

## INCORPORATION OF $^3\text{H}$ -THYMIDINE IN *MYCOBACTERIUM LEPRAE* WITHIN DIFFERENTIATED HUMAN MACROPHAGES

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### PLATE XV

**SUMMARY.** The factors influencing the incorporation of  $^3\text{H}$ -thymidine ( $^3\text{H}$ -Tdr) in the DNA of *Mycobacterium leprae* within macrophages derived from human blood have been evaluated. Fifty strains of *M. leprae* derived from skin nodules of patients with lepromatous leprosy were studied for their ability to incorporate  $^3\text{H}$ -Tdr. Control macrophages of the same donor maintained alone, or with autoclaved *M. leprae*, showed low levels of baseline  $^3\text{H}$ -Tdr incorporation. During a 15-day period of pulsing, 27 of the *M. leprae* strains incorporated  $^3\text{H}$ -Tdr at levels of 216–2834% of the incorporation by control cultures. Significant incorporation was observable by the second week of culture and cumulative increases occurred by the third week. A 24-h pulse with  $^3\text{H}$ -Tdr was inadequate for a detectable increase. A minimal duration of 4–5 days of continuous pulsing was required to obtain a significant increase in the incorporation of  $^3\text{H}$ -Tdr. Of the 50 *M. leprae* strains, 23 (46%) failed to incorporate the radiolabel. This failure was apparently not related to differences in the disease status of patients, to the transport conditions for the biopsies, to morphological indices of the extracted *M. leprae* or to the origin of the host macrophages.

### INTRODUCTION

One of the major restrictions in the understanding and treatment of leprosy has been the inability to cultivate *Mycobacterium leprae* *in vitro*. At first, assessment of the viability of this organism in the dermal lesions of patients was limited to morphological criteria (Rees and Valentine, 1962; Waters and Rees, 1962; Edwards, Draper and Draper, 1972). With the advent of the mouse-footpad model (Shepard, 1960; Rees 1964, 1966), it was possible for the first time to study the kinetics of growth and the effects of drugs on *M. leprae* *in vivo*. The mouse experimental model has been invaluable in the evaluation of drug sensitivity (Shepard, 1967; Rees, Pearson and Waters, 1970; Levy and Peters, 1977), but its usefulness is limited by the period of 6–9 months that is

required for the logarithmic growth phase of the bacteria. Animal-house facilities for normal or immunodeficient mice infected with *M. leprae* are not available in many areas of endemic leprosy. Extensive investigations of drug-resistant strains in the field and the assessment of potential anti-leprosy drugs require a rapid and quantitative method for the assessment of the viability and growth of *M. leprae*.

The use of tissue cultures of Schwann cells and other host cells to support the growth of *M. leprae* (Palmer, Rees and Weddell, 1961; Fildes, 1974; Pattyn, 1974; Lalitha, Bapat and Dastur, 1977) had only limited success because the multiplication of *M. leprae* within the cells could not be assessed with accuracy. The use of radioisotope labelling was introduced by Drutz and Cline (1972) who reported the incorporation of  $^3\text{H}$ -thymidine ( $^3\text{H}$ -Tdr) in *M. leprae* found in the infected macrophages of patients with lepromatous leprosy. Subsequently, Talwar, Krishnan and Gupta (1974) confirmed the successful incorporation of radioactive thymidine in macrophage cultures containing fresh *M. leprae* derived from skin nodules of bacilliferous leprosy patients. They also showed by electron autoradiography that the radioisotope was limited to *M. leprae* and was not present in the host cells. Moreover, further investigations with  $^3\text{H}$ -DOPA and  $^3\text{H}$ -Tdr showed uptake of radioactivity by *M. leprae* maintained in a cell-free synthetic medium (Ambrose, Antia and Khanolkar, 1974; Ambrose, Khanolkar and Chulawalla, 1978).

The present investigation was undertaken to evaluate the factors influencing uptake of  $^3\text{H}$ -Tdr in *M. leprae* maintained in macrophages obtained from normal human peripheral blood. Fifty strains of *M. leprae* obtained from the skin nodules of patients with lepromatous leprosy were studied during a period of 2 years.

#### MATERIALS AND METHODS

*Macrophage preparation.* Peripheral blood was collected aseptically from healthy donors, was heparinised (preservative-free heparin, Upjohn, Kalamazoo, USA; 10 units/ml of blood) and was allowed to sediment under the effect of gravity at 37°C for 30–40 min. The leucocyte-rich plasma was mixed with equal volumes of tissue-culture medium and dispensed into Leighton tubes in 1.5-ml portions. The medium used was RPMI 1640 (Gibco, Detroit, USA) with Hepes buffer (Gibco) at an initial pH of 7.2–7.4. The cell suspension was incubated for 18–24 h at 37°C to permit the adherence of cells to the glass. The tubes were then agitated gently, and the medium containing the non-adherent cells was removed. The cultures were washed once with warm RPMI 1640, kept at 37°C, and the tubes were refilled with maintenance medium (RPMI 1640 with 50% pooled AB serum). Antibiotics were not used in the media. After 5–6 days at 37°C, most neutrophils were detached and well differentiated macrophages could be seen (fig. 1).

Coverslips were included in a few Leighton tubes only for the study of the morphological, histochemical and phagocytic properties of the cultivated macrophages; cultures used for radioisotope uptake did not contain coverslips. All cultures were examined daily by phase contrast in an inverted microscope. Coverslip preparations were fixed and stained as described below for the various observations.

*Macrophage morphology.* Coverslips were fixed in methanol for 10 min and stained by Giemsa (Dacie and Lewis, 1966). For assessment of baseline phagocytosis, coverslips containing macrophages that had been fed "viable" or autoclaved *M. leprae* were fixed with 10%

buffered formalin and stained by the Fites modification of the Ziehl-Neelsen technique with cold carbol-fuchsin (Parameswaran, 1973).

**Macrophage histochemistry.** Nonspecific esterase was used as a marker for macrophages by the methods of Yam, Li and Cosby (1971). In brief, the coverslips were fixed with 2% glutaraldehyde (TAAB Labs, UK) in phosphate-buffered saline (PBS) for 20 min, and  $\alpha$ -naphthyl butyrate (Sigma Chemical Co., Poole, Dorset) was used as a substrate.

**Macrophage viability** was assessed initially by a dye-exclusion test with 0.2% (w/v) trypan blue in PBS. Because loss of viability was shown to be associated with detachment from the glass, daily assessment of viability was made on the basis of the glass adherence seen on microscopic examination of the Leighton tubes.

**Preparation of bacteria.** Skin biopsies from untreated lepromatous leprosy patients were sent in ice by air freight, and received within 6–18 h. Dr L. M. Hogerzeil, Victoria Hospital, Dichpalli, Dr C. G. S. Iyer, Central Leprosy Research and Training Institute, Chingleput and Professor Rattan Singh, Lok Nayak Jayaprakash Narayan Hospital, Delhi, kindly donated the biopsies. *M. leprae* was isolated from the skin biopsies essentially by the method of Rees (1964). In brief, the epidermis was removed from the skin and the dermis was cut into fine pieces and homogenised in glass homogenisers in isotonic saline with 1% (v/v) bovine serum albumin. The bacilli were counted essentially by the method of Hanks and Lechat (1964) and resuspended in RPMI 1640 with 50% AB serum at a concentration of  $5 \times 10^7$  bacilli/ml, except where otherwise stated. Extreme care was taken to maintain sterility during the removal of skin biopsies, and the subsequent extraction of the bacilli. The skin was cleaned repeatedly with alcohol and iodine and the tissue was removed under surgical operating-theatre conditions. Extraction of bacilli was done either in a tissue-culture room or on a vertical laminar-flow sterile bench. All bacterial suspensions were screened at 37°C for common contaminants on nutrient agar, and for saprophytic cultivable mycobacteria on Löwenstein-Jensen medium (Cruickshank, 1968), with an incubation time of 3 weeks. The morphological index (MI) of the extracted bacilli was assessed by the criteria of McRae and Shepard (1971). The preparations of *M. leprae* were inoculated into macrophage cultures within 24–36 h of removal from the patient. For the negative control experiments, bacilli of the same strain were first autoclaved at 15 lb/in<sup>2</sup> (1 kg/cm<sup>2</sup>) pressure for 15 min.

**Maintenance of macrophages and inoculation with bacteria.** From 6 to 7 days after establishment of the monolayers, 100  $\mu$ l of medium containing  $5 \times 10^6$  bacilli were added to each Leighton tube. The tubes were incubated for 12–18 h at 37°C. Subsequently, the monolayers were vigorously rinsed with warmed RPMI 1640. Fresh maintenance medium containing in addition 2  $\mu$ Ci of <sup>3</sup>H-Tdr (specific activity 42 Ci/mmol; Radiochemical Centre, Amersham, Bucks) was added to each culture.

The monolayers were maintained at 37°C for 24 days from the start of the experiment except where otherwise stated. The maintenance medium was replaced at weekly intervals or earlier if the pH fell below 6.5. Control cultures consisted of either non-infected macrophages from the same donor, or macrophages infected with autoclaved *M. leprae* of the corresponding strain. Replicates of five monolayers were used in each experiment. Coverslip preparations of infected macrophages were stained with Ziehl-Neelsen stain to assess the morphological state of *M. leprae* in each culture. Macrophage cultures were examined by inverted microscopy at regular intervals.

**Estimation of the uptake of <sup>3</sup>H-Tdr.** Cultures were harvested on the 24th day after inoculation, i.e., 15–16 days after the phagocytosis of *M. leprae*. On the day of harvesting, the medium was removed from the Leighton tubes and replaced with 1 ml of ice-cold RPMI 1640 containing 1% triton X-100 (Sigma Chemical Co., Poole, Dorset). The tubes were incubated for 30 min at 37°C. The cells were scraped from the surface of glass with a silicone rubber "policeman" and each culture was then harvested on Whatman No. 3 paper discs under vacuum. The paper discs were serially rinsed with agents at 4°C: twice with isotonic saline containing unlabelled thymidine 1 mg/ml, twice with 5% trichloracetic acid and twice with methanol. Subsequently, they were air dried at ambient temperature, placed in vials containing scintillation mixture (4 g of 2,4-diphenyloxazole (PPO) and 0.5 g of 1,4-bis C2-C5-phenyloxazolyl benzene (POPOP)/L of toluene), and counted in a liquid scintillation counter (Packard Scintillation Counter 3320).

Incorporation of  $^3\text{H}$ -Tdr was expressed in terms of mean counts per minute (cpm) in 3–5 replicate cultures. Percentage incorporation of  $^3\text{H}$ -Tdr into *M. leprae* was calculated as follows:

$$100 \times \left[ \frac{\left( \begin{array}{l} \text{Mean cpm of cultures} \\ \text{with "viable" } M. leprae \end{array} \right) - \left( \begin{array}{l} \text{Mean cpm of cultures} \\ \text{with autoclaved } M. leprae \end{array} \right)}{\text{Mean cpm of cultures with autoclaved } M. leprae} \right]$$

In short, the protocol for in-vitro assessment of  $^3\text{H}$ -Tdr in *M. leprae* within human macrophages was as follows. On day 7 of culture,  $5 \times 10^6 M. leprae$  were inoculated into each Leighton tube. On day 8 non-phagocytosed *M. leprae* were removed and 1.5 ml of RPMI + 50% AB serum containing 2  $\mu\text{Ci}$  of  $^3\text{H}$ -Tdr were added. Subsequently, medium containing  $^3\text{H}$ -Tdr was replaced at weekly intervals. On day 24, cultures were harvested and counted.

*Chromatographic analysis of radiolabel in culture supernatants.* Supernates from macrophage cultures labelled with 2  $\mu\text{Ci}/\text{ml}$  of  $^3\text{H}$ -Tdr, obtained at the time of harvest were analysed. Whatman No. 1 papers were charged with 25- $\mu\text{l}$  quantities of each of the supernates, the  $^3\text{H}$ -Tdr stock (0.5  $\mu\text{Ci}/\text{ml}$ ) and unlabelled thymidine (10 mg/ml), and placed in a chamber saturated with n-propyl alcohol: ammonia (sp. gr. 0.907 at 20°C), in a ratio of 7:3. After the solvent reached the top of the paper, this was air dried and examined for fluorescent areas under ultraviolet light. At the level that unlabelled thymidine appeared as a spot, the same areas of the papers used for  $^3\text{H}$ -Tdr stock and for culture supernate were cut, put in vials containing scintillation fluid and counted in a liquid scintillation counter, as described earlier. As a further check of possible degradation of the isotope label in the culture supernate, strips of the paper were cut along the path of this material for the assessment of radioactivity, as described above.

*Statistical analysis.* For convenience of the graphical and tabular representation, cpm values for replicates were expressed as mean  $\pm$  standard error. For the assessment of significant values, nonparametric statistics by the Mann–Whitney U test were used, by ranking individual cpm values of test and control cultures (Siegel, 1956).

## RESULTS

### *Characteristics of human macrophage cultures*

Macrophage cultures were examined by phase-contrast microscopy during the experimental period. Macrophages derived from different subjects showed variability in the degree of glass adherence and the kinetics of differentiation *in vitro*. Neutrophils were observed in cultures up to 48–72 h (fig. 1). Subsequently, the cell population consisted of mononuclear cells whose cytoplasm stained diffusely for nonspecific esterase (fig. 2). By day 7, more than 95% of cells had abundant well spread cytoplasm, long cytoplasmic processes and large vacuoles (fig. 3). These cells became confluent during a period of 8–15 days in different cultures. Occasionally, adherent small mononuclear cells with scanty cytoplasm were also observed. Some of these showed diffuse cytoplasmic staining for nonspecific esterase. Some replicate cultures which initially had well differentiated macrophages showed rounding of cytoplasmic processes. Such cells had a tendency to become nonadherent and nonviable within 24–48 h. These cultures were discarded.

### *Time kinetics of $^3\text{H}$ -Tdr incorporation in *M. leprae* within human macrophages*

Preliminary experiments indicated that a minimum of 4 days' pulsing with  $^3\text{H}$ -Tdr was required for detection of incorporation in this system. Table I

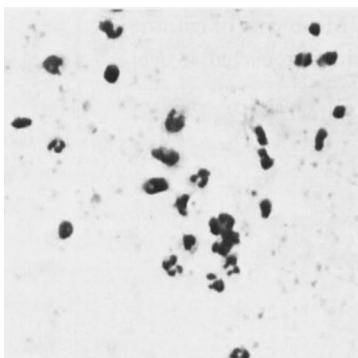
<sup>3</sup>H-THYMIDINE INCORPORATION IN *M. LEPRAE*

Fig. 1.—Culture of leucocyte-rich plasma in RPMI 1640 with 50% AB serum. At 18-24 h the glass-adherent cells are neutrophils, monocytes and platelets. Giemsa.  $\times 66$ .

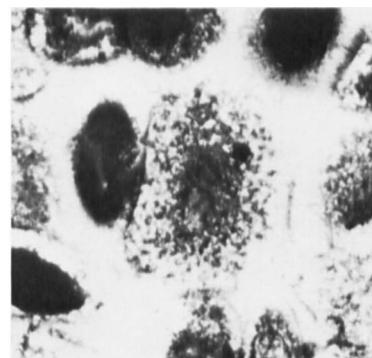


FIG. 2.—Seven-day culture, showing mainly macrophages with abundant non-specific esterase distributed in a diffuse or granular pattern throughout the cytoplasm. Histochemical stain for non-specific esterase with  $\alpha$ -naphthyl butyrate as substrate.  $\times 266$ .

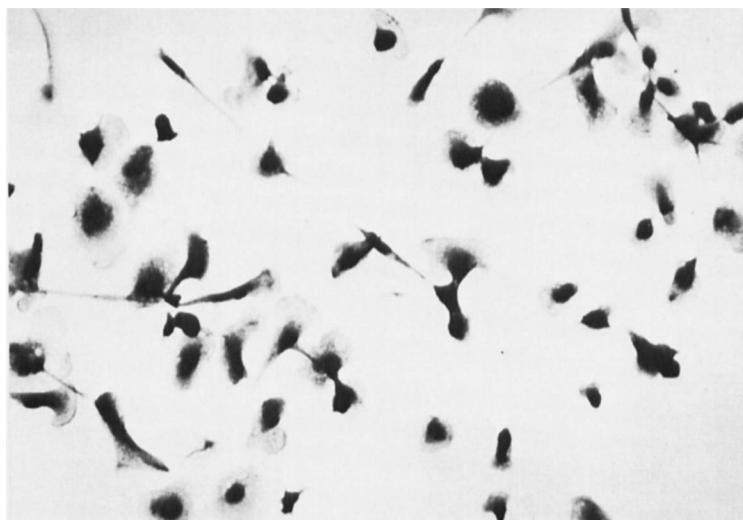


FIG. 3.—Typical 7-day culture, showing a monolayer of macrophages with well spread cytoplasm and vacuolation. Finely drawn cytoplasmic processes are evident in some cells. Contamination with other cells is negligible. Giemsa.  $\times 66$ .



TABLE I

Duration of <sup>3</sup>H-thymidine pulse for incorporation into *M. leprae* within human macrophages

Duration of <sup>3</sup> H-Tdr pulse (days)	Number of days with <sup>3</sup> H-Tdr	Scintillation cpm $\pm$ SE	Percentage incorporation of <sup>3</sup> H-Tdr	Significance (p)
6th-7th	1 day	33.3 $\pm$ 5.3	nil	...
7th-12th	5 days	57.9 $\pm$ 14.7	nil	...
13th-18th	5 days	303.2 $\pm$ 108.9	215.3	<0.05
19th-24th	5 days	313.8 $\pm$ 29.8	222.9	<0.05
14th-24th	10 days	684.0 $\pm$ 94.6	485.8	<0.004
23rd-24th	1 day	210.2 $\pm$ 27.1	148.0	NS

NS = not significant.

shows the results of one typical experiment. Pulsing at intervals for 24 h failed to show incorporation above the control levels. Pulsing for 5 days resulted in significant uptake of <sup>3</sup>H-Tdr in the second and third week of culture, i.e., from the 13th to 18th and 19th to 24th days, respectively. Continuous pulsing for 10 days (14th to 24th days) resulted in cumulative cpm values in the infected cultures. It was uniformly observed that incorporation did not occur during the first week of culture.

Because the purpose of the study was to obtain maximal incorporation of <sup>3</sup>H-Tdr, pulsing was prolonged to assess the optimum period of cumulative radioactivity in the cultures. Fig. 4 shows cpm values in cultures with <sup>3</sup>H-Tdr

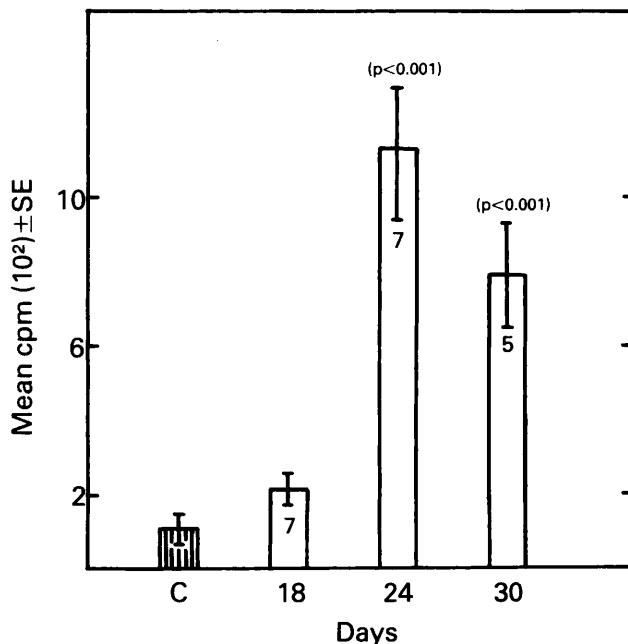


FIG. 4.—Time kinetics of the incorporation of <sup>3</sup>H-thymidine into *M. leprae* within cultivated macrophages derived from blood. C = control macrophages with autoclaved bacilli of same strain.

for 18, 24 and 30 days. Maximal uptake of  $^3\text{H}$ -Tdr was observed on the 24th day.

*Relationship between incorporation of  $^3\text{H}$ -Tdr and viability of *M. leprae**

Uninfected macrophage cultures showed baseline mean cpm values ranging from  $37.4 \pm 7.9$  to  $1206 \pm 388$ . Cultures containing autoclaved *M. leprae* in the corresponding donor macrophages showed marginal increase in cpm values which were statistically insignificant. Table II shows data on 11 strains of *M. leprae* when macrophages from identical donors were inoculated with autoclaved and freshly extracted "viable" *M. leprae* of corresponding strains. Cultures with "viable" *M. leprae* showed significantly increased incorporation of  $^3\text{H}$ -Tdr. The percentage incorporation ranged from 216.1 to 2051.2 in cultures with "viable" *M. leprae*.

*Inoculum of *M. leprae**

Three experiments were conducted in which  $1 \times 10^6$ ,  $5 \times 10^6$  and  $10^7$  *M. leprae* were added to replicate macrophage cultures. It may be noted from fig. 5 that a significant and similar level of incorporation of  $^3\text{H}$ -Tdr was observable at doses of  $1 \times 10^6$  and  $5 \times 10^6$  bacilli/tube. In two of the experiments maximal incorporation was evident at a dose of  $10^7$  bacilli.

*Relationship of  $^3\text{H}$ -Tdr incorporation with the morphological index of *M. leprae**

Attempts were made to correlate the MI of the extracted *M. leprae* with the extent of  $^3\text{H}$ -Tdr incorporation. Fig. 6 shows that some bacilli with a MI of less than 2% also showed incorporation. Of the 20 strains of *M. leprae* studied,

TABLE II  
*Incorporation of  $^3\text{H}$ -thymidine in "viable" and autoclaved *M. leprae**

Experiment no.	Mean cpm $\pm$ SE in macrophages containing		
	autoclaved <i>M. leprae</i>	"viable" <i>M. leprae</i>	Percentage incorporation
1	343.67 $\pm$ 67.6	1092.03 $\pm$ 123.9	317.8
2*	114.85 $\pm$ 46.25	2355.8 $\pm$ 280.5	2051.2
3	166.5 $\pm$ 12.1	895.6 $\pm$ 142.06	537.9
4	277.5 $\pm$ 46.03	1293.67 $\pm$ 107.37	466.2
5	276.0 $\pm$ 35.68	638.33 $\pm$ 31.29	231.3
6	140.8 $\pm$ 12.58	684.0 $\pm$ 94.64	485.8
7	138.2 $\pm$ 16.67	361.13 $\pm$ 75.0	261.3
8	116.96 $\pm$ 19.0	1455.33 $\pm$ 270.65	1244.3
9	135.8 $\pm$ 18.4	488.9 $\pm$ 53.02	360.0
10	303.4 $\pm$ 55.85	655.8 $\pm$ 52.5	216.1
11	263.5 $\pm$ 27.0	977.1 $\pm$ 103.5	370.8

\* Mean cpm of only two cultures; all other results are the mean from 3-5 cultures.

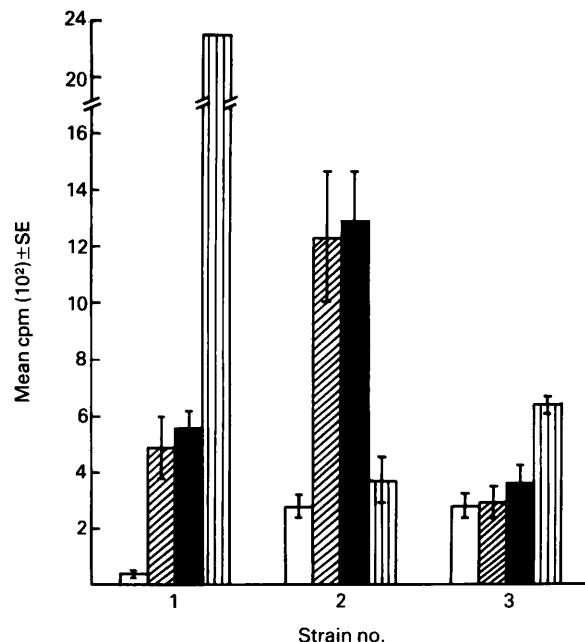


FIG. 5.—Relationship of the number of *M. leprae* in the inoculum to the incorporation of <sup>3</sup>H-thymidine in three strains of *M. leprae*. □=Control macrophages; ■=macrophages with 1 × 10<sup>6</sup> *M. leprae*; ▨=macrophages with 5 × 10<sup>6</sup> *M. leprae*; ▨=macrophages with 10<sup>7</sup> *M. leprae*.

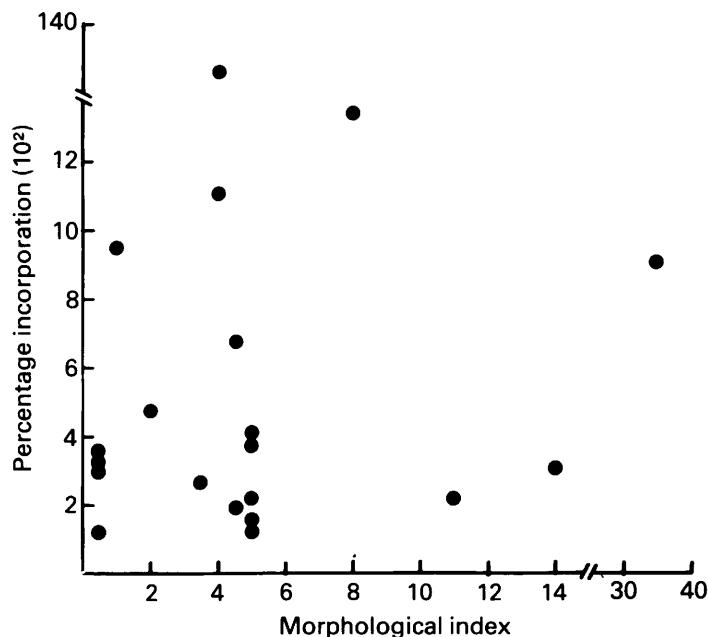


FIG. 6.—Relationship of morphological index to the percentage incorporation of <sup>3</sup>H-thymidine in 20 human-derived strains of *M. leprae*.

variation in the percentage of incorporation was noted at all levels of MI. In eight experiments in which the bacilli had a narrow range of MI, 4–6%, the  $^3\text{H}$ -Tdr incorporation varied extensively.

*Stability of  $^3\text{H}$ -Tdr in the experimental period*

Because the duration of pulse with high specific activity of  $^3\text{H}$ -Tdr was long (7 days), it was considered possible that the radiolabel had become dissociated from thymidine and incorporated into non-DNA components of *M. leprae*. On chromatographic analysis it was observed (fig. 7) that the maximal radioactivity from culture supernates was at the same level as that of the control  $^3\text{H}$ -Tdr solution. Furthermore, the trail of the supernatant showed negligible radioactivity.

*Pattern of  $^3\text{H}$ -Tdr incorporation in 50 strains of *M. leprae**

Fifty strains of *M. leprae*, derived mainly from the skin nodules of patients with untreated lepromatous leprosy, were screened for their ability to

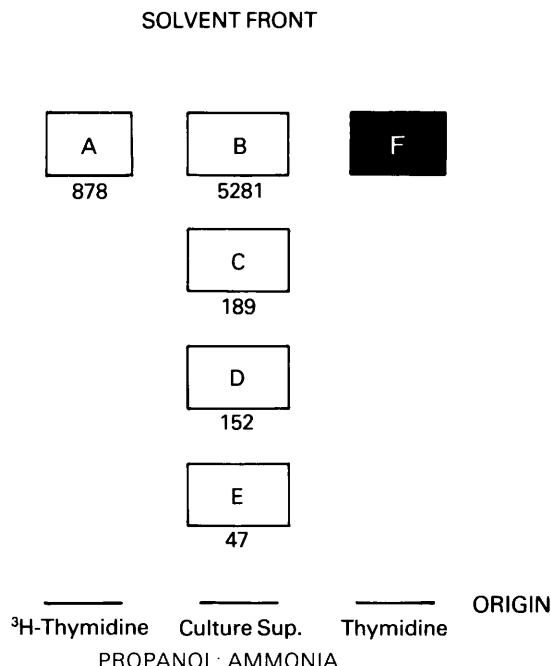


FIG. 7.—Diagrammatic representation of paper chromatogram run in a chamber with propanol: ammonia (7:3) and containing 25  $\mu\text{l}$  of the various samples of thymidine applied in a linear manner at the bottom of the paper marked as "origin". A represents 0.5  $\mu\text{Ci}/\text{ml}$  of  $^3\text{H}$ -thymidine. B represents supernate from macrophage cultures containing *M. leprae* and pulsed 7 days previously with  $^3\text{H}$ -Tdr 2  $\mu\text{Ci}/\text{ml}$ . C, D and E represent the trail of culture supernate. F represents thymidine 10 mg/ml. The numbers below the rectangles represent the cpm values detected by liquid scintillation counting of the area of the paper. The maximal counts of the culture supernate were obtained at the level of the unlabelled thymidine and control  $^3\text{H}$ -thymidine.

incorporate <sup>3</sup>H-Tdr. Fig. 8 illustrates the individual data on these strains. It is evident that considerable variation in the extent of incorporation was observed in these strains. As mentioned earlier, non-infected macrophages from different donors also showed a variation in baseline cpm values. In general, it was observed that cultures containing "viable" organisms showed an increase in incorporation of <sup>3</sup>H-Tdr that was statistically significant ( $p < 0.05 - < 0.001$ ), when there was a twofold increase or more above the control cultures containing autoclaved *M. leprae* of the same strain. In the successful cultures the mean cpm of infected cultures ranged from  $361.1 \pm 75$  to  $2566 \pm 423$ .

By the above criteria, of twofold increase in <sup>3</sup>H-Tdr incorporation and a significance of  $p < 0.05$ , 27 strains showed successful incorporation ("incorporators"), whereas 23 *M. leprae* strains failed to incorporate <sup>3</sup>H-Tdr ("nonincorporators") (figs. 8 and 9). Percentage incorporation of <sup>3</sup>H-Tdr in the incorporators ranged from 216 to 2834. In some unsuccessful experiments, the cpm values of macrophage cultures infected with *M. leprae* showed inhibition of uptake of <sup>3</sup>H-Tdr, compared with control cultures (figs 8 and 9).

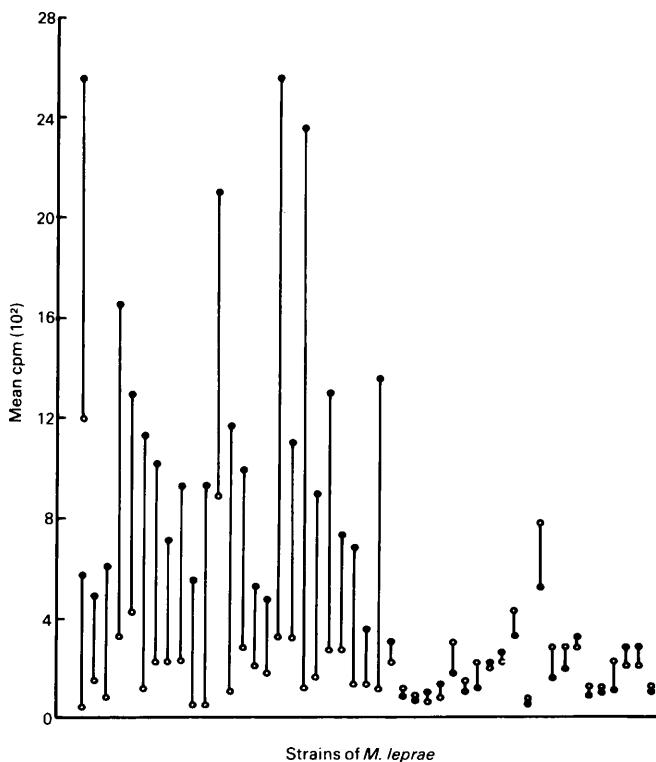


FIG. 8.—Incorporation of <sup>3</sup>H-thymidine in 50 human-derived strains of *M. leprae*. Mean cpm of macrophage cultures with autoclaved *M. leprae* (○) and with freshly extracted "viable" *M. leprae* (●) of the same strain are shown.

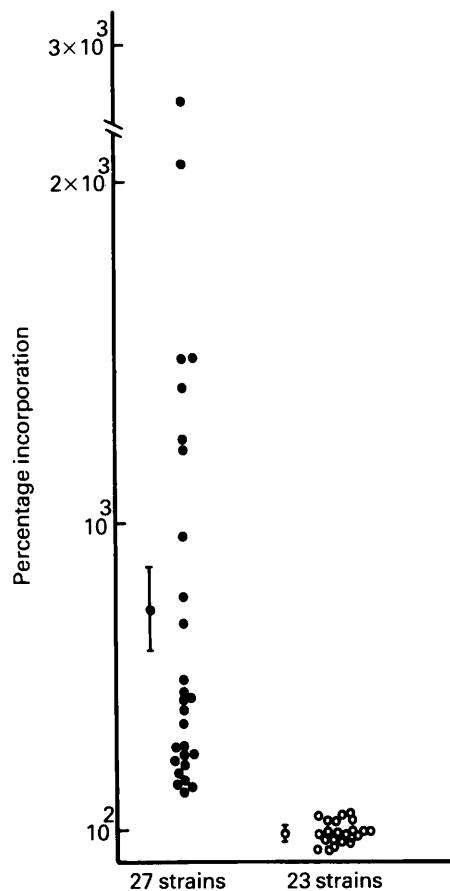


FIG. 9.—Percentage incorporation of  $^3\text{H}$ -thymidine in 50 strains of human-derived *M. leprae*. ● Indicates a strain incorporating  $^3\text{H}$ -thymidine at 216% or more ( $p < 0.05$ ); ○ indicates a strain showing insignificant incorporation. Mean percentage incorporation  $\pm$  SE is indicated by the circle and bar on the left of each group.

Absence of incorporation of  $^3\text{H}$ -Tdr in some strains of *M. leprae* was further studied. Transport and shipping of biopsies were standardised, and five experiments were conducted in which several biopsies were processed in an identical manner in the same donor macrophages (fig. 10). In each experiment, all macrophages were obtained from one donor. Different strains of *M. leprae* processed simultaneously in an identical manner were inoculated into the same batch of macrophages on the same day. Fig. 10 shows that four of the five experiments showed incorporator and nonincorporator strains of *M. leprae*. It should be noted that the bacilli used in these experiments were from untreated lepromatous patients with MI ranging from 4 to 5%.

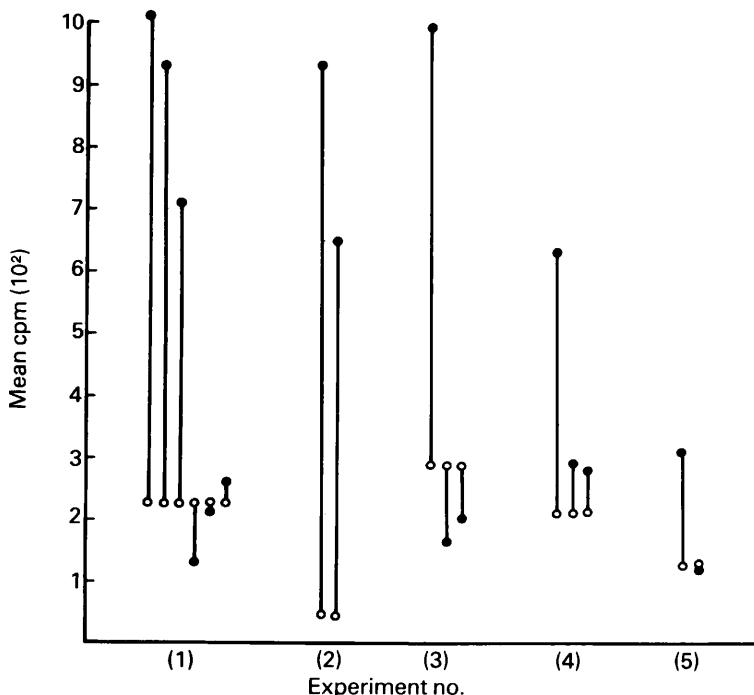


FIG. 10.—Relationship between the type of donor macrophages and the incorporation of <sup>3</sup>H-thymidine. In each of the five experiments shown, macrophages derived from the same donor were inoculated with different strains of *M. leprae* in identical conditions. Mean cpm of macrophages with autoclaved *M. leprae* (○) and macrophages with freshly extracted "viable" *M. leprae* (●).

## DISCUSSION

This study confirms earlier reports of the selective incorporation of <sup>3</sup>H-Tdr in *M. leprae* within macrophages derived from blood (Drutz and Cline, 1972; Talwar *et al.*, 1974). *M. leprae* has a predominant affinity for macrophages in the various tissues of the body and has been observed in the blood monocytes of lepromatous patients (Shankar Manja *et al.*, 1972). Monocytes from the peripheral blood of normal donors differentiate into macrophages *in vitro*. A nondividing host cell that could be maintained for a few weeks would be an ideal system for assessing the differential uptake of <sup>3</sup>H-Tdr by an obligately intracellular, noncultivable organism, such as *M. leprae*. A fast-growing cell system for this pathogen, which has a long generation time of 12–13 days (Shepard and McRae, 1965), would result in the dilution of the organism by the rapid division of the host cells and made it difficult to differentiate the DNA synthesis of the host cells from that of *M. leprae*, thus interfering with the use of radiolabelled precursors.

In these experiments, macrophages obtained from blood from normal

human donors could be maintained for several weeks in serum-enriched medium and showed low baseline  $^3\text{H}$ -Tdr incorporation. Of the 50 strains of *M. leprae* analysed, successful and significant levels of  $^3\text{H}$ -Tdr uptake were noted in 27 strains. The percentage of  $^3\text{H}$ -Tdr incorporated in successful experiments ranged from 216 to 2834. Caution was exercised to rule out the possibility of macrophage cultures being contaminated by organisms other than *M. leprae*. One of the inbuilt controls of the system lay in the fact that these cells became nonadherent and stripped from glass surfaces even when minimal bacterial or fungal contamination was accidentally introduced. Such cultures were excluded.

The incorporation of the radioactive label was rarely noted in the first week after the inoculation of macrophages by *M. leprae*. The second and third weeks appeared uniformly to show significant levels of radioactivity. A 24-hr pulse was inadequate for measurable incorporation and a minimum period of 5 days was required for significant incorporation of  $^3\text{H}$ -Tdr. Prolongation of pulsing to 15 days (with repeated changes of medium containing  $^3\text{H}$ -Tdr) resulted in cumulative levels of radioactivity in the infected cultures and the method was therefore adapted to reveal maximal incorporation of  $^3\text{H}$ -Tdr.

The requirement for a long period of radiolabelling for successful incorporation may be due to several factors. The *M. leprae* derived at a given time from a particular patient may be asynchronous and in different stages of cell cycle and viability. The generation time of *M. leprae* as reported in the mouse model appears to be long (Shepard and McRae 1965). The kinetics of *M. leprae* growth in the experimental model depend on the number of viable bacilli in the inoculum (Shepard, 1967).

It was considered that the incorporation of  $^3\text{H}$ -Tdr in *M. leprae* might not be related to DNA synthesis. The dissociation of the radiolabel from thymidine during the long experimental period and its subsequent incorporation into non-DNA components was ruled out by the chromatographic analysis of the culture supernates. The possibility of nonspecific adsorption of the label to the bacilli was also analysed. In parallel experiments, cultures containing "viable" freshly extracted *M. leprae* showed a 2.2- to 20.5-fold increase in  $^3\text{H}$ -Tdr uptake, compared with the cultures with corresponding strains "killed" by autoclaving. Moreover, the time kinetics of incorporation also showed clearly that "viable" bacilli incorporated the radiolabel in a cumulative manner in certain periods of the culture. The same cultures in the first week showed no incorporation. A dose-dependent increment in cpm was found when increasing numbers of bacilli were inoculated into the macrophage preparations. The failure of some strains of *M. leprae* to incorporate  $^3\text{H}$ -Tdr when present in macrophages is further evidence that nonspecific adsorption of the radiolabel did not occur.

Similar studies in our laboratory with mouse macrophages have confirmed the above findings. Moreover, in experiments in which macrophage cultures with *M. leprae* were subjected to treatment with DNase, evidence was obtained to indicate that the radiolabel was preferentially incorporated in the

mycobacterial DNA and not in the host-cell DNA (Satish and Nath, 1981). It thus appears that <sup>3</sup>H-Tdr incorporation in the present system is a selective feature of "viable" *M. leprae*. Macrophages, being end cells, do not possess thymidine kinase and this is further corroborated by the low background <sup>3</sup>H-Tdr incorporation noted in our control experiments. The incorporation of tritiated thymidine in cultures containing *M. leprae* draws attention to the possibility of thymidine kinase being present in this mycobacterium.

The reasons for our failure to incorporate <sup>3</sup>H-Tdr in 41% of the cultures were further considered. Transport and temperature differences were equalised, large batches of macrophages from the same donor were used from all the biopsies at a given time and "viability" of *M. leprae* was assessed by the MI. Even after the standardisation of these factors, some *M. leprae* failed to incorporate <sup>3</sup>H-Tdr. We concluded that the type of donor macrophages may not play a critical part in the incorporation of <sup>3</sup>H-Tdr in the bacilli within them. It cannot be ruled out that some of our patients had received treatment before attending our clinics, and the organisms from them may have been "non-viable". Experiments are under way to compare the macrophage system with the mouse-footpad model. These may throw more light on the state of viability of the organisms that did not incorporate <sup>3</sup>H-Tdr. Recent evidence indicates that macrophages are capable of releasing thymidine and other substances that inhibit the uptake of <sup>3</sup>H-Tdr in lymphocytes (Opitz *et al.*, 1975). The macrophages used in our studies were derived from Mantoux-positive individuals. If a crossreacting mycobacterium such as *M. leprae* stimulated the host cells to release thymidine or inhibiting substances, this might result in non-incorporation of <sup>3</sup>H-Tdr in the bacilli. This might be due to the thymidine produced by the culture cells competing with the radiolabelled thymidine for incorporation into the DNA of the bacillus, or to the inhibition of radiolabelled thymidine uptake by other substances released by the macrophages.

The limitations of the present method lie in the requirement of a larger number of *M. leprae* ( $10^6$ ) than for the mouse-footpad system ( $10^4$ ), the need for strict aseptic precautions to avoid contamination by cultivable organisms, antibiotic-free medium to support *M. leprae* growth, which increases the likelihood of contamination by other organisms, and the failure of incorporation of <sup>3</sup>H-Tdr in some experiments.

On the other hand, there are several advantages of this method. Screening for growth of *M. leprae* is rapid; quantitative results are available by 4 weeks, compared with the 6-9 months required for a similar evaluation in the mouse-footpad model. The sensitivity of the system is adequate; bacilli with 1% MI have also shown incorporation. Experiments indicating significant incorporation at the second week with a 5-day pulse may further decrease the time required for this test and thus greatly increase its utility. Larger numbers of *M. leprae* strains can be tested at a time, facilitating field studies. The cost of screening many patients in this way may in the long run be cheaper than maintaining animal-house facilities for mice. This is of particular importance in countries where leprosy is endemic, where tropical conditions and lack of

inbred mice are major problems. The quantities of drugs required for screening for their anti-leprosy effect would be small.

Even at the present level of efficiency, the use of radiolabelled precursors of DNA for the quantitative assessment of *M. leprae* multiplication has useful applications. Dapsone resistance is a serious emerging problem in the areas of endemic leprosy (Petit and Rees, 1964; WHO, 1977). Recognition of drug-resistant strains, and assessment of the incidence of this problem, are hampered by the non-availability of rapid methods to assess growth or metabolism of *M. leprae*. Studies are under way in our laboratory to evaluate the present method as a marker for drug sensitivity and for the diagnosis of drug-resistant strains. An in-vitro method for assessing the bactericidal and bacteriostatic effects of known and potential anti-leprosy drugs would greatly improve the therapy of leprosy.

This investigation received support from the Chemotherapy of Leprosy (THELEP) component of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. H.K.P. received support from the Indian Council for Medical Research. Thanks are due to Dr L. M. Hogerzeil for the constant supply of biopsies, and to Professor G. P. Talwar and Dr R. J. W. Rees for technical advice. Mr Kishore Kumar gave laboratory assistance.

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