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Autoradiographic and Metabolic Studies of *Mycobacterium leprae*

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Highly purified suspensions of *Mycobacterium leprae* show a progressive increase in incorporation of [^{3}H]thymidine and [^{3}H]DOPA in short-term cultures as shown by scintillation counting. The intact bacilli are known to have a high permeability barrier. The experiments described suggest that [^{3}H]DOPA becomes trapped within this barrier and oxidized inside the bacilli. Tests by pre-treatment with diethyl dithiocarbamate (DDC inhibitor of DOPA), cold DOPA or hyaluronidase distinguish the uptake of [^{3}H]DOPA by bacilli from the effects of connective tissue contamination. Similar increases in labelling of bacilli by scintillation counting of cultures, have been observed by autoradiography of the organisms.

The scintillation method shows promise for rapidly identifying drug resistance in lepromatous patients relapsing while on treatment with dapsone (DDS), rifampicin, clofazimine or other anti-leprosy drugs.

Introduction

In the previous communication (Khanolkar, Antia and Ambrose, 1976) the application of short-term pulse labelling of suspensions of *Mycobacterium leprae* combined with high resolution autoradiography was described. In order to apply such methods to a systematic investigation of the biological and biochemical characteristics of *M. leprae*, it is necessary to know the dynamic aspects of uptake of various labelled metabolites during various periods of culture and also to know the extent to which bacterial and tissue contaminants play a role in the incorporation of metabolites. In this communication, the use of scintillation counting in place of autoradiography is described. This method makes possible the carrying out on a regular basis of systemic and quantitative investigations of metabolism and biosynthesis of leprosy bacilli. Therefore it has clinical applications, particularly for the study of dapsone (DDS) resistance and the development of alternative drug combinations for the treatment of new patients. Basic studies of the metabolic requirements of the organism and hence the improvement of the culture medium may also be investigated.

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Materials and Methods

PREPARATION OF BACTERIAL SUSPENSIONS

The methods used for preparing bacterial suspensions have involved the minimum possible number of operations in order to avoid the risk of contamination by airborne organisms. After preparation, all suspensions are tested on nutrient agar for absence of contaminants. Each separate culture is also tested on nutrient agar and Lowenstein-Jensen medium at the end of the culture period. We have used the method for preparing suspensions from human nodules as described by Talwar, Krishanan and Gupta (1974). This was modified later during the present studies. The chopped up tissue was placed in ice-cold distilled water for 0.5 h and subsequently processed according to the method of Talwar *et al.* This we call the Modified Talwar method. After preparation of the suspension, microscopical checks were made on a standard sized drop coating a constant area of slide, to give the bacteriological and morphological (MI) index. The same slide was then observed through a microscope with a square eyepiece graticule, subdivided into 25 small squares. The average area covered by a single bacillus after acid-fast staining, as the average of a large number, was found to be 1/57th of the area of a small square. Twenty fields of the large square were counted for total number of bacilli. In addition, the areas of small squares covered by tissue fragments, mainly stained blue, by the methylene dye, were observed. Stopping down the condenser was sometimes helpful for determining these areas. The purity of a given suspension could then be assessed quantitatively as the percentage of total area covered by bacilli to the percentage covered by tissue.

MICRO-METHOD FOR AUTORADIOGRAPHY

The agar film technique, as previously described (Khanolkar *et al.,* 1976) has been modified as shown in Fig. 1. The coverslips are first dipped vertically in 1% agar and dried on filter paper to give a thin coating of transparent agar to assist adhesion. The reinforcement with a strip of cloth helps to preserve the agar film when culturing up to 12 days.

SCINTILLATION COUNTING

Microtubes, as shown in Fig. 1 (g) and (h), 40 mm × 8 mm (6 mm int. diam.) were used. They were convenient for processing 1×10^6 bacilli, and 100 or more replicate cultures could be set up from a single nodule. The cultures are pulse-labelled with 1 μCi/ml of [^{3}H]thymidine for 24 h, or for 6 h with DOPA. The tubes are then centrifuged for 10 min at 4000 rev/min. The visible pellet is washed 3 times with saline, treated with cold TCA and processed for scintillation counting according to standard methods.

CULTURE MEDIA

The problem of finding a medium suitable for the long-term culture of *M. leprae* is well known. Therefore, the absence of biochemical data concerning suitable sources of energy for *M. leprae,* alternative sources, such as citrate and pyruvate have been supplied as well as essential amino acids, particularly

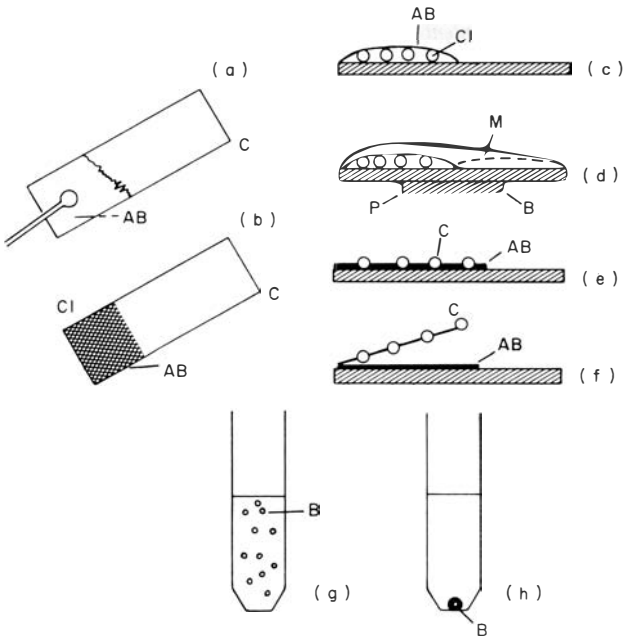

Fig. 1. *Culture methods for autoradiography and scintillation counting.* (a) Equal volumes of bacilliary suspension and 1% molten agar (maintained in water bath at 55°C) are mixed with wire loops on coverslips. (b) and (c) A small strip of loosely woven cloth is placed on molten agar to reinforce the gel. (d) The coverslip may be placed in a Leighton tube. For small volumes, to save labelled metabolites, it can be placed on a glass block (B) coated with a film of liquid paraffin (p) and incubated in a Petri dish in a humid atmosphere. After fixing it must then be washed in detergent to remove paraffin — (e) and (f). After processing and drying, the cloth can be peeled off leaving the dry agar film intact. (g) Microtube holding 0.5 ml. of culture medium and 1×10^6 bacilli for scintillation studies. (h) After pulse labelling and centrifuging a distinct pellet can be observed. This is washed and processed for counting.

asparagine and nucleotides. The new medium of Murohashi and Yoshida (1975) is simple and seems to satisfy most requirements for our short-term culture assay.

With this medium we find that, unless the MI is very low, the counts on pulse labelling after maintenance for 9 days show an increase as compared with controls labelled at day 0. On the basis of this finding we have used this medium for all the investigations supported here. However, the scintillation assay could be used to investigate modification of this medium or other possible media.

Experimental Results

PURITY OF BACTERIAL SUSPENSIONS

The micro-scanning method indicates that in the Talwar method of preparing suspensions, the tissue contamination is in the range of 30–40%. But with

TABLE 1
Analysis of bacterial content in suspensions prepared by the modified method

Patient OPD No.	Bacilli %	Tissue fragments %
99620	85	15
99722	84	16
61655	86	18
98571	94	6
30357	99	1

preparations made by the modified Talwar method as shown in Table 1, the tissue contamination is much less when expressed as the relative areas covered by tissue and bacilli. The relative dry masses will not be identical but are not expected to differ markedly from these figures. The extent of contamination with hyaluronic acid has also been assessed (Khadapkar *et al.,* 1977). A range of concentrations of a purified sample of human umbilical cord hyaluronic acid were used as standards. The test samples were supernatants obtained during centrifugation of the bacterial suspensions and the final purified suspension of *M. leprae,* obtained from the nodule. Drops of known volume were placed on standard areas of slides and allowed to dry slowly. These were then covered with a standard sized drop of toluidine blue solution as used in histochemistry. Metachromasia was observed to an extent depending on the concentration of acid mucopolysaccharide present. With the standard solutions, showing a colour range, the test samples were matched colorimetrically against a standard drop. The first washing from the bacilli was found to contain the equivalent of approximately 0.2 mg/ml of hyaluronic acid. The amount in the washed bacilli was below the detectable limit, at least 100 times less than that in the washings.

AUTORADIOGRAPHIC STUDIES OF CULTURED BACILLI

These studies were made as a further check on the localization of the labelling within the bacilli, prior to adopting the scintillation counting method.

With the Murohashi and Yoshida medium, the concentration of labelled metabolite can be reduced to 1 μCi/ml giving a low background count, not more than 18 background grains were observed per microscopic field. The average area covered by a bacillus being 1400 times less than the field area, the probability of a background grain lying over a bacillus will be 0.01. For 2 grains it will be 0.0001 and for 3 grains 0.000001. Photomicrographs taken both by transmitted light and with the polarizing vertical illuminator have been shown previously (Ambrose *et al.,* 1974; Khanolkar *et al.,* 1976). In Fig. 2 is shown a detailed analysis of a 12-day culture checked at high magnification for a group of granular bacilli and a group of labelled bacilli recorded from a camera lucida drawing. Due to slight variations in focal-plane it is difficult to record and present such detailed information by photography. Due to the porosity of the dry agar film, emulsion penetrates uniformly so that grains are developed immediately adjacent to bacilli and strictly in a straight line along the length of a bacillus. A comparison between labelling of bacilli and the morphological type of the acid-fast staining in the case of 5 untreated

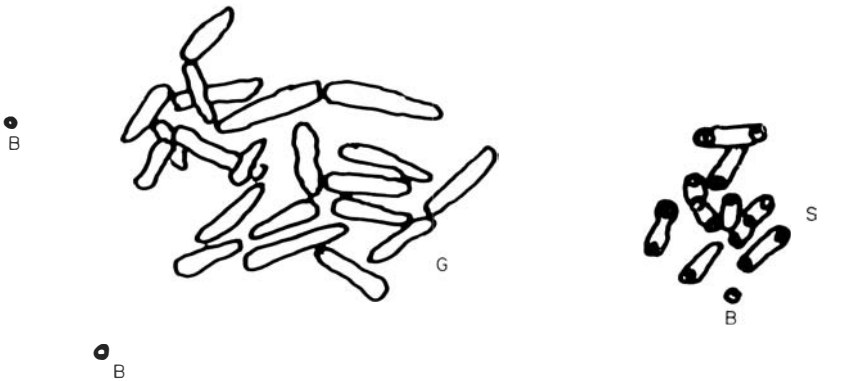

Fig. 2. Autoradiograph after maintaining for 12 days in culture (from Camera lucida drawing made at ×3000 mag.). G, Group of unlabelled and granular bacilli. S, Group of solid bacilli showing up to 3 grains per bacillus. B, Background grains. Labelled for 48 h with 1 μCi/ml of [^{3}H]DOPA.

lepromatous leprosy patients showed that labelling is almost entirely restricted to solid bacilli. In Fig. 3(a) is shown a histogram obtained in a culture maintained for 9 days before labelling with [^{3}H]DOPA. The frequency of clusters containing labelled bacilli is shown in Fig. 3(a), upper, and of

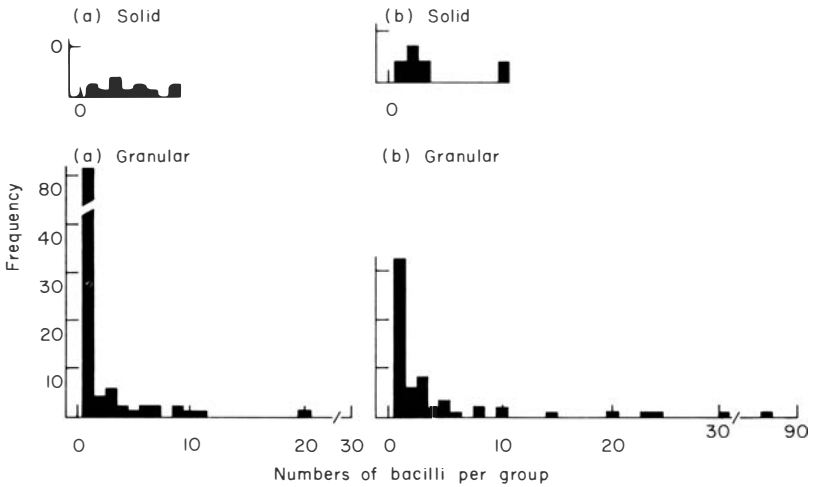

Fig. 3. Effect of added hyaluronic acid on the labelling of *M. leprae* with [^{3}H]DOPA as seen in autoradiographs (a) (upper, left). Solid bacilli. Histogram showing frequency of occurrence of clusters containing different numbers of bacilli (abscissa), after labelling for 48 h with [^{3}H]DOPA. These are the bacilli which show one or more silver grains along their length (mainly solid). (a) (lower, left). Granular bacilli. Similar histogram for the unlabelled bacilli on the same slide (mainly granular). (b) (upper and lower, right). Histograms for solid and granular groups similar to (a), but in this case 0.3 mg/ml of hyaluronic acid had been added to the medium before labelling. The general pattern of labelling is unchanged; adsorption of hyaluronic acid on previously unlabelled bacilli has not caused them to be labelled.

unlabelled granular bacilli, Fig. 3(a), lower. At day 0 labelling is restricted mainly to single bacilli, but at day 9 labelled clusters are also seen.

In Fig. 3(b) is shown a similar result when 0.3 mg/ml of hyaluronic acid had been added initially to the culture media. Although many grains were seen in the background due to oxidation catalysed by hyaluronic acid, there were mainly in different focal planes from bacillary clusters and it was possible to count this slide. The presence of hyaluronic acid does not affect the general pattern of labelling.

Due to high rate of incorporation, [^{3}H]DOPA is useful for autoradiography, although [^{3}H]thymidine also shows a small number of labelled bacilli.

THE ROLE OF TISSUE CONTAMINANTS IN THE INCORPORATION OF LABELLED METABOLITES

In Table 2 is shown the rate of labelling with [^{3}H]thymidine for fresh and heated bacilli. Twenty-four hours' labelling gives a good uptake, general absorption by tissue, as indicated in the heated specimens, being low. Absence of bacterial contaminants is checked on nutrient agar and on Lowenstein-Jensen medium.

In earlier studies with [^{3}H]DOPA, penicillin was not added to the medium. In autoradiographs non-acid-fast bacterial contaminants were sometimes seen, but none of these were labelled. A fungus, apparently *Aspergillus,* which is known to contain DOPA oxidase showed heavy labelling. Penicillin 100 i.u./ml is now introduced routinely into the culture medium. Cultures of *M. tuberculosis, M. phlei* and *M. smegmatis* in their respective growth media showed no grains in autoradiographs following [^{3}H]DOPA treatment. The role of connective tissue hyaluronic acid in the bacterial suspensions has been checked by 4 independent methods following the observations of Kato, Ishaque and Adopoe (1976) that this tissue can catalyse the oxidation of DOPA. The capacity of connective tissue to oxidase DOPA is not affected by heating (Kato *et al.,* 1976). In Table 3(a) the incorporation of [^{3}H]DOPA is

TABLE 2

Rate of labelling with [^{3}H]*thymidine as shown in scintillation counting*

Morphological Index	p mol of [^{3}H]thymidine incorporated per 10^7 bacilli		
	Time (h)		
	6	24	48
Fresh bacilli	$\times 10^{-3}$	$\times 10^{-3}$	$\times 10^{-3}$
4	9.78	15.3	16.6
4	76.4	67.6	102
5	80.8	72.0	106
Heated bacilli			
4	0.32	0.22	1.3
4	1.00	1.3	3.4

TABLE 3

(a) *Analysis of labelling of suspensions of* M. leprae *heated and pre-treated with DDC**

Patient	MI (%)	Ratio of bacilli/ tissue	Time of incu-bation (h)	pmol of [^{3}H]DOPA incorporated per 10^7 bacilli			
				Fresh bacilli		Heated bacilli	
				Column 1 control	Column 2 with DDC	Column 3 control	Column 4 with DDC
OPD No. 99355	5	Low	1	97.1	50.3	54.1	60.3
		(Talwar	6	291.1	98.7	157.4	148.7
		method)	8	162.9	132.0	117.0	90.0
L L Untreated	5	High	1	186.5	72.9	65.1	59.5
OPD No. 99620		(Modified	6	375.0	97.1	86.5	93.2
		Talwar					
LL Untreated		method)	8	223.2	70.0	61.8	61.5

* Control suspensions were incubated for 8 h in phosphate buffer, washed and incubated for a further 8 h before labelling with 2 μCi/ml [^{3}H]DOPA prior to counting at 1, 6 and 8 h. DDC-treated suspensions were also given similar washing after 8 h treatment.

(b) *Effect of pre-treatment with hyaluronidase on composition of bacterial suspensions*

Patient	Estimated by scanning of microdrop		Estimated by pre-treatment with hyaluronidase	
	Bacilli (%)	Tissue fragments (%)	Bacilli (%)	Tissue fragments (%)
OPD No. 99912	62	38	58	42
OPD No. 99620	85	15	75	25

*Control suspensions were incubated with [^{3}H]DOPA. Hyaluronidase-treated suspensions were incubated for 2 h and washed before incubation with [^{3}H]DOPA.

shown for a suspension prepared by the Talwar method (OPD No. 99355) and by the modified Talwar method (OPD No. 99620). In column 1 is shown the uptake by the fresh suspension which reached a peak at 6 h. In column 3 is shown the uptake by a heated preparation. In column 2 is shown the effect of treating fresh bacilli with diethyl dithiocarbamate (DDC), the copper chelator and inhibitor of diphenol-oxidase, for 8 h followed by washing, and further incubation for 8 h prior to labelling with [^{3}H]DOPA. The effect of DDC persists after washing, but in the case of the heated specimens it shows no inhibitory effect. The residual levels as seen in column 2 and 4 are similar, the amounts being reduced in the purified suspension prepared by the new method.

Kato *et al.* (1976) have shown that pre-treatment with hyaluronidase completely arrests the capacity of connective tissue to oxidase DOPA. In Table 3(b) is shown, in the case of a heavily contaminated sample (OPD No. 99620), the effect of pre-incubation with 0.2 mg/ml of Sigma hyaluronidase for 2 h followed by washing and labelling. Controls were similarly treated but without enzyme. Pre-treatment with cold DOPA followed by washing prior to labelling also inhibited uptake of [^{3}H]DOPA. Finally the effect of ascorbic

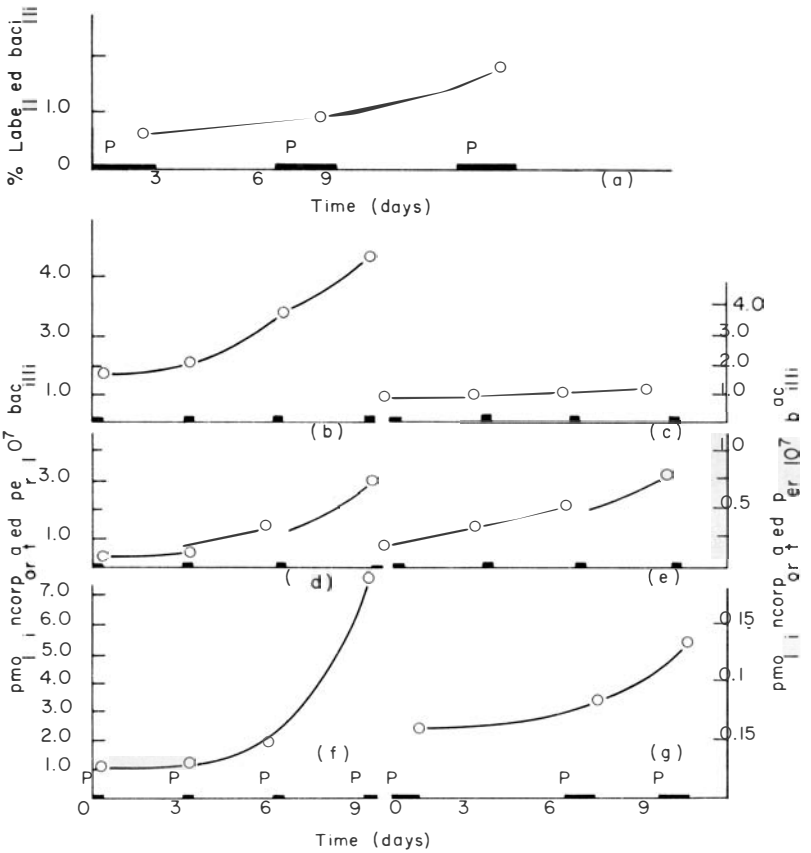

Fig. 4. Growth curves obtained by autoradiography and scintillation counting. (a) Increase in labelling of replicate autoradiographs pulse-labelled for 48 h with 1 μCi/ml of [^{3}H]DOPA. Untreated LL patient with MI 3%; P = pulse. (b) Similar growth curve obtained by scintillation counting for a highly purified suspension of *M. leprae* (only 1% tissue contamination, Case 30357 Table 1). Relapsed LL patient; MI 2%. (c) Heated suspension (60°C for 1 h). Supension prepared by Talwars method with considerable tissue contamination. (d) Growth curve for untreated LL patient; MI 4%; pulse-labelled with [^{3}H]DOPA. Rate of growth expressed as ratio of incorporation at day 9/incorporation at day 0 = 5.6. (e) Suspension of same nodule as (d). Including sediments removed at 500 rev/min prior to preparing suspension shown in (d), it is highly contaminated with tissue debris (38%). In this case 4 mM ascorbic acid was added immediately before pulse-labelling to inhibit all oxidation of [^{3}H]DOPA by tissue contaminants. Rate of growth = 5.8, is similar to (d). Repeated experiments have shown similar results with ascorbic acid. (f) Growth curve for untreated LL case, MI 6% — pulse-labelled with [^{3}H]DOPA. (g) The same suspension as shown in (f) but pulse-labelled with [^{3}H]thymidine.

acid was shown to give results similar to the previous 2 tests. Adding ascorbic acid even at 4 mM, only reduced the incorporation of [^{3}H]DOPA to the extent expected in proportion to the amount of tissue contamination [Fig. 4(e)]. Addition of ascorbic acid to reaction mixture of *M. leprae* reverses the reaction from purple pigment to pinkish indole 5:6 quinone (Prabhakaran,

1973). On the other hand it reverses completely in the case of non-enzymatic oxidation of DOPA to a colourless solution.

GROWTH CURVES

For the purpose of this communication growth is defined as an increase in the incorporation of [^{3}H]thymidine or [^{3}H]DOPA into the bacilli. Four growth curves were obtained by autoradiography, one being shown in Fig. 4(a). All showed a progressive increase in labelling at the end of 6 and 12 days, of replicate autoradiographs. In Fig. 4(b) is shown a similar curve obtained by scintillation counting in the case of a highly purified suspension of *M. leprae* (OPD No. 30357 of Table 1). In Fig. 4(c) is shown a result for a suspension containing tissue contamination heated to 60° C for 1 h. In Fig. 4(d) and 4(e) are shown results for the same suspension, but that shown in Fig. 4(c) was the preliminary sediment centrifuged at 500 rev/min, while 4(d) was from the usual supernatant centrifuged at 4000 rev/min according to the modified method. The sample 4(e) contained 38% of tissue and was treated with ascorbic acid to eliminate tissue oxidation of DOPA. Figure 4(f) and 4(g) show growth curves obtained with the same suspension using [^{3}H]DOPA and [^{3}H]thymidine. Figure 5 shows a reasonably good correlation between the morphological index and the growth rate as determined for the last 8 biopsies processed for culture.

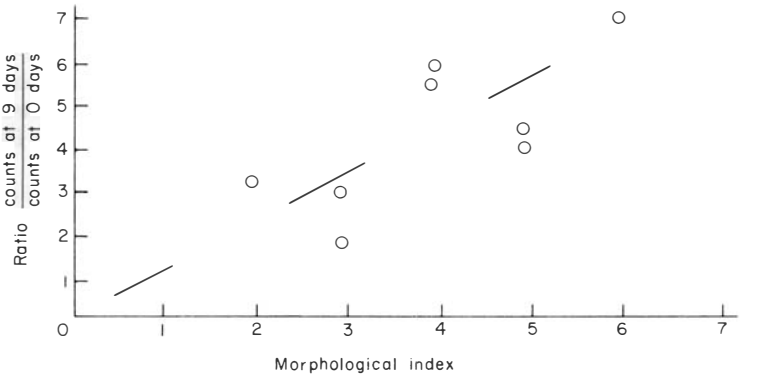

Fig. 5. Relationship between morphological index and the increase in counts at day 9 over counts at day 0, for a sequence of 8 cases tested recently.

In all cases the morphological index was checked independently at the A.L.H. (R.G.C.) and at FMR (S.R.K.).

Discussion

The use of labelled metabolites to assess metabolic activity and growth potential has already been shown to be of value with human tumours to measure drug response, effects of antisera etc., although most tumour cells do not survive for more than 1 or 2 weeks in the culture media at present

available. The approach adopted here has been basically similar for studies of *M. leprae*. Labelled thymidine is well recognized as a marker for DNA synthesis by living cells. The incorporation of this metabolite by *M. leprae* already ingested by human macrophages has been described by Drutz and Cline (1972), and by Talwar, Krishanan and Gupta (1974). The suspensions used for scintillation studies, as shown in Fig. 4(g), were pre-treated with distilled water which causes lysis of all mammalian cells. Tests for contaminating bacteria on nutrient agar and Lowenstein-Jensen medium were also negative, strongly suggesting that thymidine was also being incorporated into *M. leprae* in these cultures. Another widely used test for viability of mammalian cells has been the dye exclusion test. The high permeability barrier of the intact plasma membrane of living cells prevents the penetration of dyes such as lissamine green, but dead cells are rapidly stained. Such tests are not needed with bacteria which grow readily in culture, but are of value with *M. leprae*. The evidence presented in this paper supports other evidence that these bacilli also possess a high permeability barrier, which is only penetrated slowly by molecules such as DDC or cold DOPA. Once DOPA has entered the bacillus and been oxidized to quinone, this molecule cannot leak out before further stages of oxidation to insoluble products or incorporation into macromolecules has occurred. The radioactivity remaining will be due to those atoms which are not released during oxidation as $[^3H]_20$. Results obtained with ascorbic acid also indicate that special conditions exist within the leprosy bacilli which are not affected by the presence of this reducing agent in the surrounding medium. As shown in Fig. 4(b), a bacterial suspension containing a negligible amount of tissue contamination (OPD No. 30357 Table 1) still shows high incorporation of [^{3}H]DOPA at day 0 and subsequent increases up to day 9. Chatterjee (1977) has obtained some strains of acid-fast bacilli in long-term cultures obtained from leprosy nodules, which are also DOPA oxidase positive.

The various tests for the effect of tissue contamination on the initial counts i.e. scanning of microdrops pre-heating of suspensions, pre-treatment with DDC, pre-treatment with hyaluronidase (Tables 1 and 3) and the effect of ascorbic acid [Fig. 4(c)] give similar values for the effect of tissue contamination on the initial background count. This does not exceed 25–30% with the modified method of preparation and is generally much less than this.

The autoradiographic studies indicate that the solid bacilli incorporate sufficient labelled metabolites to produce grains in the autoradiographs. When embedded in the agar the penetration of the metabolite, and probably oxygen exchange, is further reduced so that 48 h is required for labelling. Earlier studies with the mouse footpad (Shepard and McRae, 1965) and clinical studies of decreases in the proportion of solid bacilli during the first 3 months of DDS treatment (Waters and Rees, 1962), favour the view that the solid bacilli are the viable and most actively growing fraction. The possibility that other morphological types are moribund, but recover their viability under favourable conditions cannot be excluded. The increase in the average number of grains per labelled bacillus, seen after 12 days in cultures, suggests that the increase in count with DOPA labelling is at least partly due to increased rate of metabolism, in which some previously moribund bacilli might be involved.

Nevertheless, the progressive increase in thymidine labelling, as shown by the curve of Fig. 4(g), suggests that bacterial replication may also be involved. There have been claims that other forms of *M. leprae* including coccal forms can multiply in the mouse footpad (Desikan, 1976). This problem has also been discussed by Dharmendra (1977). The acid-fast character of the bacilli obtained in clusters as seen in autoradiographs after 12 days, the bacterial specificity for DOPA labelling (Prabhakaran, 1973) and the absence of growth as double-checked, before and after culture, on nutrient agar and on Lowenstein-Jensen media, favour the view that this technique can be used for dynamic studies of the biological properties of *M. leprae*. This is borne out by data recently obtained on the response to DDS, rifampicin and clofazimine in these cultures (to be published). With untreated lepromatous leprosy cases, inhibition has been obtained in the range of concentration of DDS used in mouse footpad i.e. more than 100 times the sensitivity shown by any known contaminant. With relapsed cases DDS resistance has been observed up to concentrations corresponding to those observed in patients receiving between 100–200 mg daily dose of DDS, including cases already shown to be DDS resistant in the mouse footpad. Good response to rifampicin with the labelling test has been observed in 2 DDS-resistant cases who have now shown good clinical response to this drug.

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