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THE EFFECT OF LEVAMISOLE ON ENERGY METABOLISM IN EHRLICH ASCITES TUMOUR CELLS *IN VITRO*

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Abstract—It has been found that levamisole, an anthelmintic drug, used also as an immunomodulator in human cancer therapy, is a strong inhibitor of tumour aerobic glycolysis. *In vitro*, in Ehrlich ascites tumour (EAT) cells and supernatants it diminishes glucose uptake and lactate formation. It does not, however, exert a similar inhibitory effect on glycolytic activity in normal liver and muscle supernatants. Metabolic and enzymatic studies have shown that levamisole directly inhibits tumour phosphofructokinase decreasing ATP, as well as 2-phosphoenolpyruvate and pyruvate as further glycolytic intermediates.

L-Cysteine used for comparison also as another inhibitor of tumour aerobic glycolysis, decreasing glucose uptake and lactate formation and diminishing pyruvate and ATP levels, differs in the accompanying increase in 2-phosphoenolpyruvate concentration. This crossing-over in metabolite concentration, only seen in tumour material, points to tumour pyruvate kinase as an isoenzyme sensitive to cysteine inhibition. Direct enzymatic studies have confirmed this suggestion.

Some similarities in the influence on the metabolism of both compounds studied have been discussed, as well as the role of the effects observed in understanding the mechanisms of levamisole action (also in worms).

In the group of biologically active sulphur compounds special attention should be focused on levamisole (Fig. 1A). It was initially discovered as a stereospecific drug with anthelmintic activity [1, 2], causing muscular spastic contractions, paralysis and the passive elimination of nematodes [1, 3, 4]. Later, it became distinguished as an immunomodulator [5–8], particularly in human cancer therapy [5, 9–11].

Levamisole, when hydrolysed, loses the thiasolidine ring, and a thiol group is formed at the aliphatic chain in the vicinity of nitrogen and the carbonyl group (Fig. 1B) [1, 3, 4]. In this way it might achieve a certain resemblance to those sulphur compounds which exert a stereospecific inhibitory effect on tumour aerobic glycolysis, and restore Pasteur's effect *in vitro* [12, 13]. The compounds mentioned include L-cysteine and those of its derivatives which have L-configuration at the alpha carbon, and the thiol group in the beta position (penicillamine), or are able to restore them *in vivo* (L-cystine, 3-mercaptopyruvate, L-serine following incubation with homocysteine) [13].

The purpose of the present study was to investigate whether levamisole, like L-cysteine, is able to inhibit the abnormally elevated aerobic glycolysis in Ehrlich ascites tumour (EAT) cells, and especially in their supernatants, where glycolysis is the only pathway of energy production. For comparison, the effect of levamisole upon glycolysis in supernatants of some normal mouse tissues (liver, muscles) was also studied.

In accordance with this, an inhibitory effect of levamisole on tumour aerobic glycolysis has been found, localized at the stage catalysed by phosphofructokinase, earlier than in the case of L-cysteine, which affects tumour pyruvate kinase [13].

MATERIALS AND METHODS

Ehrlich ascites tumour (EAT) cells were taken from Swiss albino mice on the 10th day following intraperitoneal implantation, as well as normal livers and skeletal muscles. Tumour cell pellets, obtained by centrifugation (1000 *g* for 5 min) of ascitic fluid, were washed twice in physiological saline, and used in further procedures. Part of the cell pellet (approx. 0.3 ml of the packed cells per ml of medium) resuspended in Krebs–Ringer phosphate buffer (pH 7.4), fortified with 30 mmol/l Tris and 10 mmol/l glucose (final conc.), was immediately used for incubations. Another part of the tumour cell pellet as well as normal livers and skeletal muscles were homogenized in a Potter–Elvehjem glass homogenizer with 20 mmol/l Tris–HCl buffer containing 115 mmol/l KCl, 10 mmol/l $MgCl_2$ and 2 mmol/l EDTA, and centrifuged at 100,000 *g* at 4° for 15 min. The supernatants obtained (approx. 2–8 mg of proteins per ml of the medium) were incubated in 50 mmol/l Tris–HCl buffer (pH 7.4) containing 2.5 mmol/l $KHCO_3$, 2.4 mmol/l K_2HPO_4, 6.7 mmol/l $MgCl_2$, 70 mmol/l KCl, 1 mmol/l ATP, 0.3 mmol/l NAD^+ (La Page buffer), 10 mmol/l glucose and levamisole (Janssen Pharmaceutica R-12564) in various concentrations, 0.01–1 mmol/l or L-cysteine (Reanal) 0.5–5 mmol/l. In the case of metabolic studies incubations were performed in a water bath (37°). At zero, 30 and 60 min intervals aliquots of the incubation mixtures were added to

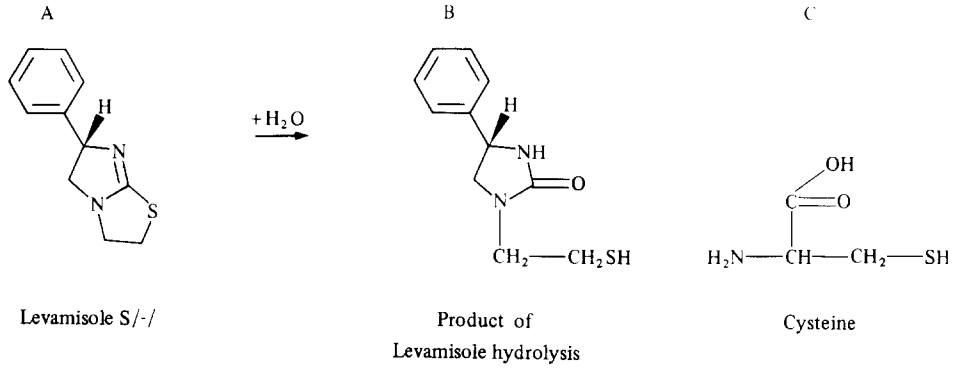

Fig. 1. The structure of levamisole and cysteine.

0.6 mol/l perchloric acid. After deproteinization, samples were taken for determinations of lactate [14] and glucose [15]. After 1 hr of incubation the remaining cell suspensions were centrifuged. The cell pellet was homogenized in 0.6 mol/l perchloric acid and the extracts obtained were adjusted to pH 7.4 with solid $KHCO_3$ and used for ATP [16], pyruvate [17] and 2-phosphoenolpyruvate [18] determinations.

Enzymatic studies were performed in supernatants obtained from Ehrlich ascites tumour cells after 1 hr incubation *in vivo* with the compounds studied. In supernatants obtained from non-incubated EAT as well as from normal skeletal muscles and livers the direct effects of the studied compounds on enzyme activities were also measured.

The hexokinase, phosphofructokinase, pyruvate kinase and lactate dehydrogenase activities were determined spectrophotometrically at 340 nm as described previously [19] in conditions ensuring pseudo-zero-order kinetics, during a 3 min period at 1 min intervals.

Protein concentration was assayed according to Lowry *et al.* [20]. The results were analysed statistically using Student's *t*-test.

RESULTS

Levamisole like L-cysteine in selected concentration of 1 mmol/l inhibited both lactate production and glucose consumption in a EAT model with a high rate of aerobic glycolysis (Table 1). The inhibitory effect was more pronounced in supernatants after removal of subcellular particles than in suspensions of intact cells. With increasing levamisole or cysteine concentrations the activity of glycolysis

Table 1. The effect of levamisole and L-cysteine on aerobic lactate production and glucose utilization in Ehrlich ascites tumour cells and their supernatants

Substance investigated	Concentration (mmol/l)	EAT cells			EAT supernatants		
		$\bar{x}$	±SD	% of control	$\bar{x}$	±SD	% of control
Lactate production							
Control	—	1.39	0.14	100.0	1.04	0.03	100.0
Levamisole	0.1	1.21	0.06	87.4†	0.71	0.14	68.3*
	1.0	0.95	0.08	68.5*	0.66	0.27	63.5‡
L-cysteine	0.1	1.13	0.03	81.1*	0.77	0.18	74.0*
	1.0	0.83	0.05	59.8*	0.65	0.18	62.5*
Glucose utilization							
Control	—	0.89	0.10	100.0	0.76	0.04	100.0
Levamisole	0.1	0.82	0.06	92.4	0.63	0.04	82.9*
	1.0	0.70	0.14	78.3‡	0.59	0.18	77.6
L-cysteine	0.1	0.84	0.16	94.6	0.51	0.06	67.1*
	1.0	0.60	0.14	67.4*	0.48	0.16	63.2*

Cells (in Krebs–Ringer phosphate buffer) and supernatants (in La Page buffer) were incubated in a water bath (37°) for 60 min with the investigated compounds and in the presence of glucose 10 mmol/l. Lactate production and glucose utilization were expressed in μmol/hr and per mg of protein (N = 10).

*$P < 0.001$.
† $0.002 < P < 0.005$.
‡ $0.005 < P < 0.01$.

Table 2. The effect of levamisole and L-cysteine on aerobic lactate production in EAT, liver and muscle supernatants

Substance investigated	Liver		Skeletal muscle		EAT	
	$\bar{x}$	% of control	$\bar{x}$	% of control	$\bar{x}$	% of control
Lactate production						
Control	0.33	100.0	0.72	100.0	1.04	100.0
Levamisole	0.36	109.1	0.64	88.8	0.66	63.5
L-cysteine	0.30	90.9	0.68	94.4	0.65	62.5

Supernatants in La Page buffer (pH = 7.4) were incubated in a water bath (37°) for 60 min in the presence of investigated compounds 1 mmol/l and glucose 10 mmol/l. Lactate production was expressed in μmoles/hr and per mg of protein (N = 3).

in intact cells decreased to approx. 50% of the control activity. No further changes were seen.

Comparative studies of the effect of levamisole on glycolysis in normal mouse tissue (liver, muscles) and EAT supernatants have shown that levamisole inhibits only excessive aerobic glycolysis in neoplastic material, reducing it to the values characteristic of muscle glycolysis in our case, but did not exert such an effect upon glycolytic activity in normal tissue supernatants (Table 2).

Parallel to the inhibition of lactate production and glucose consumption, levamisole distinctly diminished the ATP level in incubated EAT cells to approx. 40% of the control, whereas cysteine reduced it to about 60% of the control value (Table 3). In addition to this, levamisole also caused a decrease in pyruvate and 2-phosphoenolpyruvate concentrations. In this respect it caused a different effect from cysteine, which decreased ATP and pyruvate concentrations but increased their precursor level, i.e. 2-phosphoenolpyruvate. This suggested that the inhibition of glycolysis by cysteine is localized in the stage catalysed by pyruvate kinase.

Although the maximal changes in lactate and ATP levels were observed in the same 0.1–1 mmol/l concentrations of both compounds studied, more detailed dose-effect determinations (Fig. 2) indicate that comparable metabolic effects can be obtained with 50-times lower concentrations of levamisole (0.01 mmol/l) than with L-cysteine (0.5 mmol/l). Corresponding amounts of D-cysteine in the same experimental system had no effect.

Investigations on some selected glycolytic enzymes have shown that following the incubation of EAT cells with levamisole, the activities of phosphofructokinase, pyruvate kinase and lactate dehydrogenase were markedly lowered, whereas in the case of cysteine—in accordance with the results of metabolic studies—only the pyruvate kinase activity was decreased (Table 4). Studies of the direct effects of the substances investigated on enzyme activities *in vitro*, in comparison with the studies of enzyme activities following cell incubation with the same substances *in vivo*, make it possible to distinguish their direct inhibitory influence from the changes caused indirectly by accumulated endogenous signal metabolites.

Cysteine acting directly on tumour supernatants (Table 5) inhibited pyruvate kinase only. On the other hand, levamisole directly inhibited both key

Table 3. The effect of levamisole and L-cysteine on ATP, pyruvate and 2-phosphoenolpyruvate levels in Ehrlich ascites tumour cells

Substance investigated	ATP		Pyruvate		2-Phosphoenol-pyruvate	
	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD
Control values						
nmol/mg protein	12.1	1.59	2.19	0.07	1.26	0.12
percentage	100.0	13.1	100.0	3.2	100.0	9.5
Levamisole	40.0	21.6†	83.7	5.2†	66.7	18.3†
L-Cysteine	66.7	25.9§	44.7	9.6*	222.6	64.5†

Cells in Krebs–Ringer phosphate buffer (pH = 7.4) were incubated in a water bath (37°) for 30 min with 1 mmol/l of investigated compounds and 10 mmol/l of glucose. The results were expressed as the percentage of the control values ± SD (N = 6).

*$P < 0.001$.
† $0.001 < P < 0.002$.
‡ $0.01 < P < 0.02$.
§ $0.02 < P < 0.05$.

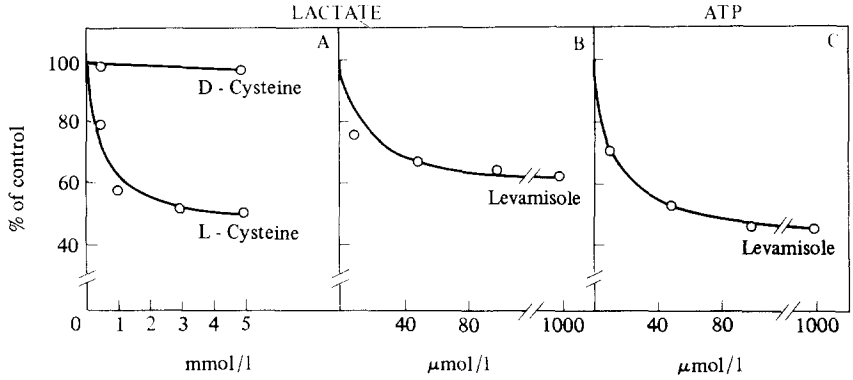

Fig. 2. The effects of various concentrations of studied compounds on lactate formation (A, B) and ATP level (C) in Ehrlich ascites tumour cells. The results are expressed as a percentage of control values.

Table 4. Activity of some glycolytic enzymes in supernatants obtained after incubation of Ehrlich ascites tumour cells with levamisole or L-cysteine

Enzyme estimated	Control		Levamisole		L-Cysteine	
	$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD
Hexokinase	44.7	8.3	40.8	5.6‡	37.2	13.3‡
Phosphofructokinase	72.8	21.2	44.2	1.1‡	60.4	26.4‡
Pyruvate kinase	7083.0	1817.0	4367.0	80.0†	6260.0	87.6‡
Lactate dehydrogenase	7673.0	909.0	4134.0	886.0*	8668.0	798.0‡

Cells in Krebs–Ringer phosphate buffer (pH = 7.4) were incubated in a water bath (37°) for 60 min in the presence of investigated compounds 1 mmol/l and glucose 10 mmol/l. The results were expressed in mIU per mg of protein (N = 4).

* $0.005 < P < 0.01$.

† $0.02 < P < 0.05$.

‡ $0.05 < P$.

Table 5. The direct effect of levamisole and L-cysteine on activity of some glycolytic enzymes in supernatants of Ehrlich ascites tumour cells and normal mouse tissues (muscle and liver)

Enzyme and material	N	Control (mIU/mg protein = 100%)		Percentage of control Levamisole		L-Cysteine	
		$\bar{x}$	±SD	$\bar{x}$	±SD	$\bar{x}$	±SD
Phosphofructokinase	4						
EAT		40.4	17.2	67.6	18.0*	103.7	6.4
Muscle		35.9	20.4	97.7	3.5	110.5	16.5
Liver		25.2	8.9	101.4	3.9	102.5	21.6
Pyruvate kinase	6						
EAT		3943.0	970.0	52.6	3.4†	65.9	8.7*
Muscle		4552.0	1833.0	105.4	5.6	101.4	13.2
Liver		435.0	155.0	98.7	4.3	111.2	13.9
Lactate dehydrogenase	5						
EAT		5643.0	1182.0	96.0	7.3	103.2	3.9
Muscle		6183.0	1203.0	101.0	8.5	103.6	13.9
Liver		2163.0	792.0	96.0	6.5	85.6	11.0

The investigated compounds (0.05 mmol/l final concentration) were added to the reaction mixture. Enzyme activities were measured spectrophotometrically at 340 nm (25°) for 3 min at 1 min intervals. The results, calculated in mIU per mg protein, were expressed as the percentage of the control value ± SD.

* $0.02 < P < 0.05$.

† $0.05 < P < 0.01$.

enzymes—pyruvate kinase and phosphofructokinase. This suggested that the inhibition of lactate dehydrogenase observed after cell incubation, was caused indirectly by the endogenously formed metabolites. Like cysteine, levamisole inhibited enzyme activities only in neoplastic material, whereas enzyme activities in normal tissue supernatants remained unchanged.

DISCUSSION

As has been previously shown [13] cysteine (0.1–1 mmol/l) stereospecifically inhibits aerobic glycolysis in neoplastic cells. Levamisole, which after hydrolysis *in vivo* might restore the structure containing a fragment of cysteine with free thiol group in the molecule [1, 3, 4] (Fig. 1b), in the same concentrations also inhibits aerobic glycolysis, measured both by lactate formation and glucose consumption. Yet certain differences are clearly visible in respect to the site of inhibition. In neoplastic material the L-cysteine causes a drop in the pyruvate and ATP concentrations, accompanied by an increase in the 2-phosphoenolpyruvate level, which indicates that the inhibitory effect of L-cysteine is localized at the stage catalysed by tumour pyruvate kinase. On the other hand, the levamisole-evoked drop in EAT pyruvate level, accompanied by a simultaneous decrease in 2-phosphoenolpyruvate and ATP concentrations, suggests that inhibition of tumour glycolysis, is related to, or even precedes the formation of the first ATP molecule. On the basis of study now reported it is impossible to exclude the effect of levamisole on 3-phosphoglyceraldehyde dehydrogenase as a thiol enzyme [21]. Direct enzymatic studies, however, have shown the inhibitory effect of levamisole on tumour phosphofructokinase.

Inhibition by levamisole of glucose catabolism in neoplastic cells at the stage catalysed by phosphofructokinase, preceding the formation of the first ATP molecule, consequently disturbs the fundamental glycolytic process of energy formation also in anaerobic conditions. Inhibition of this key enzyme, limiting and regulating glycolysis [22], causes a decrease not only in the ATP level but also in the other intermediates, including 2-phosphoenolpyruvate, a further substrate for ATP, as well as in pyruvate and lactate. This also explains the decrease in the pyruvate kinase and lactate dehydrogenase activities. It seems that the phenomenon of forward control depending on the stimulatory effect of fructose-1,6-diphosphate on pyruvate kinase [23] does not operate in the case of phosphofructokinase inhibition. Although the maximal metabolic effects of levamisole are similar to those after L-cysteine, dose–effect studies show that levamisole is able to inhibit glycolysis and diminish ATP level even in a concentration 50 times lower than that of L-cysteine. It also seems that they are not directly dependent only on the presence of the thiol group, since D-cysteine in the same experimental system has no effect, so it looks like an allosteric effect of a signal molecule.

Special attention should be paid to the selective ability of cysteine and levamisole to inhibit only neoplastic enzymes. Tumours also contain, in addition to the normal enzymatic pattern, some abnormal isoenzymes with different sensitivities to signal molecules [12, 13], which seem to be responsible for increased aerobic glycolysis. Therefore levamisole or L-cysteine even in high concentrations inhibit glycolysis to about 50% by blocking only these tumour isoenzymes.

In worms it was assumed that the thiol group of levamisole, obtained after its hydrolysis *in vivo*, interacts stereospecifically with the thiol groups of species-specific fumarate reductase (succinate dehydrogenase) and blocks the active centre of this enzyme by forming stable S—S bonds [1, 24–27]. Thus, during the anaerobic catabolism of glucose it blocks the reduction of fumarate to succinate, which is the source of ATP molecule [28, 29], and decreases the energy level. Recently it has been shown that levamisole has multiple sites of action in the respiratory chain also [30].

Muscular paralysis in worms has been explained as due to changes in the parasympathetic and sympathetic ganglia [31, 32]. It is not known whether phosphofructokinase activity might also be inhibited by levamisole in worms. If so, the changes in their muscular tension might also be a result of a drop in ATP. Irrespectively of the inhibition of fumarate reductase in some immature or adult nematodes [26, 27] which diminish the ATP level, it seems that much greater changes might originate from the inhibition of glycolysis, considered as a universal energy formation pathway, in anaerobic condition.

The energy required for neuromediator synthesis, cell membrane potential formation, and the maintenance of normal muscular tension is normally derived from glycolysis. Further studies in this field should verify this hypothesis.

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