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THE BEHAVIOUR OF LDH-3 IN PATIENTS WITH MALIGNANT DISEASES DURING THERAPY WITH CYTOSTATIC DRUGS AND PREDNISONE, STUDIED BY LDH-ISOENZYME ELECTROPHORESIS ON CELLULOSE ACETATE

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Summary

A modified method used for the quantitative estimation of LDH-isoenzymes in serum after electrophoresis on cellulose acetate is described.

Total LDH-activity and isoenzyme distribution in serum samples of capillary blood are compared to those in samples collected by venipuncture.

Total LDH-values and LDH-distributions both in 29 normal children and 14 children with malignant diseases are given.

When studying the LDH-distribution in serum samples of children with malignant diseases a significantly decreased LDH-3 was found in many patients. LDH-4 and LDH-5 were also reduced. It was noted that these children all received therapy with prednisone. Possible explanations are discussed.

Introduction

In many patients with malignant diseases serum LDH is increased and an abnormal isoenzyme distribution occurs. In leukemia an elevated LDH-2 fraction has been reported [1] and in patients with other tumors often, but not invariably, elevated LDH-3, LDH-4 and LDH-5** fractions were described [2].

In those patients in which the LDH-isoenzymes reflect LDH release from tumor cells, enzyme analysis may provide a useful parameter for pro- or regres-

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** European nomenclature: LDH-5 closest to the cathode.

sion of the tumor in connection with the therapy. For this reason we decided to investigate LDH-isoenzymes in such patients. We observed that in many children with malignant diseases under treatment a low LDH-3 was present. The study of this phenomenon is the subject of the present paper. The cause of the LDH-3 abnormality cannot be attributed to the method of investigation. The electrophoretic technique used is a modification of the method reported by Preston et al. [3], which has been improved at several points. The isoenzyme distributions obtained in reconvalescent children were similar to the normal distribution values given in the literature [2,4—7].

Materials

Investigations were carried out in samples of 29 reconvalescent children, 26 patients with malignant diseases before and during therapy with cytostatic drugs and prednisone. The group of patients investigated included 6 patients with lymphoblastic leukemia, 1 patient with monocytic leukemia, 3 patients with lymphosarcoma, 3 patients with rhabdomyosarcoma, 1 patient with histiosarcoma, 7 patients with Wilms' tumor and 5 patients with neuroblastoma.

Capillary blood samples were collected in polythene microtubes (Beckman EET-23). All blood samples were centrifuged at 15 000 rpm in a Beckman Microfuge for 45 seconds. The clear serum was removed from the residual erythrocytes. If any indication of hemolysis was observed the specimen was discarded. With a few exceptions, the estimation of the total LDH-activity and the analysis of isoenzymes were accomplished within 5 hours.

Methods

Electrophoresis

LDH-isoenzymes were separated on cellulose acetate support medium using the Gelman Sepratek Electrophoresis System (Gelman Instrument Co., Ann Arbor, Michigan). Electrophoresis was carried out by a modification of the method previously described by Preston et al. [3].

Usually two strips were floated per chamber, in freshly prepared Tris—Barbital buffer, pH 8.8, ionic strength 0.075, to wet them. Then the strips were equilibrated in the cold buffer (4°) for at least 10 minutes (maximum 3 h). The strips were lightly blotted with filter paper to remove excess of moisture and fixed on each side of the membrane support bridge. The bridge was then placed into the electrophoresis tank. Normally, 10 μl of serum were sufficient to obtain a clear LDH-isoenzyme pattern.

Per strip two samples were applied near the cathode bridge by means of the wire applicator filled with the serum samples on positions 1, 3, 6 and 8. Usually five applications were performed. Electrophoresis was carried out with previously cooled Tris—Barbital buffer (pH 8.8, I = 0.075) at 100 V, initially 2.0 mA per strip for 45 minutes. Temperature during electrophoresis increased from 16 to 18°.

Detection of the LDH-isoenzymes

In order to localize the isoenzyme bands each electrophoretic strip was

equilibrated with a second 2.5 cm × 7.5 cm cellulose acetate strip (Gelman No. 51107) impregnated with a substrate and staining mixture according to a modification of the procedure described by Barnett [8,9].

Immediately prior to use, the substrate strips were saturated by floating them onto the staining mixture which contained 0.19 M sodium lactate, 0.19% NAD (nicotinamide adenine dinucleotide), 0.057% NBT (nitro blue tetrazolium), 0.006% PMS (phenazine methosulphate) and 0.023 M phosphate buffer pH 7.5 [3,9,10]. Following electrophoresis, each electropherogram was placed onto a glass slide with the application side facing upward. After removing excess of moisture an impregnated strip was superimposed exactly onto the electropherogram. Then it was overlaid with a second glass slide making a sandwich; air bubbles between strips and glass slides should be avoided. The sandwich was wrapped in filter paper in order to protect the incubation mixture from light. The sandwich was then transferred to a petri-dish containing moistened filter paper and the closed petri-dish was incubated for 50 minutes at 37°.

During preparation of the incubation mixture and during incubation, exposure to direct light was prevented as much as possible.

Clearing procedure

Following incubation the sandwich was disassembled carefully and the upper glass slide removed. Then the strips were marked so that after clearing they could by superimposed onto each other exactly. Both strips were fixed in 10% formaline for approximately 10 minutes. Excess of moisture was removed and then the strips were soaked for 10 minutes in a clearing solution of dimethylformamide—acetic acid—water (30 : 12 : 58, v/v). Then the substrate strip was placed centrally onto a clean glass slide and overlaid with the electropherogram according to the previously signed marks. Air bubbles, if present, were removed carefully and the glass slide was placed quickly in an oven at 100°. After 15 minutes the combined strips were cleared, heat-sealed and mounted on the glass slide.

Quantitation

Scanning of the cleared and combined strips was done in a Digiscreen-R automatic scanning densitometer with integrating recorder (Gelman Instrument Co., Ann Arbor, Michigan) at 575 nm and the percentual ratio of the isoenzyme fractions was calculated.

Determination of total serum LDH activity

Total LDH was determined by a modification of the method of Wroblewski and La Due [11,12] using the LKB 8600 reaction rate analyzer (LKB Instruments, Sweden). Reaction conditions: Tris—EDTA buffer 0.10 M, pH 8.0, NADH 0.10 M, sodium pyruvate 0.87 M, temperature 35°. The LDH activity was measured by continuous monitoring of the absorbance at 340 nm.

Comments to the Methods

Cellulose acetate electrophoresis was the method of choice for the fractionation of the LDH-isoenzymes for the following reasons:

(1) a short migration time of the isoenzymes on cellulose acetate as the support medium;

(2) excellent resolution of the individual isoenzyme fractions;

(3) a stable and strongly colored zymogram is achieved, which can be stored for documentation easily;

(4) electrophoretic separation and staining can be obtained in 110 minutes. The entire procedure, including fixation and clearing of the electropherogram and quantitative densitometric determination of the fractions can be completed in 160 minutes;

(5) the same apparatus, the same support medium and the same buffer can be used for the determination of the serum proteins.

The use of a buffer of higher ionic strength in the underlying method afforded sharpening of the electrophoretic zones. Because of the low voltage used, heat production and evaporation were negligible and no cooling of the electrophoresis apparatus was necessary. Migration time was reduced to 45 minutes. The use of cold buffer and the short migration time prevented inactivation of the labile LDH-4 and LDH-5. Color development according to the sandwich technique of Barnett [8,9] was accomplished more simply by enclosing the electrophoretic strip and the substrate strip between glass slides [13]. This prevented curling of the cellulose acetate. During incubation the sandwich was placed in a moist petri-dish to prevent drying of the sandwich and losing of active staining. Incubation time was prolonged to 50 minutes, resulting in better staining. The sandwich was wrapped in filter paper in order to protect the staining mixture from light, because phenazine methosulphate is very sensitive to light. Until fixation this part of the procedure was performed in a dimly-lit room. This resulted in minimal background stain, which greatly

Fig. 1.

Electropherograms of LDH-isoenzymes in serum of two normal children: a and b are duplicates.

Serum	LDH-1 (%)	LDH-2 (%)	LDH-3 (%)	LDH-4 (%)	LDH-5 (%)	Total LDH (I.U.)
I	35	47	10	7	1	227
II	31	52	9	5	3	115

facilitated densitometric evaluation. Clearing of the strips yielded completely transparent stained electropherograms without any turbidity. By scanning of the two cleared, combined strips a greater sensitivity was achieved. In Fig. 1 the electropherograms of LDH-isoenzymes in serum of 2 normal children are given.

Reproducibility

Two serum samples were analysed in 10-fold. The mean values and standard deviations are summarized in Table I. The standard deviations are calculated according to the formula.

$$\text{S.D.} = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

TABLE I

MEAN VALUES AND STANDARD DEVIATIONS OF TOTAL LDH-ACTIVITY AND ISOENZYME DISTRIBUTION OF SERUM SAMPLES OF TWO PATIENTS

Serum	LDH-1 (%)	LDH-2 (%)	LDH-3 (%)	LDH-4 (%)	LDH-5 (%)	Total LDH (I.U.)
I (n = 10)	44 ± 2.7	34 ± 1.5	12 ± 1.0	7 ± 2.0	3 ± 1.5	119 ± 0.9
II (n = 10)	33 ± 2.8	43 ± 1.7	16 ± 2.4	6 ± 1.4	2 ± 0.5	938 ± 6.8

Results in normals and patients

The distribution of the LDH-isoenzymes expressed as percents in serum samples of 29 reconvalescent children are listed in Table II. The isoenzyme fractions were estimated in duplicate. The values calculated for each fraction were averaged and for each fraction the standard deviation was determined. Total LDH-activity and isoenzyme distribution in serum samples obtained from capillary blood and from blood samples collected by venipuncture were compared. The results are summarized in Table III. It can be concluded that there is no significant difference between the isoenzyme distribution of capillary collected blood and samples collected by venipuncture. In total LDH there were differences between the two kinds of samples: in seven cases venipuncture blood yielded lower values, in three cases, however, higher values than capillary blood.

In Table IV, some examples of isoenzyme distributions and total LDH-values in patients with malignant diseases are given. Only those patients are included in which the determinations were performed before therapy was started. In six patients a strongly elevated total LDH-activity was found, the isoenzyme pattern being more or less abnormal. In patient D.B. liver abnormalities existed, resulting in increased LDH-4 and LDH-5 fractions. Only one patient (C.M.) had a strongly elevated total LDH combined with a practically normal isoenzyme distribution. Three patients had a slightly elevated total LDH-activity. Two of them had a normal; one (F.C.) had an abnormal spectrum. Four patients had a normal total LDH-activity. In three of them the isoenzyme distribution was practically normal, in one (L.U.) it was definitely abnormal, probably due to liver damage. In Fig. 2A the isoenzyme distribu-

TABLE II

MEAN VALUES, STANDARD DEVIATIONS AND RANGES OF LDH-ISOENZYMES IN 29 RECONVALESCENT CHILDREN

Fraction	Mean value	S.D.	Theoretical range (M ± 2 S.D.)	Actual range
LDH-1 (%)	41.6	7.2	27–56	25–54
LDH-2 (%)	42.9	5.1	33–53	35–53
LDH-3 (%)	12.6	5.2	3–23	5–25
LDH-4 (%)	1.9	2.3	0– 7	0–10
LDH-5 (%)	1.0	1.2	0– 3	0– 4

tions in 13 patients (mean ± S.D.) on therapy with cytostatic drugs and prednisone are compared with the normal distribution (mean ± S.D.) given in Table II. Only those patients receiving cytostatic drugs and more than 10 mg/m^2 prednisone per day (orally) are included. One patient (M.v.H., see Tables IV and VI) who developed severe liver disease during therapy was excluded. Figure 2B shows the isoenzyme distributions in 19 patients (mean ± S.D.) under treatment with cytostatic drugs without prednisone in comparison with the normal distribution (mean ± S.D.). None of these patients had severe liver damage. It can be seen that in patients on prednisone treatment a shift to the fast-moving fractions occurs. In such patients abnormally low LDH-3 levels are found. Patients under treatment with cytostatic drugs alone did not show this

TABLE III

TOTAL LDH-ACTIVITY AND ISOENZYME DISTRIBUTION IN SERUM SAMPLES OBTAINED FROM CAPILLARY BLOOD (c) AS COMPARED WITH THOSE FROM BLOOD SAMPLES COLLECTED BY VENIPUNCTURE (v)

Patient	LDH-1 (%)	LDH-2 (%)	LDH-3 (%)	LDH-4 (%)	LDH-5 (%)	Total LDH (I.U.)
1 v	58	33	5	3	1	168
c	56	34	5	4	1	199
2 v	29	33	17	18	3	479
c	24	34	21	16	5	451
3 v	43	38	9	7	3	202
c	41	39	11	7	2	239
4 v	52	36	7	4	1	234
c	53	30	8	6	3	218
5 v	54	32	7	4	3	207
c	54	31	7	5	3	196
6 v	56	27	9	6	2	185
c	47	36	12	4	1	228
7 v	56	29	8	5	2	200
c	44	40	9	5	2	229
8 v	33	32	15	12	6	182
c	39	32	16	8	5	207
9 v	47	28	12	8	5	217
c	46	29	12	9	4	237
10 v	38	36	14	10	2	200
c	35	34	14	12	5	248

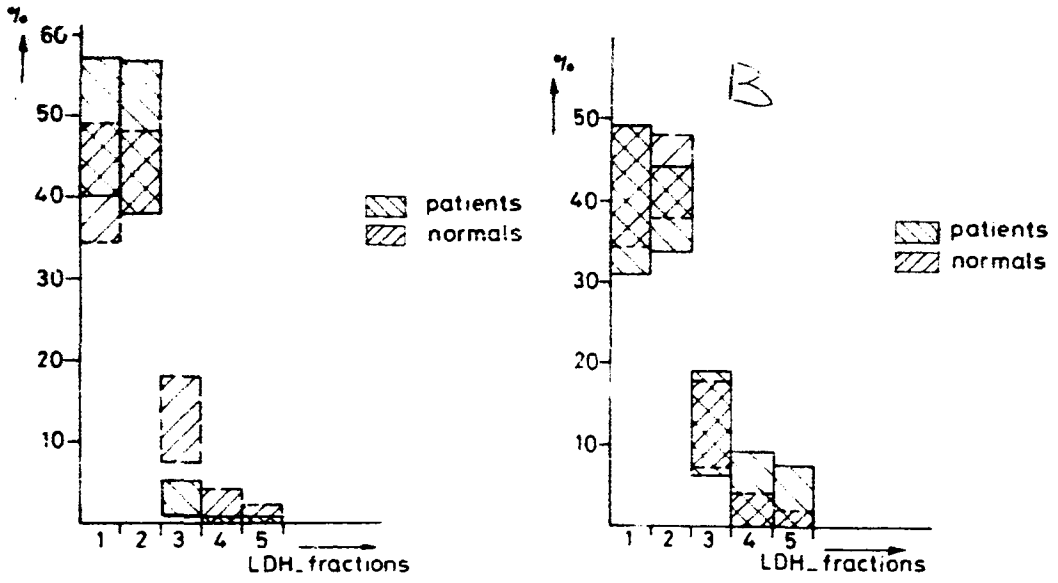

Fig. 2. A. LDH-isoenzyme distribution (%, mean ± S.D.) in patients on therapy with cytostatic drugs and prednisone (n = 13) in comparison with normals (n = 29). B. LDH-isoenzyme distribution (%, mean ± S.D.) in patients on therapy with cytostatic drugs without prednisone (n = 19) in comparison with normals (n = 29).

shift. On the contrary they showed slightly elevated LDH-4 and LDH-5 fractions, probably due to slight liver abnormalities.

Table V gives the mean LDH-distribution values and standard deviations of the two patient groups in comparison with the normal distribution. Also the calculated ratios of the H-monomer to the M-monomer are given. It can be seen

TABLE IV

ISOENZYME DISTRIBUTIONS (%) AND TOTAL LDH-VALUES (I.U.) IN PATIENTS WITH MALIGNANT DISEASES

SGPT and γ-GT are reported for information about the condition of the liver.

Patient	Age (years)	Diagnosis	LDH-1 (%)	LDH-2 (%)	LDH-3 (%)	LDH-4 (%)	LDH-5 (%)	Total LDH (I.U.)	SGPT (I.U.)	γGT (I.U.)
B. B.	10	Lymphatic	25	58	17	0	0	1364	4	10
D. B.	13	leukemia	19	23	23	20	15	3030	61	15
C. M.	4	Neuro-	33	36	21	8	2	1410	10	23
F. V.	3	blastoma	31	34	20	11	4	1870	8	30
M. v. H.	3		21	38	27	12	2	1185	5	10
H. H.	11	Lympho-	39	51	8	0	2	99	8	8
L. U.	7	sarcoma	51	20	9	7	13	69	35	6
S.	9	Rhabdomyo-sarcoma	35	42	20	1	2	193	7	10
A. R.	13	Histio-sarcoma	38	54	8	0	0	107	12	10
A. v. d. M.	6	Wilms' tumor	34	48	10	7	1	227	22	39
B. K.	8		48	42	10	0	0	276	—	6
M. H.	2		17	32	21	22	8	1423	3	10
F. C.	2		28	31	20	16	5	400	6	6

TABLE V

MEAN VALUES AND STANDARD DEVIATIONS OF LDH-ISOENZYME DISTRIBUTIONS AND H/M RATIOS IN PATIENTS UNDER TREATMENT WITH CYTOSTATIC DRUGS WITH PREDNISONE (I) WITHOUT PREDNISONE (II) AND IN CONTROLS (RECONVALESCENT CHILDREN) (III)

Patient group	n	LDH-1 (%)		LDH-2 (%)		LDH-3 (%)		LDH-4 (%)		LDH-5 (%)		H/M	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
I	13	48.5	8.3	47.2	9.4	3.1	2.1	0.7	0.8	0.5	0.8	6.1	0.8
II	19	40.2	9.2	39.3	5.1	12.6	6.4	4.5	4.8	3.4	4.2	3.8	1.4
III	29	41.6	7.2	42.9	5.1	12.6	5.2	1.9	2.3	1.0	1.2	4.2	1.0

that the H/M ratio in patients under prednisone treatment is significantly higher than in normals. This is not the case in patients on therapy with cytostatic drugs without prednisone.

In Table VI the total LDH-activity, the isoenzyme distribution and the ratio of the H-monomer to the M-monomer of patients without prednisone and during therapy with prednisone are listed. Serum glutamate-pyruvate transaminase (GPT; EC 2.6.1.2) and γ-glutamyltranspeptidase (GT; EC 2.3.2.1) are reported as indicators for liver damage.

Discussion

The isoenzyme distributions as found by us in reconvalescent children are in accordance with the distribution patterns given in the literature [2,4—7]. There is a good correlation between the fractional values of isoenzyme distribution patterns made on cellulose acetate and agar.

TABLE VI

ACTIVITY OF LDH-ISOENZYMES (%), TOTAL LDH (I.U.) AND THE CALCULATED RATIO OF THE ACTIVITY OF THE H-MONOMER TO M-MONOMER IN 7 PATIENTS WITHOUT THERAPY WITH PREDNISONE (−) AND DURING THERAPY WITH PREDNISONE (+)

SGPT and γ-GT are reported for information about the condition of the liver.

Patient	Age (years)	Diagnosis	Data	P	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5	Total LDH	SGPT (I.U.)	γ-GT (I.U.)	H/M
P. V.	11	Lymphoblastic	18- 7-73	+	44	48	6	1	1	282	15	11	4.9
		leukemia	6-12-73		22	34	24	16	4	432	3	13	1.7
A. R.	13	Histio-	30- 7-73	−	38	54	8	0	0	108	12	10	4.7
		sarcoma	10-10-73	+	35	65	0	0	0	171	5	8	5.2
			16-11-73	+	46	50	3	0	0	167	—	—	6.0
E. S.	12	Lymphoblastic	7- 9-73	+	36	60	4	0	0	661	27	12	4.9
		leukemia	16-11-73	−	26	36	24	13	1	2100	29	—	2.1
B. B.	11	Lymphoblastic	4-10-73	−	25	58	17	0	0	1364	4	10	3.3
		leukemia	18-10-73	+	45	52	2	1	0	221	26	14	5.8
R. F.	3	Lymphoblastic	27-11-73	−	31	31	22	20	6	349	9	6	1.5
		sarcoma	9- 1-74	+	62	33	1	2	2	148	9	16	7.2
M.v.H.	3	Neuro-	27-12-73	−	21	38	27	12	2	1185	4	10	1.9
		blastoma	23- 1-74	+	36	44	9	5	6	882	124	33	3.0
F. V.	3	Neuro-	14- 3-74	−	31	34	20	11	4	1870	8	30	2.3
		blastoma	30- 5-74	+	36	44	13	5	2	433	—	—	3.3

Although the differences between capillary and venous samples of total LDH are not striking, capillary values seem to be somewhat higher in most cases. This may reflect a slight hemolysis in capillary sampling as the LDH content of erythrocytes is very high [2].

In practice capillary samples can be used for LDH analysis in pediatric laboratories, unless visible hemolysis is observed. As can be seen from Table IV, serum LDH-activity is often, but by no means always increased in malignant diseases. In many patients the slow moving isoenzyme fractions contribute to a greater extent. These observations agree with data given in the literature [1,2,14–16].

It is known that administration of cytostatic drugs first produces a steep rise in LDH-activity (necrotic phase), followed by a gradual decline to a normal level (cytostatic phase) [2,17,18]. The reduction of LDH-3 as shown in Figure 2A and of total LDH-M-monomers as given in Table V and VI in patients receiving prednisone can not be explained definitely. From Figure 2B it follows that it cannot be a cytostatic effect, resulting from a diminished tumor cell production, leading to a decrease of the number of tumor cells and to a smaller release of LDH into the circulation. Such a cytostatic effect of prednisone has been described in patients with lymphogranulomatosis [19–21].

Possible explanations are:

(1) Inhibition of the synthesis of LDH in the tumor cells by prednisone. Inhibition of the synthesis of M-monomers is less probable, for LDH-2 does not decrease at all in patients receiving prednisone (Fig. 2A).

(2) Membrane stabilization in the tumor cells [22] leading to a decreased release of LDH from that tissue.

(3) Necrosis of the adrenals in which the concentration of LDH-3 is up to 60% [16]. Finally this may result in a decrease of LDH-3 in the circulating plasma. But in that case first an increase must follow the administration of prednisone, which has not been investigated.

In this work the LDH and its isoenzymes were investigated in order to look for suitable parameters for the evaluation of tumor therapy. It seems that LDH is rather non-specific for this purpose. A better result may be expected from enzymes which are more specific for the type of tissue the tumor belongs to. Chemical research in this direction has to be stimulated as much as possible.

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