Lepr. Rev. (1976) **47**, 267–273

Autoradiographic Studies of *Mycobacterium leprae*

S. R. KHANOLKAR AND N. H. ANTIA

Tata Department of Plastic Surgery, J.J. Group of Hospitals, Bombay, India

and

E. J. AMBROSE

University of London and Foundation for Medical Research, Worli, Bombay, India

A method is described for the radioactive labelling of fresh bacilli of *Mycobacterium leprae* using tritium labelled *o*-dihydroxyphenyl alanine as a metabolite. A reasonably good correlation has been obtained between the Morphological Index and the Labelling Index in the case of suspensions obtained from the nodules of a group of leprosy patients. A method for organ culture of the intact tissue of leprosy nodules has been developed. Thin sections have been prepared from these organ cultures for autoradiography using [^{3}H]-DOPA. With the aid of a polarizing vertical illuminator, the distribution of the metabolizing organisms within the tissues has been demonstrated.

Introduction

In view of the difficulty of maintaining the bacilli of *M. leprae* in culture for extended periods, the application of a short term assay system which offers possibilities for systematic laboratory investigation related to metabolism, immunological and chemotherapeutic studies has recently attracted attention (Druts and Cline, 1972; Ambrose *et al.*, 1974; Talwar *et al.*, 1974). By the use of high resolution autoradiography as described in the brief note by Ambrose *et al.* (1974), it is possible to observe the localization of silver grains immediately above individual bacilli. This method has now been extended to an investigation of the relationship of the Labelling Index to the Morphological Index and to a study of bacilli within living tissues obtained from the leprosy nodules.

Methods

AGAR FILM TECHNIQUE FOR SUSPENSIONS OF BACILLI OF *M. LEPRAE*

The bacterial suspension was prepared according to the method of Nishiura *et al.* (1969). A nodule from an untreated lepromatous leprosy patient was chopped

Received for publication 5 April, 1976.

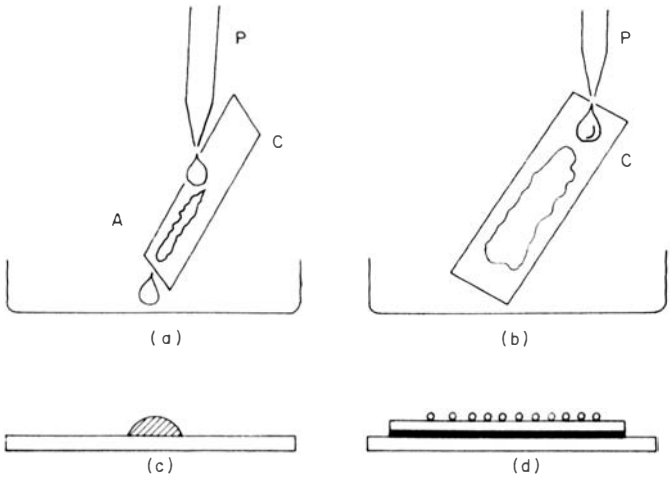

Fig. 1. Stages in the pouring of agar onto a glass coverslip to produce a thin layer. (a) The agar layer is prepared with first pipette. (b) Drop of suspension of *M. leprae* is placed on the agar, with a second pipette. P—pipette; C—coverslip. (c) Cross-section showing agar layer before drying. (d) Dried coverslip mounted with wax on a slide prior to dipping in emulsion. Bacilli lie on the upper surface.

up into small fragments with scissors, teased, and left overnight in a refrigerator in a cavity slide. The supernatant suspension containing bacilli was withdrawn the next morning, tissue debris having settled to the bottom of the slide. This procedure was performed under sterile conditions and a sample of the nodule plated on blood agar and Lowenstein-Jensen medium to rule out the presence of cultivable organisms. A 1% solution of agar was prepared and melted at 50° C in a water bath. A drop of the melted agar was poured on to a sloping coverslip followed immediately by a drop of bacillary suspension (Fig. 1). The coverslip was then placed horizontally in a Leighton tube to set. In this way a uniformly thin coat of agar was obtained containing embedded bacilli. One ml of culture medium (MEM + 10% human adult serum + 100 μ/ml pencillin) was added to the Leighton tubes, 2 of which were maintained as controls. To the other 2 tubes 5 μCi/ml of tritiated DOPA, [^{3}H]-DOPA, was added.

The Leighton tubes were incubated at 37° C for 48 h following which the coverslips were removed and washed 3 times with saline and then fixed in 10% formal saline overnight. They were then washed 6 times with distilled water and air dried. A melted drop of 50/50 paraffin and vaseline mixture was placed on a microscope slide and the coverslip mounted on it with the bacillary surface facing upwards.

K5 Ilford nuclear emulsion was diluted with an equal volume of water in a beaker placed in a water bath at 50° C. This was done in a dark room with a low intensity lamp. The slides with the mounted coverslips were dipped in the thin emulsion, following which they were dried with a fan and placed in a sealed box for 12-14 days in a refrigerator. They were subsequently developed for 5 min with Kodak D19 developer and fixed for 1 min in Amfix with hardener. After washing in running water for 20 min, the slides were air dried. The wax was removed with

xylol. The coverslips were stained in the usual way for acid-fast bacilli by the Ziehl-Neelsen technique and mounted, with the bacilli facing downwards on the slide, with D.P.X. mounting fluid.

ORGAN-CULTURE METHOD FOR INTACT TISSUE

Fragments not larger than 1 mm^3 were prepared from the biopsy and set up for organ culture on expanded stainless steel grids as shown in Fig. 2. The same medium incorporated with tritiated DOPA was employed for these experiments. Control cultures were maintained with the isotope.

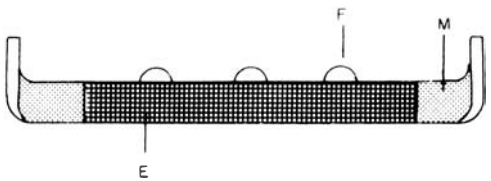

Fig. 2. Organ culture of small tissue fragments of a leprosy nodule. F—fragments; E—expanded metal grid; M—culture media.

The cultures were incubated for 48 h in a 5% CO_2 gassed incubator. They were subsequently washed with saline, fixed with 10% formol saline and embedded in paraffin blocks for histological sectioning. One μm thick sections were mounted on slides and autoradiographs were prepared by coating with K5 Ilford nuclear emulsion as before. The slides were stained by the Fite Faraco technique.

EXAMINATION OF THE AUTORADIOGRAPHS

For viewing by the usual method with transmitted light, light staining of the bacilli is desirable, and the agar film also needs to be comparatively thin. For clear identification of silver grains, particularly in well stained preparations and in the thin sections, the polarizing vertical illuminator M74 of Vicker Instruments, as developed by Rogers (1973) for autoradiography, is an added advantage as shown in Fig. 3.

With this system, the specimen can first be viewed with transmitted light and then with a dark field using polarized light and vertical illumination. Silver grains are clearly seen as bright spots on a dark field. Dye particles do not produce noticeable depolarizing reflections.

Experimental Results

There was a high degree of localization of silver grains immediately above the individual bacilli of *M. leprae* using the agar technique as already described (Ambrose *et al.*, 1974). In the earlier work the agar was poured on the horizontal surface of the coverslips. In some cases DOPA was difficult to wash out completely from the agar when the film was thick, resulting in a low background count. With the thinner agar preparation as shown in Fig. 1, the background count is extremely low. Some fragments of debris containing human cell melanin may get labelled but these can easily be distinguished from *M. leprae* by their morphology and staining. Compact bacilli are found to be labelled with 1 or 2

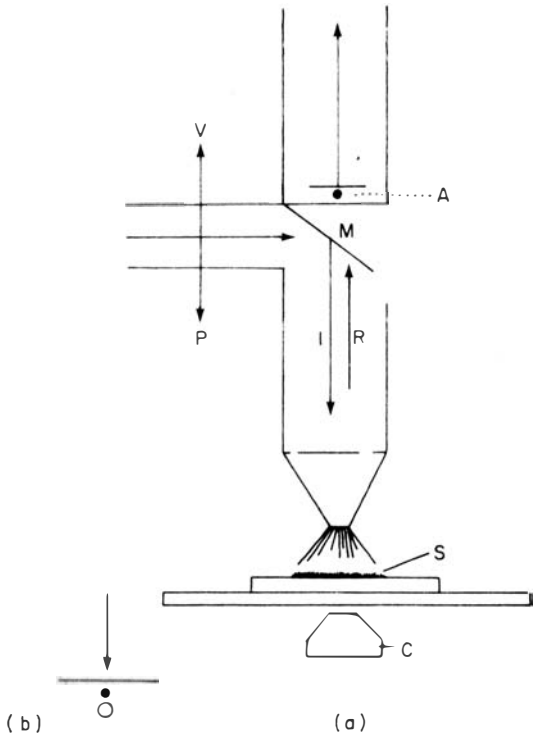

Fig. 3. Polarizing vertical illuminator used examine autoradiographs under dark field. V—vector of incident polarized light; M—partially reflecting mirror; I—polarized light incident on the specimen S; R—light back scattered and depolarized by silver grains; A—analyser with electric vector polarized perpendicular to the plane of the paper.

grains. A comparison of the Morphological Index, the Bacteriological Index and the Labelling Index for untreated patients is given in Table 1. In the third column data are given for 1000 bacilli counted in the usual transmission microscope. A comparison of this figure with that obtained using the combined transmission and vertical illuminator is given in the last column as a double check.

Within the thin sections of intact lepromatous tissue, extremely low background counts were obtained. Groups of the labelled bacilli against a completely dark field could readily by identified. It was found in general that the groups of labelled bacilli tended to appear in clusters in the tissue. Many areas containing stained bacilli were unlabelled. A typical field of labelled bacilli is shown in transmitted light field and in dark field with polarized light in Fig. 4 (a) and (b). Quantitative data obtained with a group of patients are summarized in Table 2.

Discussion

The work of Rees and Valentine (1962) has already indicated from statistical data, using the mouse foot-pad, that a relationship exists between the viability of

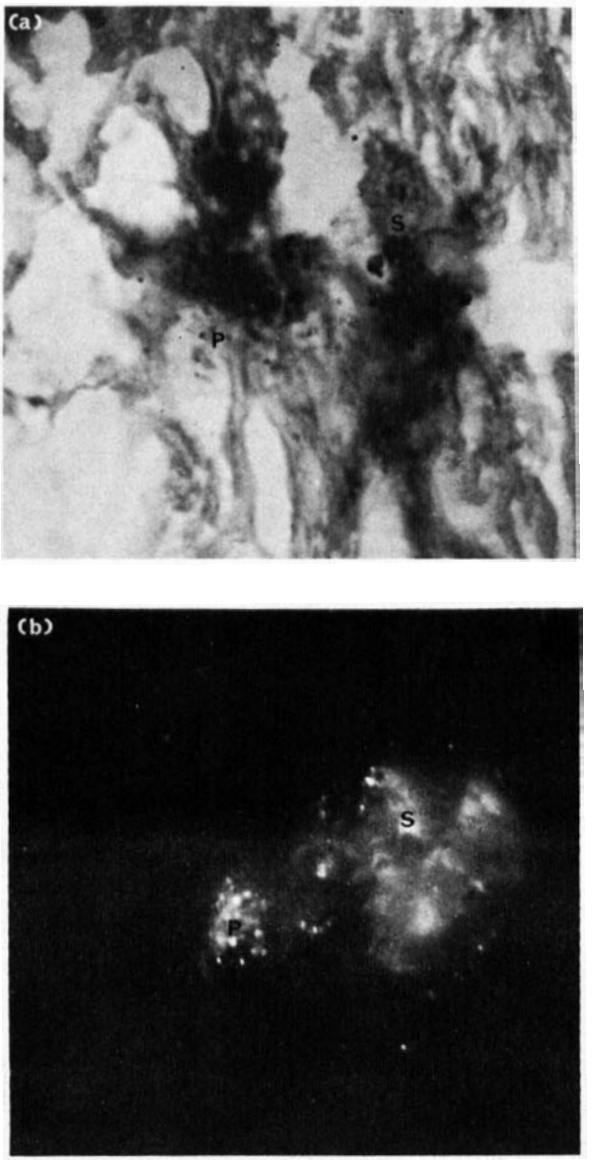

Fig. 4. Autoradiograph of the sections obtained from an organ culture of a leprosy nodule after incorporation of [^{3}H]-DOPA. (a) As seen in transmitted light. A packet of labelled bacilli is clearly visible at P. Many scattered bacilli at S. (b) The same as seen in the polarizing vertical illuminator. The silver grains adjacent to bacilli at P make the rod-shaped bacilli clearly visible. Some diffuse illumination of the tissue is produced by the dense grains at S. These were the only two labelled packets visible in the entire section. The extremely low background counts (dark field) can be seen surrounding the areas P and S.

TABLE 1

Comparison between Morphological Index and Labelling Index by incorporating [^{3}H]-DOPA into M. leprae

Specimen No.	Clinical diagnosis	BI	MI (%)	LI (%)	LI By polarizing microscope
1	LL	3+	4	3	5
2	LL	3+	4.5	5	8.5
3	LL	4+	5	6	3
4	LL	4+	0-1	1.5	0
5	LL	4+	4	4.5	4
6	LL	4+	2	3.1	2.2
7	LL	4+	4	N.D.	4[a]
8	LL	3+	1.4	3	3
9	LL	3+	3	4.7	6.2
10	LL	3+	4[a]	5	6

[a] Sample from early experiments with high background counts before agar pouring method (Fig. 1) was used, not suitable for counting in transmitted light.
BI = Bacteriological Index.
LI = Labelling Index.
MI = Morphological Index.
LL = Lepromatous Leprosy.
N.D. = Not done.
Heat-killed bacilli were used as a control. No silver grains were detected on the killed bacilli.

TABLE 2

Uptake of [^{3}H]-DOPA in thin sections of lepromatous nodules

Specimen no.	Clinical diagnosis	BI	MI (%)	Remarks
1	LL	4+	4	++
2	LL	4+	1	–
3	LL	2+	0	–
4	LL	4+	1.4	+
5	LL	4+	3	++
6	LL	5+	3.3	++
7	LL	3+	1	–
8	LL	5+	3	++

++ = Moderate level grains count.
+ = Low level grains count.
– = Grains count similar to the surrounding area.

M. leprae and the Morphological Index. For the present experiments, using the high level of labelling with [^{3}H]-DOPA of 5 μCi for 48 h, conditions are favourable for a saturation labelling of the organisms. Earlier studies (Ambrose *et al.*, 1974) have already shown that these conditions were optimal for a high level of the Labelling Index, as shown in Table 1. This data provides independent evidence that a relationship exists between the Morphological Index and the number of actively metabolizing bacilli.

The slightly higher values obtained before the use of thin agar techniques for some of the earlier cases may be due to the presence of the background labelling.

Evidence that the uptake of [^{3}H]-DOPA occurs with fresh bacilli of *M. leprae* has been recently obtained by Harris and Prabhakaran (1975). The uptake of [^{3}H]-DOPA by *M. leprae* and melanocytes which contain *o*-diphenoloxidase and by turtle heart cells which may be expected to exhibit catecholamine metabolism was demonstrated by scintillation counting of labelled cell suspensions. But *M. phlei* and armadillo fibroblasts, which do not contain *o*-diphenoloxidase, failed to incorporate DOPA. Treatment with diethyl dithio-carbamate, a copper chelating agent, prevented the incorporation of DOPA by *M. leprae*. DOPA is to be expected to become first attached at the specific receptor sites of *o*-diphenol-oxidase, which is a copper-containing enzyme. The eventual fate of the tritium atoms of DOPA cannot at present be decided, but that pigmented products are not the final product in the case of living bacilli is suggested by the complete absence of detectable pigmentation in suspensions obtained from patients. That DOPA is nevertheless metabolized is suggested by the autoradiographic studies described in the communication. The slides have by necessity to be subjected to extensive washing in water. To prepare slides for autoradiography which will retain water soluble compounds of low molecular weight such as DOPA, special techniques, as described by Rogers (1973) are likely to be required. It is distinctly possible that the tritium becomes incorporated, at least into large peptides, oligonucleotides or oligosaccharides, if not into cellular macromolecules.

The absence of grain counts over heat-killed bacilli strongly suggests that the active metabolism of the organisms is required for DOPA incorporation.

In the case of the bacilli located in the whole tissues, the Morphological Index is not easy to assess. The autoradiographic techniques may be of considerable value in assessing the relative viability of organisms located in intracellular spaces and within various tissues known to retain packets of viable bacilli.

Acknowledgements

This study was supported by grants from the Tata Companies and Trusts, Bombay, and the World Health Organisation, Geneva, and the Tropical Medicine Research Board, Ministry of Overseas Development, U.K. We wish to acknowledge the help of Mr Pote for the histology sections and Miss Jyoti Chogle for technical assistance.

References

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

Druts, 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.

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

Nishiura, M., Okada, S., Izumi, S. and Takizawa, H. (1969). An electron microscope study of the band structure of leprosy bacillus and other mycobacteria. *Int. J. Lepr.* **30**, 225.

Rees, R. J. W. and Valentine, R. C. (1962). The appearance of dead leprosy bacilli by light and electron microscopy. *Int. J. Lepr.* **30**, 1.

Rogers, A. W. (1973). *Techniques of Autoradiography*, 2nd ed. Amsterdam/London/New York: Elsevier Scientific Publishing Company.

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 macrophages. *Infect. Immun.* **9**, 187.