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An *in vitro* system to study drug sensitivity of *Mycobacterium leprae* using infected human tissue

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Abstract. A reliable screening technique for assessing the sensitivity of *Mycobacterium leprae* to drugs has been developed. The method is based on the susceptibility or otherwise of *M. leprae*–infected tissues from lepromatous leprosy patients to the action of diaminodiphenyl sulphone (dapsone) or rifampicin on the incorporation of [^{14}C]-acetate into lipids. The extent of inhibition or lack of inhibition correlated very well with the drug sensitivity or resistance of the bacteria isolated from the patients to the above drugs. A similar trend was observed when the incorporation into individual fractions of neutral lipids was measured. There was no incorporation by heat-killed tissues. This method correlates well with the 3,4-dihydroxyphenylalanine uptake studies.

Keywords. Drug sensitivity/resistance; *Mycobacterium leprae;* [^{14}C]-acetate uptake.

Introduction

Successful development of antimicrobial compounds is the end result of a rational screening programme against known microbes by simple and quick *in vitro* systems, followed by preliminary toxicological studies on the compounds which show promise. All microbes that can be cultivated can be subjected to these screening tests. *Mycobacterium leprae* which has resisted *in vitro* cultivation despite efforts by several workers, cannot be tested by these methods. The mouse foot pad model of Shepard (1960) and Rees (1964) is the most widely used method, but is limited by the long time period of 6 to 9 months required for assay.

There are currently two *in vitro* tests in use, one developed in our laboratory by Ambrose *et al.* (1974, 1978) using uptake of labelled L-3,4-dihydroxy (ring-2,5,6-^{3}H) phenylalanine (DOPA) by partially purified *M. leprae* and the other using incorporation of [^{3}H]-thymidine by *M. leprae* inside the macrophages (Drutz and Cline, 1972; Talwar *et al.,* 1974; Prasad and Nath, 1981; Nath *et al.,* 1982).

Initial studies in our laboratory showed that *Mycobacterium lepraemurium* infected liver and spleen tissue from mice, when incubated with [^{14}C]-acetate for one week in Minimal Eagle's medium containing serum, incorporated the precursor into the bacterial-bound lipid portion, identified as mycolic acids. The incorporation decreased in the presence of drugs like isoniazid, rifampicin and streptomycin. These observations formed the basis to study the incorporation of [^{14}C]-acetate into the lipids of *M. leprae* in human biopsy material, freshly obtained from patients, in the presence and absence of known antileprosy drugs namely

Abbreviations used: DOPA, L-3, 4-Dihydroxyphenylalanine; dapsone, diaminodiphenyl sulphone.

diaminodiphenyl sulphone (dapsone) and rifampicin. In this paper we describe the effect of dapsone and rifampicin on the incorporation of [^{14}C]-acetate into experiments to study the synthesis of the various lipid components by *M. leprae* contained in biopsy tissue obtained from leprosy patients and the effect of dapsone and rifampicin.

Materials and methods

Uptake of [^{14}C]-acetate

Human skin biopsis from *M. leprae* infected patients were obtained from the Acworth Leprosy Hospital, Bombay. The biopsies were collected from lepromatous leprosy patients from the ear lobe or young skin lesions, under asceptic conditions. It was processed within 3 h after collection. The tissue was washed with saline followed by a wash with Minimal Essential Medium containing penicillin (50 units/ml). It was chopped into small pieces and each piece was transferred to a 10 ml flask containing Eagle's Minimal Essential Medium, containing additional 20% AB type human serum.

Two controls were used, one untreated tissue and the other heat-killed tissue. The drug-treated tissues included dapsone (10 µg/ml) and rifampicin (5 µg/ml). The reaction mixture contained 0.5 µCi/ml of l,2-[^{14}C]-acetate (specific activity 58.6 mCi/mmol; obtained from Isotope Division, Bhabha Atomic Research Centre, Bombay). Penicillin (100 units/ml) and Mycostatin (50 units/ml) were added to the medium as antibacterial and antifungal agents. The cultures were incubated at 34±1°C and the flasks were kept in a dessicator with 100% humidity and maintained for 11 days, the medium containing [^{14}C]-acetate was changed every 3 days.

On completion of this period, the tissue was suspended in about 3 ml of saline and autoclaved at 15 lbs pressure for 30 min. The tissue was homogenized in a power driven Potter-Elvehjem type homogenizer fitted with a teflon pestle for 2 min and the homogenate was centrifuged at 182 *g* in a Sorvall refrigerated centrifuge to remove the tissue debris. The supernatant, containing the bacilli was centrifuged at 11,700 *g* for 15–20 min. The sediment, containing bacilli was washed with saline and after recentrifugation, suspended in an appropriate volume of saline. Bacillary lipids were extracted by the method of Folch *et al.* (1957). Radioactivity in each preparation at various stages was determined using a Kontron MR 300 Liquid Scintillation spectrometer after mixing the sample with Bray's scintillation fluid (Bray, 1960).

The extracted lipids were separated by thin layer chromatography using Silica Gel G (BDH) (0.25 mn thickness) and a solvent system containing hexane: ether: glacial acetic acid (80:20:1 v/v). The separated neutral lipids were visualised with iodine and identified by comparison with standards. The radioactivity associated with the various lipids was measured by scrapping the gel corresponding, to the standard spots from the plate and suspending in Bray's scintillation fluid and measuring the radioactivity. Normal skin biopsies obtained from non-leprosy individuals was also processed in a similar manner. All reagents used were of the analytical grade.

Uptake of [3H]-DOPA

Eight biopsies were processed for drug trials by the method described above and

by the method developed earlier in our laboratory using L-3,4-dihydroxy (ring-2,5,6-^{3}H) phenylalanine (specific activity 1 Ci/mmol; obtained from Amersham, England) uptake as an index of bacterial viability and drug sensitivity of *M. leprae* (Ambrose *et al.,* 1974, 1978).

Results

Effect of drug on [^{14}C]-acetate incorporation in normal tissues

Before one could study the uptake of [^{14}C]-acetate in lepromatous tissue one should know the type of incorporation of the precursor into lipids of normal skin. The uptake of [^{14}C]-acetate by the normal skin tissue and its incorporation into lipids in the presence and absence of dapsone is shown in table 1. The relative incorporation in the lipids under both the experimental condtions was similar suggesting that dapsone had no significant effect on the lipid synthesis of cells in normal skin tissue.

Table 1. Relative incorporation of [^{14}C]-acetate into lipids of normal skin in presence and absence of dapsone.

	Incorporation of acetate into lipids (%)[a]	
Biopsy No.	Control	Dapsone (10 µg/ml)
1	35	44
2	50	53
3	16	16
4	15	18
5	20	29
	27[b]±6 (mean ±SE)	32[b]±4

[a] Results expressed as percentage of radioactivity incorporated into lipids in relation to total uptake of the precursor.
[b] $P<0.05$.

Bacterial lipid synthesis in leprosy tissues

Lepromatous tissue obtained from four different patients were studied for the incorporation of [^{14}C]-acetate in the tissue as well as in the bacteria present in the tissue (table 2). It is evident that both the tissue and bacteria incorporate [^{14}C]-acetate into lipids. Such incorporation was drastically reduced in the heat killed tissue. If the incorporation in total lipids is a relevant metabolic event, then one should be able to identify the various lipid components with the [^{14}C]-label. In table 3 the radioactivity associated with the various lipid components separated by thin layer chromatography are shown. It is clear from this, while incorporation is distributed in several lipid components, the pattern of incorporation is different in normal and infected skin. In infected skin there was much less incorporation into cholesterol as well as diglycerides. An unidentified spot of radioactivity was seen in

Table 2. Incorporation of [^{14}C]-acetate into total lipids of *M. leprae* and its host tissue.

			Tissue lipids from		Bacillary lipids from	
Biopsy number	Bacterio-logical Index[a]	Morpho-logical Index[b]	Control tissue (cpm)	Heat killed tissue (cpm)	Control tissue (cpm)	Heat killed tissue (cpm)
325	5+	2%	47295	2405	17505	1300
335	6+	3%	49250	800	19440	780
402	6+	3%	12045	1000	15455	1030
408	6+	3%	11720	600	3550	260

[a] Bacteriological Index is calculated as nos of bacilli/field

1-10/100/fields 1+
1-10/10 fields 2+
1-10/1 field 3+
1-100/field 4+
1-1000/field 5+
1000/field
clumps 6+

[b] Morphological Index is calculated as percentage of stained bacilli as solid forms: an indication of viable proportion of the total bacilli.

Table 3. Radioactive distribution of [^{14}C]-acetate in the neutral lipids of normal and *M. leprae* infected skin.

	cpm (%)	
Lipids	Normal skin	Infected skin
Cholesterol	12.3	2.3
Unknown	—	5
Triglycerides	36.3	20.5
C_{12}–C_{14}		
Palmitic	19	11.8
Stearic		
Cholesterol	8.8	34.5
Mono- and diglycerides	3	2.7
Glycolipids/phospholipids	20.3	23.5
	99.3	99.3

infected skin, which was not seen in normal skin. It will be shown later that this spot is probably derived from bacteria.

Effect of drugs on lipid synthesis by bacteria

Lepromatous tissue containing *M. leprae* was exposed to the precursor in presence and absence of the drug dapsone using eleven different infected tissues (table 4) and with rifampicin for five infected tissues (table 5). It was observed that the total

Table 4. Effect of dapsone on the incorporation of [^{14}C]-acetate into lipids of *M. leprae.*

	[^{14}C]-Acetate incorporation into total lipids[a] of dapsone-sensitive *M. leprae*				[^{14}C]-Acetate incorporation into total lipids[a] of dapsone-resistant *M. leprae*		
Biopsy No.	Control	Dapsone (10 μg/ml)	Bacteriological load[b]	Biopsy No.	Control	Dapsone (10 μg/ml)	Bacteriological load
277	14	0	4+/2%	261	32	63	4+/4%
282	36	3	—	306	11	27	5+/1%
295a	47	25	5+/3–4%	325	35	39	5+/2%
298	25	20	6+/4%	340	63	72	—
299	50	37	6+/4%	382	39	64	6+/3%
302	12	5	2+/1%	397	100	100	6+/1%
315	29	13	3+/1%	408	54	70	6+/3%
317	40	31	6+/3%	Mean±SD	48±28[d]	62±24[d]	
332	96	68	5+/6%				
335	53	0	6+/3%				
402	87	74	6+/3%				
Mean±SD	44±27[c]	25±28[c]					

[a] Ratio of incorporation into bacteria to that into the tissue (%).
[b] Bacterological Index / Morphological Index
[c] $P<0.05$.
[d] $P<0.001$.

Table 5. Rifampicin effect on the incorporation of [^{14}C]-acetate into lipids of *M. leprae.*

	[^{14}C]-Acetate incorporation[a] into total lipids of *M. leprae*, sensitive to rifampicin				[^{14}C]-Acetate incorporation[a] into total lipids of *M. leprae*, resistant to rifampicin		
Biopsy No.	Control	Rifampicin (5 μg/ml)	Bacteriological load[b]	Biopsy No.	Control	Rifampicin (5 μg/ml)	Bacteriological load[b]
295a	47	26	5+/3–4%	261	32	36	4+/4%
317	40	28	6+/3%	277	14	18	4+/2%
332	96	68	5+/6%	282	36	94	—
397	100	62	6+/1%	302	12	52	3+/1%
335	53	49	6+/3%	306	11	14	5+/1%
Mean±SD	67±28	47±19[c]		325	35	41	5+/2%
				340	63	70	—
				382	39	66	6+/3%
				402	87	100	6+/3%
				408	54	56	6+/3%
				Mean±SD	38±24[d]	55±29[d]	

[a] Ratio of incorporation into bacteria to that into the tissue (%).
[b] Bacterological Index / Morphological Index
[c] $P<0.05$
[d] $P<0.001$.

synthesis of lipids in the bacteria in the presence of dapsone or rifampicin was drastically reduced, in all these cases. The difference in the amount of incorporation between the control and drug treated tissue was statistically significant. If one were to look at lipid components form the bacteria separated from these tissues under the above conditions, it would be clear that the total reduction in lipids was also reflected in the reduced level of incorporation in the presence of dapsone. Virtually most components showed lower level of incorporation in the presence of dapsone and rifampicin.

However, if the lepromatous tissues are derived from patients suspected to harbour resistant bacteria to the drugs under administration or have been proved to have relapse, then the total incorporation into lipids of *M. leprae* is not reduced in the presence of the drug. The data in tables 4 and 5 provide the total incorporation of the label into lipids of the bacteria obtained from suspected drug resistant patients for dapsone and rifampicin respectively. It is clear that the drug has no effect on the corporation into lipids of bacteria indicative of the resistance. The levels of synthesis in the control and drug treated, are not statistically significant.

The control value from the 7 and the 11 samples in table 4 were comparable, but drug treated samples markedly differed showing sensitivity of the bacteria for dapsone in the 11 cases and resistance of the bacteria to the drug in the 7 cases studied. The level of incorporation of the precursor into lipid components was higher whenever drug resistant strains were tested as compared to incorporation in the absence of this drug. We have no explanation for this observation at present. But a clearer idea could be obtained when the drug resistant bacteria are studied in a cell-free system.

Correlation of DOPA method and acetate method

M. leprae from the tissues of the 8 patients were also tested for their sensitivity/resistance to the drugs rifampicin and dapsone by another method developed in our laboratory (Ambrose *et al.,* 1974; 1978) using labelled DOPA. Uptake of DOPA and its conversion to other metabolities is one of the characteristic feature of live *M. leprae* (Prabhakaran *et al.,* 1968; Prabhakaran, 1973). We observed that the drug reduced drastically the uptake of DOPA in the sensitive strains, while it had very little or no effect on DOPA uptake by the resistant strains. These results corroborate well with the drug sensitivity/resistance in the bacteria when the uptake of labelled acetate is measured in the presence of the drugs.

Bacteria from, one of the tissues (FMR 299) were tested for sensitivity to dapsone by the mouse footpad technique (Shepard, 1960). Harvests were carried out after 6, 8 and 10 months of growth in the mice. The results indicate that the bacteria from the patient were sensitive to dapsone as was indicated by the [^{14}C]-acetate uptake system.

Discussion

Experiments carried out with *M. lepraemurium*-infected liver and spleen tissue showed that acetate could be incorporated into mycolic acids but this was not the case with *M. leprae* obtained from skin of infected human subjects. Labeling in mycolic acids could not be obtained in the human bacilli probably for two reasons: a

slow metabolic turnover of this lipid or the pathway for the biosynthesis of these acids involves a different precursor.

But we found that there was a significant incorporation in the chloroform-methanol extractable lipids in tissues containing *M. leprae* as well in the components separated after thin layer chromatography. Furthermore, the observation that dapsone and rifampicin decreased the percentage of label incorporated in the lipid portion of tissues containing *M. leprae* from patients resistant to the drug, while in resistant strains, the acetate incorporation into lipids was not inhibited correlated with the clinical status of the patient.

The absence of a significant difference in the incorporation of label in the control and drug treated normal skin indicated that the drug was probably effecting bacilli and not the metabolism of the tissue components. Biopsies which were examined for [^{14}C]-acetate incorporation into lipids, were also used to study [^{3}H]-DOPA uptake method to test drug sensitivity. The correlation between these two methods was quite good. Bacilli from one of the patients were inoculated into the mouse foot pad and the sensitivity to dapsone was shown even in the mouse.

Screening for drug sensitivity using [^{14}C]-acetate is rapid and results can be obtained within a period of 15 days. It is more informative than the method using [^{3}H]-DOPA uptake because acetate can be traced in the various lipid fractions, especially triglycerides and fatty acids. There is no ambiguity in the results and reproducibility appears to be good. The level of radioactive incorporation is fairly good unlike the incorporation of precursors into DNA (Nath *et al.,* 1982). Bacilli with a Morphological Index of even 1% shows an uptake of the precursor.

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