Considerations of the Application of the Foot Pad Technic in Leprosy Research

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I would like to discuss some of the advantages and disadvantages of the mouse foot pad system for *M. leprae* and note how these affect the application of the system in research on human leprosy. The passage of nearly eight years since work first started with *M. leprae* in the foot pad allows us some perspective.

Regularity. This chief advantage was obvious at the time of the first publications ([1, 2]), when it was noted that it should be possible to study drugs and vaccines against leprosy. Some idea of the regularity may be gained from the experience in our laboratory, where 79 isolations have been made from biopsy specimens and 75 from nasal washings, and over 300 passages have been successfully completed. The number of strains completing eight or more passages is 19, and one strain has completed 13 passages, during the course of which there has been a bacillary increase of 1.3×10^{31}-fold. Multiplication in mice is consistent if the inoculum contains some solidly staining *M. leprae*. We had some irregularities in 1963, however, apparently as a result of a "drift" in technic, which led to a carry-over of antiseptic into the syringes; our procedure for sterilizing syringes has been changed to eliminate exposure to antiseptics.

To be useful in the study of vaccines, an experimental infection must be regular and consistent, so that the differences among control groups will be distinctly less than differences between vaccinated and control groups. The general principle is that an infection is produced in which there is multiplication of the infectious agent, and what is observed is the effect of the vaccination on the agent's multiplication. In some instances where the multiplication produces death of the experimental animal or observable pathology, these changes can be used to measure the effect of the vaccine. In other instances it is necessary to measure

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the multiplication directly. The latter is the case with the *M. leprae* infection, and here the organisms are counted microscopically. The effect we have observed is a reduction in numbers of *M. leprae* on the order of 10- to 100-fold. It is useful to carry out two harvests, one shortly after growth of *M. leprae* in the controls has passed 10^6 per mouse (this will often be at about 7 months) and again three months later. By this schedule it is possible to differentiate between two effects produced by the vaccine, one a delay of bacterial growth, the other an apparently permanent reduction in numbers of bacteria. It is never certain that the results in an experimental animal can be reproduced in man, because the immunization response and the infectious processes may be different in the two species, and that is why properly designed field trials must always be carried out to test vaccines. Field trials, however, are laborious and cumbersome and not adaptable to the investigation of more than one or two factors at a time. The mouse infection with *M. leprae* was helpful in that it allowed us to compare a number of mycobacterial species for the protection they might afford against *M. leprae*, and has let us examine the effect of route, dosage, viability of vaccine, etc. The results of these studies, which were recently published (4), were that clear-cut immunity could be produced in mice by immunization with cultivable mycobacteria, that tubercle bacilli and BCG were as good as any mycobacterial species tried, and that the intracutaneous route of BCG inoculation was the most practical procedure. In more recent, unpublished work we have found that when the schedule of the vaccine in the mouse is well controlled, the regularity of vaccine protection is very much greater. Since, in general, immunity in man lasts much longer than in mice, the schedule of vaccination in man would not be expected to be as important a variable as it is in mice.

In testing drugs for their activity against *M. leprae* the requirement again is that the experimental infection be consistent. The design Dr. Chang and I have used is one weighted in favor of the drug. All drugs are more active in preventing multiplication than they are in killing bacilli in an infection already established; so the drug is started the same day the mice are infected, and it is given at the highest dose the mice will tolerate. Using this technic 12 drugs have been studied in our laboratory for their activity against *M. leprae* (3, 5). Our present routine in following the results in drug tests is the same as that used in vaccination experiments. The growth curve of *M. leprae* in the controls is followed by monthly harvests, and when the concentration in the controls first passes 10^6, the first harvest by groups is carried out to see if the drug has delayed bacterial growth at all. A second harvest by groups is made three months later to see if the *M. leprae* have broken through any particular drug or concentration. In mice receiving an effective drug there is no evidence of multiplication of *M. leprae* at all.

In working with new drugs evidence of toxicity at higher dosages offers some assurance that the drug is being absorbed. In evaluating results with new chemotherapeutic agents, one is influenced by the two historic "misses" for mycobacteria, both on the basis of dosage. First is the case of DDS, the use of which was delayed in leprosy for 10 years for what seem in retrospect to be inadequate reasons. Even though it had been reported in 1938 that it was 30 times as active as sulfanilamide against streptococci in mice and only 15 times as toxic, DDS was tried in man in vast over-dosage, and the drug was called too toxic. Yet the dosage was apparently selected entirely on a weight basis predicted from the toxicity of sulfonamides and substituted sulfones. Second is the case of cycloserine. Although this drug is not the giant in leprosy that DDS is, its experimental history is also instructive. It was called inactive *in vivo* because it had no effect when given parenterally against tuberculosis in mice. Yet no attempt was made at that time to see if adequate blood levels were produced in mice, and it was only some years later that it was learned that the drug was metabolized so rapidly in the mouse that adequate blood levels were present for only 30 minutes after each infection.

In general it may be difficult to translate results with a new drug from one species

of animal to another, but in the case of chemotherapeutic agents that have been found by *in vitro* searches the correct translation is often made very much easier by knowledge of the concentration of the drug in tissues and blood. This arises because drugs may be metabolized and excreted in very different ways in different species of animals, and often it is not even known whether the drug is active as the administered form or as one of its metabolites. However, in the case of drugs known to be active *in vitro*, it is usually possible to analyze for the active form of the drug, and in general the MIC (minimal inhibitory concentration) of a drug against a microorganism will be the same in the test tube and *in vivo* in different animal species. It is possible now, although laborious because of the slowness of the experimental infection, to learn the MIC of drugs against *M. leprae* in the mouse and use this fundamental piece of information as a guide in treatment in man.

Sensitivity. Related to consistency of the infection is sensitivity, i.e., the number of *M. leprae* required to produce an infection. The minimal infectious dose is on the order of 10 solidly staining bacilli in CFW mice so that mouse inoculation is approximately 100 times as sensitive as the microscopic method. This sensitivity makes possible a number of studies, for example, one that we have been unable to carry out because of the nonavailability of appropriate clinical material, viz., how long must treatment with DDS be continued in order to render the *M. leprae* in the tissues nonviable as measured by mouse inoculation? Waters and Rees' evidence ([7]) from the solid ratios indicates that the proportion of viable bacilli falls to something less than 5 per cent, in six months of DDS treatment in the usual dosage. The mouse inoculation method would detect many fewer viable bacilli and do it unambiguously even when the solid ratio was very low.

Perhaps it would be worthwhile to state a working hypothesis that is, I believe, fairly widespread. It is that in treating the patient the clinician is faced with two problems, which need to be separated temporarily. One is the killing of *M. leprae* with an efficient drug such as DDS; the other is treatment of reactions and short term changes. From the public health standpoint, the first aspect cannot be neglected. Probably the situation in leprosy is the same as it is in tuberculosis, where six months of chemotherapy is enough to kill most of the bacilli, but several years of uninterrupted treatment are needed to kill the remaining few persisting bacilli and thus prevent relapse. For this reason many leprologists feel that DDS should be continued during reactions if it is at all possible, and that the reactions ought to be treated by corticosteroids, for example, to make it possible to continue the DDS. We do not fully understand the basis underlying reactions, or the various clinical worsenings that can occur over short intervals (a few months). They are probably immunologic, perhaps in the broadest sense of reactions to "non-self" materials, since they are not seen after the patient's tissues have become free of (dead and living) *M. leprae*. It seems quite probable that they are not caused by multiplication of *M. leprae*, since they can occur in the face of adequate DDS intake in patients whose large population of *M. leprae* contains so few viable forms that they are not demonstrable even by inoculation of mice. To stop DDS in these patients would allow a very few persisting bacilli to begin to multiply again and only add to the long period of uninterrupted treatment that is eventually necessary. It would also add to the number of bacterial bodies that the tissue needs eventually to get rid of. Perhaps the two therapeutic problems are illustrated best by the action of corticosteroids, which most leprologists agree are efficacious in controlling reactions. Cortisone promotes the growth of *M. leprae* in untreated mice ([6]) and presumably also does so in man. Thus, when corticosteroids are given, it would appear especially important to continue DDS (even when the clinical manifestations appear to be lessening in response to corticosteroids). Another implication is that short term clinical progress of the patient does not serve the clinician as a reliable index of the response of the patient's bacilli to the administered drug.

Mice vs. other species. Some people have suggested that the mouse is not a good experimental animal because it is so small, and that larger species would be preferable. However, no other mammal is so readily available in the laboratory, and in such genetically uniform state and disease-free condition. One can be assured that the variation between individual mice even in a pen-bred stock is much less than that for elephants or chimpanzees, and that the requirement for numbers of animals to make a result free from sampling error from this cause would accordingly be less in the case of mice. In addition, a number of genetically uniform, inbred strains are readily available nowadays. We might offer thanks for the size of this species; in our laboratory we use 4,000 to 5,000 mice a year in work on leprosy, and the housing problem would be more appreciable with larger mammals.

Specificity. This infection with *M. leprae* is, as far as we know, entirely specific. Many mycobacteria do not grow in the foot pad. *M. marinum (balnei)* and *M. ulcerans* do, but they produce swollen, red feet with granulocytic infiltration and necrosis, and they can be cultivated on artificial medium at 33°C. *M. extracellularis* grows about twice as rapidly as *M. leprae* and produces large extracellular spherical masses of mycobacteria at one stage. *M. lepraemurium* grows more rapidly than *M. leprae* also and in time metastasizes to the peritoneal cavity. It never produces globi, whereas *M. leprae* does very frequently. The specificity combined with the sensitivity discussed above would suit the method to epidemiologic searches for *M. leprae* in unusual situations, e.g., in asymptomatic human beings or in extra-human locations such as arthropods or dirt.

Slowness. Most prominent of the disadvantages, to my notion, is the slowness of the infection. Although one can gain time by working with inocula containing bacilli of higher solid ratios, four months usually elapse from inoculation to harvest even in the more fortunate experiments. Many experiments take a year; one that we started in December 1962, is still in progress. The slowness is a real obstacle to progress, but it is something that may be an unchangeable characteristic of *M. leprae*. Still I cannot help thinking about the good old days with T bacteriophages, when a preliminary assessment of a day's experiment could be gained after dinner, and a final reading could be made the next morning.

Yield of bacteria. With CFW mice the average harvest at the end of the logarithmic phase is about 2×10^6 bacilli per foot pad. At the end of the second logarithmic phase this may rise above 10^7. With 10- to 100-fold more organisms many new approaches would be possible, such as *in vitro* serology, preparation of lepromins, preparation of vaccines with *M. leprae*, and perhaps even studies in the Warburg apparatus. Moreover it would simplify cultivation attempts too if the laboratory supply of *M. leprae* contained a high concentration of bacilli, since in order to follow numbers of AFB and solid ratios conveniently one needs a minimum concentration of about 5×10^5 bacilli per ml. In our laboratory we continue to look for ways to improve yields. So far we can only describe several insufficiently successful approaches, e.g., changes in ambient temperature (described earlier in the symposium), and administration of cortisone ([1]).

Perhaps the outstanding disadvantage for those who would like to study tissue changes, is the limited extent of the mouse infection, and the failure of *M. leprae* to involve the nerves as severely as it does in man. Whether this is a function of the route of infection, the neuroanatomy of the mouse, the time-scale of the infection, or other factors, is not clear. These might be interesting points for investigation.

I would like to close by noting that these advantageous and disadvantageous features have been viewed against the general background of microbiologic approaches to infectious diseases and against the specific background of leprosy, in which there are no other technics as convenient for the experimental growth of the etiologic agent. We can all look forward with pleasure to the day when other, hopefully *in vitro*, procedures will be available for exploitation and side-by-side comparison.

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DISCUSSION

Dr. Mason. Dr. Shepard's paper is open for discussion:

Dr. Knight. I have had no experience at all with Dr. Shepard's technic, but I would like to ask a quite obvious question. What does an infected foot pad look like? What is the procedure in examination?

Dr. Shepard. Different people approach this in different ways. Our routine is to start examining tissue sections after about three months by taking a mouse from the group every month. This gives us a signal when to harvest. I think Dr. Rees starts harvesting every month or so, about six months after inoculation. Probably Dr. Pattyn does the same. I see them both nodding their heads.

Dr. Middlebrook. I hope that what I have to say will not be taken amiss by those who are very ambitious to cultivate *M. leprae* in a cell-free medium. But it does seem important to recognize that many investigators of *M. tuberculosis* and experimental tuberculosis have turned back from their success in *in vitro* cultivation of those organisms to cultivation *in vivo*, for the very reason that they are suspicious that their *in vitro*-grown parasites are not comparable in one way or another to those that actually exist in the host-parasite relationship. So I think, at a time even long after success is achieved in cultivating *M. leprae* in a cell-free medium, there will be as much interest, if not more interest than there is now, in the study of these organisms as they are cultivated in cells.

Dr. Hanks. Dr. Middlebrook is exactly right as to the point regarding tubercle bacilli produced in a given physiologic state and then injected into animals. The problem is to get clean, usable bacilli free from host components. Then we can proceed with many of the greatly needed studies in pathogenesis.

Dr. Mason. We shall now proceed with the next paper, by Dr. Hilson, "Observations on the inoculation of *M. leprae* in the foot pad of the white rat."