# Screening of Drugs for Activity Against Mycobacterium leprae

Ji Baohong, Y. Matsuo, and M. J. Colston\*

A World Health Organization Study Group has recommended (29) that multibacillary leprosy be treated with combined regimens of bactericidal drugs, the regimens to consist of two drugs in addition to dapsone (DDS), each drug acting by a different antimicrobial mechanism. At this moment, however, only three such drugs-rifampin (RMP), clofazimine (CLO), and a thioamide, ethionamide (ETH) or prothionamide (PTH)-are available. And the use of two of these-CLO and a thioamide-is accompanied by undesirable side effects. CLO is not well accepted by light-skinned patients because of the skin pigmentation that results from its use. Administration of a thioamide may be associated with hepatotoxicity (1, 12, 18) or gastric intolerance. Therefore, the number of alternative threedrug regimens is limited; this represents one of the factors contributing to the failure of programs of leprosy control to employ multidrug therapy.

The development of new bactericidal drugs for the treatment of leprosy is important not only to provide additional alternatives to three-drug regimens employing DDS, RMP, CLO and ETH or PTH. Doubly resistant strains of Mycobacterium leprae have already been recognized (7, 10, 28). Relapsing patients who harbor such strains are difficult to treat and, without effective treatment, will become sources of infection with these strains. The emergence in the future of such strains of M. leprae could threaten efforts to control leprosy. Development of new drugs with bactericidal mechanisms entirely different from those of the already available drugs is therefore a matter of some urgency.

# Techniques for drug screening

Because of our inability to cultivate M. leprae in artificial media or in cell culture, the mouse foot-pad technique (<sup>26</sup>) remains the most reliable system for screening drugs for antimicrobial activity against the organism. All of the effective antileprosy drugs have been demonstrated to exert at least bacteriostatic activity in this system, whereas no compound shown to be inactive in mice has been demonstrated to exert definite therapeutic effects in leprosy patients (<sup>25</sup>).

Immunologically normal mice are most commonly employed in drug screening, because the infection with *M. leprae* is very consistent, and because genetically uniform mice are widely available. Moreover, normal mice are cheap to produce and easy to maintain, and large numbers are not required for statistical analysis of the results of drug-screening experiments.

The *M. leprae* may be obtained from skinbiopsy specimens of untreated lepromatous patients, or from the foot pads of infected mice. The organisms recovered from the foot pads are more suitable for drug screening, because the susceptibility of the strains of organisms employed may be well characterized in advance. In addition, growth of a given strain may be well characterized as the result of previous experiments, facilitating the planning of experiments and the timing of harvests. Moreover, if organisms are obtained soon after multiplication in the mouse foot pad has become maximal, the proportion of viable organisms will be maximal, and much greater than that of M. leprae obtained from biopsy specimens, and the sensitivity of the screening techniques will be increased.

As in work with any other pathogen *in vivo*, some basic information about the drug should be assembled before the drug is tested in the mouse foot-pad system. It would be helpful to know something about drug tox-

<sup>\*</sup> Ji Baohong, Leprosy Unit, World Health Organization, 1211 Geneva 27, Switzerland. Y. Matsuo, 2-5-8 Kohnan, Naka-ku, Hiroshima 730, Japan. M. J. Colston, Laboratory for Leprosy and Mycobacterial Research, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

## 55, 4 (Suppl.)

icity, stability, absorption, distribution, biotransformation and excretion (<sup>23</sup>). However, unless the drug has been commercially developed, or has already been studied in mice infected with other pathogens, this information is frequently not available, and may be expensive to acquire.

Initial screening should be carried out employing the maximal tolerated dosage of a compound. If the compound is screened at some lower dosage, and no antimicrobial activity is demonstrated, one cannot be certain that the compound would not have been active, had it been administered in a larger dosage. To find the maximal tolerated dosage of a completely new compound, assuming that no relevant information is available from the scientific literature or from the pharmaceutical firm, one should first determine its acute toxicity by the oral and systemic (intraperitoneal or intravenous) routes; greater toxicity by the systemic route suggests that the drug is incompletely absorbed from the gastrointestinal tract. This is accomplished most easily by administering a dose to five or six mice and, if all of the mice survive, to administer double the original dose to another group of mice. The procedure of doubling the dose, and administering the larger dose to a new group of mice is continued until a dose is found which kills all or most of the mice. The number of mice killed by each dose is then plotted as a function of the logarithm<sub>10</sub> of the dose, and the dose that kills 50% of the mice (the " $LD_{50}$ ") is determined from the resulting graph. One then administers daily to one group of 10 mice one tenth the  $LD_{50}$ , and to additional groups sub-multiples (one hundredth, one thousandth, etc.) of the  $LD_{50}$ . If mice of the group administered the largest dose die, its administration is terminated, and administration of a dose one tenth the smallest dose is begun to an additional group of mice. The desired dose is the largest dose that permits survival of all of the mice after administration for one month.

If the drug is absorbed from the gastrointestinal tract, it should first be administered in the largest tolerated dosage, either incorporated in the mouse diet, or by means of an esophageal cannula (by "gavage"). Because mice eat more-or-less continuously throughout the day and night, administra-

tion of the drug incorporated into the diet provides more constant blood and tissue levels of the drug. Administration of the drug by gavage produces high peak blood concentrations, which are achieved quickly, followed by elimination of the drug at a rate characteristic of the drug. Therefore, although one may administer the same daily dosage by both methods, the results of the screen may differ somewhat, depending upon whether the drug has been administered incorporated into the diet or by gavage. When the compound is not well absorbed from the gastrointestinal tract, it may be administered by injection subcutaneously, intramuscularly, intraperitoneally, or even intravenously. In every case, the drug should be administered in the maximal tolerated dosage for initial screening.

The sequence of studies performed in the course of screening drugs for activity against *M. leprae* is the following: 1) determination of the activity of the compound administered in the maximal tolerated dosage; 2) determination of the minimal effective dose (MED) of the compound; 3) characterization of the activity of the compound as bacteriostatic or bactericidal, and determination of the minimal bactericidal dose (MBD); 4) measurement of concentrations of the compound in blood, and determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC).

Three basic methods are employed to screen drugs for antimicrobial activity against *M. leprae* in mice: 1) the continuous method ( $^{24}$ ); 2) the kinetic method ( $^{20-22}$ ); and 3) the method of proportional bactericide ( $^{3}$ ).

**Continuous method.** The drug is administered continuously from the day mice are inoculated until the experiment is completed. Multiplication of *M. leprae* in untreated control mice is followed by interval harvests. When the evidence of multiplication is unequivocal—i.e., the number of organisms per foot pad of the control mice has increased from an inoculum of  $10^{3.7}$ – $10^4$  to at least  $10^{5.7}$  (500,000), harvests of *M. leprae* are performed from the foot pads of both control and treated mice. If, on two consecutive occasions, approximately one month apart, the number of *M. leprae* har-

Strain of	No. of M. leprae**	No. of <i>M</i> with thia	MED			
M. leprae	(×10 <sup>4</sup> )	0.0003	0.01	0.03	0.1	(g/100 g)
SBL 16325	96	49	9.3	1.2	<1.0	0.03
SBL 16220	81	34	12	1.0	<1.0	0.03
SBL 15337	112	87	75	21	1.0	0.1
TG	250	36	8.3	1.8	1.0	0.03
9593	99	75	14	12	<1.0	0.1

TABLE 1. Sensitivity of M. leprae to thiambutosine; estimation of MED.

\* Mean of 4 foot pads of treated mice harvested 6 months after inoculation.

\*\* Mean of 4 foot pads of control mice harvested 6 months after inoculation.

vested from treated mice does not fall within the range of the results of four simultaneous harvests from control mice, the drug can be stated to have been active (the probability that these results would have occurred by chance is only 0.04). Having established by this method that the drug exhibits antimicrobial properties, one may, by the same method, proceed to establish the MED. The MED is determined by administering the drug in a range of concentrations. An example of the application of the continuous technique to determination of the MED, in which five strains of M. leprae were tested for susceptibility to thiambutosine, is shown in Table 1. Mice were inoculated, each with 10<sup>4</sup> organisms in one hind foot pad, and drug was administered from the day of inoculation in a concentration of 0.003, 0.01, 0.03, or 0.1 g per 100 g mouse diet. Twenty mice were administered a drug-free diet, and five mice were included in each drug-treated group. At monthly intervals, beginning three months after inoculation, four control mice were sacrificed, and harvests of M. leprae were performed from the inoculated foot pads. After six months, when the number of organisms per foot pad reached 106, drugtreated mice were sacrificed, M. leprae were harvested, and the MED determined.

The continuous method permits demonstration of the activity of a drug against M. *leprae*, and is necessary for determination of the MIC. However, the continuous method cannot distinguish between merely bacteriostatic and bactericidal activity. Many drugs are bacteriostatic against M. *leprae*, whereas only a few are bactericidal; only bactericidal drugs are useful in the therapy and control of leprosy (<sup>25</sup>). Another disadvantage of the continuous method is that it requires a large quantity of the drug; a minimum of 18–20 g will be needed for screening. Pharmaceutical firms are not usually able to provide such large quantities of compounds, unless the compounds are being produced commercially or are undergoing active development. And special syntheses of compounds may be quite expensive.

Two methods have been employed to determine whether the activity of a compound against *M. leprae* is bacteriostatic or bactericidal.

Kinetic method. The drug is administered for a limited period, usually 60 days, beginning about 60 or 70 days after inoculation, when the M. leprae are in early or midlogarithmic growth. Multiplication of the organisms in untreated control mice is followed by interval harvests. When administration of the drug is stopped, harvests of *M. leprae* are performed from the foot pads of both control and treated mice. Additional harvests are performed, usually at intervals of 28 or 56 days, until the organisms have multiplied to the level of  $10^{6.0}$ - $10^{6.3}$ per foot pad of treated mice, or until no mice remain. The logarithmic phase of the growth curve of M. leprae is determined for each group of mice, and the growth delay for each treated group is measured graphically, by comparison of each growth curve with that for control mice. Because earlier during logarithmic multiplication, fewer organisms are counted, and the numbers per foot pad are subject to greater sampling variation, the duration of the growth delay should be determined late during logarithmic growth, usually at the times that the growth curves pass 10<sup>5.7</sup>-10<sup>6.0</sup> M. leprae per foot pad.

#### 55, 4 (Suppl.)

Dava	Strain of M. Jonnan	Dose**	Growth delay	Excess delay***	
Drug	Strain of M. leprue	(g/100 g)	(days)	(days)	
Thiambutosine	SBL 16220	0.03	20	_	
		0.1	75	15	
		0.2	103	43	
	TG	0.03	23	_	
		0.1	91	31	
		0.2	88	28	
Protionamide	TG	0.03	76	16	
		0.1	285	225	
		0.2	>332	>272	

TABLE 2. Effect of administration of graded doses of thiambutosine and protionamide for limited periods of time on the growth of M. leprae in the foot pads of mice.\*

\* Adapted from references no. 4 and 5.

\*\* Drugs were administered for 60 days, beginning from the day of inoculation.

\*\*\* Total growth delay minus the delay attributable to presence of drug.

Theoretically, a purely bacteriostatic drug will inhibit multiplication of the organisms for as long as the drug is administered. Thus, the growth curve of M. leprae in mice treated with a purely bacteriostatic drug should be parallel to that in the control mice, but should lag behind the control growth curve by a length of time no greater than that during which the drug was administered. The absence of bactericidal activity is reliably demonstrated by this approach. The failure of bacterial multiplication to resume immediately following cessation of drug administration may represent evidence that M. leprae were killed during treatment. On the other hand, a growth delay longer than the period of drug administration may indicate only prolonged bacteriostasis (also termed "bacteriopause"), which may indicate persistence of the drug in the tissues (3) or within the organism, or it may reflect the recovery of organisms that have been damaged reversibly. In these instances, the growth delay will be longer than the duration of drug administration. Thus, the kinetic method can distinguish between purely bacteriostatic and so-called "bactericidal-type" activity (25). However, this method cannot distinguish between bacteriopausal and bactericidal activity, unless there is total failure of resumption of bacterial multiplication, after drug administration has been stopped.

An example of the application of the kinetic method to the determination of the MBD is shown in Table 2.

Although the continuous method is the most sensitive screening technique, the kinetic method is commonly employed for initial screening, in order not to overlook what might be interesting activity (22, 23). Even though a purely bacteriostatic drug is unlikely to be employed in the treatment of leprosy, such activity may represent an important lead, when it is exerted by a compound, the chemical structure of which is entirely different from the structures of the existing antileprosy drugs. Such a drug may suggest the possibility of activity against a different target enzyme of M. leprae and, by means of the study of quantitative structure-activity-relationships (QSAR), it may be possible to propose bactericidal derivatives of the drug. The kinetic method is particularly suitable for QSAR studies, because it provides data that are continuously distributed (22).

**Proportional bactericide method.** The second method for determining whether the activity of a compound is bactericidal or bacteriostatic is the method of proportional bactericide.

An inoculum containing  $10^{3.7}-10^4 M$ . *lep-rae* per 0.03 ml is prepared in the usual way, and diluted serially in 10-fold steps, so that the final dilution contains 0.5 or 1 organism per 0.03 ml. Each dilution is used to inoculate groups of five to ten mice. Except for the control group, the mice are treated for a period of time that varies, depending upon the drug, from one to 60 days. The mice are then held for at least one year, a period of

TABLE 3. Proportional bactericide test: bactericidal activity of thiocarlide, thiambutosine, thiacetazone, DDS, RMP and CLO against M. leprae (strain TG).\*

Drug and concentration (g/100 g diet)	No. M. leprae inoculated per foot pad				MPN**	Survival
	104	10 <sup>3</sup>	10 <sup>2</sup>	101		(%)
Control	10/10***	10/10	9/9	6/8	1600	_
DDS (0.01)****	10/10	7/7	8/8	2/7	350	22
RMP (0.003)	2/8	1/9	0/8	0/9	0.2	0.01
CLO (0.003)	8/9	5/8	3/7	0/8	14	1
Thiocarlide (0.1)	9/9	8/8	10/10	8/10	1600	100
Thiambutosine (0.1)	10/10	10/10	8/8	10/10	>1800	112
Thiacetazone (0.1)	9/9	7/7	7/7	4/8	920	58

\* Adapted from reference no. 3.

\*\* Most probable number of viable M. leprae per 10<sup>4</sup> organisms.

\*\* No. of foot pads showing multiplication of M. leprae

No. of foot pads harvested

\*\*\*\* All drugs were adminstered for 60 days.

time theoretically sufficient for one surviving organism to multiply to 106. At the end of the year, harvests of M. leprae are performed from individual foot pads; if the harvest yields at least  $10^5$  organisms, the M. leprae are considered to have multiplied, indicating that the inoculum provided at least one viable organism. The numbers of M. leprae surviving the treatment are calculated by estimating the most probable number (MPN) of viable organisms (8. 27). However, the estimation of the MPN is based on the assumptions that M. leprae are distributed randomly in an inoculum, and that one viable organism is sufficient to give rise to infection of the foot pad. In the case of M. leprae and the mouse foot pad, these assumptions are probably untenable; the preferred alternative is to calculate the median infectious dose (ID<sub>50</sub>; the number of organisms required to infect 50% of the animals) (22).

The results of an application of the proportional bactericide method are presented in Table 3. In this experiment, groups of 10 mice were inoculated, each with  $10^4$ ,  $10^3$ ,  $10^2$  or 10 *M. leprae* in one hind foot pad. Treatment with the regimens indicated was begun on the day of inoculation, and stopped after 60 days. One year later, the mice were sacrificed, and harvests of *M. leprae* were performed from the inoculated food pads. The MPN of viable organisms was determined for each mouse group, and the percent of organisms surviving each treatment was calculated. Although the proportional bactericide method requires more mice and more time than do either of the other methods, it is the most reliable method for detecting bactericide, and permits determination of the degree of bactericidal activity. On the other hand, the method is incapable of detecting bacteriostatic or bacteriopausal activity. Therefore, the proportional bactericidal method is not routinely employed for initial screening.

Having established the MED and the MBD, the next step in drug screening is to correlate these values with the concentrations of drug found in the plasma of mice administered the drug in the corresponding concentration (the method for determining the plasma concentration of a drug obviously depends upon the nature of the drug under investigation); the MIC is the plasma concentration provided by the MED, and the MBC is the plasma concentration provided by the MBD. In Table 4 are presented the concentrations of RMP in mice administered the drug in the MED-0.01 g per 100 g diet. It can be seen that the MIC of RMP for this strain of M. leprae is approximately 6 µg per ml. A similar experiment carried out on mice administered the MBD would reveal the MBC.

The results obtained from drug screening in mice are relatively clear cut. However, some discrepancies have been noted between laboratories. For example, Shepard and Chang reported (<sup>24</sup>) that thiambutosine was inactive and thiacetazone only partially

### 55, 4 (Suppl.)

active against *M. leprae*, when the drugs were administered in a concentration of 0.1 g per 100 g, whereas Colston and his coworkers found (<sup>4</sup>) that, administered in this concentration, both drugs completely inhibited multiplication of the organisms. Because every laboratory employs different strains of *M. leprae*, each strain of course having been isolated from a different patient, one must be cautious in extending to all strains of the organism the results of studies on a single strain.

#### Prospects for drug screening

Although the mouse foot-pad technique represents the only reliable method for studying drug activity against M. leprae outside the patient, the technique possesses many disadvantages. It is time consuming, and requires many mice and gram amounts of the compounds to be tested, and can therefore be employed to investigate only limited numbers of compounds representing a very few selected classes. On the other hand, the search for active compounds representing a wide variety of classes requires a rapid screening method that will yield results within days or, at most, a few weeks, and that requires only milligrams rather than gram amounts of the compounds. Ideally, drugs should be screened in vitro; however, M. leprae cannot yet be cultivated in vitro.

Within the last decade, several systems have been reported to be suitable for rapid determination of the viability of M. leprae in vitro. These techniques include uptake of tritiated thymidine (3HTdR) by M. leprae in a macrophage monolayer (17); incorporation of <sup>3</sup>HTdR and tritiated dihydroxyphenylalanine by M. leprae in a cell-free medium (13); measurement of the bacterial content of adenosine triphosphate (6); fluorescence staining by fluorescein diacetate and ethidium bromide (14); measurement of changes of Fc receptors on the surface of macrophages (11, 15); changes of the ratio of cholesterol to cholesterol esters within macrophages that have ingested viable M. leprae (16); and measurement of the intrabacterial sodium : potassium ratio (19). Although all of these systems require further investigation and independent verification, it appears likely that, in the near future, some methods will be shown to possess the spec-

TABLE 4. Serum RMP concentrations inmice administered 0.01 g RMP per 100 gdiet.\*

Period of drug adminis- tration (days)	No. mice examined	Range of serum RMP concentra- tions (µg/ml)	Mean serum RMP concentration (µg/ml ± SE)
1	3	2.1-4.8	$3.3 \pm 1.4$
2	5	3.5-8.1	$5.9 \pm 1.9$
4	5	4.4-9.5	$6.4 \pm 1.9$
7	5	3.4-7.5	$5.8 \pm 1.6$
14	6	3.4-7.7	$4.9 \pm 1.6$
21	6	2.4-7.7	$5.7 \pm 1.6$
45	6	3.0-6.6	$4.2 \pm 1.7$
66	6	1.9-4.1	3.1 ± 1.4

\* Adapted from reference no. 9.

ificity and sensitivity needed to permit their application to drug screening.

Finally, as the result of developments in the field of molecular biology ( $^{2, 30}$ ), it may soon be possible to clone and express those genes of *M. leprae* that code for enzymes likely to be the targets of drug activity. Once this has been accomplished, the recombinant microorganisms—e.g., *Escherichia coli*—or the purified enzymes could then be employed for rapid drug screening. This entirely new approach is still in an early stage of development, but appears to possess great potential; it should therefore be the focus of future work.

#### REFERENCES

- CARTEL, J. L., MILLAN, J., GUELPA-LAURAS, C. C. and GROSSET, J. Hepatitis in leprosy patients treated by a daily combination of dapsone, rifampicin and a thioamide. Int. J. Lepr. 51 (1983) 461.
- CLARK-CURTISS, J. E., JACOB, W. R., DOCHERTY, M. A., RITCHIE, L. R. and CURTISS, R. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. J. Bact. 161 (1985) 1093.
- COLSTON, M. J., HILSON, G. R. F. and BANNERJEE, D. K. The "proportional bactericidal test". A method for assessing bactericidal activity of drugs against *Mycobacterium leprae* in mice. Lepr. Rev. 49 (1978) 7.
- COLSTON, M. J., HILSON, G. R. F., ELLARD, G. A., GAMMON, P. T. and REES, R. J. W. The activity of thiacetazone, thiambutosine, thiocarlide and sulphamethoxypyridazine against *Mycobacterium leprae* in mice. Lepr. Rev. 49 (1978) 101.
- 5. COLSTON, M. J., ELLARD, G. A. and GAMMON, P. T. Drugs for combined therapy. Experimental

studies on the antileprosy activity of ethionamide and prothionamide, and a general review. Lepr. Rev. **49** (1978) 115.

- DHOPLE, A. M. Adenosine triphosphate content of *Mycobacterium leprae* from leprosy patients. Int. J. Lepr. 52 (1984) 183.
- GUELPA-LAURAS, C.-C., GROSSET, J., CON-STANT-DESPORTES, M. and BRUCKER, G. Nine cases of rifampicin-resistant leprosy. Int. J. Lepr. 52 (1984) 101.
- HALVORSON, H. O. and ZIEGLER, N. R. Application of statistics to problems in bacteriology. I. A means of determining bacterial population by the dilution method. J. Bact. 25 (1933) 101.
- HOLMES, I. B. and HILSON, G. R. F. The effect of rifampicin and dapsone on experimental *Mycobacterium leprae* infections: minimum inhibitory concentrations and bactericidal activity. J. Med. Microbiol. 5 (1972) 251.
- JACOBSON, R. R. and HASTINGS, R. C. Rifampicin resistant *M. leprae.* Lancet 2 (1976) 1304.
- 11. JAGANNATHAN, R. and MAHADEVAN, P. R. Synergistic activity of dapsone and brodimoprin (a dihydrofolate inhibitor) against *Mycobacterium leprae.* IRCS Med. Sci. **13** (1985) 265.
- JI, B., CHEN, J., WANG, C. and KIA, G. The hepatotoxicity of combined therapy with rifampicin and daily protionamide for leprosy. Lepr. Rev. 55 (1984) 283.
- KHANOLKAR, S. R., AMBROSE, E. J., CHULAWALA, R. G. and BAPAT, C. V. Autoradiographic and metabolic studies of *Mycobacterium leprae*. Lepr. Rev. 4 (1978) 187.
- KVACH, J. T., MUNGULA, G. and STRAND, S. H. Staining tissue-derived *Mycobacterium leprag* with fluorescein diacetate and ethidium bromide. Int. J. Lepr. 52 (1984) 176.
- MANKAR, M. V., JAGANNATHAN, R. and MAHADE-VAN, P. R. In vitro drug screening system using membrane alteration in macrophages by *Mycobacterium leprae.* J. Biosci. 6 (1984) 709.
- NAIR, I. and MAHADEVAN, P. R. An in vitro test using cholesterol metabolism of macrophages to determine drug sensitivity and resistance of *Mycobacterium leprae*. J. Biosci. 6 (1984) 221.
- NATH, I., PRASAD, H. K., SATHISH, M., SREEVATSA, DESIKAN, K. V., SESHADRI, P. S. and IYER, C. G. S. Rapid, radiolabeled macrophage culture method for detection of dapsone-resistant *Mycobacterium leprae*. Antimicrob. Agents Chemother. 21 (1982) 26.

- PATTYN, S. R., JANSSENS, L., BOURLAND, J., SAYLAND, T., DAVIES, E., GRILLONE, S., FERRACCI, C. and THE COLLABORATIVE STUDY GROUP FOR THE TREAT-MENT OF LEPROSY. Hepatotoxicity of the combination of rifampicin-ethionamide in the treatment of multi-bacillary leprosy. Int. J. Lepr. 52 (1984) 1.
- SEYDEL, U., LINDNER, B. and DHOPLE, A. M. Results from cation and mass fingerprint analysis of single cells and from ATP measurements of *M. leprae* for drug sensitivity testing: a comparison. Int. J. Lepr. 53 (1985) 365.
- SHEPARD, C. C. A kinetic method for the study of the activity of drugs against *Mycobacterium leprae* in mice. Int. J. Lepr. **35** (1967) 429.
- SHEPARD, C. C. Further experience with the kinetic method for the study of drugs against *Mycobacterium leprae* in mice. Activities of DDS, DFD, ethionamide, capreomycin and PAM 1392. Int. J. Lepr. **37** (1969) 389.
- SHEPARD, C. C. Statistical analysis of results obtained by two methods for testing drug activity against *Mycobacterium leprae*. Int. J. Lepr. 50 (1982) 96.
- SHEPARD, C. C. Experimental leprosy. In: *Leprosy.* Hastings, R. C., ed. Churchill Livingstone, 1985, pp. 269–286.
- SHEPARD, C. C. and CHANG, Y. T. Effect of several anti-leprosy drugs on the multiplication of human leprosy bacilli in foot-pads of mice. Proc. Soc. Exp. Biol. Med. **109** (1962) 636.
- SHEPARD, C. C., VAN LANDINGHAM, R. M. and WALKER, L. L. Recent studies of antileprosy drugs. Lepr. Rev. 39 (1971) 340.
- SHEPARD, C. C., VAN LANDINGHAM, R. M. and WALKER, L. L. Recent studies of antileprosy drugs. Lepr. Rev. 54 Special Issue (1983) 23S.
- TAYLOR, J. The estimation of numbers of bacteria by ten-fold dilution series. J. Appl. Bact. 25 (1933) 101.
- WARNDORFF-VAN DIEPEN, T. Clofazimine-resistant *M. leprae* a case report. Int. J. Lepr. 50 (1982) 139.
- 29. WHO STUDY GROUP. Chemotherapy of leprosy for control programmes. WHO Tech. Rep. Ser. 675, 1982.
- YOUNG, R. A., MEHRA, V., SWEETSER, D., BUCHANAN, T., CLARK-CURTISS, J., DAVIS, R. W. and BLOOM, B. R. Genes for the major protein antigens of the leprosy parasite *Mycobacterium leprae*. Nature 316 (1985) 450.