INTERNATIONAL JOURNAL OF LEPROSY

Volume 49, Number 2
Printed in the U.S.A.

The Uptake of ^{3}H-Thymidine in *Mycobacterium leprae* Inoculated Mouse Macrophage Cultures as a Rapid Indicator of Bacillary Viability. Factors Influencing the Specificity of the *in vitro* Assay[1]

M. Sathish and Indira Nath[2]

The inability to culture *Mycobacterium leprae in vitro* and the lack of precise methods to assess the viability of this organism have been major obstacles in the development of suitable therapeutic measures in leprosy. In the last decade the mouse foot pad model, first described by Shepard in 1960 ([13]), reproduced in many laboratories of the world and subsequently modified ([9,10]), has proved to be an invaluable tool for the evaluation of drug sensitivity ([4,12,14]), diagnosis of dapsone resistance ([7,11]), and kinetics of drug action ([3,11,14]). However, this method calls for large numbers of mice and adequate air conditioned animal house facilities, features which are lacking in many developing countries where leprosy is endemic. Moreover, a period of 6–12 months is required for screening in this system. Thus both financial and time constraints limit the widespread use of this animal model in field studies. With the emergence of dapsone resistance ([16]), an urgent need is being felt for a rapid method of screening for resistance and for screening newer antileprosy drugs.

In 1965, Pasquier ([6]) reported the incorporation of radioactive isotopes in mycobacteria by autoradiography. Drutz and Cline ([1]) applied this to *M. leprae* and showed radiolabeled bacilli in the peripheral blood monocytes of lepromatous patients. Talwar, *et al.* ([15]) extended this further and reported the incorporation of tritiated thymidine (^{3}H-Tdr) in *M. leprae* residing in human macrophage cultures. This method has been further refined, standardized, and found reproducible in 60–70% of *M. leprae* strains screened in our laboratory ([8]).

Though the human macrophage culture system using radiolabeled precursors for DNA synthesis has been found to be useful for the assessment of limited multiplication of *M. leprae*, it may not prove to be feasible for field studies involving large numbers of patients. Moreover, 5–6 days of culture are required for peripheral blood monocytes to differentiate into macrophages. Thus a week's prior warning is necessary for the preparation of macrophage cultures.

Keeping this drawback in view, the present study was undertaken with murine macrophages. Here we report successful incorporation of ^{3}H-Tdr in human-derived *M. leprae* residing in macrophages obtained from the peritoneal exudate cells of BALB/c mice. Some of the factors influencing this methodology have been indicated. Further refinements of this technique and its application to drug screening and to the identification of dapsone resistant strains are under current investigation.

MATERIALS AND METHODS

Extraction of *M. leprae*. Skin biopsies from bacilliferous, lepromatous patients were kindly supplied by Drs. C. G. S. Iyer and P. S. Seshadri, Central Leprosy Training and Research Institute, Chingleput, and Dr. L. M. Hogerzeil, Victoria Hospital, Dichpalli. The tissues were shipped by air

[1] Received for publication on 1 July 1980; accepted for publication in revised form on 19 January 1981.

[2] M. Sathish, M.Sc.; I. Nath, M.D., Department of Pathology, All India Institute of Medical Sciences, New Delhi-110 029, India. Correspondence to Dr. Nath.

on wet ice and received within 6–18 hr after removal. *M. leprae* were extracted as described earlier ([8]). In brief, the epidermis was removed and the tissue was homogenized in glass homogenizers in RPMI 1640 (GIBCO Biocult). Care was taken to minimize the amount of tissue contamination. The extracted bacilli were screened for other contaminating bacteria. The extracts were plated and incubated at 37°C on a) nutrient agar for 72 hr and b) Löwenstein-Jensen medium for 8 weeks. *M. leprae* were counted by the method of Hanks, *et al.* ([2]). The bacilli were inoculated into macrophage cultures within 48 hr of removal from the patient.

Macrophage cultures. Nonstimulated peritoneal exudate cells (PEC) from BALB/c mice were removed by washing out the peritoneal cavity with 2–3 ml of cold RPMI 1640 containing 10 units per ml of preservative free heparin (Upjohn Co., Kalamazoo, Michigan, U.S.A.). The peritoneal exudate from individual mice was diluted with an equal amount of RPMI 1640 supplemented with 20% fetal calf serum (FCS) and 50 units per ml of Mycostatin (GIBCO Biocult). One ml of cell suspension was delivered into each Leighton tube and incubated at 37°C for 2 hr. Subsequently, nonadherent cells were gently removed, and the medium was replaced. The macrophages were maintained at 37°C and used 48 hr later for the support of *M. leprae*.

Inoculation with *M. leprae* and assessment of ^{3}H-thymidine uptake. After checking for secondary bacterial contaminants, 1×10^6 to 5×10^6 *M. leprae* were inoculated into each Leighton tube. After 24 hr incubation, the unphagocytosed bacteria were removed, the cultures were gently washed with warm medium, and the medium was replaced with RPMI 1640 containing 30% FCS and 0.5 to 1.0 μCi of high specific activity ^{3}H-thymidine (42 Ci/mmol; Radiochemical Centre, Amersham). Cultures were incubated at 37°C for 14 days. Medium containing the radiolabel was changed every 4–5 days to avoid degradation of thymidine. Control cultures consisted of a) noninfected macrophages from the same batch of mice and b) macrophages infected with autoclaved *M. leprae* of the corresponding strain. Replicates of five Leighton tubes, after randomization of initial macrophage cultures, were used in each group. During the culture period some tubes were discarded due to stripping of macrophages. Results of such cultures are expressed as mean counts per minute (CPM) of a minimum of three replicates.

Cultures were harvested on day 14 at 4°C. The macrophages were scraped with the help of a rubber policeman and transferred to Whatman glass fiber discs under vacuum. Serial washing of the discs was done with saline containing cold thymidine (1 mg/ml), twice each with 5% trichloroacetic acid and methanol. The dried discs were counted in a liquid scintillation counter (Packard & Co.). The mean counts per minute (CPM) of replicates were calculated. This incorporation index was assessed as:

$$\frac{\text{Mean CPM of cultures infected with freshly extracted } \textit{M. leprae}}{\text{Mean CPM of cultures with autoclaved } \textit{M. leprae}}$$

DNase treatment of cultures containing *M. leprae*. Three macrophage cultures containing freshly extracted *M. leprae* and the radiolabel were harvested on day 14. Each experiment containing an individual *M. leprae* strain had two replicates of pooled cells from two Leighton tubes. The cells were lysed by repeated freeze-thawing and divided into two equal fractions. Each aliquot was treated with 250 Kunitz units of DNase (Type I, Sigma Chemical Co.) dissolved in 0.15 M NaCl containing 0.005 M $MgSO_4$ for 18 hr at 37°C. Subsequently, the extracts were collected on glass fiber discs as before and counted in a liquid scintillation counter.

Controls consisted of 72 hr old concanavalin A (Pharmacia Ltd., Uppsala) stimulated lymphocytes of normal donors. Mitogen cultures were put up as described before ([5]). Replicates of 10^5 cells were freeze thawed and treated as above.

Statistical analysis. Nonparametric statistics were used for tests of significance. Mann Whitney's U test was employed by ranking individual CPM values of replicates of test and control cultures. Mean values have been given in figures and tables to simplify visualization and expression of multiple experiments having three to five replicates.

Nonspecific esterase. Macrophages were stained for nonspecific esterase essentially by the method of Yam, *et al.* using alpha-naphthyl acetate as substrate and 2% glutaraldehyde (Taab Labs) as a fixative ([17]).

RESULTS

Macrophages derived from BALB/c mice were maintained in culture for a period of 2–3 weeks. Morphologically, these cells were spindle shaped with a small reniform nucleus and abundant cytoplasm. Unlike human macrophage cultures, binucleate and multinucleate cells were rare (personal observations). Mitotic figures were never observed. A few adherent round mononuclear cells with scanty cytoplasm were seen in every culture. Both types of cells were diffusely stained for nonspecific esterase. None of the 17 strains of *M. leprae* plated on Löwenstein-Jensen medium at the time of the skin biopsies showed evidence of mycobacterial growth at the end of 8 weeks.

Pattern of ³H-Tdr uptake in *M. leprae* strains maintained in mouse macrophage cultures. Seventeen strains of human-derived *M. leprae* were screened for the uptake of ³H-Tdr. Figure 1 shows the mean CPM values observed in control and infected macrophage cultures for each individual strain of bacillus. It may be noted that the baseline DNA synthesis of each batch of macrophages showed a variation which ranged from 319 ± 266 to 4439 ± 357 (mean ± S.E.) CPM. The overall increment in the uptake of ³H-Tdr in culture containing freshly extracted *M. leprae* varied from 145 ± 30 to 14,152 ± 5162 CPM. It may also be observed that some cultures with freshly extracted bacilli showed a) insignificant or b) negative incorporation of the radiolabel as compared to control cultures. On comparison of individual CPM values of replicates of control and test cultures, statistical significance of 5% or less was obtained when test cultures in general showed an increment of ³H-Tdr uptake at or above two-fold the mean values. Using these criteria it was found that 10 of the 17 strains (58.8%) incorporated ³H-Tdr at significant levels varying from $p < 0.05$ to $p < 0.001$ (Fig. 2).

Time kinetics of ³H-Tdr incorporation. The relationship of the duration of pulse to the incorporation of ³H-Tdr was studied in three experiments using different strains of *M. leprae*. A representative experiment is depicted in Fig. 3. In all of the experiments uptake of ³H-Tdr above control levels was not observed by the seventh day. Prolongation of the pulse period to 14 days showed significant incorporation of the radiolabel in cultures containing freshly extracted bacilli.

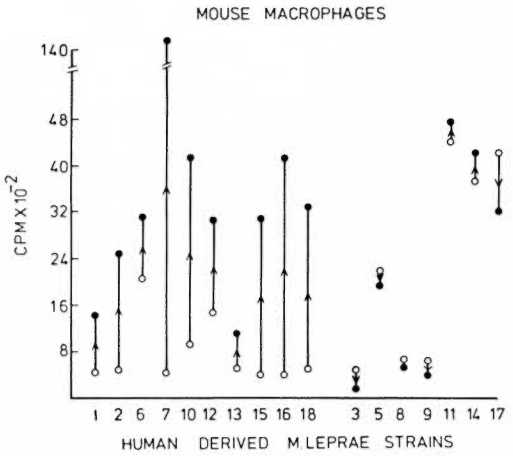

FIG. 1. Incorporation of ³H-Tdr in 17 human derived *M. leprae* strains maintained in mouse macrophages for 14 days *in vitro*. Mean counts per minute (CPM) of cultures with autoclaved (○) and freshly extracted *M. leprae* (●) inoculated cultures of individual strains. Numbers along the ordinate refer to *M. leprae* strains.

Relationship of viability of *M. leprae* to the uptake of ³H-Tdr. Cultures containing autoclaved *M. leprae* showed some increase in CPM values as compared to the incorporation of ³H-Tdr in uninoculated macrophages (Table 1). However, it may be noted that macrophage cultures containing freshly extracted unkilled bacilli showed a significant increment ($p < 0.05$ to $p < 0.001$) in ³H-Tdr uptake above the values obtained with the autoclaved bacilli of the identical strain. Stimulation indexes and CPM values expressed subsequently were calculated by using cultures with autoclaved bacteria as the controls.

Specificity of ³H-Tdr incorporation in *M. leprae*. Three sets of experiments containing individual *M. leprae* strains were subjected to DNase treatment under conditions which would degrade mammalian DNA. It may be observed from Table 2 that 85% of

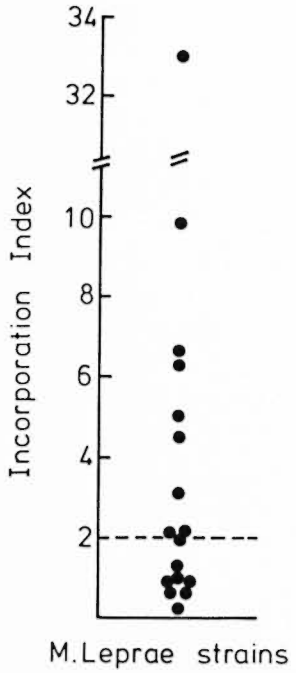

FIG. 2. Incorporation Index of ^{3}H-Tdr incorporation in 17 human derived *M. leprae* strains maintained in mouse macrophage cultures for 14 days *in vitro*. *Incorporation Index*:

$$\frac{\text{Mean CPM of cultures inoculated with freshly extracted } M.\ leprae}{\text{Mean CPM of cultures inoculated with autoclaved } M.\ leprae}$$

Statistical significance ($p < 0.05$ to $p < 0.001$) was observed in cultures which showed Incorporation Index of 2 (- - - - -) and above. Mann Whitney U test was employed by ranking individual replicates of control and test cultures.

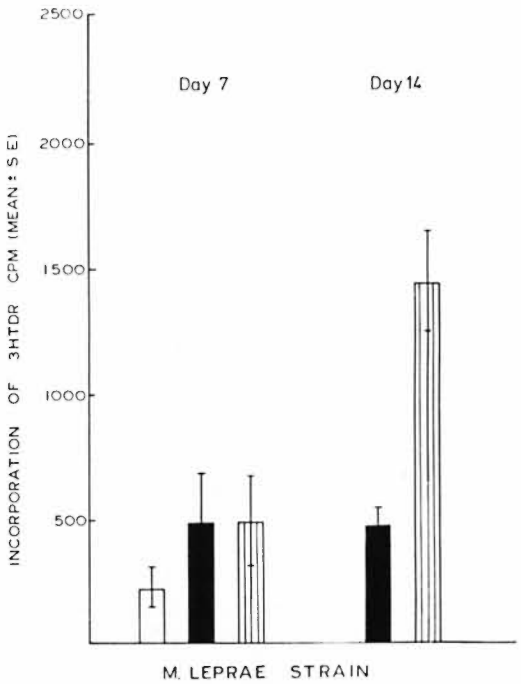

FIG. 3. A typical experiment showing time kinetics of incorporation of ^{3}H-Tdr in one human derived *M. leprae* strain maintained in mouse macrophage cultures for 7 and 14 days *in vitro*. □ Macrophages only; ■ Macrophages + 5×10^6 autoclaved *M. leprae*; ▥ Macrophages + 5×10^6 freshly extracted *M. leprae*.

the CPM values due to mitogen stimulated lymphocyte DNA synthesis was inhibited by DNase. In contrast, the incorporation of ^{3}H-Tdr in macrophage cultures containing freshly extracted *M. leprae* was only mildly inhibited by DNase, thereby indicating that the CPM values obtained in these cultures were predominantly due to bacterial DNA synthesis.

Factors influencing nonincorporation of ^{3}H-Tdr. As shown earlier (Fig. 2), 41% of the *M. leprae* strains screened in the *in vitro* macrophage culture failed to incorporate ^{3}H-Tdr. The factors responsible for this feature were analyzed further.

a. Dose of bacillary inoculum in macrophage cultures. Strains 8, 9, 13, 15, and 16 were inoculated into Leighton tubes at a dose of 1 million (Fig. 1). It may be observed that of these strains, 8 and 9 did not incorporate ^{3}H-Tdr whereas the other three strains showed an incorporation index above 2. Similar observations were made when 5 million bacilli per tube were used. Thus the size of the bacillary inoculum used in the present protocol did not appear to be the contributive factor for the unsuccessful incorporation of thymidine (Fig. 1).

b. Concentration of the radiolabel. Both 0.5 μCi and 1 μCi of ^{3}H-Tdr per culture were used. Strains 15-18 had the lower concentration while the others had the higher concentration of the radiolabel in the culture system. Both successful and unsuccessful incorporations were observed at these levels of the radiolabel (Fig. 1).

c. Morphological Index (MI) of the extracted *M. leprae*. The MI of the bacilli used

TABLE 1. *Incorporation of ³H-thymidine by* M. leprae *residing in mouse macrophages. Data expressed as mean values of counts per minute of replicate cultures ± standard error.*

Strain no.	Mø[a] alone	Mø + autoclaved *M. leprae*	Mø + freshly extracted *M. leprae*
1	214 ± 60	463 ± 80	1439 ± 239
2	290 ± 140	492 ± 163	2475 ± 793
7	427 ± 47	678 ± 255	14,152 ± 5162
10	680 ± 151	941 ± 264	4142 ± 262
15	319 ± 266	421 ± 402	3092 ± 500
18	424 ± 362	495 ± 400	3270 ± 136

[a] Mø = peritoneal exudate macrophages from BALB/c mice.

in the culture varied from 0.45 to 12%. No relationship was observed between the MI and the failure of incorporation of ³H-Tdr.

d. Batches of host macrophages. All experiments were done on macrophages derived from BALB/c mice. Batch to batch variation in DNA synthesis was observed in these cells. Thus the macrophage cultures derived from the same batch of mice were randomized and two strains of *M. leprae* obtained from different individuals, but transported and handled in an identical manner, were inoculated into the same set of macrophages. Figure 4 shows the results of five such experiments. It may be observed that in the same set of host cells, the *M. leprae* strains varied in their ability to incorporate ³H-Tdr.

DISCUSSION

The current study utilizing *M. leprae* infected mouse macrophage cultures confirms our earlier findings that some human-derived strains of *M. leprae* incorporate ³H-Tdr *in vitro* ([8]). Macrophages derived from the nonstimulated PEC of BALB/c mice could be maintained in culture for varying periods of time and successfully supported *M. leprae in vitro*. An adaptive period of 1 week was required before the incorporation of thymidine could be observed in the infected macrophage cultures. Fourteen days of pulsing with ³H-Tdr appeared to be required for obtaining two-fold or more increments in CPM values of the infected cultures over control cultures.

The uptake of ³H-Tdr appeared to be related to the viability of *M. leprae* and was not due to the nonspecific adsorption of the radiolabel by the mycobacteria. This was indicated in experiments showing a) a differential uptake of ³H-Tdr in unkilled as compared to the corresponding autoclaved

TABLE 2. *Effect of DNase treatment on ³H-thymidine uptake by* M. leprae *resident in murine macrophage cultures. Data is presented as mean counts per minute of triplicate cultures ± standard error.*

Experiment no.	Mø cultures + *M. leprae* + ³H-thymidine			% Inhibition[c]
		Untreated (CPM)	DNase treated (CPM)	
1	a[a]	3740	3043	19
	b[b]	3516	2645	25
2	a	3059	3063	−0.13
	b	2714	2746	−0.18
3	a	495	442	11
	b	451	414	8
	Con A stimulated 72 hr human lymphocytes			
4		9670 ± 120	1450 ± 46	85

[a,b] Replicates of cultures with same *M. leprae* strain.

[c] % inhibition = 100 × CPM of DNase treated ³H-Tdr incorporated cultures/CPM of untreated ³H-Tdr incorporated control cultures.

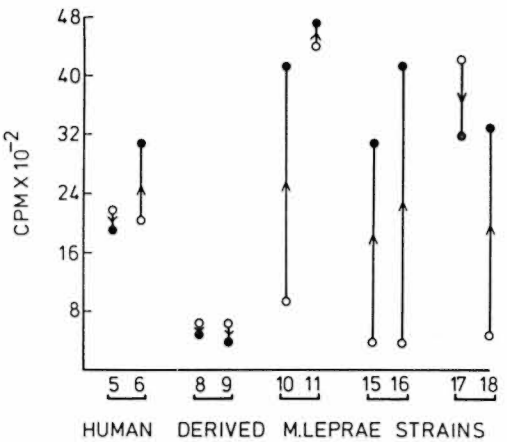

FIG. 4. Five paired experiments showing incorporation of ³H-Tdr in *M. leprae* strains derived from different patients and tested in an identical manner are depicted. Numbers along the ordinate refer to *M. leprae* strains inoculated simultaneously in the same set of murine macrophages. Mean counts per minute (CPM) of replicate cultures inoculated with autoclaved (○) and freshly extracted *M. leprae* (●).

strain; b) a time lag of 1 week required for the uptake of ^{3}H-Tdr, indicating an *in vitro* adaptation of *M. leprae*; and c) a failure in 41% of *M. leprae* strains (treated in an identical manner) to incorporate thymidine.

The location of the radiolabel appears to be in mycobacterial DNA as indicated by experiments conducted with DNase under conditions which markedly degrade mammalian DNA. In three individual macrophage cultures containing different strains of *M. leprae* it was found that the radiolabel resisted DNase treatment for 18 hr (Table 2). Earlier data on human macrophage cultures ([8]) had shown that the ^{3}H-Tdr label used up to 7 days in culture remained stable and associated with thymidine. These two features taken together would thus suggest that ^{3}H-Tdr is being preferentially incorporated into the DNA of *M. leprae* rather than the mammalian host cell. Though macrophages in the end cell stage do not appear to possess thymidine kinase ([15]), data is not available in the literature regarding the definitive presence of this enzyme in *M. leprae*. Since this enzyme is necessary for the incorporation of thymidine into DNA, our findings would indirectly suggest that thymidine kinase is present in *M. leprae*.

Some of the reasons for the unsuccessful incorporation of ^{3}H-Tdr in certain strains of *M. leprae* have been ruled out. Investigations are being currently undertaken to study this phenomenon further. It was observed that a) the size of the bacillary inoculum used currently, b) the dose of the radiolabel, c) the differences in batches of host macrophages, and d) the MI variability of the extracted bacilli were not the incriminating factors for the differential uptake of ^{3}H-Tdr. It is possible that the incorporation of this precursor of DNA may be an inherent property of some of the bacilli in the extracted inoculum. This feature may be linked to the stage of the cell cycle of the bacilli. It is probable that the extracted bacilli in the skin biopsies would be asynchronous and in various stages of their cell cycle. Thus only those bacilli which are in the S-phase during the limited period of culture would incorporate ^{3}H-Tdr. It is therefore possible that cultures containing bacilli predominantly in the non-S phase may show undetectable uptake of ^{3}H-Tdr.

The current report presents preliminary evidence to indicate that the mouse macrophage culture system used simultaneously with a radiolabeled DNA precursor is useful for a rapid, sensitive, and highly quantitative assessment of *M. leprae* viability. This *in vitro* method takes 14 days as compared to 6–12 months in the mouse foot pad. With further refinement and increase in efficiency it may facilitate the early diagnosis of dapsone resistant strains and screening for antileprosy drugs. The use of mouse macrophages as compared to human macrophages further increases the feasibility of screening large numbers of *M. leprae* strains with minimum inherent variation in host cells.

SUMMARY

Mouse peritoneal macrophages derived from BALB/c were used as host cells for 17 human-derived *M. leprae* strains. Simultaneous pulsing with ^{3}H-thymidine (^{3}H-Tdr) showed uptake of the radiolabel in 58.8% of the bacilli over a 14 day period. Preliminary data of three *M. leprae* strains indicate that the ^{3}H-Tdr is preferentially incorporated into the mycobacterial DNA and not into the mammalian host cell DNA. This *in vitro* assay provides a rapid assessment of *M. leprae* viability. The factors influencing the uptake of ^{3}H-thymidine are described.

RESUMEN

Se usaron macrófagos peritoneales de ratones BALB/c como células huésped para 17 cepas de *M. leprae* aisladas de humanos. El marcaje simultáneo con timidina tritiada (H^3-Tdr) reveló la incorporación de la marca en el 58.8% de los bacilos, a lo largo de un periodo de 14 días. Los datos preliminares con 3 cepas de *M. leprae*, indicaron que la H^3-Tdr se incorpora preferentemente en el DNA micobacteriano y no en el DNA de la célula huésped. Este ensayo *in vitro*, proporciona una prueba rápida de la viabilidad del *M. leprae*. Se describen los factores que influyen en el consumo de la H^3-Tdr.

RÉSUMÉ

On a utilisé des macrophages de péritoine de la souris, venant de la souche BALB/c, comme cellules-hôte pour 17 souches de *M. leprae* obtenues chez l'homme. Les pulsations simultanées avec de la thymidine-H^3 (H^3-Tdr) a montré une absorbtion du produit radioactif marqué, dans 58,8% des bacilles, au cours d'une période de 14 jours. Les données préliminaires obtenues sur trois souches de *M. leprae* indiquent que la thy-

midine-H^3 est incorporée de façon préférentielle dans le DNA mycobactérien, et non dans le DNA de la cellule hôte de mammifères. Ces épreuves *in vitro* permettent une évaluation rapide de la viabilité de *M. leprae*. Les facteurs qui influencent l'incorporation de thymidine-H^3 sont décrits.

Acknowledgments. The investigation received financial assistance from the Chemotherapy of Leprosy (THELEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. M. Sathish was supported by the British Leprosy Relief Association (LEPRA).

REFERENCES

1. Drutz, D. J. and Cline, M. J. Incorporation of tritiated thymidine by leprosy bacilli in cultures of human lepromatous macrophages. J. Infect. Dis. **125** (1972) 416–419.
2. Hanks, J. H., Chatterjee, B. R. and Lechat, M. F. A guide to the counting of mycobacteria in clinical and experimental materials. Int. J. Lepr. **32** (1964) 156–157.
3. Holmes, I. D. and Hilson, G. R. F. The effect of rifampicin and dapsone on experimental *Mycobacterium leprae* infections : minimum inhibitory concentrations and bactericidal action. J. Med. Microbiol. **5** (1972) 251–261.
4. Levy, L. and Peters, J. H. Some characteristics of the action of dapsone on multiplication of *Mycobacterium leprae* in the mouse. Lepr. Rev. **48** (1977) 237–245.
5. Nath, I., Curtis, J., Sharma, A. K. and Talwar, G. P. Circulating T-cell numbers and their mitogenic potential in leprosy—correlation with mycobacterial load. Clin. Exp. Immunol. **29** (1977) 393–400.
6. Pasquier, J. F. Utilization of labelled carbon ^{14}C in the study of mycobacteria and experimental tuberculoses. In: Pasquier, J. F., Trinka, L., Urbanak, R., *The Use of Radioactive Isotopes in Tuberculosis Research*. Pasquier, J. F., Trinka, L. and Urbanak, R., eds. New York: Pergamon Press, 1965, p. 43.
7. Pettit, J. H. S. and Rees, R. J. W. Sulphone resistance in leprosy : An experimental and clinical study. Lancet **2** (1964) 673.
8. Prasad, H. K. and Nath, I. Factors influencing the incorporation of ^{3}H-thymidine in *M. leprae* residing in differentiated human macrophages. J. Med Microbiol. (in press).
9. Rees, R. J. W. Limited multiplication of acid fast bacilli in the foot pads of mice inoculated with *M. leprae*. Br. J. Exp. Pathol. **45** (1964) 207–218.
10. Rees, R. J. W. Enhanced susceptibility of thymectomised and irradiated mice to infection with *M. leprae*. Nature (Lond). **211** (1966) 657–658.
11. Rees, R. J. W. Drug resistance of *M. leprae* particularly to DDS. Int. J. Lepr. **35** (1967) 625–638.
12. Rees, R. J. W., Pearson, J. M. H. and Waters, M. F. R. Experimental and clinical studies on rifampicin in treatment of leprosy. Br. Med. J. **1** (1970) 89–92.
13. Shepard, C. C. The experimental disease that follows the infection of human leprosy bacilli into foot pads of mice. J. Exp. Med. **112** (1960) 445–454.
14. Shepard, C. C. A kinetic method for the study of activity of drugs against *M. leprae* in mice. Int. J. Lepr. **35** (1967) 429–435.
15. Talwar, G. P., Krishnan, A. D. and Gupta, P. D. Quantitative evaluation of the progress of intracellular infection *in vitro*: Incorporation of ^{3}H-thymidine into deoxyribonucleic acid by *M. leprae* in cultivated blood monocytes. Infect. Immun. **9** (1974) 187–194.
16. WHO. Expert Committee on Leprosy, 5th Report. WHO Tech. Rep. Series, No. 607, Geneva: World Health Organization, 1977.
17. Yam, L. T., Li, C. Y. and Crosby, W. H. Cytochemical identification of monocytes and granulocytes. Am. J. Clin. Pathol. **55** (1971) 283–290.