Lepr. Rev. (1977) **48**, 27–34

Failure of *Mycobacterium leprae* to Incorporate Tritiated Thymidine Administered *In vivo*

MICHAEL J. EVANS*

Life Sciences Division, Stanford Research Institute, Menlo Park, California 94025, U.S.A.

and

LOUIS LEVY

Leprosy Research Unit, Public Health Service Hospital, San Francisco, California 94118, U.S.A.

Tritiated thymidine ([^{3}H]TdR) was administered to mice infected with *Mycobacterium leprae* in attempts to label the *M. leprae in vivo*; injections were made either intraperitoneally or locally into the infected foot-pads. Although labelling of the tissue cells was heavy, indicating that [^{3}H]TdR was available to *M. leprae*, no labelled *M. leprae* were observed in either of the 2 studies conducted.

Introduction

Labelling of *Mycobacterium leprae in vitro* with a radioisotope has been accomplished in 4 laboratories. Drutz reported (Drutz and Cline, 1972) the incorporation of tritiated thymidine ([^{3}H] TdR) by *M. leprae* already resident in cultured macrophages derived from blood monocytes of bacteraemic patients with lepromatous leprosy. Talwar and his co-workers (Talwar *et al.*, 1974) subsequently reported incorporation of [^{3}H] TdR by *M. leprae* that had been inoculated into human macrophage cultures. Ambrose *et al.* (1974) described radiolabelling of *M. leprae* in cultures of human macrophages following the addition of [^{3}H] TdR or of [^{3}H] dihydroxyphenylalanine ([^{3}H] DOPA) to the culture. Finally, Prabhakaran (Harris and Prabhakaran, 1975) reported binding of [^{3}H] DOPA by *M. leprae in vitro.* No workers have reported success in attempts to label *M. leprae in vivo.*

In our first experiment, we were unable to demonstrate radiolabelling of *M. leprae,* which had multiplied in the mouse foot-pad, by [^{3}H] TdR administered intraperitoneally. Therefore, we carried out a second experiment in which

* Requests for reprints should be addressed to Dr Evans.

Received for publication 23 June 1976.

[^{3}H]TdR of high specific activity was administered repeatedly into the infected foot-pad tissues during the period of logarithmic multiplication of *M. leprae*.

Materials and Methods

Methyl-labelled [^{3}H]TdR was purchased from New England Nuclear Corp., Boston, Massachusetts. Locally-bred BALB/c mice and thymectomized, irradiated, and bone-marrow reconstituted B6C3F ([C57Bl/6♀ x C3H/AnF♂] F_1) hybrid mice (supplied by C. C. Congdon, Oak Ridge Laboratory, Oak Ridge, Tennessee) were inoculated in the hind foot-pads with *M. leprae* of the strain used in most experiments in these laboratories.

In the first experiment, 2 BALB/c mice inoculated 112 days earlier with $10^{3\cdot7}$ *M. leprae* and 2 thymectomized, irradiated B6C3F_1 mice inoculated 356 days earlier with $10^{3\cdot7}$ organisms were given an intraperitoneal injection of 0.1 mCi of [^{3}H]TdR (specific activity, 6.7 Ci/mmol) every hour for 10 h; a third animal from each group served as a control. One hour after the last injection, the mice were killed; both hind foot-pads were removed, fixed in 2% glutaraldehyde, postfixed in OsO_4, and embedded in Araldite (Evans *et al.*, 1973*b*). One-micron sections were cut for light microscopic autoradiography and coated with Ilford L-4 nuclear emulsion. After the sections were exposed for up to 10 weeks, the autoradiographs were developed and stained with toluidine blue. For electron microscopic autoradiography, gold sections were placed on a grid and covered with Ilford L-4 nuclear emulsion. After exposure times of up to 8 months, these sections were developed and viewed under a Philips 200 electron microscope (Evans *et al.*, 1973*a*).

In the second experiment, 60 BALB/c mice inoculated in both hind foot-pads with $10^{3\cdot7}$ *M. leprae* were divided into 4 groups. One group served as a control. Approximately 0.05 mCi [^{3}H]TdR [0.05 ml of a solution containing 1 mCi ^{3}H (specific activity, 50.8 Ci/mmol) per ml] was administered daily, 5 days per week, into each infected foot-pad of the mice of the 3 experimental groups (Groups A, B and C). The mice of Group A were injected daily for 10 days between day 77 and day 93 after inoculation, those of Group B were injected with [^{3}H]TdR daily for 10 days between day 93 and day 108 after inoculation, and the mice of Group C were injected daily for 20 days between day 77 and day 108 after inoculation with *M. leprae*. For control mice, *M. leprae* were harvested from the pooled tissues of 4–8 foot-pads at intervals by published methods (Shepard, 1960; Shepard and McRae, 1968). For [^{3}H]TdR-treated mice, *M. leprae* were harvested from the foot-pads at intervals of 121, 156 and 197 days after inoculation. At the 2 earlier intervals, the organisms were recovered by differential centrifugation from each tissue homogenate and washed 3 times with Hanks' balanced salt solution. An 0.8-ml aliquot of each suspension of washed *M. leprae* was placed in a liquid scintillation vial, 14.2 ml of NCS solubilizer (Nuclear Chicago, Chicago, Illinois) was added, and the radioactivity of the organisms together with tissue debris was measured in a Nuclear Chicago Series 720 liquid scintillation spectrometer. In addition, portions of the bacterial suspensions resulting from the harvests and the foot-pad soft tissues of additional mice were processed for autoradiography; the methods used were the same as those described for the first experiment.

Results

INTRAPERITONEAL ADMINISTRATION OF [^{3}H]TdR

Organisms were found in small infiltrates of mononuclear cells in the foot-pads of the mice inoculated 112 days earlier with *M. leprae*. Light microscopic autoradiography revealed heavy nuclear labelling in cells of the infiltrate and epidermis. Very little background labelling was present. Although numerous *M. leprae* were observed, none were labelled. Electron microscopic autoradiography also showed no labelled *M. leprae*. In the group of thymectomized, irradiated

mice inoculated 356 days earlier, organisms were found mainly in mononuclear cells; however, *M. leprae* were occasionally seen in other connective tissue and muscle cells. As in the study of immunologically normal mice, nuclear labelling of various cell types was observed, but no labelled *M. leprae* were observed with either light or electron microscopic autoradiography. Interestingly, no mononuclear cells containing *M. leprae* showed nuclear labelling.

ADMINISTRATION OF [^{3}H]TdR INTO THE FOOT-PAD TISSUES

The results of harvests of *M. leprae* from the control and [^{3}H]TdR-treated mice are shown in Fig. 1, in which the common logarithm of the number of acid-fast bacilli (AFB) per foot-pad is plotted as a function of time after inoculation. The least-squares line fitted to the results of harvests performed from control mice between days 77 and 156 after inoculation represents the growth curve of *M. leprae* in untreated mice. Multiplication of *M. leprae* appears to have been inhibited by [^{3}H]TdR administration, as shown by the results of harvests performed 121 days after inoculation. However, between days 156 and 197, the organisms multiplied in the foot-pads of mice of Groups A and B at the same rate as that in control mice.

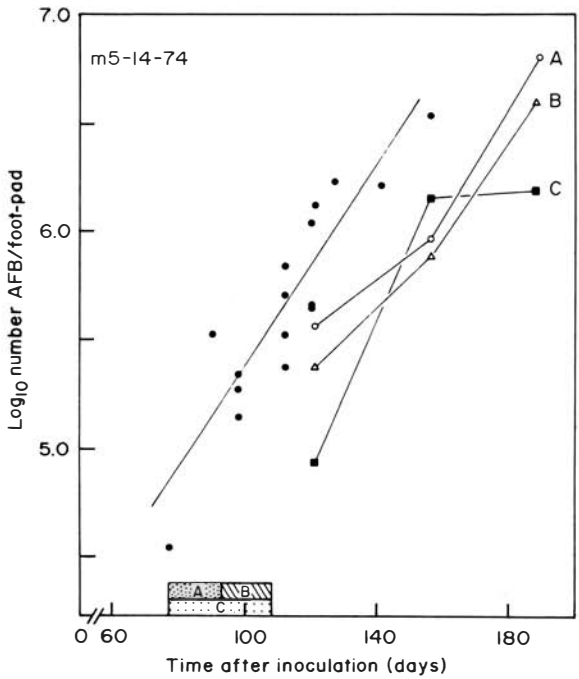

Fig. 1. Log_{10} number of AFB per foot-pad as a function of the time after mice had been inoculated with *M. leprae*. The closed circles represent harvests from untreated control mice; the straight line drawn through these points is the regression line representing the logarithmic phase of multiplication of *M. leprae* in control mice. The horizontal bars on the abscissa represent the periods of administration of [^{3}H]TdR; the letters within the bars identify the corresponding bacterial growth curves.

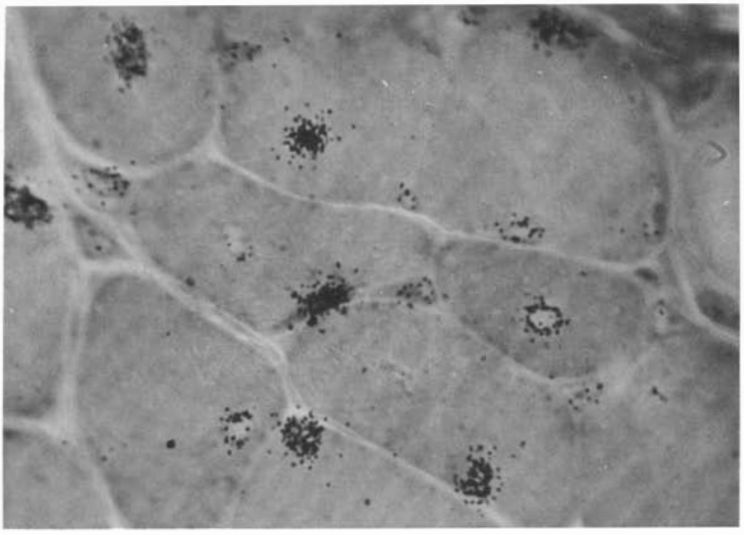

Fig. 2. Labelled nuclei in muscle from mouse foot-pad; Group A at 121 days. x 1250.

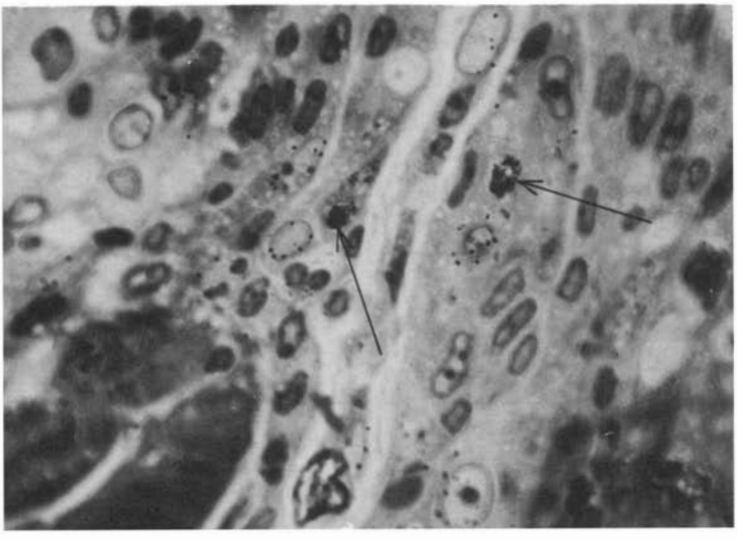

Fig. 3. Labelled nuclei and unlabelled globi (arrow) in mouse foot-pad; Group C at 197 days. x 1250.

In the autoradiograph prepared from the tissues of mice killed 121 days after inoculation, we observed moderate cellular infiltrates but few organisms. Almost all nucleated cells in the tissues from Groups A, B and C were labelled (Fig. 2). Very little background labelling was present, and although most nuclei were labelled, diffuse cytoplasmic labelling was not observed. At no time were labelled *M. leprae* observed.

In the preparations made from the tissues of mice killed 156 days after inoculation, cellular infiltrates were present together with many organisms. Again, almost all of the nucleated cells in the tissues of Groups A, B and C mice were labelled. Diffuse cytoplasmic labelling was observed in the tissues of mice from Group C. Organisms were observed both in globi and singly within cells, but none were labelled. Again, no cells containing *M. leprae* organisms were labelled.

Fig. 4. Labelled tissue fragment in suspension of mouse foot-pad; Group B at 156 days. x 1250.

In the tissues of mice killed 197 days after inoculation, large cellular infiltrators and many *M. leprae* organisms were present. Many cells were labelled but not as many as in the tissues obtained at earlier intervals. Many mononuclear cells contained *M. leprae* in globi. No labelled *M. leprae* were observed (Fig. 3).

The results of liquid scintillation spectrometry of the suspensions of *M. leprae* resulting from harvests performed on day 121 and day 156 after inoculation are summarized in Table 1. These results suggest that there had been some incorporation of ^{3}H into the particulate material of the suspensions. The very small quantity of ^{3}H detected in the filtrates of these suspensions after filtration through an 0.22 μm pore-size membrane filter indicates that washing was complete. The quantity of the radioisotope incorporated appears to be the same for all harvests, although it is perhaps a little greater in the material harvested from Group B mice. Certainly, there does not appear to have been a decrease in the quantity of isotope incorporated between the day-121 and day-156 harvests.

TABLE 1

Incorporation of [^{3}H]TdR into foot-pad tissue homogenates

Mouse group	Counts per min* Day of harvest 121	156
Control†	< 1	< 1
A	855 ± 9‡	934 ± 9
B	1333 ± 13	1404 ± 14
C	950 ± 10	934 ± 10
Filtrates	12	4

* Corrected for background.

† Control mice were mice that had been inoculated with *M. leprae* but received no [^{3}H]TdR; *M. leprae* were harvested from control animals and experimental animals at the same time.

‡ Mean ± standard deviation.

Autoradiographs of these suspensions revealed no labelled *M. leprae* but numerous labelled tissue fragments (Fig. 4).

Discussion

The purpose of these studies was to label *M. leprae in vivo* with [^{3}H] TdR. Our first attempt was designed to expose the organisms to [^{3}H] TdR almost continually for 10 h by multiple intraperitoneal injections of the material. The numerous labelled tissue cells in the mouse foot-pad verified that [^{3}H]TdR had been available for incorporation by the organisms. However, no *M. leprae* were labelled in this experiment, whether the organisms were single or in globi. In the second experiment, multiple injections of [^{3}H] TdR were given directly into the infected foot-pads of mice over a period of days. The organisms were in logarithmic multiplication at the start of injections. Again, no labelled *M. leprae* were observed, either singly or in globi, although most tissue cells were labelled. Suspensions of washed *M. leprae* from treated mice showed more counts by liquid scintillation spectrometry than did organisms harvested from untreated controls, suggesting that [^{3}H] TdR had been incorporated. However, autoradiography of these suspensions failed to show labelled *M. leprae*, although it did show considerable labelling of tissue fragments. Unfortunately, uninfected mice had not been injected with [^{3}H] TdR; thus, no suitable control was available.

Other investigators have reported labelling *in vivo* of *M. leprae* with [^{3}H] TdR. Only Talwar and his co-workers (1974) presented data not entirely based on autoradiographs. By means of liquid scintillation spectrometry, these workers observed a large increase in [^{3}H]TdR incorporation, and they found autoradiographic evidence to suggest that the counts were coming from labelled *M. leprae*. Drutz and Cline (1972) observed an increased grain count over globi compared to cells without globi. Ambrose *et al.* (1974) reported successful labelling of *M. leprae* with [^{3}H]TdR. Unfortunately, all these reports presented insufficient information about controls. For example, it is important to exclude *Mycoplasma* contamination of macrophage cultures, which can produce spurious incorporation

of [^{3}H]TdR (Paul, 1975). The above studies would have been more convincing if they had included autoradiographs of uninfected cultures exposed to the isotope.

The problems encountered in attempting to label *M. leprae* were recently reviewed by Drutz (1975). Our own failure to show labelling of *M. leprae in vivo* may have been caused by several factors. First, it may be that *in vivo M. leprae* do not utilize thymidine in the form injected, as has been suggested by Wayne (L. G. Wayne, personal communication). If *M. leprae* utilize exogenous thymidine for DNA synthesis, it is possible that, in our experiment, not enough [^{3}H]TdR was incorporated by the organisms to be apparent by autoradiography. This could have resulted from dilution of [^{3}H]TdR in the body of the animal or from a slow rate of DNA synthesis. Another possibility is that only a few *M. leprae* synthesize DNA at any one time. In the studies by Drutz and Cline (1972), only 1–2% of the globi were labelled. If that were the case *in vivo,* it is conceivable that the few labelled *M. leprae* could have been missed during examination of the slides.

In our second experiment, failure to obtain labelled organisms could have resulted from several additional factors. First, the growth curves of Fig. 1 suggest that multiplication ceased during the period of multiple [^{3}H]TdR injections, so that the *M. leprae* were not synthesizing DNA at the time [^{3}H]TdR was available. The reason for cessation of multiplication is not clear; however, it was recently demonstrated that repeated administration of saline into the foot-pad tissues of mice produces an inflammatory reaction that is associated with cessation of multiplication of the *M. leprae* (L. Levy and T. C. Merigan, unpublished data). Second, if the organisms did incorporate [^{3}H]TdR, subsequent multiplication may have diluted the label to an undetectable level. Re-utilization of the radiolabelled compound would have been beneficial in this study, because it would have increased the opportunity for labelling of *M. leprae.* Because of the large numbers of labelled tissue cells present and the time course of this study, re-utilization must have been occurring; nevertheless, *M. leprae* were not labelled.

An interesting result of this study was that, despite the many labelled tissue cells present, the vast majority of those containing *M. leprae* were not labelled. This is in agreement with previous observations (Evans, 1974) and raises the question of how mononuclear cells increase in number to accommodate multiplication of *M. leprae* in the mouse foot-pad.

Acknowledgement

This research was supported in part by the U.S. Leprosy Panel of the U.S.-Japan Co-operative Medical Science Program administered by the Geographic Medicine Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014, U.S.A. (Grant R22 A1 07801).

References

Ambrose, E. J., Antia, N. H. and Khanolkar, S. R. (1974). Uptake of radioactive DOPA by *M. leprae*. *Nature* **249**, 854.

Drutz, D. J. (1975). Progress in the radiolabeling of *M. leprae*. *Int. J. Lepr.* **44**, 65.

Drutz, D. J. and Cline, M. J. (1972). Incorporation of tritiated thymidine by leprosy bacilli in cultures of human lepromatous macrophages. *J. Infect. Dis.* **125**, 416.

Evans, M. J., Cabral, L. J., Stephens, R. J. and Freeman, G. (1973*a*). Renewal of alveolar epithelium in the rat following exposure to NO_2. *Am. J. Path.* **70**, 175.

Evans, M. J., Newton, H. E. and Levy, L. (1973*b*). Early response of mouse foot-pads to *M. leprae*. *Infect. Immun.* **7**, 76.

Evans, M. J. (1974). Mononuclear cell turnover in mouse foot-pads infected with *M. leprae*. Proceedings of the U.S.-Japan Leprosy Conference. Kyoto, Japan. 16–18 October.

Harris, E. B. and Prabhakaran, K. (1975). Uptake of radioactive DOPA by *Mycobacterium leprae in vitro*. *Microbiol.* **12**, 119.

Paul, J. (1975). *Cell and Tissue Culture,* p. 484. Edinburgh, London and New York: Churchill Livingstone.

Shepard, C. C. (1960). The experimental disease that follows the injection of human leprosy bacilli into foot-pads of mice. *J. Exp. Med.* **112**, 445.

Shepard, C. C. and McRae, D. H. (1968). A method for counting acid-fast bacteria. *Int. J. Lepr.* **36**, 78.

Talwar, G. P., Krishnan, A. D. and Gupta, P. D. (1974). Quantitative evaluation of the progress of intracellular infection *in vitro*: Incorporation of [^{3}H] thymidine into deoxyribonucleic acid by *Mycobacterium leprae* in cultivated blood monocytes. *Infect. Immun.* **9**, 187.