ANTILEISHMANIAL ACTIVITY OF MYCOBACILLIN

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Mycobacillin, a cyclic polypeptide antifungal antibiotic effectively inhibits the growth of promastigote form of the protozoal organism, *Leishmania donovani*, strain 81 in liquid medium. Oxygen uptake by intact cells of the protozoa with exogenous glucose is appreciably reduced within first 30 minutes in presence of the antibiotic at a concentration of 15 μg/ml. Appreciable leakage of intracellular 260 nm and 280 nm absorbing materials takes place from the protozoan cells incubated with the antibiotic at similar concentration.

Mycobacillin, an antifungal antibiotic discovered by MAJUMDER and BOSE[1] from the culture filtrate of a selected strain of *Bacillus subtilis* is a cyclic polypeptide consisting of 13 residues of 7 different amino acids. Its structure was reported earlier[2,3,4]. *Leishmania donovani* is a member of the group of haemoflagellates and is responsible for visceral and cutaneous leishmaniasis in man. In the present paper the authors are reporting about the inhibitory effect of mycobacillin on the growth, respiration *etc.* of the promastigotes of *Leishmania donovani*.

Materials and Methods

Strain, media and maintenance

The organism used throughout this work was *Leishmania donovani*, strain 81. The strain was originally obtained from Dr. J. C. ROY, Director, Immunobiological Laboratory, Calcutta, India. It was grown at 22±1°C on slants of slightly modified RAY's medium,[5] 100 ml of which contained glucose 1 g, sodium chloride 0.6 g, oxoid peptone 1 g, agar 2.5 g, beef-heart extract 50 ml and hemolysed rabbit-blood 10 ml. The pH of the medium was 7.2. Luxuriant growth of the organism was found within three days after subculture. The organism was maintained by subcultures made at intervals of 48 hours.

The liquid growth medium contained everything except the agar used in the solid growth medium.

Cell-suspension

The cells after 72-hour growth were gently scraped by a glass loop from the slant surface and suspended in cold (10±1°C) saline (0.155 M, pH 7.2). The suspension was then centrifuged quickly in a refrigerated centrifuge (5±1°C) at 2,000×g for 10 minutes. The cells were then washed twice with cold normal saline in the same way. Finally the packed cells were suspended in cold normal saline (pH 7.2) and used in the experiments.

The cells were checked for motility as an indication of viability before each experiment. Only data obtained from suspensions retaining good motility before the experiments were used.

Saline-phosphate buffer

Saline-phosphate buffer (pH 7.2) containing 85 μmoles of sodium chloride and 100 μmoles of

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sodium phosphate per ml was prepared by mixing measured volumes of 0.1 M saline and 0.2 M sodium phosphate buffer (pH 7.2).

Antibiotic solutions

Solution of mycobacillin (5 μg/ml) was prepared by dissolving it in 0.1 M sodium bicarbonate solution. Solutions of streptomycin sulphate, tetracycline hydrochloride and sodium penicillin G of the same concentration were prepared by dissolving them in glass distilled water.

Cell-count

Number of cells present per ml was determined by counting the cells on a Neubauer improved haemocyatometer plate after making proper dilution of the cell-suspension. Before counting, the plate was held briefly over steam in order to stop movement of the protozoan cells.

Estimation of protein

Protein contents of the cell-suspensions were determined by biuret method.[6] One mg of cell-protein was found to be equivalent to 1.6×10^9 cells.

O_2-Uptake studies

Oxygen uptake studies were done by conventional manometric techniques in a Warburg apparatus[7].

Cell-free extract

About 400 mg of *L. donovani* cells washed with cold normal saline (pH 7.2) were mixed with 0.5 g of washed and prechilled ballotini glass powder in a mortar and pestle and broken by grinding at intervals to dissipate the generated heat. The whole mass was taken up in 5 ml of precooled 0.5 M Tris-buffer (pH 7.4) and centrifuged at $5,000\times g$ for 30 minutes. The whole operation was carried out at 3°C. The supernatant was collected and used as cell-free extract. The precipitate suspended in 1 ml of the same buffer was termed 'residue'.

Measurement of release of 260 nm and 280 nm absorbing materials from the cells of *L. donovani*

Suitable aliquot from the incubation mixture containing the cells was centrifuged in a refrigerated centrifuge (5 ± 1°C) at $2,500\times g$ for 15 minutes. The clear supernatant (cell-free under microscope) was taken out with a capillary and a known volume of it was treated with an equal volume of cold perchloric acid solution (0.5 M) for twenty minutes. The precipitate if any, was separated by centrifugation at $5,000\times g$ for 10 minutes. The clear solution was taken out and its absorbancy at 260 nm and 280 nm was determined by use of a Beckman (DU) spectrophotometer.

Chemicals

Nicotinamide-adenine dinucleotide (NAD), flavine adenine dinucleotide (FAD), co-enzyme A (CoA), adenosine triphosphate (ATP) were obtained from Sigma Chemical Co. (St. Louis, U.S.A.). All other chemicals were purchased from BDH Chemical Ltd. (Poole, England).

Results

Mycobacillin at a concentration of 20 μg/ml completely inhibits the growth of the promastigote form of *L. donovani* (Fig. 1). Several other antibiotics like penicillin, streptomycin and tetracycline were found to be completely ineffective at identical concentration. It is evident from Fig. 1 that only partial inhibition of growth of the organism takes place at lower concentrations of the antibiotic.

The effect of the antibiotic on the respiration of the protozoan cells in presence of exogenous glucose is shown in Fig. 2. The oxygen-uptake of the cells is reduced by about 35% within 30 minutes of incubation with mycobacillin (15 μg/ml). However, a prolonged period of about 70 minutes is required before the respiration is completely stopped. The cells on examination under microscope after 30 minutes of such contact with the antibiotic at the same concentration appear to lose their motility to a considerable extent and after 60 minutes of contact their motility is completely lost, although they remain unaffected from morphological stand point. It is also evident from Fig. 2 that the degree of inhibition of respiration of the organism increases with increase in concentration of the antibiotic. In order to ascertain

whether the inhibition of respiration of protozoan cells is due to any direct effect of the antibiotic on glycolysis or tricarboxylic acid cycle of the organism, we studied the oxygen-uptake by the cell-free extract of the organism in presence of the antibiotic. The oxygen-uptake by the cell-free extract of *L. donovani* in presence of exogenous glucose is very low and fortification of the incubation medium with a mixture of several cofactors like NAD, FAD, CoA and ATP fails to show any significant stimulation (Fig. 3). Addition of 'residue' along with the co-factors however results in further stimulation of oxygen-uptake. Addition of phenazine methosulphate, an effective electron acceptor, however strongly stimulates the oxygen-uptake and the dye can successfully replace the requirement of the 'residue'. Mycobacillin at two different concentrations, 15 μg/ml and 30 μg/ml, has no effect on the oxygen-uptake by the cell-free extract of *L. donovani* fortified with necessary co-factors and phenazine methosulphate (Fig. 3).

It is evident from Table 1 that appreciable leakage of 260 nm and 280 nm absorbing intracellular materials takes place from the cells of *L. donovani* incubated with mycobacillin at concentrations of 15 μg/ml and 30 μg/ml. Such release of the intracellular materials continues over a period of 90 minutes. The release of such intracellular materials from the protozoan cells during this

Fig. 1. Inhibition of growth of *L. donovani* in presence of mycobacillin.

Five ml of liquid growth medium in each of different sterile test tubes containing different antibiotics in indicated concentrations was inoculated with 2.5×10^6 *Leishmania* cells at their log phase of growth. A control was run side by side. The tubes were incubated at 22°C. Aliquots were drawn at intervals of 20 hours and growth of the cells was measured by counting them as described in Materials and Methods.

A: Growth in absence of any antibiotic (control)
B: Growth in presence of penicillin (20 μg/ml)
C: Growth in presence of streptomycin (20 μg/ml)
D: Growth in presence of tetracycline (20 μg/ml)
E: Growth in presence of mycobacillin (20 μg/ml)
F: Growth in presence of mycobacillin (15 μg/ml)
G: Growth in presence of mycobacillin (10 μg/ml)

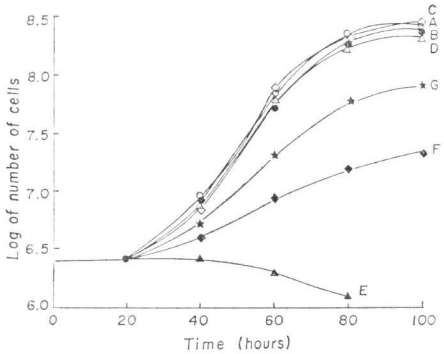


Fig. 2. Effect of mycobacillin on the respiration of *L. donovani*.

In a final total volume of 3 ml, the incubation mixture in the flasks contained 200 μmoles of Na-phosphate (pH 7.2), 250 μmoles of NaCl, 1 ml of cell-suspension (4.5 mg protein), 10 μmoles of glucose and indicated amounts of mycobacillin (where added). Glucose and mycobacillin were added from the side arms of the flasks after 60 minutes of equilibration in which the endogenous respiration was exhausted (as evidenced by the control flask). Gas phase: air. Temperature: 37°C.

A: Respiration in the absence of mycobacillin (control)
B: Respiration in the presence of 15 μg/ml of mycobacillin
C: Respiration in the presence of 30 μg/ml of mycobacillin
D: Respiration in the presence of 40 μg/ml of mycobacillin

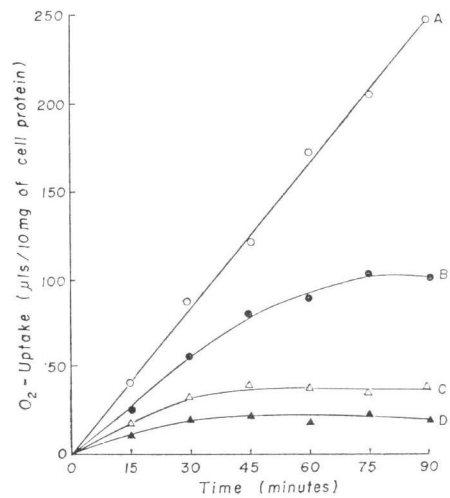
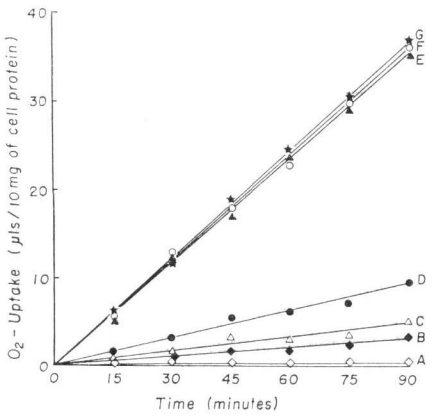


Fig. 3. Oxygen uptake by cell-free extract of *L. donovani* in presence of mycobacillin.

In a final volume of 3 ml, the incubation mixture in the flasks contained: NaCl 250 μmoles, glucose 10 μmoles, cell-free extract 1 ml (5 mg protein), Tris-buffer (pH 7.4) 200 μmoles, $MgCl_2$ 50 μmoles, 5 μmoles each of ATP, NAD, CoA, FAD (where added), phenazine methosulphate 10 μmoles (where added) and indicated amounts of mycobacillin (where added). Gas phase: air. Temperature: 37°C.

A: O_2-Uptake by cell-free extract only
B: O_2-Uptake by cell-free extract + glucose
C: O_2-Uptake by cell-free extract + glucose + co-factors
D: O_2-Uptake by cell-free extract + glucose + co-factors + 'residue' (0.5 ml)
E: O_2-Uptake by cell-free extract + glucose + co-factors + phenazine methosulphate
F: O_2-Uptake by cell-free extract + glucose + co-factors + phenazine methosulphate + mycobacillin (15 μg/ml)
G: O_2-Uptake by cell-free extract + glucose + co-factors + phenzine methosulphate + mycobacillin (30 μg/ml)



period in absence of the antibiotic is negligible.

Discussion

It is evident from the results of these studies that mycobacillin has remarkable inhibitory effect on the life of the promastigotes of *L. donovani*. Though inhibition of growth of the organism takes place at a concentration of 20 μg/ml (Fig. 1), the respiration in presence of exogeneous glucose is inhibited at concentrations lower than 15 μg/ml (Fig. 2). The failure of the antibiotic at such lower concentration to completely inhibit the growth of the organism in liquid growth medium (Fig. 1) may be due to some kind of protection afforded to the organism by the nutrients like peptone, beef-heart extract and haemolysed rabbit-blood present in it. The low oxygen-uptake by the cell-free extract of the organism (Fig. 3) in presence of exogeneous glucose may be due to disorganization of the biochemical chain of reactions resulting from loss of cellular integrity. Results shown in Fig. 3 indicate that the antibiotic has no effect on the oxygen-uptake by the cell-free extract of the organism reinforced with the necessary co-factors and phenazine methosulphate. This proves that the antibiotic in spite of having pronounced inhibitory effect on the respiration of the intact cells, has no direct effect on the reactions involved in glycolysis or TCA cycle of the organism. Continuous leakage of 260 nm and 280 nm absorbing materials from the cells incubated with mycobacillin (Table 1) indicates that a definite change in the permeability barrier of the protozoan cells is caused by the antibiotic. Such action of the antibiotic on the cell-membrane seems to cause progressive exhaustion of the cells of their essential metabolites. Further investigation is in progress and at this stage we can conclude that the inhibition of respiration and loss of motility of the organism are the secondary effects of the action of the antibiotic which acts primarily on the membrane of the protozoan cells.

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Table 1. Release of 260 nm and 280 nm absorbing intracellular materials from *L. donovani* in presence of mycobacillin.

In a final volume of 5 ml, made up with saline-phosphate buffer (pH 7.2), each tube contained 1 ml of cell-suspension (5.2 mg protein) and the antibiotics (as and where indicated). Tube Nos. 1 and 2 served as controls. The tubes were incubated at 30°C and the release of 260 nm and 280 nm absorbing intracellular materials was measured as described in Materials and Methods. Tube No. 6 measured the absorbancy for the total amount of such intracellular materials present in the cells in each tube.

Tube No.	Incubation system	Absorbancy per mg of protein									
		At 260 nm after					At 280 nm after				
		0 min.	30 min.	60 min.	90 min.	120 min.	0 min.	30 min.	60 min.	90 min.	120 min.
1	Cell-suspension	0.02	0.04	0.08	0.12	0.13	0.02	0.03	0.05	0.06	0.07
2	Cell-suspension *$NaHCO_3$ (0.1 M)	0.03	0.04	0.07	0.13	0.14	0.01	0.03	0.06	0.06	0.08
3	Cell-suspension mycobacillin (15 μg/ml)	0.04	0.25	0.45	0.56	0.57	0.04	0.25	0.37	0.45	0.47
4	Cell-suspension mycobacillin (30 μg/ml)	0.06	0.40	0.68	0.80	0.82	0.04	0.38	0.62	0.81	0.82
5	Cell-suspension penicillin (30 μg/ml)	0.03	0.04	0.07	0.11	0.12	0.03	0.03	0.05	0.07	0.08
6	Cell-suspension (0.1 M perchloric acid (4 ml))	—	—	—	—	4.60	—	—	—	—	4.85

* As present in the mycobacillin solution in tube No. 3.

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