of the result.* That such a small dosage of DDS was able to suppress multiplication of *M. leprae* was quite surprising, and we have reexamined the results repeatedly (but unsuccessfully) in search of technical errors. The control mice did not have as consistently high a level of AFB as has been observed routinely, and 2 causes for the irregularity deserve consideration. For one, the unexpectedly great sensitivity of the organism to this drug suggests how difficult it may be to prevent significant contamination of control diet with DDS. For another, BALB/C mice were used, and our previous experience with them, although favorable, is very much less extensive than with CFW mice. Nevertheless, the differences between control and treated mice were distinct. In no case did treated mice have counts that represented much more than the occasional chance encounter of persisting inoculum. The possibility of artifact due to uneven distribution of the drug in the diet, *e.g.*, occasional intake of amounts larger than nominal, was minimized by the method of mixing drug into diet. Preliminary confirmation of the very great sensitivity of *M. leprae* to DDS has been obtained with another strain of the microorganism in another experiment in which 4,4′-diacetylaminodiphenyl sulfone is being tested as a repository injection(8). (DDS, or the monoacetyl derivative is slowly released by deacetylating enzymes in the tissues.) First results indicate that the minimal effective dose is about 1-5 mg/kg when given every 2 months.

2. *Strain differences.* Little is known about genetic differences in sensitivity to very low levels of DDS among populations of *M. leprae* in the same and in different untreated patients, and experiments to test more isolates are in progress. Lowe studied groups of lep-

romatous patients treated with usual doses of DDS for periods up to 7 years and found that they all responded(1). More recently, Pettit and Rees(9) have described DDS-resistant strains of *M. leprae* isolated in mice from patients who had been treated unsuccessfully for 13 to 15 years. The level of DDS they used in mice was 0.1% in the diet; such an intake produced about 20 μg/g non-hepatic tissue in CFW mice(4). Pettit and Rees estimate that such resistant cases had occurred not more than 3 times in 5000 lepromatous cases.

3. *Estimated sensitivity of* M. leprae *to DDS.* The lower active dosages did not produce chemically detectable concentrations of DDS in the blood. In the upper ranges (but below 0.2% in the diet, at which level food consumption is reduced) the concentration of sulfone in blood and tissue was approximately proportional to the intake, and extrapolation to 0.00001% in the diet leads to an estimate of about 0.003 μg/DDS/g blood and non-hepatic tissue. Not much can be said about the accuracy of the estimate since it involves an extrapolation over a hundred-fold range and little is known about the induction levels of conjugation enzymes(10), the amounts of which would be important parameters. Although the endpoint was not reached, this concentration of drug may be compared to reported minimal inhibitory concentrations (MIC) of sulfones and sulfonamides for other microorganisms. Karlson(11) studied a number of mycobacterial cultures on Lowenstein-Jensen medium; some of the cultures, including most of the *M. avium* were sensitive to 3.1 μg/ml, the lowest concentration tested. The MIC of the more active sulfonamides for sensitive non-acid fast bacteria is about 0.5 to 1.0 μg/ml(12). The MIC for DDS against *Streptococcus agalactiae* is also about 0.5 μg/ml; against group A streptococcus infections in mice DDS is active at an intake of 0.00625% in the diet(13), which would produce about 0.5 μg/ml in the blood. The suppressive dose of DDS for blood-induced *Plasmodium berghei* infections in mice is 0.275 mg/kg/day(14) a dose that corresponds to about 0.0003% in the diet, and an estimated 0.1 μg/DDS/g tissue. Thus *M. leprae* is apparently much more sensitive to DDS than other microorganisms so far studied, suggesting the presence of unusual aspects of the folate (or sulfone) metabolism or distribution.

4. *Concentration of DDS in infected tissue.* The concentration of sulfone in non-hepatic tissue has usually been reported as about the same as that in the blood of humans(15,2) and mice(4). However, Chatterjee and Poddar(16) reported that the concentration of S^{35} labelled DDS after a single dose was about 10 times higher in leprous skin than in normal skin. Although the mice in the present experiment did not receive high enough intakes of DDS, those in a previous experiment(4) did, and infected foot pad suspensions had been preserved at −60°C. The concentration was found to be 18, 6 and less than 12 μg DDS/g foot pad, respectively, in 3 different harvests of mice receiving 0.2% DDS in the diet. The average concentrations in blood and muscle had been found to be 22 and 20 μg/g, respectively, in the same experiment(4). Thus there was no suggestion that DDS is more concentrated at the infected site in mice under the stated experimental conditions.

5. *Implications of the result in human leprosy.* In the experimental design used here, what is tested is suppression of multiplication of a small bacterial inoculum. In the leprosy patient many of the bacilli probably are multiplying only slowly, so they would be less susceptible to DDS. Also the bacterial population in the patient is very much larger, thus increasing the possibility of the presence of bacilli resistant to the amount of DDS to which they are exposed. Hence it would be expected that the level of DDS needed for effective human therapy is higher than that needed to suppress infection in the mouse. Nevertheless the difference between the tissue levels produced in humans with the accepted therapeutic dose of DDS and the levels found effective in this study are very large (about a thousand-fold). In apparently the only published experiment to learn the minimal effective dose of DDS in human leprosy, Lowe(2) treated 6 tuberculoid leprosy patients with increasing doses, starting each at 15 mg/day and stepping up the dose of each

every 2 months to a maximum of 100 mg/day. He noted clinical improvement with 15 mg, but said it was less rapid than that with 50 and 100 mg/day. However, the clinical picture is not generally considered to be consistent enough to allow such fine discrimination in drug response, and in most clinical trials it has been found necessary to assess the drug by the bacteriologic response in groups of lepromatous cases, each group on a particular dosage for a year or more.

Thus it seems indicated to test the therapeutic effect in human leprosy of lower dosages of DDS and less frequent administration. The margin between toxic level and therapeutic level of the drug appears to be so large as to allow considerable innovation in the regimen. DDS is excreted only slowly and Ross(15) has reported detectable concentrations in blood 2 to 4 weeks after the last administration of sulfone. The results suggest that DDS might have an especially useful role in chemoprophylaxis, a contral measure potentially adaptable to many leprosy endemic areas.

Summary. 1. The activity against *Mycobacterium leprae* of 4,4'-diaminodiphenyl sulfone (DDS) was tested in mice by feeding a series of diets containing the drug in concentrations ranging from 0.03 to 0.00001%. Multiplication of *M. leprae* was completely suppressed at all levels. 2. The lowest DDS intake was a hundred times less that the least amount required to produce chemically detectable amounts in the blood (0.2 to 0.3 μg/ml).

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An Anomalous Effect of Theophylline on ACTH and Adenosine 3',5'-Monophosphate Stimulation.* (31283)

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The mechanism of action of a set of peptide and amine hormones in their target tissues has been shown to involve a coupling of the primary hormone reaction with effector systems by adenosine-3',5'-monophosphate (3',5'-AMP)(1-4).

In general 3 criteria have been used to establish an intermediary role for 3',5'-AMP in hormone action. First the hormone is shown to have a specific effect to increase the concentration of 3',5'-AMP in the target tissue; second, 3',5'-AMP is shown to simulate the effect of the hormone on its target tissue; third, inhibition of the breakdown of 3',5'-AMP in target tissues by methyl xanthines is shown to either stimulate or potentiate the hormonal action(5).

The participation of 3',5'-AMP as an inter-

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