Mycobacterium tuberculosis H37Rv Grown *in vivo:* Nature of the Inhibitor of Lactic Dehydrogenase of *Mycobacterium phlei.* (26419)

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Previous reports from this laboratory(1, 2,3,4) have shown that cells of *Mycobacterium tuberculosis* H37Rv grown *in vivo* (LH37Rv) adsorb from lung tissue of tuberculous mice a substance which inhibits electron transport system in cell-free extracts of *Mycobacterium tuberculosis* H37Rv, *Mycobacterium tuberculosis* BCG and *Mycobacterium phlei* as well as in lung homogenates of normal mice. Cell-free extracts of *M. phlei* were subsequently used as an indicator system for detection of the above inhibitor. It has been found that extracts from various organs of both infected and normal mice displayed an inhibitory activity similar to that of LH37Rv cells, this activity being much more pronounced in organs of tuberculous mice. To ascertain whether the inhibition brought about by extracts from the various organs of normal and infected mice and by LH37Rv cells was due to the same substance, study to elucidate the nature of the inhibitor was undertaken. This is reported here.

Materials and methods. The organisms used were: *Mycobacterium tuberculosis* LH37Rv, *Mycobacterium tuberculosis* H37 Rv and *Mycobacterium phlei.*

M. tuberculosis H37Rv and *M. phlei* were grown in media previously described(2). *M. tuberculosis* LH37Rv was obtained from lungs of moribund mice infected with *M. tuberculosis* H37Rv. Tubercle bacilli were separated from lungs by a procedure described elsewhere(2). For experiments on inhibition of lactic dehydrogenase of *M. phlei,* the tubercle bacilli grown *in vivo* after separation from lungs were washed twice in a 0.1 M phosphate buffer pH 7.2 and resuspended in the same buffer to a density corresponding to 3-4 mg dry weight per 1 ml. Cells of *M. phlei* were harvested by centrifugation, washed twice in a 0.1 M phosphate buffer pH 7.2 and resuspended in the same buffer, 50 mg dry weight of cells per 1 ml of buffer. The resulting thick suspension was disintegrated for 45 minutes in a 9 KC Raytheon magnetostriction oscillator. Cell debris and unbroken cells were removed by centrifugation at 9,000 rpm in an MSE high speed centrifuge in the cold. The cell-free supernatant containing 12 to 15 mg protein per 1 ml was used throughout. Protein content was estimated by the biuret method. Cell-free extracts of *M. phlei* are occasionally referred to as enzyme preparation. When required, the enzyme preparations were dialyzed in the cold for 24 hours against frequent changes of distilled water. Lactic dehydrogenase activity of cell-free extracts of *M. phlei* was determined by reduction of triphenyltetrazolium chloride under aerobic conditions. Reaction mixture contained: 0.5 ml of enzyme preparation; 0.5 ml of 0.1 M phosphate buffer pH 7.2 (or 0.5 ml of various additions in the same buffer); 0.1 ml of 0.3 M sodium lactate and 0.1 ml of 1% solution of triphenyltetrazolium chloride. Time of reaction was 10 minutes, temperature 37°. The reaction was arrested by addition of 2 ml of *iso*-butanol, formazan was extracted twice with the same solvent and its color intensity read in a Coleman Jr. spectrophotometer at 485 mμ. For preparation of extracts from organs of mice, the various organs were homogenized in a teflon homogenizer run at top speed for 2 minutes, and centrifuged at 7,200 × g for 10 minutes in the cold. The supernatant was used throughout. All procedures were carried out in the cold; 4% homogenates were used. The extracts can be stored for weeks at −10° without losing their inhibitory activity.

DPNase (Pyridine transglycosidase) was assayed by the method of Kaplan *et al.*(5), based on the cyanide reaction of diphosphopyridine nucleotide (DPN). Reaction mixture contained: 0.4 parts of enzyme; 0.4

TABLE I. Some Physical Properties of Inhibitor of Lactic Dehydrogenase of *M. phlei*.

Previous treatment of preparations containing inhibitor	Lactic dehydrogenase of cell-free extracts of *M. phlei* (O.D. of formazan) in presence of			
	LH37Rv cells	Lung extracts	Spleen extracts	Control
Non-treated	.28	.60	.40	2.80
Heated at 80° for 5 min.	2.85	2.70	2.70	
Dialyzed	.16	.48	.40	

For experimental conditions see *Materials and methods*.

parts of 0.1 M phosphate buffer pH 7.2 and 0.2 parts of 0.003 M DPN (purchased from N. B. Co., Cleveland, Ohio). Before and after various time intervals of incubation at 37°, 1 ml samples were withdrawn, 3 ml of 1.0 M KCN were added and the mixture read at 325 mμ in an Unicam spectrophotometer. Samples with KCN without DPN served as blanks. When LH37Rv cells were used as the source of the enzyme, the cells were removed by centrifugation after addition of KCN.

Results. Some physical properties of the inhibitor. The inhibitor of the electron transport system of *M. phlei,* found in the extracts from various organs of mice and on the surface of *M. tuberculosis* H37Rv grown *in vivo* is thermolabile and nondialyzable. Heating of LH37Rv cells, lung or spleen extracts at 80° for 3 to 5 minutes completely destroyed their inhibitory activity. Dialysis, on the other hand, did not affect their inhibitory activity (Table I).

Kinetics of inhibition. It has been previously shown(3) that rate of inactivation of lactic dehydrogenase of *M. phlei* by the various preparations containing the inhibitor depends on concentration of the inhibitor in reaction mixture. Inactivation of lactic dehydrogenase of *M. phlei* by concentrated preparations of the inhibitor was apparent immediately after addition of the latter. On the other hand, diluted preparations of the inhibitor inactivated lactic dehydrogenase of *M. phlei* only after preincubation.

To study in detail the kinetics of inhibition we have used LH37Rv cells, lung or spleen extracts (from either normal or tuberculous mice) in such amounts as did not inhibit lactic dehydrogenase of the enzyme preparations when added to the reaction mixture simultaneously with lactate and tetrazolium. The various preparations containing the inhibitor were incubated with the enzyme preparation at 37° and 4°, at various time intervals samples were withdrawn, lactate and tetrazolium were added and lactic dehydrogenase of the enzyme preparations was determined. The results are illustrated in Fig. 1. Incubation of lung and spleen extracts and of LH37Rv cells with the enzyme preparation caused a gradual decrease in lactic dehydrogenase activity of the latter. The decrease in enzyme activity was only apparent on incubation at 37°. Incubation of the enzyme preparation with the inhibitor at 4° left lactic dehydrogenase activity of *M. phlei* unimpaired.

Restoration of dehydrogenase activity in inactivated extracts of M. phlei. Results of the above experiments suggested that inhibition of lactic dehydrogenase in cell-free extracts of *M. phlei* was caused by destruction of some component of lactic dehydrogenase system of the above organism. To prove this, we have performed experiments on restoration of dehydrogenase activity in the inactivated enzyme preparation. These experiments were carried out as follows: Lactic dehydrogenase in cell-free extracts of *M. phlei* was inactivated by incubation of the enzyme preparation with LH37Rv cells, spleen or lung extracts. When LH37Rv cells were used they were removed by centrifugation from the enzyme preparation after inactivation of the enzyme was completed. Dialysed or boiled extracts of non-treated *M. phlei* were added to the inactivated enzyme preparation and the effect of these additions on lactic dehydrogenase activity of the latter was determined. The results of these experiments (Table II) clearly show that addition of boiled extracts from non-treated *M. phlei*

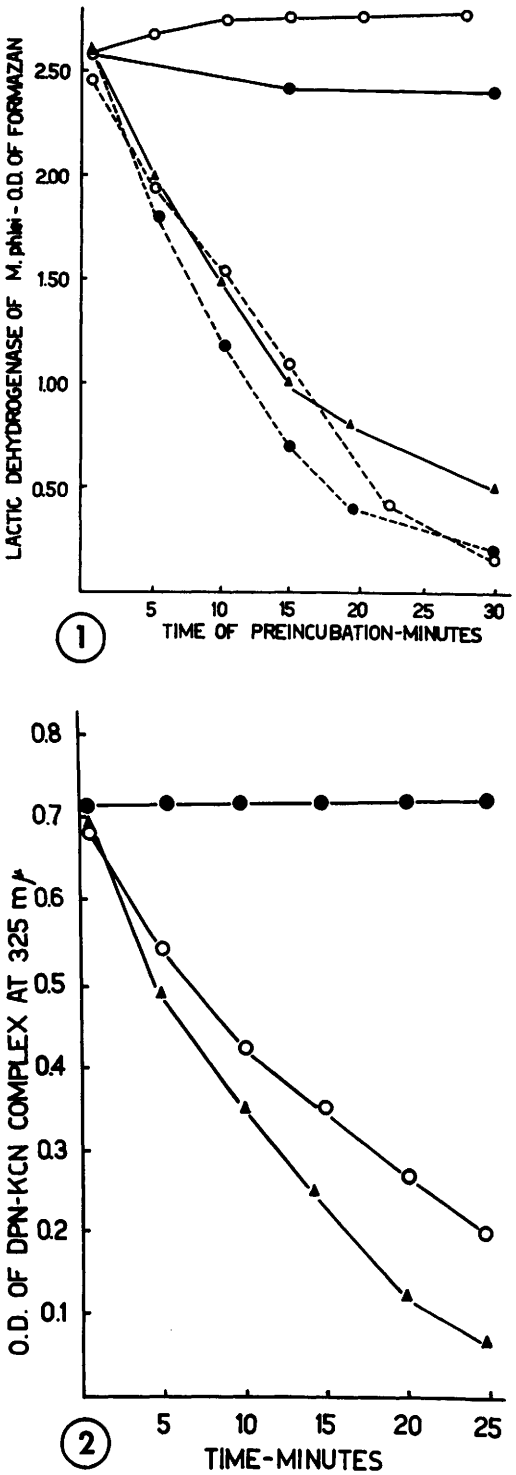

FIG. 1. Effect of preincubation of inhibitor with cell-free extracts of *M. phlei* on lactic dehydrogenase activity of the latter. Reaction mixture contained: 0.5 ml of cell-free extracts of *M. phlei*; 0.5 ml of lung extract (when present); 0.2 ml of spleen extract (when present); 0.5 ml of LH37Rv cells (when present). After preincubation for time indicated in Fig., 0.1 ml of 0.3 M sodium lactate and 0.1 ml of 1% solution of triphenyltetrazolium chloride were added. Formazan was measured after 10 min. incubation at 37°.

○- - -○ Lactic dehydrogenase activity of cell-free extracts of *M. phlei* preincubated at 37° with LH37Rv cells.
▲——▲ *Idem* with lung extract.
●- - -● " " spleen " .
○——○ " , non-treated (control).
●——● Lactic dehydrogenase activity of cell-free extracts of *M. phlei* preincubated with LH37Rv cells at 4°.

Since preincubation of cell-free extracts of *M. phlei* with lung or spleen extracts at 4° gave identical curves to that of preincubated extracts with LH37Rv cells, they are omitted from figure.

FIG. 2. DPNase activity of H37Rv cells, LH37-Rv cells, and spleen extracts of mice. For experimental conditions see *Materials and methods*.

○——○ LH37Rv cells
●——● H37Rv "
▲——▲ Spleen extract

completely restored dehydrogenase activity of the inactivated enzyme preparation. On the other hand, addition of dialyzed extracts of *M. phlei* failed to restore the enzymatic activity in inactivated extracts of *M. phlei*. It should be pointed out that the various additions to the enzyme preparation inactivated by spleen or lung extracts were made in presence of these extracts. This was permissible since the amount of inhibitor used was so small that it did not inhibit the lactic dehydrogenase of the enzyme preparations without preincubation. It was then shown that boiled extracts of non-treated *M. phlei* lost their restorative property after incubation with the various preparations containing the inhibitory substance. Addition of boiled extracts so treated, to the inactivated enzyme preparation was done after destroying the inhibitor by boiling the reaction mixture for 5 minutes. This was permissible since the factor inactivated by the inhibitor was thermostable.

Having ascertained that the inhibitory effect of LH37Rv cells, lung or spleen extracts of mice, both normal and infected, was caused by inactivation of some heat stable component of lactic dehydrogenase system of *M. phlei*, we determined the effect of known coenzymes of lactic dehydrogenase on

TABLE II. Restoration of Lactic Dehydrogenase Activity in Cell-Free Extracts of *M. phlei* Inactivated with LH37Rv Cells, Lung or Spleen Extracts.

Additions	Lactic dehydrogenase of cell-free extracts of *M. phlei* (in O.D. of formazan) inactivated by			
	LH37Rv cells	Lung extract	Spleen extract	Control of non-treated extracts of *M. phlei*
No additions	.51	.46	.21	3.10
Dialyzed extracts of non-treated *M. phlei*	.61	.58	.18	5.10
Boiled extracts of non-treated *M. phlei*	3.20	3.14	3.21	3.51
Idem after incubation with inhibitor	.32	.48	.16	3.10
DPN	3.20	3.10	3.40	3.40
DPN after incubation with inhibitor	.34	.50	.16	3.10

Lactic dehydrogenase of *M. phlei* was inactivated by procedure described in text and Fig. 1. For lactic dehydrogenase assay see *Materials and methods.*

restoration of enzymatic activity in the inactivated enzyme preparations. Addition of diphosphopyridine nucleotide to extracts of *M. phlei* inactivated by LH37Rv cells, lung or spleen extracts completely restored their dehydrogenase activity (Table II). Moreover, it has been shown that DPN after incubation with LH37Rv cells, spleen or lung extracts completely lost its restorative property.

Nature of the inhibitor. Since destruction of DPN may be accomplished by the action of one of 2 enzymes: the nucleotide pyrophosphatase or pyridine transglycosidase (DPNase), we performed experiments to determine which enzyme is operative in this case. For this purpose LH37Rv cells as well as lung or spleen extracts were incubated with DPN and hydrolysis of DPN was followed spectrophotometrically by determination of DPN level by cyanide. The results (Fig. 2) show clearly that DPN was hydrolyzed at the nicotinamide ribose linkage. It has therefore been concluded that the inhibition of lactic dehydrogenase of *M. phlei* brought about by LH37Rv cells, lung and spleen extracts of mice is due to cleavage of DPN by DPNase present in all these preparations.

Discussion. Discussing the possible role of the inhibitor adsorbed on tubercle bacilli grown *in vivo* in the pathogenesis of tuberculosis we have assumed(3) that the tubercle bacilli upon ingestion by phagocytes introduce the inhibitory substance into the phagocytizing cell. The result is inhibition of phagocyte respiration and eventually its death. Results presented here show that the inhibitor is DPNase. Enzymes destroying DPN have been found in plague toxin(6) and in streptococcal culture supernates(7). A remarkable correlation between leukotoxicity and capacity of streptococci to produce DPNase was observed(7). The authors assumed that leukotoxicity may be due to destruction of DPN of phagocytes by DPNase. If these assumptions are proved to be true, then *M. tuberculosis* H37Rv grown *in vivo* will provide an interesting example of a microorganism which employs host enzyme for destruction of host cell. The effect of *M. tuberculosis* H37Rv grown *in vivo* on phagocytes and the role of DPNase in destruction of the latter is being investigated.

Summary. Some physical properties and kinetics of the action of the inhibitor of lactic dehydrogenase of *M. phlei,* present in spleen and lungs of mice and on the surface of *M. tuberculosis* H37Rv grown *in vivo* have been studied. The inhibitor was identified as DPNase.

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Studies of Fibroblastic Cells Cultivated from Bone Marrow of Leukemic and Non-Leukemic Patients.* (26420)

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Technics employed for *in vitro* preparation of human bone marrow cell cultures have been reviewed by Osgood(1), Reisner(2) and Berman(3). The present report describes a procedure for cultivating cells from leukemic and normal bone marrows on feeder layers of human amnion cells. Employing this technic, the susceptibility of normal and leukemic bone marrow cell cultures to certain viruses was investigated. The fluorescent antibody staining technic was also applied in attempts to detect a specific antigen in leukemic bone marrow cell cultures and efforts were made to isolate a cytopathogenic agent from leukemic marrow aspirates.

Materials and methods. Sternal bone marrow aspirates were withdrawn aseptically in 1-3 ml amounts from patients undergoing open cardiac surgery and from children with acute leukemia. The latter specimens were obtained during the course of diagnostic and follow-up aspirations performed at the Tumor Therapy Clinic of Children's Hospital Medical Center. To each ml of aspirated material was added 0.0025 mg of sterile heparin(4). Aspirates were suspended in bovine amniotic fluid (BAF) medium(5) containing 20% horse serum which had been inactivated for a half hour at 56°C. Nucleated cell counts were performed on the final suspensions.

Primary monolayer cultures of trypsinized human amnion cells (HA) were prepared in 150 × 16 mm test tubes as previously described(5). The monolayers were nourished with BAF medium containing 5% inactivated horse serum. In some experiments 5-15-day-old HA monolayers were x-irradiated with 3,500 r. They were exposed for 12 minutes and 40 seconds at a distance of 40 cm from a Siemens x-ray tube operating at 250 KV and 15 ma, filtered with 1 mm of aluminum. The dose rate was 277 r/min. X-irradiation was of sufficient intensity to arrest cellular multiplication(6). Cell viability, however, appeared to be unaffected as judged by morphologic appearance and acid production.

Viruses. The behavior of the following viruses was studied in bone marrow cell cultures:

Measles, Edmonston strain, 28th human kidney cell passage(7).

Mumps, Enders strain, 47th chick amniotic sac passage.

Sindbis, Strain AR339, 10th chick embryo cell passage(8).

Poliovirus Type I, Brunhilde strain, passed 1× in human uterine and 2× in human amnion cell cultures.

Herpes simplex, L strain, passed 5× in chick embryo amniotic sac and 3× in chick embryo cell cultures(9).

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