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Application of the Mouse Foot-Pad Technique in Immunologically Normal Mice in Support of Clinical Drug Trials, and a Review of Earlier Clinical Drug Trials in Lepromatous Leprosy

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The current era of chemotherapy of leprosy was ushered in by Shepard's first published descriptions in 1960 of the mouse foot-pad technique ([23, 24]), and also by a series of publications, the first of which appeared in 1962 ([35]), in which Rees and his co-workers established the measurement of the morphological index (MI) as a useful means of evaluating the response of the patient to chemotherapy.

Certainly, there had been earlier efforts to assess various chemotherapies. Beginning in 1941 with Promin®, Faget and his coworkers at Carville examined a variety of substituted dapsones ([8]). Dapsone (DDS) itself was first used in 1946 by Cochrane, who employed the drug in a large parenteral dose, and reported it too toxic for use ([1]).

The earliest critical clinical report is that reported in 1954 by Lowe ([14]). Of 109 patients with multibacillary leprosy available for analysis, arrest of the process, defined as a minimum of 24 months of DDS as monotherapy with smears remaining negative for 12 months, had been achieved in 97. In the same paper, Lowe described the results of a study of relapse. Of 130 patients with multibacillary disease arrested after an average of 28 months of DDS as monotherapy, 124 were found free of all evidence of relapse after an average of 22 months without maintenance chemotherapy.

A series of large, carefully designed trials was subsequently conducted by the Leonard Wood Memorial in The Philippines and elsewhere, employing as the principal measures of patient-response periodic clinical assessment and measurements of the bacterial index (BI). Although most of the patients had received some prior sulfone therapy, a comparison of DDS with placebo after 48 weeks of treatment demonstrated the superiority of DDS ([7]).

Because only imprecise methods for assessment of the efficacy of an antimicrobial drug were available—clinical assessment, measurement of the BI, and the rate of relapse after termination of chemotherapy, these early studies involved large numbers of patients, who were treated—and therefore exposed to the risks of drug toxicity and of ineffective chemotherapy—for long periods of time, with disappointingly small yield. Nevertheless, these controlled trials, together with a number of anecdotal reports and the results of uncontrolled trials, established that DDS as monotherapy was efficacious in the treatment of multibacillary leprosy. These workers were certainly aware of the risk of drug resistance in the course of long-continued monotherapy, but were reassured by the apparent absence of relapses associated with the emergence of DDS-resistant *Mycobacterium leprae.*

Morphological index. Following the lead of earlier workers, who had called attention to the altered morphology of the *M. leprae* of treated patients ([6, 14, 15]), Rees and his coworkers produced experimental evidence that the morphologically altered organisms were dead ([21, 22]), and undertook a series of trials of chemotherapy among patients with multibacillary leprosy in Sungei Buloh. These trials demonstrated that the MI decreased to baseline values within a few months of beginning treatment of previously untreated patients with DDS in full dosage ([34, 36]), with or without the addition of macrocyclon ([32, 36]) or ditophal ([34]), and similar results were reported after beginning treatment with DDS in much smaller dos-

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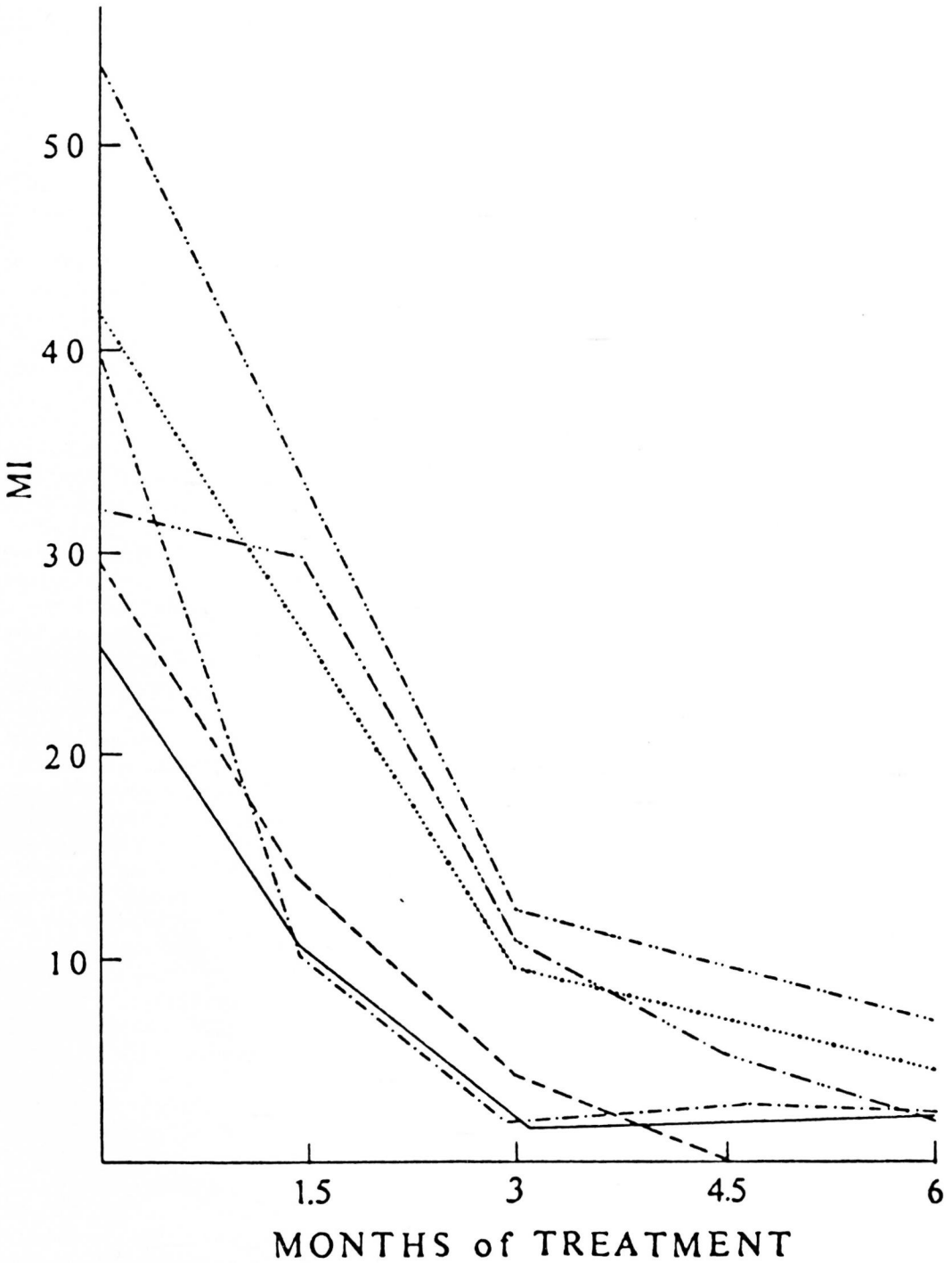

FIG. 1. MI as a function of duration of treatment. Treatment with: dapsone, 300 mg twice weekly, mean of 32 LL patients, adapted from ref. 34 (-···-); dapsone, 300 mg twice weekly, adapted from ref. 35 (-····-); dapsone, 300 mg twice weekly, mean of 4 BL patients, adapted from ref. 34 (·····); clofazimine, 300 mg daily six days per week, adapted from ref. 18 (-·-·-); clofazimine, 300 mg daily six days per week, adapted from ref. 20 (——); clofazimine, 100 mg twice weekly, adapted from ref. 32 (-----).

ages ([16, 19]) or clofazimine (CLO) in dosages of 300 mg six days weekly ([20]) or 50 mg twice weekly ([33]).

Thus, clinical trials based on changes of the MI permitted recognition of effective drugs after treatment of small numbers of patients for only a few months. On the other hand, as shown in Figure 1, the MI appeared to decrease at much the same rate, whether the drugs were employed in full dosage or only in some small fraction of this dosage. Repeated measurement of the MI was capable of demonstrating the efficacy of drugs in a qualitative fashion, but appeared incapable of discriminating between more and less active regimens. Because an average patient with lepromatous leprosy begins treatment with no more than 10% of his *M. leprae* solidly staining, and therefore presumed viable, and because it is difficult to examine and score as solid or non-solid many more than 50–100 organisms, disappearance of solidly staining organisms from the patient's smears indicates merely that 90% of the viable organisms originally present have been killed; further killing of *M. leprae* simply cannot be observed by this technique.

Mouse foot-pad technique. The application of Shepard's mouse foot-pad technique to measure the rate at which *M. leprae* are killed during effective DDS treatment of lepromatous patients, reported in 1968 ([27]), provided for the first time a reasonably quantitative and sensitive means of evaluating the antimicrobial activity of individual drugs. In this application, mouse inoculation is employed in much the same way that sputum culture is employed in assessing the efficacy of an antituberculosis drug.

A skin lesion large enough to permit several biopsies is chosen. A biopsy is performed before the start of treatment, the resulting 50- to 100-mg specimen providing 10^6 to 10^8 organisms if the patient is lepromatous and previously untreated. *M. leprae* are recovered from the biopsy specimen, counted and diluted so as to provide an inoculum of $10^{3.7}$ to 10^4 acid-fast bacilli (AFB) per foot pad, and the hind foot pads of 10 to 20 normal mice are inoculated.

According to Shepard's technique, one mouse is sacrificed every month, beginning about three months after inoculation, and the inoculated hind foot pad is fixed, lightly decalcified, and processed for histopathological examination, after which paraffin sections are stained by an acid-fast stain. The presence in a section of a "significant lesion"—one filling at least one fourth of a 540× microscope field with brightly staining AFB, with or without a typical infiltrate—indicates the end of the "incubation period" (IP), the time elapsed between inoculation and appearance of an important number of AFB in a histopathological section, and the signal for performing a harvest. Additional mice are killed, and *M. leprae* are harvested from the pooled tissues, usually of four inoculated foot pads. The AFB are enumerated, and the "generation time" (G) is calculated as if multiplication of *M. leprae* had occurred at a constant rate from the day of inoculation until the day of harvest.

Additional biopsy specimens are obtained in the course of treatment, mice are inoculated, and killing of *M. leprae* is indicated by progressive increases of the values for IP and G, as shown in Figure 2, which illustrates the application of the technique to a patient with previously untreated lepromatous leprosy treated with DDS in a daily dose of 50 mg. An IP >12 months or G ≥ 100 days is evidence of failure of the *M. leprae* to multiply in the mice. In the uppermost panel of this figure, the number of AFB recovered from skin biopsy specimens, obtained at intervals during treatment, may be seen to have decreased only slightly in the course of about one year of treatment. In the second panel, the solid ratio, very low to begin with, fluctuated between 0 and 5 solids per 100 AFB, before solid organisms finally disappeared about eight months after beginning treatment. Infectivity of this patient's *M. leprae* for the mouse decreased during the first several months of treatment, as shown in the lower panels.

The result at 90 days is characteristic of the irregular results encountered as the infectivity of the organism for the mouse becomes marginal. *M. leprae* appear to have multiplied in the single mouse sacrificed for histopathological study 11 months after inoculation (therefore, IP = 11 months), but not in the four mice sacrificed for harvest at that time, yielding G ≥ 100 days.

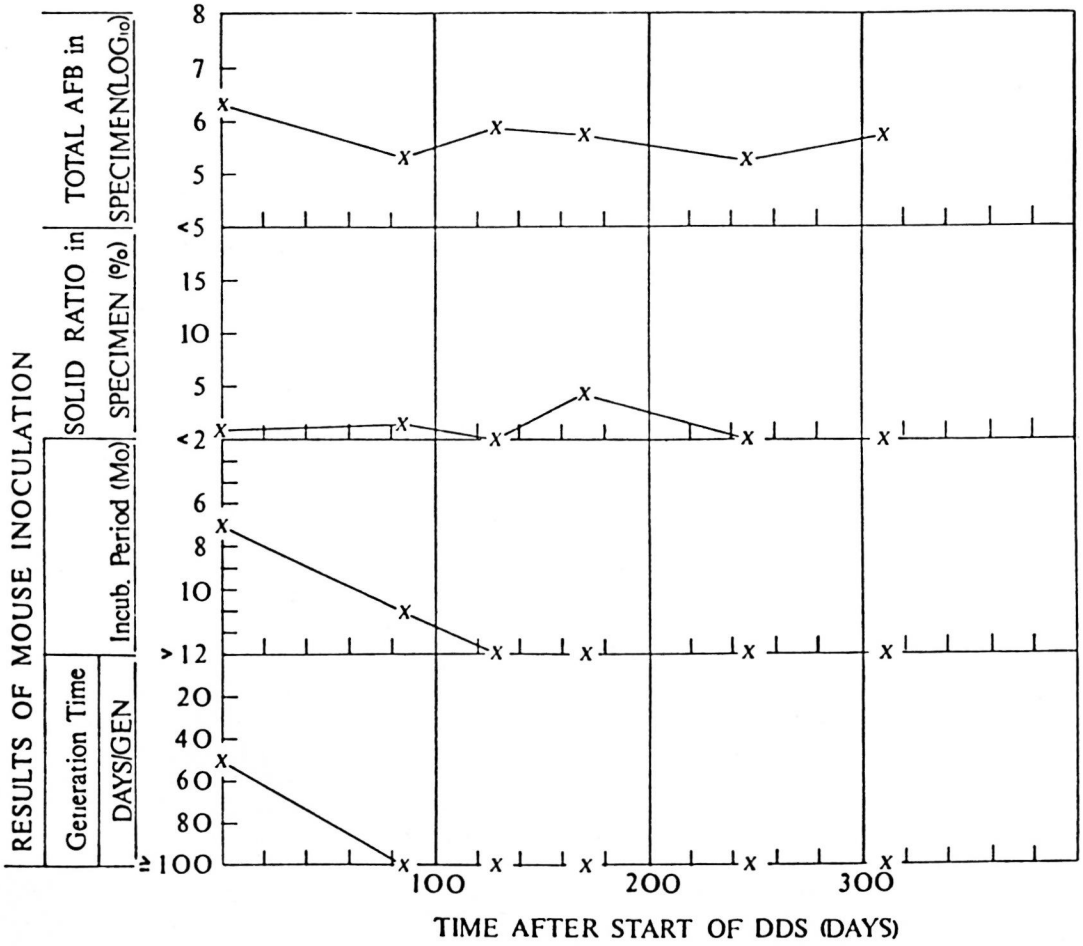

FIG. 2. Results of treatment with dapsone of a patient with previously untreated lepromatous leprosy (adapted from reference no. 27).

Application of the mouse foot-pad technique to measurement of the rate at which patients with multibacillary leprosy respond to antimicrobial chemotherapy, first reported in 1968 ([27]), provided a technique more sensitive and discriminating than that of the MI. Because the technique involves examination of a much larger number of organisms than does measurement of the MI, killing of at least 99% of the viable *M. leprae* originally present can be recognized with precision; inoculation of mice with organisms recovered from biopsy specimens obtained at intervals during treatment enables measurement of the rate of bacterial killing, and permits discrimination between more and less effective regimens—i.e., those associated with more and less rapid killing.

Employing this technique, a number of clinical trials were carried out, most of them among patients with previously untreated lepromatous leprosy, both in San Francisco and Cebu. The first trial, carried out in San Francisco among both previously treated and new patients with multibacillary leprosy, involved a comparison of DDS, 50 mg daily, with CLO, 100–200 mg daily ([20]). Among eight patients, all but one previously untreated, who were demonstrated to harbor *M. leprae* susceptible to DDS, G increased in direct relation to the duration of treatment, reaching the value of 100 days after 105 days of treatment. By contrast, among five patients with DDS-resistant *M. leprae* who were treated with CLO, G reached the value of 100 days only after

TABLE 1. *Course of events during dapsone monotherapy of multibacillary leprosy.**

Duration of treatment (mo)	Results			Interpretation	
	BI	MI	Mouse inoculation	Total *M. leprae*	Viable *M. leprae*
0	4+	10%	+	10^{11}	10^{10}
1	4+	1%	+	10^{11}	10^{9}
2	4+	<1%	+	10^{11}	10^{8}
3	4+	<1%	−	10^{11}	$<10^{8}$
12	3+	<1%	−	10^{10}	$<10^{7}$
24	2+	<1%	−	10^{9}	$<10^{6}$
36	1+	<1%	Not possible	10^{8}	$<10^{5}$

* Adapted from reference no. 9.

treatment for 150 days. In fact, the value for G increased at the same rate in both groups of patients, but only after a delay of approximately 50 days among the CLO-treated patients.

The apparent delay of the onset of bacterial killing during treatment with CLO suggested a requirement for loading the patient with drug, before it exerted bactericidal effects. To test this hypothesis, a trial of a number of intermittent CLO regimens was mounted among new multibacillary patients in Cebu ([3]). Patients were allocated to treatment with CLO 200 mg daily six days weekly, 100 mg three times weekly, 300 mg once weekly, 600 mg once every two weeks, or 600 mg on two consecutive days once every four weeks. All five regimens proved to be effective, but the degree of effectiveness appeared to be inversely proportional to the degree of intermittency, contrary to what was expected if loading were required.

Trials of acedapsone, 225 mg intramuscularly every 11 weeks, were also carried out both in San Francisco and Cebu ([4, 28]). In both trials, carried out among patients with previously untreated multibacillary leprosy, the rate of response varied widely from patient to patient; some patients responded as rapidly as did the average patient treated with DDS in full dosage, whereas other patients responded much more slowly. Neither the acetylator status of the patient ([17]), nor the susceptibility of the patient-strain of *M. leprae* to concentrations of DDS (in the mouse diet) smaller than 0.0001 g per 100 g diet ([10]), nor the concentration of *M. leprae* in the pretreatment biopsy specimen ([11]) appeared to determine the rate of patient response.

Finally, a number of trials of rifampicin (RMP) were carried out in both San Francisco and Cebu ([2, 4, 13, 29, 30]). These trials demonstrated the extremely potent and rapid bactericidal effect of the drug; *M. leprae* recovered from biopsy specimens three–four days following a single 600–1500 mg dose of RMP failed to multiply in mice, whereas RMP in a daily dose of 300 mg was somewhat less effective than the drug in a daily dose of 600 mg.

An important limitation of the mouse foot-pad technique as applied to the detection of viable *M. leprae* in normal mice is its insensitivity. Although it is capable of detecting a very small number of viable organisms (perhaps as few as one), it is incapable of detecting even larger numbers of viable organisms, if these organisms are diluted by some much larger number of dead *M. leprae*—i.e., if the viable organisms represent only a very small proportion of the total.

As shown in Table 1, a heavily infected patient with lepromatous leprosy begins treatment with a total population of 10^{11} *M. leprae,* which is consistent with a BI of 4+. [Shepard estimated ([25, 26]) that a 60-kg man has a skin surface-area of 1.70 m^2, and that the skin is involved to a depth of 2 mm. Assuming the skin to be 30% involved, that 1 cm^3 of skin weighs 1 g, and that a BI of 4+ is equivalent to a concentration of 10^8 AFB per g skin, one may calculate that the total population of *M. leprae* of such a patient is of the order of 10^{11}.] The MI is 10%, indicating that 10% of this patient's organisms are viable, and the *M. leprae* recovered from a pretreatment biopsy specimen infect mice. [A biopsy specimen from this patient

might contain 10^7–10^8 *M. leprae,* of which 10^6–10^7 are viable ([5]).]

At the end of the first month of DDS therapy, 90% of the viable *M. leprae* have been killed; accordingly, the MI has decreased to 1%. As the inoculum of $10^{3.7}$ to 10^4 organisms contains about 50 to 100 viable *M. leprae,* this patient's organisms remain infective for mice. By the end of the second month of treatment, 90% of the remaining viable *M. leprae* have been killed, and no solidly staining organisms are seen when 100 are examined in the course of measuring the MI. By this time, the inoculum contains only the minimal number of organisms required to infect mice, and some mice escape infection, rendering the results of mouse inoculation irregular.

From the end of the third month of treatment, the patient's total bacterial population, the great majority of which is dead, does not decrease appreciably until the end of the first year of treatment, by which time it has decreased by 90%. At this time, the patient's *M. leprae* are no longer infective for mice, simply because the proportion of viable organisms has become so small that none is likely to be included in the inoculum of 5000. Despite the loss of infectivity of the organisms for the mouse, however, the patient may by no means be considered to have been cured. The small proportions of viable *M. leprae* may be detected by inoculation of immune-deficient rodents with $>10^4$ organisms ([31]).

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