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A Kinetic Method for the Study of Activity of Drugs Against *Mycobacterium leprae* in Mice[1, 2]

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Most studies of the activity of drugs against *Mycobacterium leprae* in mice from this laboratory ([4,5,6,7,11]) and from others ([1,2,3]) have followed a procedure in which administration of the drugs is begun on the day of infection and continued until the termination of the experiments. The infectious inoculum is diluted so that if no bacillary multiplication occurs, the concentration of AFB in the foot pad tissues remains below microscopically detectable limits. Only inhibition of multiplication is observed, and the killing of *M. leprae* by the drug is not measured.

With Y. T. Chang we have made an attempt to measure bactericidal activity by DDS, by allowing the *M. leprae* to multiply to the usual plateau level above 1 x 10^6 per mouse before starting the drug ([8]). At intervals, treated and control mice were then sacrificed, the *M. leprae* counted microscopically, and their viability measured by subinoculation into new groups of mice. Although this approach had the advantage of mimicking the usual therapeutic situation in the human patient, it had the great disadvantage of being very laborious and time-consuming. In addition, determination of the rate of killing was made difficult by the cycles of death and growth that occur during the plateau phase of the bacillary growth curve in the untreated mouse ([9]).

The paper presented here describes a kinetic method that takes advantage of the amount of accuracy available in the logarithmic phase of the growth curve of *M. leprae*. Drug is given only for a limited period early in the growth curve, and the effect of drug is noted by the subsequent delay in appearance of the logarithmic phase of growth.

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[2] The following abbreviations are used throughout: AFB = acid-fast bacteria; DADDS = 4,4′-diacetyldiaminodiphenyl sulfone; DDS = 4,4′-diaminodiphenyl sulfone; DPT = diphenylthiourea (thiambutosine, Ciba-1906); INH = isoniazid; PAS = *p*-aminosalicylic acid; SM = streptomycin.

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MATERIALS AND METHODS

The mice were of the CFW strain, raised at the National Communicable Disease Center. They were caged, and cages randomized, and all the mice infected with 5 x 10^3 *M. leprae* from a strain (B2602) in fourth mouse passage from an untreated lepromatous patient. The diets and injections were given as shown in Table 1. The control diet was unpelleted commercial chow. The drug diets were prepared by adding drugs to it as follows: 0.1% DDS, 0.01% DDS, 0.1% DPT, and 0.6% PAS as dry powder; and 0.00001% DDS and 0.01% INH as ethanolic solutions. The liquid-solid twin-shell blender ([11]) was employed; precautions were taken to prevent carry-over of drug.

The methods for harvesting foot pads ([6,7]) and for counting AFB ([10]) have been described.

RESULTS AND INTERPRETATION

The growth curve in control mice was monitored by harvests from pools of four mice from different control groups at monthly intervals, starting at three months after inoculation. Soon after the bacterial population had increased to normal plateau levels above $10^{6.0}$, harvests were carried out on pools of four mice from each treated and control group, first at five months (161-162 days) and again at eight months (250-251 days). The results are given in Table 1 and Figure 1. It is apparent that some of the treatments delayed the growth of *M. leprae*, but none was able to kill all the organisms.

A graphic analysis of the results is given in Figure 1, where the growth curves are idealized into lag, logarithmic, and plateau phases. Graphically the lag phase assumes pure persistence of the inoculum until the

TABLE 1. *The effect of temporary administration of drugs on subsequent growth of* M. leprae

Mouse number	Diets[a] and injections	Harvest (AFB/mouse)	
		5 months	8 months
1–20	Control	1.2×10^6	1.7×10^6
21–40	DDS, 0.1%	$<1.8 \times 10^4$	2.2×10^6
41–60	DDS, 0.01%	1.6×10^4	1.9×10^6
61–80	Control	4.0×10^6	2.1×10^6
81–100	DDS, 0.00001%	4.8×10^6	1.4×10^6
101–120	DADDS, 100 mgm./kgm. at 30 days only[b]	4.6×10^5	1.9×10^6
121–140	Control	2.5×10^6	3.2×10^6
141–160	SM, 2 mgm. thrice weekly, 30 to 86 days[b]	2.7×10^5	1.4×10^6
161–180	INH, 0.01%	4.6×10^6	2.2×10^6
181–200	Control	1.2×10^6	1.3×10^6
201–220	DPT, 0.1%	1.5×10^5	1.1×10^6
221–240	PAS, 0.6%	3.8×10^6	2.8×10^6
241–260	Control	2.2×10^6	2.2×10^6
261–280	DDS, 0.01% + SM 2 mgm. thrice weekly 30–86 days	$<1.6 \times 10^4$	1.2×10^6
281–300	DDS, 0.01% + INH, 0.01%	1.5×10^5	2.0×10^6
301–320	Control	5.1×10^6	2.0×10^6
321–340	DDS, 0.01% + DPT, 0.1%	1.7×10^4	1.8×10^6
341–360	DDS, 0.01% + PAS, 0.6%	4.7×10^5	1.8×10^6
361–380	Control	1.3×10^6	5.8×10^5

[a] Diets given from 33 to 93 days postinoculation.
[b] Control diet given.

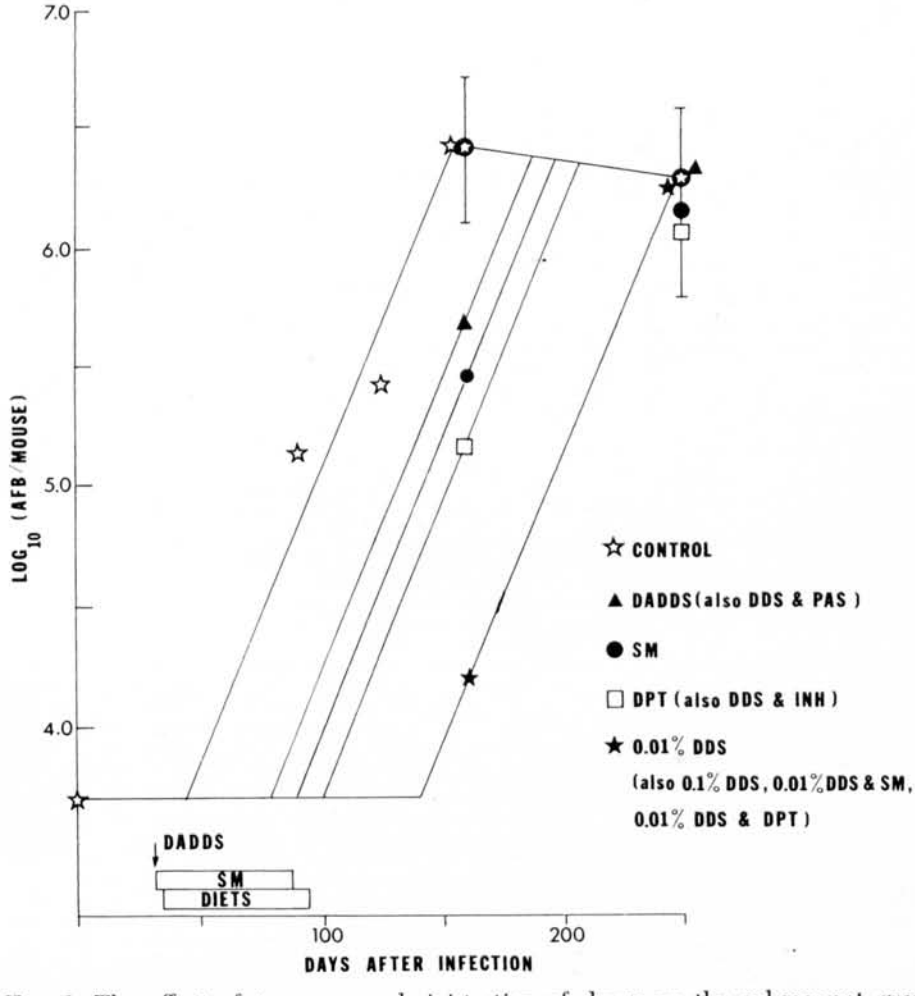

FIG. 1. The effect of temporary administration of drugs on the subsequent growth of *M. leprae* in mice. The growth curve in untreated controls (white stars) rose to normal plateau levels above $10^{6.0}$ at 156 days. Harvests were then carried out in all of the treated and control groups; harvests from all these groups were reported three months later. The average for all the control groups is indicated by the circled stars, and the range of harvests from individual control groups by the vertical lines. Also shown are the harvests from the groups whose treatment caused some delay in the growth of *M. leprae*. "DDS and PAS" refers to mice receiving a combination of DDS and PAS, "DDS and INH" to mice receiving a combination of DDS and INH, etc.

beginning of the logarithmic phase. In fact, however, death of bacilli (or loss from the site) may also occur, and the differentiation is made later on the basis of other evidence. The logarithmic phases are assumed to have generation times of 12.5 days; this is our best estimate of the growth rate in this period ([9]), and this value fits the present data well. The logarithmic phases are drawn to the best fit of experimental values in this interval and assumed to extend without interruption until they reach plateau levels. According to this analysis the control curve entered the logarithmic phase at 43 days and left it to enter the plateau phase at 156 days.

Single drugs. The simplest cases for interpretation are SM, DPT, INH, PAS, and 0.00001 per cent DDS. SM delayed the bacterial growth, but it did so only for the

period during which it was administered. DPT also delayed bacterial growth, but only for a period that extended seven days beyond the period of its administration. Hence it would also have been bacteriostatic without being significantly bactericidal. INH, PAS, and 0.00001 per cent DDS when given singly had no noticeable effect during this experiment. Since the first harvest (five months) was apparently carried out very shortly after the end of the logarithmic phase, even partial inhibition should have been detectable.

The interpretation of the results with DDS involves the persistence of this drug in the tissues. DDS, fed in a concentration of 0.01 per cent, delayed the growth of *M. leprae* 47 days beyond the period of drug administration. DDS is a slowly excreted drug and 0.01 per cent in the diet is approximately 100 times the minimal amount needed to stop multiplication ([5]), so that at least part of the 47 days would be the time required for tissue concentrations to fall below the minimal inhibitory concentration. Excretion rates are not accurately known in the mouse, but some evidence is available from other experiments where DDS was administered only periodically. In an experiment where 200 mgm. DDS/kgm. was given by injection at intervals of 0.5, 1, or 2 months intervals the results suggested that bacteriostasis persisted for three weeks after each injection ([5]). Also Rees found that the administration of 0.1 per cent DDS in the diet for one day every 14 days was enough to stop multiplication of *M. leprae* (personal communication). In the present experiment, if bacteriostatic concentrations of DDS persisted in the tissues for 14 to 21 days, there would remain 26 to 33 days of delay that could have been caused by killing of *M. leprae*. A maximal estimate of the amount of killing could be made on the basis that this amount of delay was the time required for growth at full logarithmic growth rates to replace the fraction of *M. leprae* killed by the drug. A delay of 26 to 33 days would correspond to 2.1 to 2.6 generation times, or a killing rate of 77 to 84 per cent.

The administration of a ten-fold greater amount of DDS, i.e., 0.1 per cent in the diet, did not cause significantly greater killing of *M. leprae*. Although no AFB were encountered in the count of the first harvest, the number at the second harvest showed the bacterial population to be up to normal plateau levels. Although an intake of 0.1 per cent in the diet would produce higher blood and tissue concentrations of drug ([7]), and consequently a longer period for elimination of drug after intake had ceased, it is doubtful if the greater amount of delay that would have been expected would have been measurable under the particular experimental conditions.

DADDS is a repository compound and the length of the delay it caused would reflect the period over which it released DDS (or the mono-acetyl derivative) in significant amounts. From this experiment it would not be possible to know if any of the delay should be ascribed to bacterial killing.

Combination of drugs. Several drugs were given in combination with DDS to see if they would increase the amount of killing. The concentration of DDS chosen for these mixtures was 0.01 per cent in the diet, since this intake produces blood and tissue levels that are about the same as those observed in man on standard therapeutic dosages ([7, 11]). SM and DPT when given singly were effective in this experiment, but in combination with DDS the effect was the same as that of DDS alone. PAS and INH given singly had no noticeable inhibitory effect on *M. leprae*, but the combination of each with DDS was not as effective as DDS alone.

DISCUSSION

The method described appears capable of measuring the bacteriostatic as well as the bactericidal effect of drugs. The experiment had been planned with the expectation that more *M. leprae* would be killed by two months of DDS, especially when it was administered during the logarithmic phase of growth. Presumably the bactericidal effects would have been more distinct if the drug had been given over a longer period, and experiments are in progress to determine the effect of duration of DDS administration. In the interpretation of the results here two simplifying assumptions

were made: (1) that after the beginning of administration of an effective drug the cessation of bacillary growth is rapid enough to prevent significant increase in numbers of bacilli, and (2) that as soon as an inhibitory drug disappears from the tissues bacillary growth begins at the rate observed in the controls. Current experiments seek to follow these events of the growth curve more closely, with more frequent harvests that start at the end of the period of drug administration.

Comparison with earlier results. An indication of the reliability of the present method and its interpretation can be gained by a comparison with earlier results. Reference is made here only to strains from untreated patients.

1. DDS was here found not to be inhibitory when given as 0.00001 per cent in the diet. Previously this dosage had been found to inhibit one strain ([11]), but not to inhibit four others ([5]). A dosage of 0.0001 per cent in the diet has been found to inhibit nine of nine strains tested ([11], unpublished results). Previous work with concentrations of DDS in the range of 0.01 per cent to 0.1 per cent in the diet had shown all strains from untreated patients to be inhibited ([1,3,6,7]), a finding in agreement with the present results. These earlier experiments had not sought to measure bactericidal effect. In one experiment where this was studied ([8]), killing of *M. leprae* was not detectable after 57 days of 0.1 per cent DDS in the diet, but it was distinct at 88 days and thereafter. The interpretation of that experiment had been obscured by the succession of death and growth phases that occur in the normal plateau phase, and it was not clear whether the killing began when it did because a growth phase had begun or whether the length of exposure to drug had reached a critical time. The results of the present experiment tend to support the latter explanation, but further experimentation is needed to clear this up. There appears to be no serious discrepancy between the two sets of experimental results. Waters and Rees ([13]) have described the regular decrease in normally stained *M. leprae* in lepromatous patients in their first three and six months of treatment with DDS. However, the definition of a normally stained bacillus for that study was apparently not as rigorous as the one used here for solidly stained bacilli. Nevertheless, their patients had a decline in normally stained bacilli of 78 per cent in three months, a decrease only moderately slower than the estimated maximal killing rate in the present study.

2. DADDS in the present experiment apparently delayed the growth of *M. leprae* only 36 days when given in a single injection of 100 mgm./kgm. Earlier it had been found that as little as 6 mgm./kgm. was nearly completely suppressive when given every 60 days ([5]). Before the apparent discrepancy can be interpreted, more needs to be learned of the rate at which DADDS stops growth and of the contribution made by duration of exposure to DDS.

3. SM was earlier found to inhibit the growth of *M. leprae* completely when given in a dosage of 2 mgm. five days a week ([7]). In the present experiment 2 mgm. thrice weekly for two months was found to be bacteriostatic but not bactericidal.

4. DPT, as 0.1 per cent in the diet, was found inactive against one strain of *M. leprae* ([7]), and active against four others ([1, 3]). The present results show that the same concentration of drug for two months is bacteriostatic but not bactericidal.

5. Earlier, INH had been found to inhibit the growth of *M. leprae* completely ([6]). (The concentration of INH in that reference should have read 0.01 per cent in the diet.) In the present experiment no effect was detected.

6. Previously PAS was found active at 0.6 per cent ([6]), but it was without effect here. Supposedly the discrepancies between the two experiments with regard to these two drugs were caused by strain differences in sensitivity. As mentioned above, the timing of the harvests in the present experiment was such that even partial inhibition should have been detected. The possibility that inhibition was present, but was followed by more rapid than normal logarithmic growth, would have to be ruled out by a harvest at the end of the period of drug administration.

Combination of drugs. There are no previous reports on the effect in mice of

drug combinations. Clinically such combinations have been frequently tried, inspired no doubt by the success of combinations in tuberculosis. In the present experiment SM and DPT when given singly were each inhibitory during the period of administration, but when they were given with 0.01 per cent DDS the effect was the same as with DDS alone. A synergistic effect of sulfones and SM had been noted with *M. tuberculosis* (14). INH and PAS, both inactive when given singly, were found to antagonize the antibacterial effect of DDS. Several explanations for the antagonism seem possible at present. One that might be mentioned is that INH and PAS were able to increase the excretion of DDS through inductive enzyme formation, so that the concentration of DDS was lower during the period of administration and did not persist so long afterwards. In the case of PAS an interference at the enzyme site seems possible, since both PAS and DDS probably act on mycobacteria by competing with *p*-aminobenzoic acid in the enzymatic formation of intermediates of folic acid (14). It has been observed that PAS will enter into the formation of folate analogs that will substitute for true folate compounds in certain microorganisms (12).

Further experience with this kinetic method will be needed to define its usefulness and to establish the correctness of the interpretations given here. Nevertheless, it appears obvious now that there are certain advantages over the principal method used previously. Varying degrees of antibacterial effect can be observed. Less drug is required. The period of administration is shortened, so that the work involved in making and feeding the diets is significantly decreased, as is the possibility of laboratory error.

SUMMARY

A kinetic method is described for the study of the activity of drugs against *M. leprae* in the mouse. The drug is administered for a limited period early in the infection, and the effect on the subsequent bacterial growth curve is observed. The method appears capable of detecting and measuring a drug's bacteriostatic as well as bacteridal effects on *M. leprae*.

In the experiment reported, administration of drug was limited to a two-month period that ended at 93 days after infection. Most of this period corresponded to the early logarithmic phase in the untreated controls. None of the treatments eradicated the infection. The results were interpreted as follows: SM, 2 mgm. thrice weekly, and 0.1 per cent DPT in the diet were each bacteriostatic, but they did not have important bactericidal activity. INH, 0.01 per cent, and PAS, 0.6 per cent in the diet, were each inactive. DDS, 0.01 per cent in the diet, was bacteriostatic and probably partially bactericidal; the killing rate was estimated not to exceed 77 to 84 per cent. A higher concentration (0.1% in the diet) was no more effective. The combination of 0.01 per cent DDS with either SM or DPT was no more effective than DDS alone. INH and PAS antagonized the antibacterial effect of DDS.

RESUMEN

Se describe un método kinético para el estudio de la actividad de las drogas contra *M. leprae* en los ratones. La droga es administrada en etapas tempranas de la infección por un tiempo limitado, y el efecto subsecuente en la curva de crecimiento del bacterio es observada. El método es capaz de detectar y medir los efectos bacteriostáticos de la droga como también el efecto bactericida en el *M. leprae*.

En el experimento mencionado, la administración de la droga fué limitada a un período de dos meses que terminó 93 días despues de la infección, y la mayor parte de este período correspondió a la fase logarítmica temprana en los controles no tratados. Ninguno de los tratamientos erradicó la infección. Los resultados fueron interpretados como sigue: SM, 2 mgm. tres veces a la semana, y 0.1 por ciento DPT en la dieta fueron cada uno bacteriostáticos, pero ellos no tuvieron actividad bactericida de importancia. INH, 0.01 por ciento, y PAS, 0.6 por ciento en la dieta, fueron cada uno inactivos. DDS, 0.01 por ciento en la dieta fué bacteriostático y con probabilidad parcialmente bactericida; la tasa de mortalidad se estimó que no excedió el 77 al 84 por ciento. Una mas alta concentración (0.1% en la dieta) no fué mas efectiva. La combinación de DDS al 0.01 por ciento tanto con SM o DPT no fué mas efectiva que el DDS solo. Las drogas INH y PAS antagonizaron el efecto antibacteriano del DDS.

RÉSUMÉ

On a décrit dans cet article une méthode cinétique permettant d'étudier chez la souris l'activité des médicaments contre *M. leprae*. Le médicament est administré au début de l'infection et pour une période de temps limitée. L'effet sur la courbe de croissance bactérienne qui survient ensuite est alors observé. La méthode paraît permettre de détecter et de mesurer les effets tant bactériostatiques que bactéricides d'un produit sur *M. leprae*.

Au cours de l'expérimentation relatée ici, l'administration du produit a été limitée à une période de 2 mois qui terminée a 93 jours apres l'infection. La plus grande partie de cette période correspondait à la phase logarithmique précoce chez les témoins non traités. Aucun des traitements n'a permis de supprimer l'infection. Les résultats furent interprétés comme suit : la streptomycine, à la dose de 2 mgm. trois fois par semaine, de même que l'addition de 0.1 pour cent de DPT dans la nourriture, constituaient des traitements bactériostatiques, mais ne témoignaient d'aucune activité bactéricide importante. L'INH, ajouté à la nourriture dans la proportion de 0.01 pour cent, et le PAS, administré par la même méthode dans la proportion de 0.6 pour cent, étaient tous deux inactifs. La DDS, à raison de 0.01 pour cent dans la nourriture, était bactériostatique et probablement, en partie, bactéricide; on a estimé que le taux de destruction n'excédait pas 77 à 84 pour cent. Une concentration plus élevée (0.1 pour cent dans la nourriture) n'était pas plus efficace. La combinaison de 0.01 pour cent de DDS avec, soit la streptomycine, soit le DPT, n'était pas plus efficace que la DDS seule. L'INH et le PAS témoignaient d'une action antagoniste contre l'effet antibactérien de la DDS.

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