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Lactate Dehydrogenase in *Mycobacterium leprae* Grown in Armadillo Liver[1]

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In most bacteria, including the mycobacteria, the catabolic fate of pyruvate is either oxidation in the tricarboxylic acid cycle or anaerobic conversion to lactate, by lactate dehydrogenase (LDH). Lactate may then be converted back to pyruvate or, in the presence of molecular oxygen, converted directly to acetate by lactate oxygenase, an enzyme characteristic of mycobacteria ([10]).

A complete tricarboxylic acid cycle has now been demonstrated in *Mycobacterium leprae* ([15]). Oxygen uptake and pyruvate formation were demonstrated in extracts of *M. leprae* incubated with lactate ([7, 9]) but no attempt was made in these early studies to distinguish the activity in *M. leprae* extracts from the activity in the host tissue, which may contaminate preparations of *M. leprae*. Recently, differences were observed in the LDH isoenzymes in serum ([13]) and skin biopsies ([11]) between lepromatous or borderline lepromatous and uninfected individuals or patients with paucibacillary leprosy. It was suggested that additional "isoenzymes" in skin biopsies might in fact be LDH from *M. leprae* ([11]). In this communication, we report on LDH from *M. leprae* organisms purified from armadillo tissue.

MATERIALS AND METHODS

Preparation of *M. leprae* cell-free extract. Highly purified suspensions ([17]) of bacteria were prepared by the homogenization of heavily infected armadillo liver followed by treatment with DNAase, Percoll (Pharmacia) density gradient-centrifugation, and separation on an aqueous two-phase system [7 g Dextran T500 (Pharmacia); 4.9 g polyethylene glycol 6000; 0.1 g polyethylene glycol monopalmitate; 0.01 M NaCl; 0.01 M potassium phosphate (pH 6.9); bacteria suspended in Tween buffered with 2 mM MES, pH 7; and distilled water to 100 g in total ([17])]. Contamination of these suspensions by bacteria other than *M. leprae* was assessed by examining for growth on Löwenstein-Jensen medium, on blood-agar plates, and on nutrient-agar medium. In no instances were contaminating mycobacteria detected, even after incubation for up to two months, and contamination with other bacteria never exceeded one colony forming unit per 5×10^6 *M. leprae*. Cell-free extracts of *M. leprae* were prepared by suspending 40 to 200 mg dry weight bacteria in a total volume of 10 ml 1.5 mM HEPES (adjusted to pH 7.2 with KOH) and ultrasonically treating for periods no longer than 3 min for a total of 11 min at 100 W in a Dawe Soniprobe (type 7532A), cooled on wet ice. The probe was completely enclosed in a Dawe Sealed-Atmosphere Treatment Chamber (type 7530-5A) in order to contain the aerosol formed. The disrupted material was centrifuged at 20,000 *g* (*g* max) for 10 min and the supernatant was re-centrifuged for 10 min to remove remaining bacteria and debris. The final supernatant (cell-free extract), containing 1 to 5 mg protein/ml, was stored at −80°C. It was not possible to detect acid-fast bacteria in a pellet (obtained by centrifuging at 20,000 *g* for 10 min) from the cell-free extract.

NaOH-treatment of *M. leprae*. Suspensions of pure *M. leprae* were incubated at 25°C in 1 N NaOH for 1 hr; after neutralization with 2 M HEPES, the bacteria were washed by centrifugation and resuspended in 0.1% Tween 80 (buffered with 2 mM MES, pH 7); then a cell-free extract was prepared, as described above.

Extracts of host tissue. Liver tissue from

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uninfected armadillo was homogenized in 50 mM phosphate (Na_2HPO_4/KH_2PO_4, pH 7.5) buffer with 1 mM $MgCl_2$. Extracts of infected tissues were prepared from supernatants of the first homogenate (0.1 M Tris, pH 8.7) after centrifugation at 16,000 *g* for 10 min during the preparation of pure mycobacteria. In all extracts, the protein was determined by the Lowry method.

Enzyme assay. The method used as "standard" for detecting LDH (EC 1.1.1.27; L-lactate: NAD+ oxidoreductase) was based on that used with extracts of *M. tuberculosis* ([7]), revised to avoid interference by NADH oxidizing activity ([5]). Extracts were incubated with 50 mM Tris-HCl, pH 7; 0.17 mM NAD; 0.1 U diaphorase (Sigma, from porcine heart)/ml; 0.6 mM p-iodonitrotetrazolium violet (INT), and 7 mM L-lactate (Li salt). Increase in OD_{500} for reduced INT, ($E \,|\, ^{cm}_{mM} = 12.6$) was read against controls with all the above except lactate, and units of activity (1 unit converted 1 μmol substrate/min) were calculated. Alternative assays used were: a) 50 mM Tris-HCl, pH 7.0; 0.09 mM NADP; 7 mM L-lactate-increase in OD_{340} (for NADPH, $E \,|\, ^{cm}_{mM} = 6.23$) was read; b) 0.1 M Na_2HPO_4/KH_2PO_4, pH 7.0; 0.2 mM NAD; 1 mM pyruvate (Na salt) - fall in OD_{340} (for NADH, $E \,|\, ^{cm}_{mM} = 6.23$) was read. Conditions for all assays were: 25°C; incubation time up to 30 min (calculations were based on initial velocities); volume was 500 μl.

Polyacrylamide gel electrophoresis (PAGE). PAGE was based on methods used by Saoji, *et al.* ([12]) for LDH ([11, 13]) but miniaturization was not done. Electrophoresis at alkaline pH (8.5 to 9) was done by the method of Davies ([3]); staining was by a method involving preincubating in 0.5 M Na_2HPO_4/KH_2PO_4, pH 7.4, for 15 min then incubating in the stain (lactate, NAD, NaCl, $MgCl_2$, PMS, NBT or 0.6 mM INT, Na_2HPO_4, KH_2PO_4; pH 7.4) for LDH ([4]).

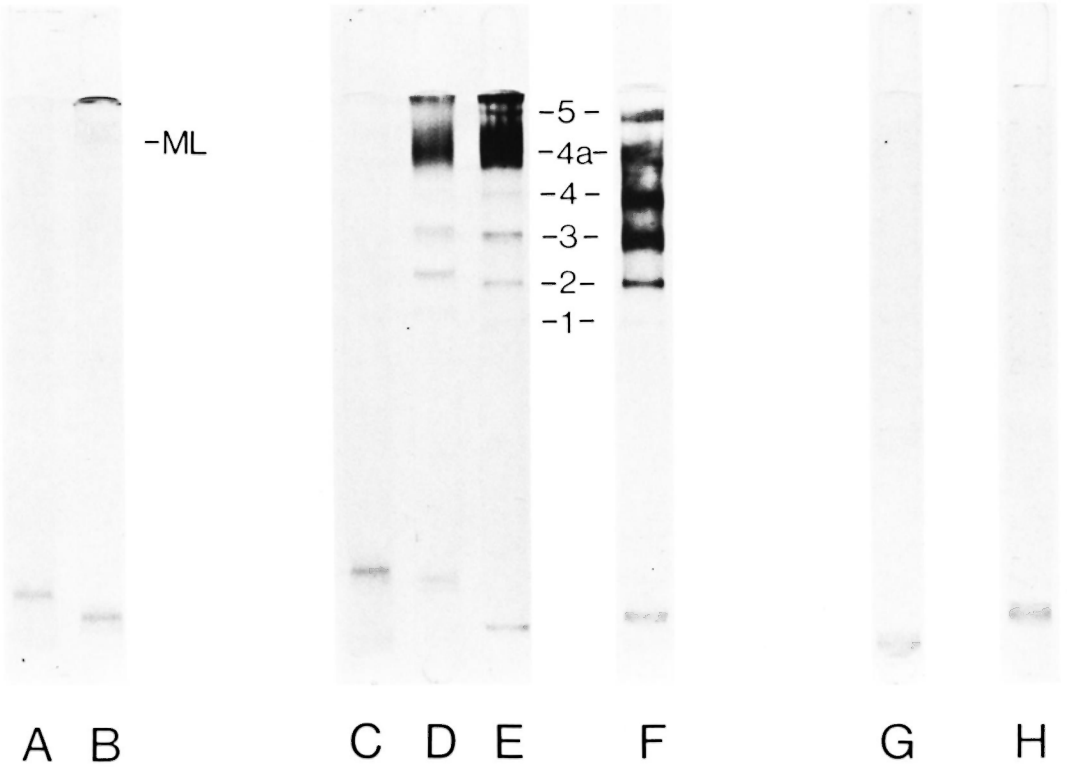
Gas electrophoresis was also used preparatively, as follows: LDH-isoenzymes were located in unpreincubated, unstained gels by comparing with stained gels. The individual isoenzymes were collected from unstained gels by slicing parts of the gel corresponding to stained bands on the stained gels, chopping, placing on top of pre-electrophoresed support gels, covering with 265 mM Tris-75 mM glycine-30% glycerol (pH 8.8) solution, overlaying with 2 M NaCl and subjecting to reverse electrophoresis ([8]). Since the most critical area of activity electrophoresed very slowly, reversed electrophoresis was performed for 3 hr at 4 mA/gel. Recovered activity was then desalted against 3 mM MES, pH 7.0, to a theoretical glycerol concentration of 0.1%, using an Amicon CF-25 ultrafiltration cone (cut off MW = 25,000).

RESULTS

Lactate dehydrogenase was detected in five cell-free extracts made from suspensions of *M. leprae* purified on five separate occasions. The leprosy bacilli were harvested from four different armadillo livers; the two suspensions derived from the same tissue were used for extracts "*M. leprae* 3" and "NaOH-treated *M. leprae* 2" (Table 1). NaOH-treatment of the purified suspensions did not diminish the LDH levels (Table 1). LDH was also detected in extracts of armadillo liver. The activity was less in the extract from infected tissue, possibly as a result of homogenization at a higher pH, than in that from the uninfected tissue.

Gel electrophoresis of the cell-free extracts revealed six LDH isoenzymes in the armadillo liver (The Figure). Essentially, the isoenzyme patterns in extracts from the infected and uninfected tissue were similar. The intensities of individual bands of LDH activity were different for extracts from infected and uninfected liver (The Figure), but the difference in R_f for any particular band was no greater than 0.02, and this variation was not related to whether or not the tissue was infected. R_f values (bromophenol blue had a value of 1.00) were: band 1, 0.44; band 2, 0.36; band 3, 0.27; band 4, 0.19; band 4a, 0.12; and band 5, 0.04. When extracts of *M. leprae* were electrophoresed, a diffuse area of LDH activity (ML in The Figure) with an R_f value of 0.04–0.07, between bands 4a and 5 from liver tissue, was observed. This band was only observed when INT was substituted for NBT in the stain. The band marked "a" in The Figure ran in the same position as albumin (R_f 0.66) and was not dependent on the inclusion of lactate in the stain; eF can be calculated from these results: $eF = (R_f/0.66)$.

LDH in extracts of *M. leprae* was less strongly inhibited by oxamate than LDH in



THE FIGURE. Polyacrylamide gel electrophoresis of lactate dehydrogenase in cell-free extracts. Tracks A, B, G & H = extracts from untreated *M. leprae* 3 (1.2 mg protein). Tracks C, D & E = extracts from uninfected armadillo liver (0.2 mg protein). Track F = extract from infected armadillo liver (0.6 mg protein).

Lactate omitted from tracks A and C and present for the other 6 tracks. The fastest running band on each gel is the marker, bromophenol blue. The final electron acceptor was INT for tracks A, B, C & D and NBT for tracks E, F, G & H.

extracts of armadillo liver (Table 2). This differential inhibition was statistically significant; p was as low as possible in the Wilcoxon (non-parametric) test ([2]) for sample sizes at all levels of oxamate (actual values; $p = 0.005$ to 0.05). NADP was reduced in the presence of lactate both with extracts of *M. leprae* and with armadillo liver, but only at an insignificant rate compared with NAD reduction in host tissue extracts (Table 2). Additionally, pyruvate reduction by NADH (the reverse reaction of LDH) occurred at similar rates to lactate oxidation in the presence of NAD in extracts from both *M. leprae* and armadillo liver (Table 2).

Fructose-1,6-diphosphate is a known activator of bacterial ([16]) and parasite ([6]) LDHs but no such activation was observed in extracts of *M. leprae* (unpublished observations). The problem with studying and interpreting this observation in crude extracts of leprosy bacilli is that there is high aldolase activity ([14]) which would result in a change of 5 μM/min of fructose-1,6-diphosphate and triosephosphates in these as-

TABLE 1. *Lactate dehydrogenase in extracts of* M. leprae *and host tissue.*

Extract	Activity (nmol/min/mg protein)[a]
M. leprae 1	0.40
M. leprae 2	0.22
M. leprae 3	0.59
NaOH-treated *M. leprae* 1	0.56
NaOH-treated *M. leprae* 2	0.64
Uninfected armadillo liver	28
Infected armadillo liver	12

[a] May also be expressed as mU (international)/mg protein. Activity was detected by "standard" method. Limit of detection was 0.01.

TABLE 2. *Effect of altering assay system or adding inhibitors.*[a]

Alteration/addition	Extract from	
	M. leprae[b]	Armadillo liver[c]
None: "standard" method[a]	100	100
Oxamate included at:		
0.25 mM	94 ± 6 (7)	70 (2)
1.00 mM	81 ± 4 (6)	47 ± 6 (5)
10.00 mM	60 ± 4 (5)	5 ± 1 (4)
NADP replaced NAD (Alternative assay a)	36 ± 3 (4)	1.4 (2)
Reverse reaction, pyruvate reduction, NADH oxidation (Alternative assay b)	90 (2)	95 (2)

[a] Activity in incubations is expressed as a percentage of activity in the "standard" method. For absolute activity in the standard method, see Table 1. Normalized data presented as mean percent of controls ± S.D. (n).

[b] Effect of these alterations were the same on extracts of untreated or NaOH-treated *M. leprae*, and results shown are the mean values from experiments with at least three extracts, including at least one of each type.

[c] Effect of these alterations were the same on extracts of infected or uninfected armadillo liver, and results shown are the mean values from experiments with at least three extracts, including at least one of each type.

says, where 250 μg bacterial protein was used in the inoculations.

There are six LDH isoenzymes in armadillo tissue, and it is possible that they may have different properties. It is therefore possible that adsorption of specific isoenzymes to *M. leprae* might explain the differences in the properties observed between extracts of *M. leprae* and armadillo liver. However, the isoenzymes from the armadillo liver which have similar mobilities to the *M. leprae* LDH (host LDH isoenzymes 4a and 5) were inhibited by oxamate as strongly as the LDH from crude extracts (Table 3) and were therefore distinct from LDH in *M. leprae* extracts (Table 2). Indeed, when LDH isoenzymes from both infected and uninfected armadillos were fractionated (into 1–4, 4a and 5), the individual fractions had similar properties with respect to oxamate inhibition, to each other, and to LDH in crude extracts (Table 3). Poor yields from reversed electrophoresis, the method used to recover individual isoenzymes, precluded assaying bands 1–4 separately, and precluded recovering activity from the single LDH band observed for *M. leprae*.

DISCUSSION

Lactate dehydrogenase was demonstrated to be an authentic enzyme of *M. leprae*. It was distinguished from possible host-derived, contaminating activity on the basis of differential inhibition by oxamate and activity with NADP, which was almost absent in LDH from armadillo liver. The presence of LDH in the NaOH-treated *M. leprae* provided supporting evidence for its authentic bacterial nature. It has, therefore, been established that *M. leprae* can catabolize carbon sources to pyruvate ([14]) and then either anaerobically, to lactate, or oxidatively, via the tricarboxylic acid cycle ([15]).

Gel electrophoresis did not effectively separate *M. leprae* LDH and LDH isoenzymes of armadillo liver. However, it was possible to distinguish the isoenzymes with R_f values similar to *M. leprae* LDH by reverse electrophoresis followed by incubation with oxamate. Since the yields of LDH obtained by reverse electrophoresis were poor relative to the yields of proteins obtained by the workers who devised the method ([8]), sufficient LDH activity could only be recovered from host LDH, which had a relatively high specific activity in crude extracts (Table 1). The explanation for the poor yields of LDH may be that high pH was used, and heating of the LDH to above 35°C occurred during reverse electrophoresis. These factors may have caused some denaturation of the LDH. No change in the qualitative pattern of host isoenzymes was observed on infection of armadillos, in contrast to the observation of additional bands in human skin biopsies ([11]). Judging by the level of staining of *M. leprae* LDH, at least 3 mg protein, from 10^{11} *M. leprae*, would

TABLE 3. *Recovery of lactate dehydrogenase isoenzymes from gels and their inhibition by oxamate.*

Extract	Band(s)[a]	% Recovery	Activity in presence of 10 mM oxamate[b]
Uninfected armadillo liver[c]	1–4	10	5 (2)
	4a		8 ± 4 (4)
	5		5 (2)
Infected armadillo liver[d]	1–4	5	ND
	4a		10 (2)
	5		14 ± 5 (3)

[a] See The Figure for numbering.
[b] As a percentage of activity in the absence of oxamate (100 − [% inhibition]). Normalized data presented as percent of controls ± S.D. (N).
[c] 0.5 mg applied/gel.
[d] 1 mg applied/gel.

be required to account for the main additional band observed in infected skin biopsies. Therefore, it appears unlikely that *M. leprae* accounts directly for the additional LDH band in skin biopsies; an increase of at least two orders of magnitude in *M. leprae* LDH levels over the level when *M. leprae* was isolated from armadillo tissue would be required, and this would all have to appear in the skin biopsy.

Nevertheless, the finding by Saoji, *et al.* that the additional LDH band(s) were observed in biopsies only from leprosy patients with borderline to lepromatous leprosy ([11]) remains of clinical interest. The eF value of *M. leprae* LDH was 0.06 to 0.10, in contrast to the mobility of the slower "anomolous" band in infected human skin, which had an eF value of 0.11 to 0.14 ([11]).

SUMMARY

Lactate dehydrogenase (LDH) was detected in extracts of untreated and NaOH-treated *Mycobacterium leprae.* Since armadillo liver LDH isoenzymes with a similar electrophoretic mobility were shown to be considerably more sensitive to inhibition by oxamate than LDH in *M. leprae* extracts, it was confirmed that *M. leprae* grown in armadillo liver has its own LDH. Neither the activity of LDH in *M. leprae* nor its electrophoretic mobility supported the tentative suggestion that an "anomolous" LDH isoenzyme in infected skin biopsies was in fact an *M. leprae*-derived enzyme.

RESUMEN

Se encontró que hay actividad de lactato deshidrogenasa (LDH) en los extractos del *M. leprae* tratado o sin tratar con NaOH. Se confirmó que el *M. leprae* crecido en el hígado de armadillo tiene su propia LDH puesto que las isoenzymas de la LDH del hígado de armadillo que tienen una movilidad electroforética similar a la LDH de los extractos del *M. leprae* son más sensibles a la inhibición por oxamato. Ni la actividad de LDH en *M. leprae,* ni su movilidad electroforética, apoyan la proposición de algunos de que en las biopsias de piel infectada se encuentra una isoenzima "anómala" de la LDH proviniente del *M. leprae.*

RÉSUMÉ

On a détecté de la lactate dehydrogenase (LDH) dans des extraits de *Mycobacterium leprae* non traités ou traités par la soude caustique (NaOH). Vu qu'on a pu démontrer que les isoenzymes LDH du foie de tatou, présentant une mobilité électrophorétique semblable, étaient considérablement plus sensibles à l'inhibition par l'oxamate que la LDH recueillie à partir de *M. leprae,* cette observation confirme que les bacilles de la lèpre qui se multiplient dans le foie de tatou ont leur propre LDH. L'activité du LDH de *M. leprae,* pas plus que sa mobilité électrophorétique, ne confirment la suggestion provisoire qui avait été émise, qu'une isoenzyme LDH anormale (anomolous), dans des biopsies de peau infectée, était en fait une enzyme dérivée de *M. leprae.*

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