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Phospholipids of ethambutol-susceptible and resistant strains of *Mycobacterium smegmatis*

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Abstract. The composition, subcellular distribution and rate of synthesis of phospholipids were compared in ethambutol susceptible and resistant strains of *Mycobacterium smegmatis.* Significant quantitative alterations in phospholipids accompanied the acquisition of resistance, whereas fatty acyl group composition of total phospholipid remained the same in ethambutol resistant and susceptible strains. Cell wall of resistant strain exhibited an accumulation of phospholipids and a decrease in the degree of unsaturation of phospholipid fatty acyl groups. Changes in the cell wall phospholipid composition may contribute to resistance of *Mycobacterium smegmatis* to ethambutol.

Keywords. *Mycobacterium smegmatis;* phospholipids; fatty acids; ethambutol; susceptible; resistant; cell membrane; cell wall.

Introduction

Ethambutol (EMB) is an effective and specific antitubercular drug which is frequently used in combination with other antitubercular drugs in chemotherapeutic regimens (Iseman and Goble, 1988). Previous reports in literature have dealt with the effect of EMB on nucleic acid metabolism (Forbes *et al.,* 1965; Bacalao and Reiber, 1972), mycolic acid Synthesis (Takayama *et al.,* 1979; Kilburn and Takayama, 1981) and phospholipid metabolism in mycobacterial species (Kilburn *et al.,* 1981; Cheema and Khuller, 1985). Kilburn *et al.* (1981) observed a decrease in phospholipid synthesis and leakage of phosphatidylethanolamine (PE) out of the cells in *Mycobacterium smegmatis,* upon EMB exposure.

Phospholipids are the major constituents which are associated with the transport of metabolites across the membrane (Fourcans and Jain, 1974). In general antimicrobial action of the drugs involve transport of the drug from external environment to some site on or with in the cell followed by certain alterations in cell membrane (Beggs and Andrews, 1973). There are several reports which suggest that microorganisms alter their lipid composition in order to resist the toxic effect of drugs (Suling and O'Leary, 1977; Gilleland *et al.,* 1984; David and Rastogi, 1985). In mycobacteria, a genus with a high percentage of lipids in cell envelope, investigations involving analysis of phospholipid metabolism of drug resistant strains have not been carried out. Hence, the present investigation was carried out to compare the phospholipid composition, distribution and metabolism in EMB-susceptible and resistant strains of *M. smegmatis* ATCC 607.

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Abbreviations used: EMB, Ethambutol; PE, phosphatidylethanolamine; TLC, thin-layer chromatography; TPL, total phospholipid; CL, cardiolipin; PIM, phosphatidylinositol mannosides.

Materials and methods

[1-^{14}C]–Sodium acetate obtained from Bhaba Atomic Research Centre, Bombay and EMB procured from Lederl Laboratories, USA were used.

Bacterial strain and cultivation

M. smegmatis ATCC 607, originally obtained from NCTC, London was used. Resistant mutant of *M. smegmatis* was isolated by replica plating technique as described by Lederberg and Lederberg (1952). Stability of the mutants was confirmed by subculturing them alternately in EMB-free and EMB containing media. The organisms were grown in modified Youman's medium on rotary shaker at 37°C. Cells were harvested in their respective mid log phases which was 48 h for susceptible strain and 72 h for resistant strain.

Isolation of cell wall and cell membrane fractions

Mycobacterial cells were disintegrated by ultrasonication, according to the method of Hill and Ballou (1966). Subcellular fractions were isolated and purified by differential centrifugation according to the method of Kearney and Goldman (1970). Purity of cell membrane was checked by measuring the activity of ATPase according to the method of Penumarti and Khuller (1983).

Labelling of phospholipids

Cells harvested in log phase were washed with normal saline and resuspended in Kreb's Ringer buffer under sterile conditions. Cells were incubated at 37°C for 1 h under shaking conditions to get a homogenous suspension. (1-^{14}C)-Sodium acetate (25 μCi/100 ml of medium) was added to cells. Incubation was continued for 90 min and at different time intervals 10 ml aliquots from each culture were transferred into tubes containing 0·5 ml of 1 M KCN (Kilburn *et al.,* 1981). The tubes were centrifuged at 2,700 *g* for 15 min. The cell pellet was recovered and lipids extracted. Radioactivity was counted in a Packard Tricarb Liquid Scintillation Counter using a toluene based scintillation fluid containing 0·4% (w/v) PPO and 0·05% (w/v) POPOP.

Extraction and identification of lipids

Lipids were extracted and purified by the method of Folch *et al.* (1957). Individual phospholipid components were seperated by thin-layer chromatography (TLC) on silica gel H plates using solvent system, chloroform: methanol: 7N ammonia (65:25:4, v/v/v). Phospholipids were quantitated by estimating lipid phosphorus according to the method of Bartlett (1959) as modified by Marinetti (1962). The acetone insoluble phospholipids were used for fatty acid analysis (Khuller and Brennan, 1972). Methyl esters of phospholipid fatty acids were prepared by thionyl chloride procedure of Prabhudesai (1978) and were resolved by Nucon Gas Chromatogram (Model 5700) on a 10% diethyleneglycol succinate (60–80 mesh chromosorb W) and OV-1 column

(60–80 mesh Gas Chrom Q) at 190°C using nitrogen as carrier gas. OV-1 was used to seperate tuberculostearic acid and oleic acid. Fatty acids were identified by comparasion of their retention times with authentic standards. The amounts of fatty acids were calculated by triangulation.

Results and discussion

As lipids constitute a major portion of the mycobacterial envelope, an attempt was made to study their role in development of drug resistance. A mutant of *M. smegmatis,* isolated by the replica plating method was found to be resistant to 200 μg/ml of EMB. Identical growth conditions were used for both parent and variant strains and they were harvested at similar phases of growth. Analysis of the total phospholipid (TPL) content of EMB-resistant mutant revealed (table 1) a significantly lower ($P \leqslant 0{\cdot}001$) level when compared to the EMB-susceptible strain. This decrease was reflected in cardiolipin (CL) content, while there was no apparent change in the PE content. Another quantitative alteration observed in the EMB-resistant strain was its increased phosphatidylinositol mannoside (PIM) content ($P \leqslant 0{\cdot}001$). Relative decrease in CL content of EMB-resistant strain is much more than augmentation in its PIM content which probably accounts for decreased TPL content of EMB-resistant strain. However, Cheema *et al.* (1986) observed no quantitative changes in phospholipid content of EMB-susceptible and EMB-resistant (resistant to 54 μg/ml EMB) strains. This inconsistency could be due to high level of resistance of mutant used in the present study.

Table 1. Comparison of phospholipid composition of EMB-susceptible and resistant strains of *M. smegmatis.*

Strain	Total phospholipids mg/g dry wt. of cells	Individual phospholipids (mg/g dry wt. of cells)		
		PIM	PE	CL
EMB-susceptible	25·89 ± 2·71	9·54 ± 1·74	3·21 ± 1·37	13·07 ± 1·94
EMB-resistant	19·76 ± 0·78[a]	13·22 ± 1·39[a]	2·59 ± 0·64	3·94 ± 0·37[a]

Values are mean ± SD from 5 different batches.
[a] $P \leqslant 0{\cdot}001$.

Analysis of phospholipid fatty acids (table 2) revealed that the relative percentage of various fatty acids of the EMB susceptible strain differed from that of the EMB-resistant strain. A significant decrease in the proportion of myristic acid and a

Table 2. Relative percentage of phospholipid fatty acids in EMB-susceptible and resistant strains of *M. smegmatis.*

Strain	Fatty acid composition (relative percentage)						
	14:0	16:0	16:1	18:0	18:1	18:Me	U/S
EMB-susceptible	7·84	39·53	11·45	11·41	9·27	20·54	0·26
EMB-resistant	5·37	33·86	9·80	11·33	10·70	28·97	0·26

U/S, Ratio of unsaturated to saturated fatty acids.
18: Me, Tuberculostearic acid.

significant increase in the proportion of tuberculostearic acid was observed in EMB-resistant strain, when compared to the EMB-susceptible strain. However the ratio of unsaturated to saturated fatty acids (U/S) remained unaltered in the EMB-resistant strain. Alberghina (1976) has also reported homogeneity in fatty acid composition of polar lipid fractions of different sensitive and resistant strains of mycobacteria. It was further suggested that differences could be found in the analysis of fatty acids in definitive subcellular fractions.

Since quantitative changes were observed in TPL content of EMB-susceptible and resistant strains, precursor incorporation studies were carried out to determine phospholipid synthesis in both the strains. Pulse labelling of lipids with [1-^{14}C]–sodium acetate followed for 90min revealed that the incorporation into phospholipids increased continuously with time in susceptible as well as EMB-resistant strains (figure 1). However, the amount of radioactivity incorporated was significantly lower in EMB-resistant cells than susceptible cells. This explains the decreased TPL content of EMB-resistant strain as discussed earlier (table 1).

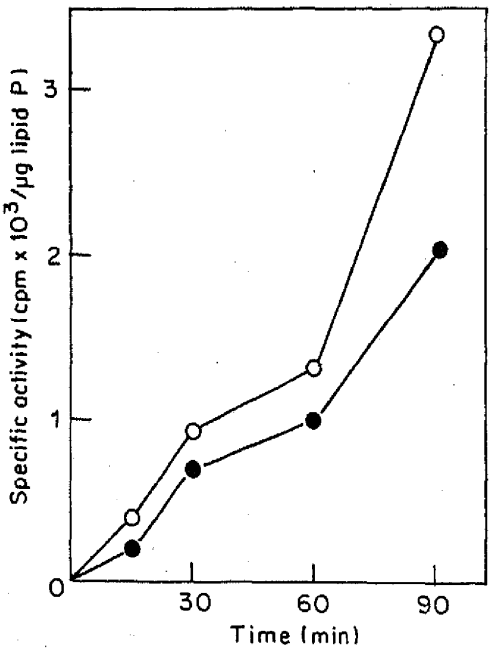

Figure 1. Change in specific activity of TPL of EMB-susceptible (O) and EMB-resistant (1) cells of *M. smegmatis* ATCC 607.

The cell membrane of the wild type strain of *M. smegmatis* contains more phospholipids than its cell wall and is enriched in PIM, as was also observed by Penumarti and Khuller (1983). On the contrary, cell wall of EMB-resistant strain contains more PL than its cell membrane. Unlike sensitive strain, PIM are equally distributed in the wall and membrane fraction of the EMB-resistant strain. Cell wall fraction of mutant strain showed an accumulation of phospholipids accompanied by decreased PL content in cell membrane, when compared with susceptible strain. Individual phospholipid content of cell wall of EMB-resistant strain was also enhanced (table 3).

Table 3. Distribution of phospholipids in cell wall and cell membrane fractions of *M. smegmatis* strains, susceptible and resistant to EMB.

Strain and subcellular fraction	TPL (mg/g dry wt. of cells)	Individual phospholipids (mg/g dry wt. of cells)		
		PIM	PE	CL
EMB-susceptible strain				
Cell wall	27·36 ± 2·97	8·99 ± 1·08	4·15 ± 0·73	14·21 ± 3·42
Cell membrane	50·67 ± 1·27	35·37 ± 1·57	3·15 ± 0·39	12·14 ± 1·20
EMB-resistant strain				
Cell wall	65·65 ± 2·46^c	29·13 ± 2·55^c	10·37 ± 1·22^c	26·25 ± 0·57^b
Cell membrane	42·54 ± 4·75^a	31·56 ± 4·13	4·59 ± 0·51^b	6·38 ± 0·25^c

Values are mean ± SD from 3 independent batches.
$^aP \leqslant 0{\cdot}05$; $^bP \leqslant 0{\cdot}001$; $^cP \leqslant 0{\cdot}001$.

These findings are analogous to those of Mackenzie and Jordon (1970) who observed an accumulation of PL in cell envelope of viomycin resistant *Rhizobium melliloti.* Phospholipid fatty acid composition of EMB resistant cell wall fraction was also different from that of EMB susceptible strain (table 4). There was a significant decrease in the level of unsaturated fatty acids in the cell wall of EMB-resistant strain accompanied by increased level of saturated fatty acids. The decrease in unsaturated fatty acids is revealed in the level of both palmitoleic acid and oleic acid. This accounts for lower U/S ratio of EMB-resistant cell wall than that of the EMB-susceptible preparation. Increase in unsaturated fatty acids is known to increase the membrane permeability, whereas increase in saturation decreases membrane permeability (McElhaney *et al.,* 1973). Thus it can be assumed that a decrease in the unsaturation of cell wall of mutant strain provides a barrier to penetration of drug resulting in decreased sensitivity to the drug. Wada *et al.* (1975) also suggested that an increase in the proportion of unsaturated fatty acids in phospholipids may be associated with increased polymyxin B sensitivity. Cell membrane of EMB-resistant strain exhibited an increase in the level of unsaturated fatty acids, particularly of oleic acid resulting in increased U/S ratio in comparison to EMB-susceptible cell membrane (table 4). Increased unsaturation of cell membrane of mutant strain compensated the decreased unsaturation of cell wall resulting in unaltered U/S ratio of whole cell, as compared to EMB-susceptible strain. David (1980) proposed that resistance in nontubercular mycobacteria is determined by the structure of bacterial

Table 4. Phospholipid fatty acid composition of subcellular fractions of EMB-susceptible and resistant *M. smegmatis.*

Strain	Subcellular fraction	Fatty acid composition (relative percentage)						
		14:0	16:0	16:1	18:0	18:1	18:Me	U/S
EMB-susceptible	Cell wall	7·59	11·59	27·32	16·61	9·97	26·91	0·59
EMB-resistant	Cell wall	9·98	26·84	10·64	35·94	Traces	9·98	0·13
EMB-susceptible	Cell membrane	5·73	23·39	6·14	11·55	8·73	22·22	0·24
EMB-resistant	Cell membrane	6·69	30·37	14·09	11·41	8·44	19·85	0·33

18: Me, Tuberculostearic acid.
U/S. Ratio of unsaturated to saturated fatty acids.

outer layers, which prevent the drugs from reaching the cytoplasmic membrane where transport actually takes place. The observations obtained in the present investigation also suggest that increase in phospholipid content of cell wall might be causing a non specific blanketing action as hypothesised by Anderes *et al.* (1971) for antibiotic resistant *Psudomonas aeruginosa*. Decreased degree of unsaturation of cell wall phospholipid fatty acids also appear to be important in providing a barrier to the penetration of drugs. As a result, the drugs are unable to penetrate into the cell which leads to decreased susceptibility towards drugs. However further studies on uptake of labelled EMB by EMB-susceptible and resistant strain are necessary to confirm this hypothesis.

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