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Effect of Anti-tuberculosis Drugs on the Iron-Sequestration Mechanisms of Mycobacteria

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ABSTRACT

The effect of sub-lethal concent-rations of isoniazid, ethambutol, rifampicin and pyrazinamide on the growth *in vitro* and the production of both exochelins and mycobactins by the high virulent and the South Indian low virulent strains of *M. tuberculosis* was examined under iron-deficient and iron-rich conditions. There was a marked decrease in the growth of both strains in the presence of increasing concentrations of all four drugs, the inhibition being total in the presence of minimal inhibitory concentrations of the drugs. It was also observed that the growth-inhibitory effect of all four drugs was slightly reversed in the presence of high concentration of iron in the medium. A significant increase was observed in the concentrations of both siderophores in the presence of all four drugs, under both iron-deficient (or) iron-rich conditions.

Keywords : Anti-tuberculosis drugs, Iron transport, Mycobacteria

INTRODUCTION

With the onset of infection and an elevation in the body temperature, the host limits the availability of essential nutrients by a process. called nutritional immunity. Of these nutrients, iron is recognised to be vital for the survival and proliferation of micro-organisms within the host. Mycobacteria also require iron for their survival within the host. To meet the demand for iron, mycobacteria synthesize and utilize specific high affinity iron-binding compounds which help them grow in the iron-restricted conditions of the host[1,2]. Two types of iron-binding compounds are produced[3] : exochelins, occurs extracellulary to act as scavenger and mycobactin, occurs on the cell wall to act as transport and as a store for iron.

Our present knowledge of several host-defence mechanisms in tuberculosis is incomplete, particularly with respect to nutritional immunity. Thus for instance, very little is known about the iron-sequestration mechanism adopted by mycobacteria during infections like tuberculosis. Among the several mechanisms proposed for the action of anti-tuberculosis drugs such as isoniazid and ethambutol against tubercle bacilli is the capacity of these drugs to chelate metal ions essential for the growth of these micro-organisms[4]. An investigation was therefore undertaken to study the effect of sub-lethal levels of these drugs on the growth *in vitro* and the production of both exochelins and mycobactins by the high virulent $H_{37}R_V$ and the South Indian low virulent (SILV) strains of *M. tuberculosis* under iron-deficient and iron-rich conditions. A similar investigation was undertaken with other anti-tuberculosis drugs such as rifampicin and pyrazinamide, which are not known to have any metal ion binding properties.

Table 1 Effect of sub-lethal concentrations of anti-tuberculosis drugs on the mean cell dry-weight (of 4 estimates) by the virulent ($H_{37}R_v$) and the South Indian low virulent (SILV) strains of *M. tuberculosis* under iron-deficient (0.02 µg/ml) and iron-rich (4.0 µg/ml) conditions.

Drug	Drug concentrations in the medium (µg/ml)	Mean cell dry-weight (g/100 ml) standard deviation according to stain and iron concentration (µg/ml)			
		$H_{37}R_v$		SILV	
		0.02	4.0	0.02	4.0
Isoniazid	0	0.109 ± 0.01	1.077 ± 0.01	0.087 ± 0.01	0.910 ± 0.02
	0.0125	0.046 ± 0.01	0.314 ± 0.01	0.045 ± 0.01	0.205 ± 0.01
	0.0250	0.012 ± 0.01	0.07 ± 0.01	0.011 ± 0.01	0.057 ± 0.01
	0.0500	NG	NG	NG	NG
Ethambutol	0	0.109 ± 0.010	1.077 ± 0.097	0.087 ± 0.007	0.910 ± 0.016
	0.5	0.069 ± 0.003	0.397 ± 0.020	0.060 ± 0.002	0.258 ± 0.037
	1.0	0.017 ± 0.002	0.093 ± 0.008	0.019 ± 0.001	0.087 ± 0.009
	2.0	NG	NG	NG	NG
Rifampicin	0	0.125 ± 0.017	1.074 ± 0.055	0.089 ± 0.003	0.897 ± 0.017
	0.0125	0.040 ± 0.002	0.283 ± 0.007	0.043 ± 0.004	0.308 ± 0.011
	0.0250	0.013 ± 0.001	0.086 ± 0.003	0.018 ± 0.001	0.073 ± 0.004
	0.0500	NG	NG	NG	NG
Pyrazinamide	0	0.125 ± 0.017	1.074 ± 0.055	0.089 ± 0.003	0.897 ± 0.017
	2.5	0.070 ± 0.001	0.309 ± 0.007	0.073 ± 0.004	0.315 ± 0.005
	5.0	0.017 ± 0.002	0.088 ± 0.005	0.020 ± 0.001	0.088 ± 0.005
	10.0	NG	NG	NG	NG

NG= no growth

MATERIAL & METHODS

Organisms and growth *in vitro:* The drug-susceptible, high virulent $H_{37}R_v$ and the South Indian low virulent (SILV) strains of *M. tuberculosis* were inoculated into a synthetic medium (pH 6.8) which was prepared in iron-free glassware. The minimal inhibitory concentrations of liquid medium were 0.05 µg/ml for isoniazid and rifampicin, 2.0 µg/ml for ethambutol and 10.0 µg/ml for pyrazinamide. The concentrations of the drugs employed in the present experiment were 0, 0.0125, 0.025 and 0.05 µg/ml fir usibuazid (Bayer Leverkusen, Germany) and rifampicin (Polfa Tarchomin, Poland), 0, 0.5, 1.0 and 2.0 µg/ml for ethambutol (Sigma, USA) and 0, 2.5, 5.0 and 10.0 µg/ml for pyrazinamide (Merck Sharp & Dohme Ltd, UK). The concentration of iron employed (Fe^{++}) in the medium were 0.02 and 4.0 µg/ml. Incubation was at 37°C for 35 days without shaking.

Determination of cell dry-weight: Cell dry-weights were determined using preweighed filters with drying to constant weight at 100°C.

Extraction and estimation of exochelins and mycobactins: Exochelins were converted to their ferri-complexes and extracted into chloroform from cell-free culture filtrates[6]. Mycobactins were isolated by ethanol extraction of freshly harvested, moist mycobacterial cells[7]. Both the siderophores were estimated gravimetrically.

The experiment was set up in quadriplicate and the estimations were statistically analysed employing students t-test (Paired and unpaired).

OBSERVATIONS

There was a significant decrease in the growth of both $H_{37}R_v$ and SILV with increasing concentrations of isoniazid in the medium ($P < 0.001$) upto the minimal inhibitory concentration of the drug under both iron-deficient and iron-rich conditions (Table 1). There was no growth when isoniazid 0.05 µg/ml (MIC) was added. to the medium under both iron-deficient and iron-rich conditions. However, the growth under iron-rich conditions was substantially higher ($p < 0.001$) than under iron-deficient conditions both in the presence and in the absence of isoniazid in the medium. The growth of SILV under iron-deficient (or) iron-rich conditions was significantly lower than that of $H_{37}R_v$ ($P<0.01$) in the presence and in the absence of isoniazid in the medium.

There was a significant increase in the release of exochelins of both $H_{37}R_v$ and SILV with increasing concentrations of isoniazid in the medium ($P<0.001$) upto the minimal inhibitory concentration of the drug under both iron-deficient and iron-rich conditions (Table 2). The release of exochelins of $H_{37}R_v$ was significantly higher ($P<0.01$) than that of SILV under both iron-deficient and iron-

Table 2 Effect of sub-lethal concentrations of anti-tuberculosis drugs on the mean Exochelin concentrations (of 4 estimates) by the virulent ($H_{37}R_v$) and the South Indian low virulent (SILV) strains of *M.tuberculosis* under iron-deficient (0.02 µg/ml) and Iron-rich (4.0 µg/ml) conditions.

Drug	Drug concentrations in the medium (µg/ml)	Mean cell dry-weight (g/100 ml) *standard deviation according to stain and iron concentration (µg/ml)			
		$H_{37}R_v$		SILV	
		0.02	4.0	0.02	4.0
Isoniazid		34.4 ± 03.2	09.5 ± 0.1	31.5 ± 4.4	08.1 ± 0.5
	0.0125	53.3 ± 08.0	19.1 ± 0.6	38.6 ± 11.3	13.4 ± 2.0
	0.0250	85.2 ± 14.1	26.1 ± 2.4	90.1 ± 11.8	17.7 ± 2.2
	0.0500	NE	NE	NE	NE
Ethambutol	0	34.4 ± 03.2	09.5 ± 0.1	31.5 ± 4.1	08.1 ± 0.5
	0.5	58.5 ± 02.2	16.5 ± 0.7	38.5 ± 6.7	13.7 ± 1.5
	1.0	74.7 ± 21.4	21.7 ± 1.9	52.7 ± 2.3	23.2 ± 2.6
	2.0	NE	NE	NE	NE
Rifampicin	0	35.2 ± 2.2	09.8 ± 0.2	30.9 ± 4.9	08.1 ± 0.5
	0.0125	49.5 ± 2.6	17.7 ± 0.4	46.6 ± 4.3	13.0 ± 0.5
	0.0250	80.6 ± 8.4	23.4 ± 0.7	55.0 ± 3.7	27.4 ± 1.5
	0.0500	NE	NE	NE	NE
Pyrazinamide	0	35.2 ± 2.2	09.8 ± 0.2	30.9 ± 4.9	08.1 ± 0.5
	2.5	46.7 ± 6.5	14.5 ± 1.6	41.0 ± 2.3	12.8 ± 0.2
	5.0	59.3 ± 6.4	22.8 ± 1.3	51.4 ± 3.4	22.8 ± 1.3
	10.0	NE	NE	NE	N E

NE=No Exochelin

Table 3 Effect of sub-lethal concentrations of anti-tuberculosis drugs on the mean mycobactin concentrations (of 4 estimates) by the virulent ($H_{37}R_v$) and the South Indian low virulent (SILV) strains of *M.tuberculosis* under iron-deficient (0.02 µg/ml) and iron-rich (4.0 µg/ml) conditions.

Drug	Drug concentrations in the medium (µg/ml)	Mean cell dry-weight (g/100 ml) ±standard deviation. according to stain and iron concentration (µg/ml)			
		$H_{37}R_v$		SILV	
		0.02	4.0	0.02	4.0
Isoniazid	0	82.6 ± 02.3	22.6 ± 0.5	54.7 ± 03.1	20.1 ± 1.1
	0.125	108.9 ± 06.9	36.1 ± 0.9	66.3 ± 03.0	30.5 ± 1.2
	0.0250	145.5 ± 36.5	42.3 ± 4.0	90.1 ± 11.8	59.0 ± 3.8
	0.0500	NM	NM	NM	NM
Ethambutol	0	082.6 ± 02.3	22.6 ± 0.5	054.7 ± 3.1	20.1 ± 1.1
	0.5	120.4 ± 03.7	30.4 ± 1.0	067.l ± 1.9	30.2 ± 1.4
	1.0	136.3 ± 19.6	40.4 ± 3.5	105.5 ± 4.5	40.l ± 3.5
	2.0	NM	NM	NM	NM
Rifampicin	0	083.4 ± 02.2	22.9 ± 0.7	053.6 ± 4.4	21.4 ± 0.7
	0.0125	104.8 ± 08.3	35.4 ± 0.8	069.8 ± 6.5	27.7 ± 2.8
	0.0250	161.3 ± 16.8	42.7 ± 4.7	110.0 ± 7.3	45.0 ± 4.9
	0.0500	NM	NM	NM	NM
Pyrazinamide	0	084.8 ± 02.2	22.8 ± 0.7	052.4 ± 4.4	21.4 ± 0.7
	2.5	103.4 ± 05.7	27.5 ± 1.3	061.3 ± 4.9	27.8 ± 1.2
	5.0	118.7 ± 12.8	34.2 ± 2.0	102.9 ± 6.8	42.6 ± 3.8
	10.0	NM	NM	NM	NM

NM=No Mycobactin

rich conditions in the presence and in the absence of isoniazid.

The production of mycobactins of both $H_{37}R_v$ and SILV also significantly increased with increasing concentrations of isoniszid in the medium ($p<0.001$) upto the MIC of the drug under. both iron-deficient and iron-rich conditions (Table 3). The production of mycobactins of $H_{37}R_v$ was significantly higher ($p< 0.001$) than that of SILV under both iron-deficient and iron-rich conditions in the presence and in the absence of isoniazid. The production of both exochelins and mycobactins was substantially less under iron-rich conditions than under iron-deficient conditions ($p<0.001$) in the presence and in the absence of isoniazid. The effect of rifampicin, ethambutol and pyrazinamide on the growth (Table 1) of $H_{37}R_v$ and SILV and the production of exochelins (Table 2) and mycobactins (Table 3) was similar to that of isoniazid.

DISCUSSION

Among the several mechanisms proposed for the action of some anti-tuberculosis drugs such as isoniazid, ethambutol, p-aminosalicylic acid, streptomycin and cycloserine is their capacity to withhold metal ions required for the growth of tubercle bacilli by chelation[4]. There is no evidence to suggest that other anti-tuberculosis drugs such as rifampicin, ethionamide (or) kanamycin have the

capacity to hold metal ions. Salicylates and dihydroxy benzoic acids have been recognized as siderophores in several bacterial and fungal species such as *M. smegmatis, Aerobacter aerogenes, E. coli* and certain species of *Salmonella*[8,9]. Ratledge and coworker have worked out in detail the action of PAS against *M. smegmatis* and *M. bovis* BCG with respect to acquisition or iron by these strains[10,11]. Under conditions of iron deficiency (0.1 µg/ml), PAS was shown to strongly inhibit the formation of mycobactin. Further, uptake of iron was also inhibited and a decline was observed in the activity of several iron-containing enzymes. Brown & Ratledge[11] have suggested that PAS might be exerting its primary inhibitory effect by blocking the transfer of iron from mycobactin to an intra-cellular acceptor.

Findings presented in this report show that there is marked decrease in the growth of both the $H_{37}R_v$ and the SILV stains in the presence of increasing. concentrations of isoniazid and ethambutol, the inhibition being total in the presence of the respective minimal inhibitory concentrations of the two drugs. It was also observed that the growth inhibitory effect was slightly reversed in the presence of a high concentration of iron in the medium. Further, an appreciable increase was observed in the production of both exochelins and mycobactins, both under iron-deficient and iron-sufficient conditons. These findings therefore, suggest a role for these two drugs in the withholding of iron from the bacteria. Surprisingly, however, a very similar pattern of findings, including a reversal of the growth-inhibitory effect in the presence of excess iron and an appreciable increase in the production of both siderophores, was observed with rifampicin and pyrazinamide, drugs which are no: known to have any capacity for holding metal ion. These findings, therefores, suggest that the iron-withholding effect of isoniazid and ethambutol is non-specific. The increase in the production of both sidephores might also be a non specific reaction of the bacilli in an attempt to survive under conditions inimical to growth.

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