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Biochemical studies with bi-resistant mutants (ethambutol plus streptomycin and isoniazid plus streptomycin) of *Mycobacterium smegmatis* ATCC 607

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Abstract. Biochemical characteristics of bi-resistant mutants (resistant to ethambutol plus streptomycin or isoniazid plus streptomycin) of mycobacteria isolated by replica plating from *Mycobacterium smegmatis* ATCC were compared with those of the drug-susceptible strains. Reduced incorporation of [^{14}C]uracil, [^{3}H]lysine and [^{14}C]acetate into RNA, protein and phospholipids respectively was seen in the resistant mutants. Total phosphorlipids were enhanced in ethambutol plus streptomycin resistant mutant and decreased in isoniazid plus streptomycin resistant mutant. There were similar changes in levels of individual phospholipids. The resistant mutants revealed an accumulation of phospholipids in the cell wall, and a marked decrease of phospholipids in the cell membrane in comparison to the susceptible strain. Several qualitative alterations in the polypeptide profile (with respect to number and molecular weight) of the crude protein extract and of different subcellular compartments were seen in the resistant mutants.

Keywords. *Mycobacterium smegmatis*; isoniazid; ethambutol; streptomycin; cell membrane; cell wall; phospholipids.

Introduction

Tuberculosis chemotherapy regimens involve the concomitant administration of two or more antitubercular drugs because the emergence of organisms resistant to two or more drugs at the same time is less likely. Double-drug combination therapy is more effective than treatment with any single drug (Mitchison, 1984; Grosset *et al.*, 1985). The combination of isoniazid with streptomycin has been demonstrated to be almost as potent as the triple-drug combination of isoniazid-streptomycin-ethambutol (Tsang *et al.*, 1978). The mode of action of each of these drugs is known (Santhanam and Subramanian, 1977). The primary action of isoniazid is its inhibitory effect on mycolic biosynthesis in sensitive cells (Winder and Collins, 1970). Streptomycin affects protein synthesis at ribosomal level and inhibits chain elongation (Shaila *et al.*, 1975). Ethambutol interferes with nucleic acid metabolism (Tsukamura and Mizuno, 1972) as well as synthesis of mycolic acid (Kilburn and Takayama, 1981). Inspite of double-drug or triple-drug regimens, increased drug resistance of mycobacteria has been reported (Mitchison, 1984). Although sufficient information is available on the biochemical aspects of different antitubercular drugs, reports regarding the mechanism of drug resistance in mycobacteria, specially

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Abbreviations used: (INH+SM)R, Isoniazid plus streptomycin resistant; (EM+SM)R, ethambutol plus streptomycin resistant; SDS, sodium dodecyl sulphate; PAGE, Polyacrylamide gel electrophoresis; CL, cardiolipin; PIMs, phosphatidylinositolmannosides; PE, phosphatidylethanolamine; TPL, total phospholipids.

double-drug-resistant mutants are limited. This communication deals with the biochemical variations in drug-resistant and susceptible strains of *Mycobacterium smegmatis* ATCC 607.

Materials and methods

Culture and growth of the organism

A culture of *M. smegmatis* ATCC 607 was maintained on Lowenstein-Jensen medium. The two bi-resistant mutants, one resistant to a combination of isoniazid (1000 μg/ml) and streptomycin (150 μg/ml), designated (INH + SM)R, and one resistant to a combination of ethambutol and streptomycin (100 μg/ml each), designated (EMB + SM)R, were isolated in the laboratory by replica plating from the wild strain (*M. smegmatis* ATCC 607) as described by Lederberg and Lederberg (1952). The stability of mutants resistant to drugs was confirmed by maintaining them on drug-free media for 2–3 months and then transferring them to medium containing drug(s). The mutants were maintained on solid Youman's medium in screw-capped vials with or without drug(s). The cultures were grown in the modified Youman's medium as stationary cultures at 37°C and harvested in the mid-log phase.

Macromolecular synthesis

The incorporation of [^{3}H]lysine (sp. activity 5600 mCi/mmol), [^{14}C]acetate (sp. activity 58·46 mCi/mmol) and [^{14}C]uracil (sp. activity 56mCi/mmol) (Bhabha Atomic Research Centre, Bombay) into proteins, phospholipids and RNA was monitored. Mid-log-phase cells were harvested, washed with physiological saline, and resuspended in sterile Youman's medium. The cells were preincubated under shaking conditions at 37°C for 30 min. Radiolabelled precursor was then added and incubation was continued for 2 h at 37°C. The reaction was terminated and processed as described earlier (Khuller *et al.,* 1984).

Extraction and identification of lipids

Lipids were extracted and purified by the method of Folch *et al.* (1957). The individual phospholipids were separated by thin-layer chromatography (TLC) on silica gel H plates using the solvent system choloroform: methanol: 7 N 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).

Isolation of cell wall and cell membrane fractions

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 the subcellular fractions was assessed by electron microscopy and by measuring the

activity of ATPase, a marker enzyme for cell membranes, according to the method of Penumarti and Khuller (1983).

Metabolism of phospholipids

Synthesis and degradation of phospholipids in mid-log-phase cells were examined by the pulse-chase technique of Kanfer and Kennedy (1963) using [^{32}P]ortho-phosphoric acid as described earlier (Mahajan and Khuller, 1984).

Sodium dodecyl sulphate Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) was done on slab gels by the method of Laemmli (1970). The gels were scanned densito-meterically on an LKB 2202 Ultroscan laser densitometer at 750 nm at a scanning speed of 80 mm/min.

Results and discussion

Development of resistance to the combination of drugs retarded the growth (figure 1) of both the mutants, *viz.,* (INH + SM)R and (EMB+SM)R. The log phase was

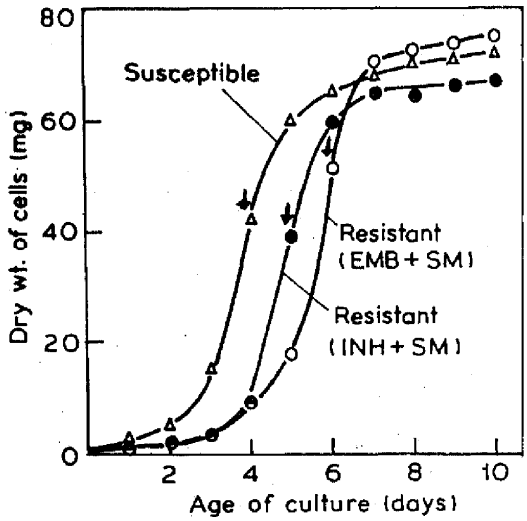

Figure 1. Growth patterns of susceptible and resistant cultures of *M. smegmatis.* Arrows indicate time of harvesting of cells.

prolonged, as has been reported for *Psuedomonas aeruginosa* resistant to quarter-nary ammonium compound (Anderes *et al.,* 1971). Therefore susceptible cultures where harvested after 4 days of growth, and (INH + SM)R and (EMB + SM)R cultures after 5 and 6 days respectively.

Incorporation of the appropriate radiotracers in mid-log-phase cultures was

monitored in order to examine macromolecular synthesis in susceptible and resistant cultures (table 1). In comparison with the susceptible strain, the (INH + SM)R

Table 1. Incorporation of [^{14}C]uracil, [^{3}H]lysine and [^{14}C]acetate into macromolecules of drug-susceptible and resistant mutants of *M. smegmatis.*

Cell type	[^{14}C]uracil (cpm/mg protein)	[^{3}H]lysine (cpm/mg protein)	[^{14}C]acetate (cpm/mg of lipid phosphorus)
Susceptible	42,950 ± 6,400	2,270 ± 120	30,410 ± 3,950
(EMB + SM)R	12,780 ± 580***	970 ± 100***	15,820 ± 580***
(INH + SM)R	5,320 ± 410***	1,110 ± 120***	14.520 ± 380***

Values are mean ± SD of 3 independent batches analysed in triplicate.
***p<0·001.

mutant showed an approximately 8-fold (~88%) decline in [^{14}C]uracil incorporation, and ~50% decrease in labelled lysine and acetate incorporation. Similarly, the (EMB + SM)R mutant revealed a 70% decrease in RNA synthesis, while protein and phospholipid synthesis were decreased by 50%. The reduced incorporation of all 3 radiolabelled precursors indicates lower metabolic status of the mutants.

The total phospholipid content of the bi-resistant mutant (INH+SM)R was significantly decreased in comparison with that of the susceptible strain (table 2).

Table 2. Phospholipid composition of drug-susceptible and resistant mutants of *M· smegmatis*

Cell type	Total phospholipid (mg/g dry wt of cells)	Phospholipid composition		
		PIMs	PE (mg/g dry wt of cells)	CL
Susceptible	25·45 ± 0·40	11·51 ± 0·93	3·07 ± 0·69	10·92 ± 0·06
(EMB + SM)R	31·02 ± 0·66	15·33 ± 0·98**	5·92 ± 0·78**	9·14 ± 0·32
(INH + SM)R	14·52 ± 0·70***	7·11 ± 1·63**	1·83 ± 0·21*	5·13 ± 1·13***

Values are mean ± SD of 3 independent batches analysed in triplicate.
*P<0·05; **P<0·01; ***P<0·001.

The decrease cannot be attributed to resistance to isoniazid or streptomycin alone as monoresistant mutants of *M. smegmatis* to INH and SM did not reveal any change in total phospholipids (Kanwar and Khuller, 1988). In contrast, in the (EMB + SM)R mutant a marked increase in the total phospholipid content was apparent. Increased lipid content has been suggested to be responsible for drug resistance in other microorganisms (Mackenzie and Jordan, 1970; Norrington and James, 1970; Chang *et al.,* 1972). Individual phospholipids analysed were significantly decreased in (INH + SM)R and increased in (EMB + SM)R, in agreement with decrease/increase in total phospholipid content. The decrease was most prominent in cardiolipin (CL), followed by phosphatidylinositolmannosides (PIMs) and phosphatidylethanolamine (PE) (table 2). Alterations in levels of individual phospholipids may be due to altered activities of the particular phospholipid metabolizing enzymes.

Since changes were observed in phospholipid composition, metabolism of phos-

pholipids was examined. Net synthesis of phospholipids was reduced in both mutants (figure 2). The distribution of radiolabel in individual phospholipids after a

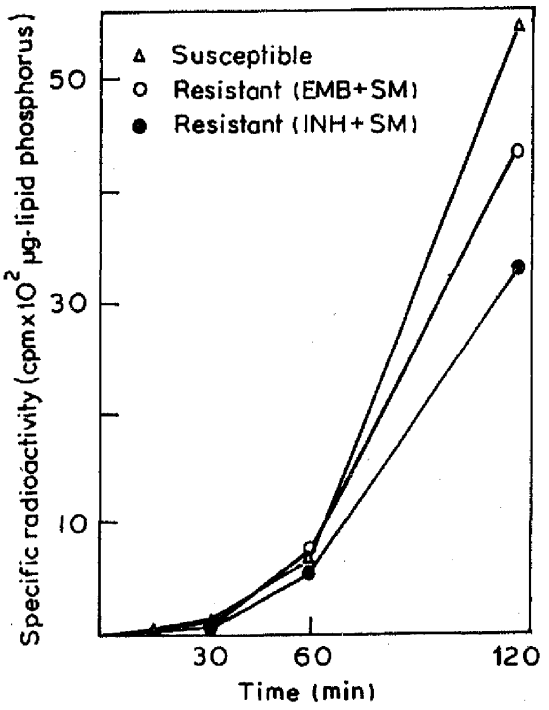

Figure 2. Specific radioactivity incorporated into phospholipids of susceptible and resistant cultures of *M. smegmatis.*

120 min pulse indicated that in the mutants PE synthesis was highest, followed by PIMs and CL; in the susceptible cells sequence (decreasing order of net synthesis) was CL, PIMs and PE, in agreement with the observations of Dhariwal *et al.* (1978). The increase in the net synthesis of PE with concomitant decrease in the net synthesis of cardiolipin in the mutants suggest that these phospholipids are possibly synthesized by a common pathway, as hypothesized for phospholipid synthesis in *E. coli* (Miyazaki *et al.,* 1985; Kobayashi *et al.,* 1986).

Following the pulse of radioactive precursor, the label was chased for 24 h after removing extracellular [^{32}P]orthophosphate. In susceptible cells, increased in corporation was observed up to 2h, followed by loss of radioactivity. In resistant cells, there was continuous incorporation up to 4–8 h, followed by loss during the subsequent period, probably because of slow utilization of labelled precursors in these cells. Increased degradation of phospholipids was seen in the (INH + SM)R mutant, compared to the susceptible strain, while the (EMB + SM)R mutant showed decreased degradation (data not presented). These results suggest that the alterations seen in total phospholipid content (table 2) are due to changes in degradation rate rather than synthesis.

The subcellular distribution of phospholipids was investigated. It is evident from table 3 that both cell wall and cell membrane of *M. smegmatis* contain phospho-

Table 3. Phospholipid composition (mg/g dry weight of cell fraction) of subcellular fractions of drug-susceptible and resistant mutants of *M. smegmatis*.

	Cell type		
	Susceptible	(EMB+SM)R	(INH+SM)R
Cell wall			
TPL	25·35 ± 1·79	63·83 ± 7·57***	39·45 ± 1·34***
PIMs	14·95 ± 1·64	43·46 ± 4·06***	25·87 ± 2·51***
PE	1·76 ± 0·31	9·40 ± 1·25***	3·46 ± 0·94*
CL	8·09 ± 1·12	10·65 ± 1·85*	11·47 ± 1·37**
Cell membrane			
TPL	60·17 ± 0·46	39·45 ± 1·34***	41·62 ± 1·57***
PIMs	37·18 ± 1·30	17·97 ± 0·53***	29·38 ± 2·40**
PE	8·30 ± 0·68	3·62 ± 0·42***	4·82 ± 10·39***
CL	14·70 ± 0·79	8·78 ± 1·22***	6·58 ± 1·00***

Values are mean ± SD of 3 different batches analysed in triplicate.
*P<0·05; **P<0·01 ***P<0·001.

lipids and that the latter has comparatively more phospholipid. This distribution of phospholipids in the wall and membrane fractions of the susceptible strain is in agreement with the earlier results of Kates (1964), Asselineau (1966) and Mahajan and Khuller (1983). As no report is available on phospholipid composition of subcellular fractions in drug-resistant mutants of mycobacteria, the present results have been compared with those for other microorganisms. Both the double-resistant mutants showed significant alterations in total and individual phospholipid content of the cell wall, like Gram-negative antibiotic-resistant bacteria (Mackenzie and Jordan, 1970). These findings in general support the hypothesis that the amount of phospholipids in the cell wall plays a role in determining the spectrum of drug sensitivity by an exclusion mechanism (Brown, 1971). In contrast to the situation in the cell wall, total phospholipid content of the cell membrane fraction was considerably decreased in the mutants, as in polymyxin-resistant *Pseudomonas aeruginosa* (Brown and Wood, 1972) and penicillin-resistant gonococci (Mavrov *et al.,* 1986).

In keeping with the alterations in the total phospholipid (TPL) content of cell wall and cell membrane in the mutants, the levels of PIMs, PE and CL were also elevated in the cell wall and decreased in the cell membrane. Elevation of PE level in the cell wall is of significance as polar lipids have a prominent role in the formation of a cell surface structure that regulates permeability (Alberghina, 1976). Alterations in PE content may possibly modulate surface charge in drug-resistant mutants.

The protein profiles of the drug-susceptible and resistant strains were analysed by SDS-PAGE. Qualitative changes were seen in the polypeptide profiles of crude protein extract, cell wall and cell membrane of the mutants, implying that these components are involved in the drug resistance. These observations are in accordance with those made on *Acromonas salmonicida* (Wood *et al.,* 1986).

The observations presented here clearly demonstrate that development of drug resistance in mycobacteria leads to alterations in the composition of cell wall and cell membrane.

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