REVERSAL OF MYCOBACILLIN INHIBITION REACTIONS BY STEROLS AND PHOSPHOLIPIDS

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Sterols like cholesterol, ergosterol, ergocalciferol and stigmasterol can antagonize various reactions of the polypeptide antibiotic mycobacillin on sensitive cells of *Aspergillus niger*, *e. g.* growth inhibition, agglutination and release of ultraviolet-absorbing compounds from the cells. Among the lipids tested *viz.* lecithin, sphingomyelin and cephalin, only lecithin is able to antagonize these antifungal reactions of mycobacillin. The growth-inhibiting property of the antibiotic on *A. niger* is antagonized by intracellular sterols and lipids isolated from the same sensitive organism. The intracellular antagonists have been characterized mainly as cholesterol and lecithin.

Several papers are available on antagonistic actions of sterols, lipids and many other chemical compounds against the activity of antibiotics. So it has been reported that cholesterol, by forming complexes with nystatin, antimycoin and filipin prevents the antifungal action of these polyene antibiotics.[1] Furthermore, there are reports of antagonistic action of phospholipids on the activity of tyrothricin, a mixture of polypeptide antibiotics[2]. Recently NOSE *et al.* have studied the reversal by phospholipids of the antibiotic activity of pyrrolnitrin, a pyrrole derivative.[3] These authors also traced back the antagonistic action of this compound to the antibiotic-lipid interaction. However, to our knowledge, there is no report on the antagonistic action of sterols and lipids on antifungal polypeptide antibiotics except those on mycobacillin[8].

The isolation of mycobacillin and the determination of its amino acid sequence have been performed by MAJUMDER and BOSE[4,5]. The mode of action of this antibiotic was studied with a sensitive strain of *Candida albicans*[6,7] and a filamentous fungus like *Aspergillus niger*[8]. Recently it was shown that the action of mycobacillin on *C. albicans* is antagonized by cholesterol[9]. The present paper deals with the protective effect of sterols and phospholipids (both commercial compounds, and compounds isolated from *A. niger*) against antibiotic reactions of mycobacillin towards the filamentous fungus *A. niger*.

Materials and Methods

A sensitive strain of *A. niger* (G_3Br) was used throughout the experiments. For agglutination reactions another strain of *A. niger* (G_4) which profusely forms spores was used. The organisms were maintained on CZAPEK agar slants. For obtaining mycelial growth CZAPEK broth was used.

Mycobacillin was isolated according to methods used by MAJUMDER and BOSE[4,5]. Sterols were obtained from Sigma Chemical Co. Lecithin was obtained from Dr. B. N. GHOSH, Serologist and Chemical Examiner to the Government of India, Calcutta-16. Cephalin and sphingomyelin were obtained from Sigma Chemical Co. All the compounds were used in ethanolic solution. A stock solution of 2,000 μg/ml was prepared and experimental solutions of desired strength were made therefrom.

Antagonistic action towards growth inhibition: Growth inhibition was measured by the standard cup-plate method using 5-days' old spore suspension of *A. niger* (G_3Br) as an inoculum.

Release of ultraviolet-absorbing compounds from mycelia of *A. niger*: Mycelia, pre-grown for 3 days in CZAPEK broth were added to each flask. The ultraviolet absorption of supernatants was taken at 260 and 280 mμ by a Beckmann (DU) spectrophotometer.

Agglutination reaction: For agglutination the reaction mixtures consisting of 4 ml CZAPEK medium, 5 ml spore suspension (Density according to Klett reading=200 at 660 mμ filter) and 1 ml solution of mycobacillin (250 μg/ml) or sterol or lipid, or the mixture of mycobacillin (250 μg/ml) and sterol or lipid in concentrations as indicated in Table 3 were shaken for 1 hour at 37°C in 50 ml conical flasks on a rotatory shaker and incubated at 37°C or 4°C for desired intervals. Observations were made visually.

Isolation of sterol and lipid from *A. niger* mycelium: Three-days' old mycelium of *A. niger* (G_3Br) was freed from medium by washing with distilled water. After removing excess water with filter paper the mycelial mass was cut into pieces, ground into a paste with quartz powder and taken up in chloroform - methanol (2 : 1) mixture. The resulting mixture was then stirred on a magnetic shaker for four hours and finally filtered. The filtrate was washed 4 times with distilled water and evaporated to dryness under nitrogen atmosphere in a flash evaporator. The residue was dissolved in chloroform and charged on a silicic acid column (25 cm long). Different fractions were collected by gradient elution using chloroform and methanol. Components of the eluates were identified by thin-layer chromatography.

In order to perform a more thorough identification of cholesterol, the above procedure for the isolation of sterol and lipid was modified to eliminate phospholipid by precipitation with acetone at an earlier step. For this purpose 60 g of freeze-dried cells of *A. niger* were heated with methanol for 5 minutes at 65°C with stirring. After addition of chloroform the mixture was again heated with stirring at 65°C for 20 minutes and was finally filtered. The filtrate was washed with distilled water and was evaporated to dryness. The residue was dissolved in a small amount of chloroform, and 20 volumes of cold acetone were added to this solution. The precipitate which contained phospholipid was discarded. In order to work up for sterol the filtrate was evaporated to dryness. The residue was dissolved in a small amount of chloroform and charged to a neutral alumina column (10 cm). The column was charged with benzene and fractions of the eluate were collected in 10-ml batches. After collection of 50 ml of eluate, further elution of the column was performed with chloroform. The eluate was now collected in 5 batches of 10 ml. All these 10 batches were tested for sterol by the thin-layer chromatographic technique using three different solvent systems and also three different spraying agents. Authentic cholesterol was used as a reference compound.

Results

Antagonistic action of sterols and lipids on the growth inhibiting property of mycobacillin:

Table 1 shows that cholesterol, ergosterol, ergocalciferol and stigmasterol antagonize the growth inhibiting action of mycobacillin. Among the lipids *viz.* lecithin, sphingomyelin and cephalin, only lecithin is able to antagonize the antibiotic effect of

mycobacillin.

Effect of cholesterol and lecithin on the releasing action of the antibiotic in presence and in absence of saccharose:

It appears from Table 2(a) and 2(b) that the releasing action of mycobacillin on mycelium of *A. niger* is antagonized by both cholesterol and lecithin although in case of the latter compound the antagonistic action is not expressed very clearly, especially when the releasing action was measured in terms of 260 mμ absorbing materials. In most cases the presence of saccharose, possibly acting as an osmotic stabilizer, has a decreasing effect on the leakage of ultraviolet-absorbing compounds from the fungal cells. But again, this minimized release is also antagonized by cholesterol and lecithin.

Agglutinating action of mycobacillin in presence of antagonists:

It is observed that the agglutinating action of mycobacillin on the fungal spores is prevented by the presence of cholesterol, ergosterol, ergocalciferol and lecithin at concentrations as indicated in Table 3.

Table 1. Antagonistic action of sterols and lipids on the growth-inhibiting property of mycobacillin against *A. niger*

Name of sterol or lipid	Concn. of sterol or lipid (μg/ml)	Ratio of mycobacillin and sterol or lipid		Diameter of zone of inhibition (mm)
		Conc. ratio	Molar ratio	
Cholesterol	125	2 : 1	8 : 1	0
Ergosterol	500	1 : 2	2 : 1	0
Ergocalciferol	500	1 : 2	2 : 1	0
Stigmasterol	500	1 : 2	2 : 1	0
Lecithin	125	2 : 1	4 : 1	0
Sphingomyelin	500	1 : 2	*	21
Cephalin	500	1 : 2	*	22

Growth inhibition was measured by the cup plate method. The concentration of mycobacillin is always 250 μg/ml. For mycobacillin (250 μg/ml) alone, the diameter of zone of inhibition is 24 mm.
'0' indicates absence of a zone of inhibition.
* The molecular weight is not known.

Table 2(a). Effect of cholesterol on the release by mycobacillin of UV-absorbing compounds from mycelia of *A. niger* in the presence and absence of sucrose [a]

Flask No.	Reagents added (ml) to 6 [b] ml of Na-phosphate buffer (pH 7) solution		Absorbancy at	
	Zero time	After 20 hours	260 mμ	280 mμ
1	Alcohol, 1,0	Mycobacillin, 1.0 Cholesterol, 1.0	0.200	0.115
A	〃	Mixture (MC), 1.0 〃	0.120	0.105
2	Mycobacillin, 1.0	Alcohol, 1.0 Cholesterol, 1.0		
B	〃	Mixture (MC), 1.0 〃	0.265 0.210	0.140 0.115
3	Mixture (MC), 1.0	Alcohol, 1.0 Cholesterol, 1.0		
C	〃	Mycobacillin, 1.0 〃	0.125 0.165	0.055 0.055
4	Cholesterol, 1.0	Alcohol, 1.0 Mycobacillin, 1.0		
D	〃	Mixture (MC), 1.0 〃	0.205 0.140	0.080 0.015

a) Flasks containing the ingredients listed in the 'Zero-time' column were shaken for 20 hours at 31℃. Substances listed under 'After 20 hours' were then added and the contents of flask filtered immediately. Beckmann readings were made of the supernatant fluids. For a base reading, all constituents were added simultaneously, filtered and the supernatant was used. The concentrations of mycobacillin and cholesterol were 100 μg/ml and 50 μg/ml respectively. Concentrations of mycobacillin and cholesterol in the mixture were so adjusted that the reaction system contained 100 μg/ml of mycobacillin and 50 μg/ml of cholesterol.
b) Flasks marked A, B, C, D contained 5 ml of phosphate buffer+1 ml of 20% sucrose solution.

Antagonization of the growth-inhibiting property of mycobacillin by a cellular extract from the sensitive organism and by purified phospholipid or sterol derived therefrom:

It appears from Table 4 that the growth inhibition of *A. niger* by mycobacillin is antagonized if some lipoidal extract prepared from the same sensitive fungal strain,

is added to the assay system. Both a phospholipid and a neutral lipid fraction (sterol), obtained on fractionation by silicic acid column chromatography with a gradient elution technique, were also able to antagonize the action of mycobacillin.

Characterization of antagonistic factors present in cell phospholipid and sterol:

Table 5 shows that the sterol fraction gives a single spot which possesses the same Rf value and color as cholesterol. The sterol fraction was further studied in three different solvent systems (Table 6). The Rf values of the unknown sterol agree with that of cholesterol in each case. This is also supported by the specific color reaction (LIBERMANN-BURCHARD reaction) of the second spraying agent. The phospholipid fraction separates into two spots. The major spot has the same Rf value as lecithin. No ninhydrin-positive spot could be detected in the chromatogram. So it does not contain sphingomyelin and cephalin.

Table 2(b). Effect of lecithin on the release by mycobacillin of UV-absorbing compounds from mycelia of *A. niger* in the presence and absence of sucrose

Flask No.	Reagents added (ml) to 6 ml of Na-phosphate buffer (pH 7) solution		Absorbancy at	
	Zero time	After 20 hours	260 mμ	280 mμ
1	Alcohol, 1.0	Mycobacillin, 1.0 Lecithin, 1.0 Mixture (ML), 1.0	0.230	0.125
A	〃	〃	0.200	0.075
2	Mycobacillin, 1.0	Alcohol, 1.0 Lecithin, 1.0 Mixture (ML), 1.0	0.285	0.150
B	〃	〃	0.280	0.155
3	Mixture (ML), 1.0	Alcohol, 1.0 Mycobacillin, 1.0 Lecithin, 1.0	0.225	0.090
C	〃	〃	0.265	0.130
4	Lecithin, 1.0	Alcohol, 1.0 Mycobacillin, 1.0 Mixture (ML), 1.0	0.300	0.175
D	〃	〃	0.235	0.120

Addition and detais of the procedure were the same as described in Table 2(a). Concentrations of mycobacillin and lecithin were 100 μg/ml and 50 μg/ml respectively. The mixture also contained mycobacillin and lecithin in same ratio.

Table 3. Antagonistic action of sterols and lecithin towards the agglutinating property of mycobacillin on spores of *A. niger* (G_4)

Concn. of mycobacillin (μg/ml)	Name of sterol or phospholipid	Concn. of sterol or phospholipid (μg/ml)	Agglutination after			
			18 hours at		24 hours at	
			37°C	4°C	37°C	4°C
0	0	0	—	—	—	—
250	0	0	+++	—	+++	—
250	Cholesterol	125	—	—	—	—
250	Ergosterol	500	—	—	—	—
250	Ergocalciferol	500	—	—	—	—
250	Lecithin	125	+	—	+	—

Agglutination was observed visually.
+ indicates agglutination.
— indicates no agglutination.

Discussion

In the present paper we have studied the antagonistic effects of phospholipids and sterols on the antibiotic action of mycobacillin against *A. niger*. It has been observed that among the commercial sterols tested, cholesterol, ergosterol and ergocalciferol can antagonize mycobacillin sensitivity reactions, *e. g.*, growth inhibition, agglutination and release of ultraviolet-absorbing materials from the fungal cells. Stigmasterol at least reversed the growth-inhibitory effect of mycobacillin. Among the three commercial lipids tested, only lecithin is able to antagonize the reactions of mycobacillin.

In order to learn if any of these antagonists is involved in the mode of action of mycobacillin on sensitive organisms, experiments were performed to see if the growth

Table 4. Prevention of the growth-inhibiting property of mycobacillin by a cellular extract from the sensitive organism *A. niger* and by purified phospholipid or sterol fractions derived therefrom

Mycobacillin (μg/ml)	Crude extract (ml)	Phospholipid fraction* (ml)	Neutral lipid fraction** (ml)	Zone of inhibition (mm)
250	—	—	—	24
250	0.05	—	—	0
250	—	0.05	—	0
250	—	—	0.05	0

Cell extract was prepared by treating 4 g of mycelia with 100 ml of chloroform-methanol (2 : 1) mixture.

'O' indicates absence of zone of inhibition.

* The phospholipid fraction was collected by elution with the solvent system chloroform methanol (1 : 9) from a silicic acid column charged with a crude extract.

** The neutral lipid fraction was collected by elution with the solvent system chloroform - methanol (9 : 1) from the silicic acid column as in a.

Table 5. Characterization of antagonistic factors present in cell phospholipid and sterol of *A. niger*

Compound	Solvent system	Spraying agent	Rf
Unknown sterol	$CHCl_3$ - C_6H_6 (95 : 5)	C_2H_5OH - H_2SO_4 - $(CH_3CO)_2O$ (8 : 1 : 1)	0.25
Cholesterol	〃	〃	0.25
Ergosterol	〃	〃	0.27
Ergocalciferol	〃	〃	0.40
Unknown phospholipid	$CHCl_3$ - MeOH - H_2O (95 : 35 : 6)	Phosphomolybdic acid in 60 % perchloric acid	0.60
Lecithin	〃	〃	0.61
Sphingomyelin	〃	〃	0.21

The plate was made of silica gel G (Sigma Chemical Co., N.Y.) of 1 mm in thickness and was activated for 1 hour at 110℃. For sterol the solvent system was allowed to run for 2 hours. In the case of phospholipid the time for solvent run was 1 hour. After spraying the plates were again heated at 100℃ for 15 minutes.

Table 6. Characterization of cholesterol

Compound	Solvent system	Spraying agent	Rf	Color of the spot
Fraction Nos. 3, 4, 5	(A) $CHCl_3$ - CH_3COCH_3 (90 : 10)	I, II, III	0.60	I yellowish brown
	(B) C_6H_6 - $CHCl_3$ (40 : 60)	I, II, III	0.50	II pink to green
	(C) C_6H_6 - $CH_3COOC_2H_5$ (60 : 20)	I, II, III	0.15	III yellow
Cholesterol	(A)	I, II, III	0.62	I yellowish brown
	(B)	I, II, III	0.49	II pink to green
	(C)	I, II, III	0.15	III yellow

Spraying agent I: Iodine vapour. II: C_2H_5OH - Ac_2O - H_2SO_4 (8 : 1 : 1). III: Phosphomolybdic acid.

inhibition of *A. niger* by mycobacillin is antagonized by a lipoidal extract of the same sensitive organism. The results show that both cell-phospholipid and cell-sterol can reverse the growth-inhibiting property of the antibiotic. The intracellular compounds were then characterized by thin-layer chromatography on silica gel and were found to be mainly cholesterol and lecithin. Thus the antagonists are present in sensitive cells themselves. Possibly, this could mean that these cell components represent the binding sites for the antibiotic whose antifungal action owes its origin to this specific interaction.

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