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Drug Susceptibility Testing of *Mycobacterium leprae*

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Until the introduction of dapsone (DDS) and other sulfones, there was no effective treatment of leprosy. The evident success of sulfone therapy led to the universal use of these drugs. Unfortunately, because of the unavailability of other effective antileprosy drugs at that time, the sulfones were necessarily employed as monotherapy.

Although resistance to DDS was suspected in a few cases as early as 1953 ([24]), no laboratory technique was available for assessment of the drug susceptibility of *Mycobacterium leprae* until Shepard's description ([18]), in 1960, of his system for cultivating the organism in the mouse foot pad. Employing this technique, secondary resistance to DDS was proved for the first time in 1964 ([17]), at which time the prevalence was estimated to be only 2 per 1000 patients at risk ([16]). Perhaps because of this low estimate of prevalence, the medical community was not alerted to the risk of secondary resistance as a consequence of monotherapy with DDS, and this treatment continued to represent the standard, despite the availability, by this time, of clofazimine (CLO), ethionamide (ETH) and, not long thereafter, rifampin (RMP) as bactericidal companion drugs.

By 1976, it was already apparent that secondary resistance to DDS had become an important problem ([3]). And, in 1977, the first cases of primary resistance to DDS were reported ([14]). Subsequently, formal surveys of DDS resistance, both secondary and primary, have been conducted in a number of leprosy-endemic areas. As the result of these surveys, secondary DDS resistance is now understood to be distributed Worldwide, with rapidly increasing prevalence and alarming annual incidence rates in some areas. In addition, primary DDS resistance has been detected with an unexpectedly great frequency. Finally, secondary resistance to RMP, CLO and ETH has been reported, and some strains of *Mycobacterium leprae* resistant to two drugs have been detected ([5]).

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Drug susceptibility testing by means of mouse foot-pad technique

Although trials of therapy can demonstrate drug resistance, and have been applied to the diagnosis of secondary DDS resistance ([12, 13, 22]), such trials may require more than five years to prove resistance of low degree, thereby delaying effective treatment of the patient. Several tests have been reported to permit rapid determination of the susceptibility of *M. leprae* to a drug *in vitro* ([1, 6, 8, 10, 11]); however, these tests appear to require further investigation and independent verification. Therefore, employment of the mouse foot-pad technique remains the standard method for testing the susceptibility of *M. leprae* to drugs.

Mice. Immunologically intact mice are preferred for this purpose. Although immune-deficient mice—i.e., adult-thymectomized, lethally irradiated and bone-marrow-reconstituted (T900R), or congenitally athymic "nude" mice—will permit multiplication of *M. leprae* from larger inocula than will intact mice, inocula larger than 5000–10,000 organisms per foot pad are not required. In fact, use of such small inocula is advantageous; because the frequency of spontaneous drug-resistant mutant *M. leprae* is probably no greater than $1{:}10^6$, the likelihood that an inoculum of 5000–10,000 organisms will contain a drug-resistant mutant is vanishingly small.

The specimen. To obtain the organisms to be inoculated into mice, one usually biopsies a lesion. Selection of the lesion to be biopsied is important; the decision is based upon the number of *M. leprae* observed in smears prepared from the lesions, and also upon the appropriateness of the site for biopsy. Normally, one chooses the lesion yielding the largest BI; the BI of the lesion must be at least 2+ to provide enough organisms for inoculation. The specimen obtained with a 5-mm skin punch is usually large enough. If the only site yielding a suf-

ficiently large BI is inappropriate for biopsy—e.g., it is located on the face or earlobe—one may be able to collect enough organisms by scraping the lesion. Once the organisms have been obtained, one may begin appropriate treatment, without awaiting the results of mouse inoculation.

Specimens to be shipped to a distant laboratory should be maintained at 4°C, and shipment should be completed as rapidly as possible, preferably with no more than 72 hours elapsing between biopsy and inoculation of mice; otherwise, the proportion of viable *M. leprae* may be substantially reduced.

It is not necessary to conduct the test of drug susceptibility during primary isolation of *M. leprae* —i.e., directly on the organisms recovered from the skin-biopsy specimen; the test may also be carried out on organisms isolated from mouse foot pads and passaged to new mice. This latter practice is more economical, especially in the study of secondary resistance, in which case the proportion of viable *M. leprae* may be so small that organisms do not multiply in mice. Because drug resistance of *M. leprae* results from the selection of drug-resistant mutants, the susceptibility of the organisms does not change in the course of repeated passage ([20]).

Inoculum size. Usually, mice are inoculated into one or both hind foot pads with 5000–10,000 *M. leprae* per foot pad. Sometimes, however, the number of organisms recovered from the biopsy specimen is insufficient. In this circumstance, one has two options: the number of mice to be inoculated may be reduced to the control group and the group to be treated with the minimal effective dosage (MED) of the drug; or the inoculum may be reduced, while inoculating the usual number of mice. In general, the first option is preferred; an inoculum smaller than 1000 per foot pad may result in such irregular multiplication in control mice that it will be difficult to interpret the results of the test.

Drug administration. After inoculation, the mice are divided among a number of groups of 10–20 mice. One group is held without treatment, and the remaining groups are administered drugs, each in two or three dosages. Treatment of the mice is usually begun immediately after inoculation, and continued until the number of *M. leprae* per foot pad of untreated control mice approaches 10^6. At the present time, susceptibility of the organisms to only the four bactericidal drugs—DDS, CLO, RMP and protionamide (PTH)—is of interest.

All four bactericidal drugs may be administered *per os,* either incorporated into the mouse diet or by gavage, because they are well absorbed from the gastrointestinal tract of the mouse. Because of the great convenience of administering the drugs incorporated into the mouse diet, this mode of drug administration is preferred. However, a common source of error in the susceptibility test is inadequate mixing of drugs into the diets. The drugs must be incorporated into the diets in as uniform a manner as possible. A liquid-solid blender is most suitable for this purpose; if no such blender is available, but only one for mixing solids with solids, one should begin by mixing a weighed portion of the powdered drug with a small quantity of some inert solid such as lactose or the powdered diet, gradually diluting the drug by adding weighed amounts of the diet to the blender. If an adequate blender is not available, exhaustive mixing by hand is necessary.

In preparing a series of drug-containing diets, one should always begin with the diet containing the smallest concentration of the drug. In this way, one may prepare diets of progressively greater concentration without stopping between diets to wash the blender, without fear of contaminating a diet with one containing a larger concentration of the drug.

For each drug, the smallest dosage to be administered should be the MED, the smallest dosage capable of inhibiting multiplication in mice of *M. leprae* obtained from previously untreated lepromatous patients before primary resistance to the drug has been recognized. The larger dosages are usually 10-fold and 100-fold multiples of the MED. The MED of DDS has been determined to be 0.0001 g per 100 g diet ([20]); the MEDs of the other bactericidal drugs have not been fully established (*vide infra*).

During administration of a DDS-containing diet, one should regularly take samples of the diet from the diet feeders in the

cages, and from the supply of diet in use, in order to assay the concentration of DDS in the diet. The assay may be performed by a simple colorimetric method ([2]). No simple methods have yet been developed for assaying the concentrations of the other bactericidal drugs in mouse diet.

Although RMP, like DDS, can be incorporated into the diet, it appears to be unstable in this situation, and its potency may diminish with time. Therefore, in testing the susceptibility of *M. leprae* to RMP, it may be better to administer freshly prepared solutions of the drug by gavage.

Harvests of *M. leprae*. In some laboratories, harvests are carried out from pools of foot-pad tissues, whereas in other laboratories, harvests are performed from individual foot pads. Approximately six months after the mice were inoculated, harvests of *M. leprae* are performed from the inoculated foot pads of two to four untreated mice, and repeated at intervals of two months, until the average number of organisms per foot pad is found to be at least 5×10^5. At this time, harvests of *M. leprae* are performed immediately from the inoculated foot pads of at least four mice from each treated group. If, after 12 months, the organisms are found to have multiplied in the control mice, but to an average of fewer than 5×10^5 *M. leprae* per foot pad, harvests should also be performed from mice of each of the treated groups.

Interpretation of the test. The criterion of multiplication of *M. leprae* in the mouse foot pad must be strictly defined. The minimal number of organisms per foot pad detectable by the usual counting techniques is rather large. A count of 2×10^4 *M. leprae* per foot pad indicates that only one or two organisms have been observed; one cannot be certain that the one or two organisms observed are not those inoculated. To be confident that multiplication has in fact occurred, it is usual to require an increase at least to 10^5 organisms per foot pad. Employing this criterion of multiplication, the results of a susceptibility test are intrepreted as follows: a) susceptible—multiplication of *M. leprae* is observed only in untreated mice, and in no treated mouse; b) resistant—the organisms are observed to have multiplied in at least one treated mouse. The degree of resistance is determined by the diet containing the largest concentration of drug that permits multiplication of the *M. leprae*. For DDS, the degree of resistance is defined as low, intermediate or high, depending on the ability of the organisms to multiply in mice administered DDS in a concentration of, respectively, 0.0001, 0.001 or 0.01 g per 100 g diet; c) inconclusive—multiplication is observed in no drug-treated mouse, and in so few control mice that, by means of the exact probability calculation, one cannot distinguish the number of control mice demonstrating multiplication from zero. In such cases, the organisms should be passaged into new groups of mice and the tests repeated.

As examples, the results of six tests of susceptibility to DDS are shown in Table 1.

Discussion

The definition of resistance to a drug is based on the behavior of "wild" strains of a bacterial species. Resistance of *M. tuberculosis* is defined as a decrease of susceptibility of a degree sufficient to make it reasonably certain that the strain is different from a sample of wild strains that have never come into contact with the drug. Thus, one must test the susceptibility of a fairly large number of wild strains, and study the distribution of values of the minimal inhibitory concentration (MIC) ([9]). The same definition should be applied to *M. leprae*.

A survey of 73 strains of *M. leprae* isolated from previously untreated patients before 1977, when the first report ([14]) of primary resistance to DDS appeared, has revealed that 44% of the strains were inhibited from multiplying by administration to the mice of DDS in a concentration of 0.00001 g per 100 g mouse diet; and all 73 strains were susceptible to 0.0001 g DDS per 100 g diet. Therefore, any strain of *M. leprae* that multiplies in the foot pad of mice administered DDS in a concentration of 0.0001 g per 100 g diet should be considered resistant to DDS. It is indeed a pity that no systematic survey involving larger numbers of wild strains, obtained from a representative sampling of geographic areas, was carried out; however, the need was not anticipated, and the first report of primary re-

TABLE 1. *DDS-susceptibility tests of six strains of* M. leprae.

Strain no.	No. mice showing multiplication/no. inoculated				Interpretation
	Concentration of DDS (g per 100 g diet)				
	0	0.0001	0.001	0.01	
1	10/10	0/10	0/10	0/10	Susceptible
2	4/9	0/10	0/10	0/9	Inconclusive
3	8/8	7/9	0/10	0/10	Low resistance
4	10/10	9/9	7/7	0/10	Intermediate resistance
5	9/9	10/10	4/10	0/9	Intermediate resistance
6	8/8	10/10	10/10	10/10	Full resistance

sistance made it clear that such a survey was no longer possible.

In the diagnosis of a drug-resistant strain of *M. tuberculosis,* one takes into account both the critical concentration of drug in the medium, and the critical proportion of drug-resistant mutants; for the first-line antituberculosis drugs, this critical proportion is 1% ([4]). However, when susceptibility to a drug is measured by means of the mouse foot-pad technique, it does not appear necessary to measure the proportion of drug-resistant *M. leprae.* Because the inoculum is no larger than 10^4 organisms, and the proportion of viable organisms recovered either from clinical specimens or from mouse passage is usually smaller than 10%, the number of viable organisms inoculated into each foot pad is no greater than 1000. Because the frequency of spontaneously occurring drug-resistant mutants is probably not larger than $1{:}10^6$, the possibility of including resistant individuals in the inoculum is negligible, unless the proportion of resistant mutants has increased by more than 1000-fold. Of course, the proportion of resistant individuals can increase passively in the course of effective treatment, without these organisms having multiplied, as the result of killing of the drug-susceptible organisms. However, because the total number of *M. leprae,* viable and dead, does not change significantly during initial treatment, and because, during initial treatment, the proportion of viable organisms decreases to a level insufficient to permit multiplication in mice ([7]), one is unlikely to detect the resistant mutants. Therefore, isolation of drug-resistant organisms in mouse foot pads necessarily indicates that the resistant mutants have multiplied ([5]).

The concentration of DDS in the plasma of mice administered 0.0001 g DDS per 100 g mouse diet is of the same order as that observed in humans receiving 1 mg DDS daily ([13]). Because the usual dosage of DDS—100 mg daily—yields a peak plasma concentration some 500-fold greater ([23]), the criterion of resistance to DDS may appear clinically irrelevant ([21]). It is clear, however, that if *M. leprae* multiply in mice administered DDS in the concentration of 0.0001 g per 100 g diet, the strain demonstrates decreased susceptibility. If the patient's strain of *M. leprae* is resistant only to this small concentration of DDS, that patient should respond to DDS administered regularly in full dosage ([13, 21]). However, resistance to DDS develops in stepwise fashion ([13]); therefore, patients whose organisms are resistant only to the smallest concentration of DDS probably harbor a small number of individual mutants resistant to a larger concentration of the drug ([22]); these patients may relapse after having initially responded to monotherapy with DDS in full dosage. Because of the limited availability of mouse foot-pad facilities during the 1970s, when DDS monotherapy was widely employed, progression from resistance of low degree to that of a higher degree was not well documented. However, some patients were recorded ([15]) whose *M. leprae* were initially resistant only to DDS administered in the MED, who relapsed after having responded to supervised treatment with DDS in full dosage for 30–37 months. Moreover, in the very paper ([21]) in which the authors had suggested revising the criterion for DDS resistance to multiplication of the organisms in mice administered 0.01 g DDS per 100 g diet, it was reported that, of five strains

that multiplied only in mice administered 0.0001 g DDS per 100 g, three later proved clinically resistant to supervised DDS monotherapy in full dosage. Thus, resistance to 0.0001 g DDS per 100 g diet is certainly not a phenomenon devoid of clinical significance. In addition, when such strains are commonly found among previously untreated patients, this finding has great epidemiologic significance, and must also be taken into account in planning community-wide programs of treatment for control.

The critical concentrations of drugs other than DDS are not as well established as that for DDS. The literature describes only a very few wild strains, the susceptibility of which to the other bactericidal drugs has been studied ([5])—fewer than 15 strains for RMP, about ten strains for CLO, and no more than five strains for ETH or PTH. Thus, the currently accepted criteria for susceptibility—0.0001 g per cent for CLO, 0.003 g per cent for RMP and 0.01 g per cent for PTH—may not be correct. A more complete study of additional wild strains may well demonstrate some that would be considered resistant according to these criteria. For example, drug susceptibility tests have been performed on ten strains isolated from previously untreated patients after three or 12 months of combined chemotherapy [Subcommittee on clinical trials of the Scientific Working Group on Chemotherapy of Leprosy (THELEP); unpublished data]; two of the strains multiplied in mice administered 0.0001 g CLO per 100 g diet, seven were resistant to 0.003 g per cent RMP, and one was resistant to 0.01 g per cent PTH. Moreover, two of these strains were doubly resistant—to both CLO and RMP. These patients had been treated with strictly supervised regimens consisting of three bactericidal drugs, and the drug susceptibility tests were carried out in a laboratory of undoubted competence. Because secondary resistance cannot possibly have emerged so soon after beginning treatment, it appears most likely that the critical concentrations of these drugs have been underestimated. Clearly, many more wild strains of *M. leprae* must be tested for susceptibility to RMP, CLO, and ETH or PTH as soon as possible.

Finally, in contrast to the situation with respect to DDS, it is not clear whether resistance to RMP, CLO or PTH results from single-step or multi-step mutations. Although the data from one strain ([4]) suggest that resistance to RMP may result from a single-step mutation, many more similar studies are needed.

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