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Effect of DDS on Established Infections with *Mycobacterium leprae* in Mice[1]

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The usual experimental design for chemotherapeutic studies of infectious agents in experimental animals is one where treatment is started at the time the animals are infected. What is tested in this design is the ability of the drug to prevent multiplication of the infectious agent. There are several advantages to such a schedule, among which are the promptness with which results are obtained and the relatively clear-cut difference between treated and control groups. We have used this approach in earlier work with *Mycobacterium leprae* in mice ([7, 8, 9, 11]).

In the natural human disease therapy is begun after the infection is already established. In this situation one wishes to be able to attack all physiologic states of the infectious agent, and not merely to prevent active multiplication. In the present study an attempt was made to reproduce the usual therapeutic situation, and administration of the drug was delayed until the bacilli had completed the first phase of active bacillary multiplication and had reached a relatively stationary level. We have confined our attention to 4,4'-diaminodiphenylsulfone (DDS).

MATERIALS AND METHODS

The plan of the experiment is described in Figure 1. The procedures have been described as follows: Counts of acid-fast bacteria (AFB) ([5]), determination of generation time (G) ([10]), histologic procedures ([6]), and administration of DDS ([8, 9]). The determination of the ratio of solidly stained AFB was made as described ([10]), except that immersion oil was also put between the slide and the condenser (N.A. 1.30), and the additional requirement was imposed that solid bacilli also be deeply stained. The latter stipulation was added in order to eliminate the occasional pale but evenly stained organism that interfered with the previous determination, especially at very low solid ratios. It should be noted that the acid-fast stains were made at room temperature, and that excessive heat was avoided during fixation. Previous experience had shown that hot stains give distinctly inferior results ([5]).

Before the beginning of treatment all mice were fed the usual pelleted commercial chow. DDS was administered as a dry addition to powdered commercial diet. The controls received the same diet without addition of drug. Sulfone determinations were performed by a slight modification ([9]) of the method of Simpson ([12]).

Two mice were pooled for each harvest until after 318 days; later there was one in each harvest. The solid ratio was not determined in the DDS mouse at 502 days because of the small number of bacilli in the smear. No mice remained in the DDS group after 502 days.

RESULTS

Number of acid-fast bacilli. Before treatment was started the total bacillary population had risen by 102 days to the normal plateau level above $10^{6.0}$. DDS was started

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in half the mice about one month later. In the treated mice after that time the number of acid-fast bacilli did not rise significantly. In the untreated controls there was the usual slow, moderate bacillary increase. Later (after 500 days) the number of acid-fast bacilli decreased in both groups.

Bacillary viability in untreated mice. At the time the experiment was begun, it was thought that the bacilli in the maximum stationary phase (at the plateau level) were in a relatively steady state. Later it was observed ([10]) that this phase is in fact marked by alternating phases in which either bacillary death or bacillary growth predominates, as determined by changes in the ratio of solidly staining bacilli. In the control mice, according to this interpretation, the period from 134 to 195 days was a death phase, and there was conversion of solid to nonsolid bacilli with concomitant changes of infectivity. A growth phase then followed and carried the number of solid bacilli to a second peak at 226 days. After 226 days a second death phase ensued, and the solid ratio fell to less than 2 per cent after 300 days. Infectious and solidly staining bacilli did not rise to detectable levels again until after 450 days. Then another growth phase was reflected by a prolonged period in which solid and infectious bacilli were present in most harvests.

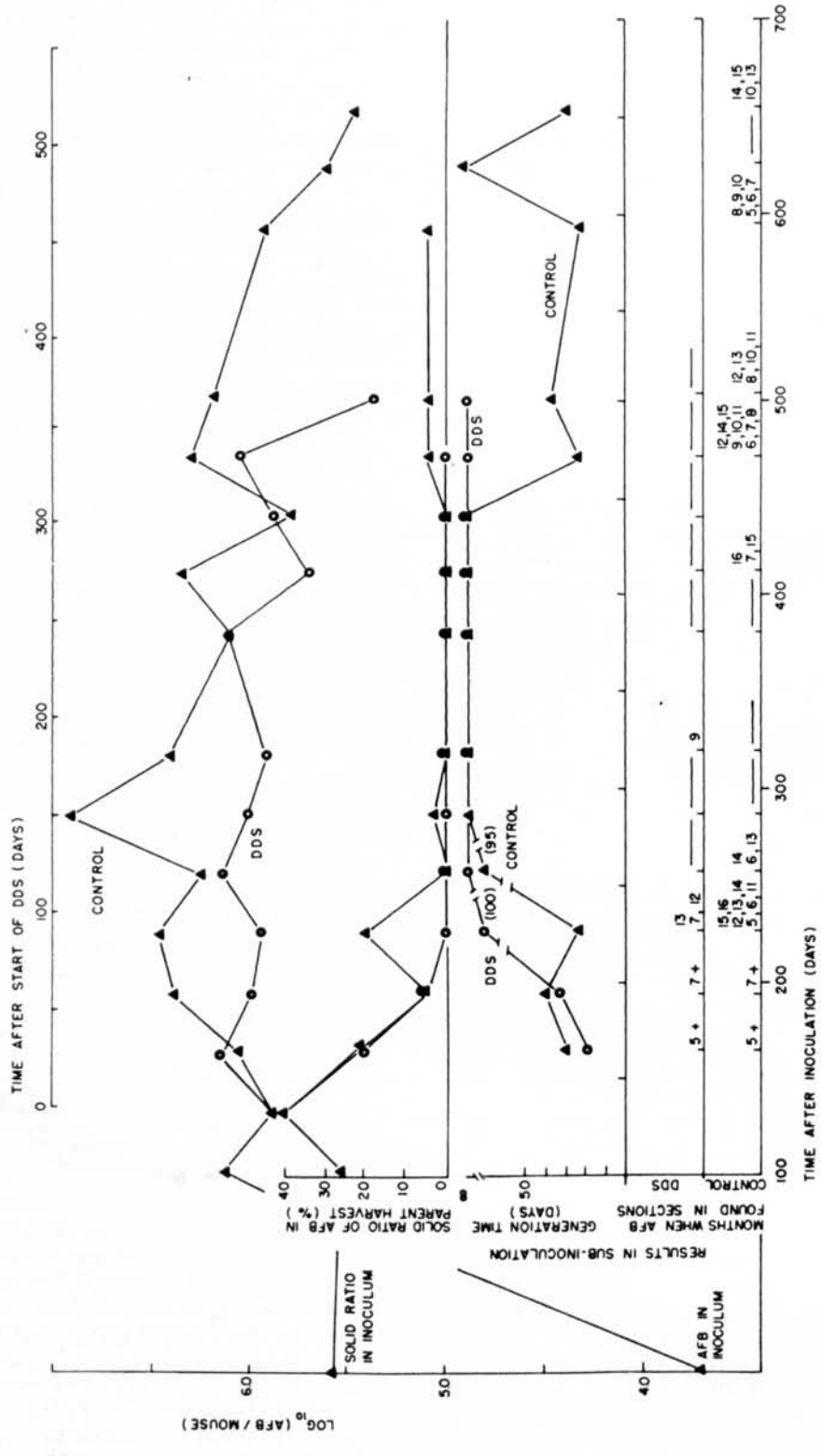
Bacillary viability in treated mice. DDS was started shortly after the beginning of the first death phase, and the first two harvests in the treated mice, after 27 and 57 days of DDS, were made during the death phase of the controls. In these harvests the number of viable bacilli, as determined by subinoculation and by staining, was little different from that in the untreated controls. The next harvest was made after 88 days of DDS (226 days after infection). The bacillary viability in the treated mice was then distinctly reduced, as evidenced in a solid staining ratio of less than 2 per cent and a subinoculation result with a prolonged generation time and fewer positive sections.

In contrast, the harvest in the untreated mice at the same time had the usual rise in solid staining ratio associated with the second growth phase. The increase in viability was also reflected by the shortened generation time in the subinoculated mice. This second growth phase had been observed in all experiments in which harvests are carried out frequently enough, i.e., at least once a month. It appeared in this experiment at the usual time, about 140 days after the peak of the first growth phase.

After that the only evidence of viable bacilli in the harvests in the treated mice was a single positive section in the mice subinoculated 318 days after infection (after 180 days of DDS). The minimal infectious dose of *M. leprae* in the mouse foot pad is on the order of 10 solidly staining bacilli ([10] and unpublished results), so that the negative results in the subinoculation indicate that the proportion of viable bacilli had fallen below 10 in 5×10^3 bacilli or about 0.2 per cent. Since about a million bacilli per mouse were present in the harvests, up to a few thousand bacilli could have been present without detection by subinoculation.

Correlation between solidly stained bacilli and their infectivity. A previous study ([10]) examined the idea emphasized in recent years by Rees and Valentine ([4]) that dead *M. leprae* do not stain solidly in the acid-fast technic. Mouse foot-pad inoculation results confirmed the proposition directly, and suggested also that a proportion of the bacilli scored solid by the criteria we then used were also dead. Accordingly the stricter definition of solid bacilli given in MATERIALS AND METHODS was set. The results of the present experiment support the new definition.

In instances where solid bacilli were detectable the rate of appearance of bacillary growth in subinoculated mice was now well predicted by the solid ratios (in terms of reference [10]), G_s did not increase at low solid ratios). Also, when the inoculum contained more than 100 solid bacilli, the results were regular. When the inoculum contained less than 100 solid bacilli the results were irregular, as evidenced by infrequently positive sections, and low or negative bacillary harvests. A single unexplained exception occurred with the control harvest at 287 days, when a solid bacillus was scored but the subinoculation


TIME AFTER START OF DDS (DAYS)
CONTROL
DDS
LOG10 (AFB / MOUSE)
SOLID RATIO IN INOCULUM
SOLID RATIO OF AFB IN PARENT HARVEST (%)
GENERATION TIME (DAYS)
RESULTS IN SUB-INOCULATION
AFB IN INOCULUM
MONTHS WHEN AFB FOUND IN SECTIONS
TIME AFTER INOCULATION (DAYS)

was negative. It is noteworthy that with the stricter definition there was no evidence that nonsolid bacilli were viable.

Amount of sulfone in blood of the mice. Blood samples of several mice being sacrificed for counts of acid-fast bacilli were saved for sulfone determinations as a check on the accuracy of drug administration. Samples from five treated mice had free sulfone values ranging from 8 to 20 μgm./ml. (average 14 μgm./ml.). Samples from nine control mice were negative.

DISCUSSION

The experimental model duplicated the main features of the natural infection. DDS was able to prevent further bacillary increase, but even after one year of continuous drug therapy there was only slight reduction in numbers of total bacilli. In contrast, the number of viable (solidly staining and infective) bacilli did decrease in the course of a few months.

It is interesting to compare the results with the clinical results of Waters and Rees ([13]), who found that the average solid ratio decreased from about 50 per cent to about 10 per cent in three months of DDS therapy, and to about 3 per cent in six months. It remained at about 3 per cent for the 18 months of their observation. There are technical differences in the preparation of slides and in their observation, and their solid ratios are not necessarily equivalent to ours. Nevertheless these results indicate that similar events occur in the human patient in about the same time period.

To our knowledge the present study is the first direct measurement of the rate at which DDS kills *M. leprae.* The time scale involved was found to be an extended one. It required 88 days of DDS before the viability of the bacilli was found to be reduced at all on comparison with the controls. This corresponds to about seven times the 12-13 day generation time estimated for *M. leprae* in the logarithmic phase ([10]). Presumably viable bacilli in reduced numbers persisted much longer, and there was a single histologically positive mouse inoculated with the harvest at 318 days.

The time of the onset of observable killing of *M. leprae* by DDS may have been governed by the onset of the second growth phase. In general, antibacterial substances

FIG. 1. Results of DDS treatment of established infection of *M. leprae* in mice. Seventy mice were inoculated in a hind foot pad with 5.0 x 10^3 acid-fast bacteria (AFB), 30% of which stained solidly. About one month after the population of AFB had grown to a level of about 10^6 (138 days after inoculation), DDS (0.1% in the diet) was started in half the mice. At intervals one or two mice from the DDS-treated and control groups were taken for harvests of infected foot pads, in which the AFB were counted (top), and the ratio of solidly staining AFB determined (next to top), as an estimate of bacterial viability. For this ratio usually 50 bacilli were scored, and the zero value refers to less than 2 per cent, i.e., no solid bacilli were encountered. The viability of the AFB in most harvests was also tested by subinoculation of about 5 x 10^3 AFB into new groups of mice. In these subinoculated groups a mouse was taken at monthly intervals for histologic sections (bottom two lines). The months in which AFB-positive sections were found are indicated ("+" signifies that sections were not made after that month, "———" that no positive sections were found). When AFB were first found in the sections, harvests of four mice were made for counts of AFB. The rapidity with which the AFB grew in the subinoculated mice is expressed as generation time (∞ indicates that no AFB were found in the harvest, or that the harvest was not carried out because the sections were always negative). The records for the harvests at 502 days were in fact reversed from that shown. It is assumed that a mix-up in the records occurred in those harvests, which came at a particularly busy period. Since bacillary viability in the treated group would have represented sulfone resistance, the bacilli harvested from the sub-inoculated group were tested for sulfone resistance by passage to mice receiving 0.01 per cent DDS in the diet. In this passage no growth was detected in the treated mice, although bacillary growth was prompt and normal in the untreated control.

are much more active during the rapidly growing phases of bacteria. Presumably DDS acts against *M. leprae* by interfering with the synthesis of folate compounds. Griffin and Brown ([2]) found that all the bacteria they studied, including *M. avium,* synthesized these compounds in two enzymatic reactions. In the first one a dihydropteridine (2-amino-4-hydroxy-6-hydroxymethyldihydropteridine) reacted with *p*-amino benzoic acid to form dihydropteroic acid. In the second reaction dihydropteroic acid reacted with glutamic acid to give dihydrofolic acid. Experiments with cell-free extracts showed that sulfonamides prevented the formation of active folate compounds by entering into the first reaction in place of *p*-aminobenzoic acid to form inactive analogs of dihydropteroic acid ([1]). It seems likely that before DDS becomes lethal a certain amount must be metabolized by the bacterium, and that the time required for lethality depends upon the level of the bacterium's metabolic activity.

Another factor that may have affected the lethal rate of the drug was its concentration. In the present study the amount of DDS given was 0.1 per cent in the diet, which produced about 10-20 μgm. free sulfone/ml. blood. Human beings receiving full dosage of DDS (100 mgm./day) have 1-5 μgm. free sulfone/ml. blood. How much difference this would make in the rate of killing is not known. These concentrations are very much greater than the 0.003 μgm./ml. or less estimated as the minimal inhibitory concentrations of DDS for *M. leprae* in the mouse ([11]).

SUMMARY

1. In an attempt to reproduce in mice the therapeutic situation in the human patient, experimental infections of mice were allowed to establish themselves before treatment was started. To accomplish this, mice were inoculated with 5,000 bacilli, and the infection was allowed to develop without therapy through the logarithmic phase of bacillary growth and into the "maximum stationary" phase, where the bacterial population is a few million. Then, a little more than one month after the end of the logarithmic phase, DDS (0.1% in the diet) was begun, and it was continued for about one year.

2. Treatment stopped any further bacillary increase. There was little change in numbers of acid-fast bacilli other than a possible decrease of several-fold toward the end of the year of therapy.

3. The number of solidly staining acid-fast bacilli in the treated mice was not significantly different from that in the untreated mice during the first 57 days of therapy. After 88 days of therapy solid bacilli decreased to undetectable levels (less than 0.2%), and did not reappear.

4. Subinoculation of the harvests into new groups of mice revealed that the number of viable (infective) *M. leprae* fell in parallel with the number of solid bacilli, as defined here. The number of solid bacilli subinoculated predicted the subinoculation result satisfactorily, except that subinoculation was able to detect lower numbers of viable bacilli.

RESUMEN

1. En un ensayo para reproducir en ratónes el esquema therapeútico de enfermos, se permitió que se produjeran infecciones experimentales de ratónes antes de iniciar el tratamiento. Para este propósito, los ratónes fueron inoculados con 5,000 bacilos y se permitió que la infección se desarrollara sin tratamiento durante la fase logaritmica del crecimiento bacilar y hasta la fase "estacionaria máxima" donde el número de bacteria es de unos pocos millones. Luego, poco después de un mes al término de la fase logarítmica, se comenzó con DDS (0.1% en la dieta), y se continuó por un año, aproximadamente.

2. El tratamiento detuvo el aumento bacilar posterior. Hubo poco cambio en el número de los bacilos ácido-resistentes, excepto una posible disminución de fases múltiples hacia el final del año de tratamiento.

3. El número de bacilos ácido-resistentes densamente teñidos en los ratónes tratados no fué significativamente diferente de aquellos en los ratónes no tratados durante los primeros 57 días de tratamiento. Después de 88 días de tratamiento los bacilos sólidos disminuyeron a niveles no detectables (menos de 0.2%), y no volvieron a reaparecer.

4. La subinoculacion de la cosecha en nuevos grupos de ratónes reveló que el número de *M. leprae* vivos (infectantes) cayeron paralelamente con el número de bacilos sólidos, como aquí se las define. El número de bacilos sólidos subinoculados predijo satisfactoriamente el resultado de la subinoculación, excepto que la subinoculación permitió detectar números mas bajos de bacilos vivos.

RÉSUMÉ

1. Avec le propos d'essayer de reproduire chez la souris la situation thérapeutique du malade humain, on a fait en sorte que des infections expérimentales s'établissent chez des souris avant e'instauration du traitement. A cette fin, on a inoculé des souris avec 5,000 bacilles, et on a laissé l'infection se développer sans traitement pendant tout le cours de la phase logarithmique de croissance bacillaire, et jusque durant la phase "stationnaire maximale" alors que la population bactérienne atteint quelques millions. A ce moment, soit un peu plus d'un mois après la fin de la phase logarithmique, on a commencé la DDS (0.1% dans la ration), ce qui fut continué pour environ une année.

2. Le traitement a arrêté toute augmentation ultérieure du nombre des bacilles. Il y a eu peu de changement dans le nombre des bacilles acido-résistants, sinon peut-être une diminution de plusieurs fois à la fin de l'année de traitement.

3. Le nombre de bacilles acido-résistants se colorant sous forme solide n'a pas été différent chez la souris traitée et chez la souris non traitée durant les 57 premiers jours de traitement. Après 88 jours de traitement, le nombre de bacilles solides a diminué jusqu'à une proportion qui ne pouvait être décelée (moins de 0.2%) et ne l'a plus dépassée.

4. L'inoculation sous-cutanée du produit de récolte à un nouveau groupe de souris a révélé que le nombre de *M. leprae* viables (dotés de pouvoir infectieux) diminue de manière parallèle au nombre des bacilles solides, tels qu'ils sont définis ici. Le nombre de bacilles solides sous-inoculés prédit de façon satisfaisante les résultats de la sous-inoculation, si l'on excepte cependant le fait que la sous-inoculation a permis de détecter des nombres plus faibles de bacilles viables.

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