Lactate Dehydrogenase Isoenzyme Pattern in Sera of Patients with Malignant Diseases

Elefteria E. Giannouli,¹ Dimitrios L. Kaliaptsi,²* Constantinos Tentas,³ and Phedon Fessas²

Total lactate dehydrogenase (LDH; EC 1.1.1.27) activity and the percentage distribution of LDH isoenzymes were determined in 127 patients with malignant diseases. A shift in the isoenzyme patterns was observed toward the M-type, with an increase in the percentage of LDH-4 and LDH-5 isoenzymes and a slight increase in total LDH activity of all patients. Serum samples from 68 of the patients contained an abnormal isoenzyme of LDH, "LDH-1ex," that, on agarose gel electrophoresis at pH 8.6, migrated between albumin and LDH-1 isoenzyme. Chemotherapy, radiotherapy, or surgical removal of the tumor was accompanied by disappearance of this abnormal isoenzyme. The heat stability of LDH-1ex isoenzyme appears to be similar to that of LDH-1 but greater than that of the other LDH isoenzymes. Statistical analysis of these data demonstrated a significant correlation between malignancy and the appearance of LDH-1ex isoenzyme (P < 0.001). In contrast, the relationship between LDH-1ex isoenzyme and metastasis or anatomical location of the malignancy is not statistically important (P < 0.1).

Additional Keyphrases: electrophoresis, agarose gel • monitoring therapy • chemotherapy • tumor markers • cancer

Lactate dehydrogenase (LDH; EC 1.1.1.27) is a tetrameric enzyme composed of two immunologically distinct subunits (M and H).² The five theoretically possible forms of LDH (H₄, H₂M₂, H₂M₃, H₂M₄, and M₄) are found in human tissues, the proportions of these isoenzymes differing in various organs. Not only are isoenzyme patterns believed to vary according to the metabolic requirements of each tissue (1), but also alterations in the proportions of the LDH isoenzymes have been observed during development, under changing biological conditions, and in response to pathological processes (2, 3). Shifts in the patterns of LDH isoenzymes have often been observed in cancers in laboratory animals and in humans (3–13).

Recently, many workers have reported the presence of additional bands in electrophoretograms of human serum. Some of these bands have been shown to be genetic variants (14), but more commonly they have been established to be immunoglobulin complexes (15–23), tumor products (23–28), or recombination products between H, M, and C monomers made during storage of samples at −20 °C (27). All of these extra bands have appeared after agarose gel electrophoresis at pH 8.6 between the normal location of LDH-1 (H₄) and LDH-5 (M₄) isoenzymes, except for two unusual isoenzymes migrating more cathodally to LDH-5, designated LD-6 and LD, (26, 27, 29). However, "LD-6" is apparently not a true lactate dehydrogenase (26, 27).

Here we report the presence of an abnormal LDH isoenzyme, LDH-1ex, located anodally to the LDH-1 isoenzyme and associated with malignant states.

Materials and Methods

Specimen Collection

We examined 33 apparently healthy individuals and 127 patients with malignant diseases, admitted to the General State Hospital "Agios Andreas"-University of Patras, during the two-year period from 1984 through 1986. The patients, 71 men and 56 women, ranged in age from 30 to 85 years. Table 1 lists the primary sites of their tumors. Of the 127 patients examined, 52 presented with metastasis of their tumor to one or more organs. Patients with co-existing myocardial infarction, liver diseases, diabetes mellitus, uremia, muscle diseases, septic conditions, or traumas were excluded. Specimens were collected before any curative surgical resection or before any radiotherapy or chemotherapy of patients, except in the follow-up studies.

Venous blood was sampled in the morning and serum was promptly separated from the clot. Serum obtained by centrifugation (20 min, 3000 × g, 20 °C) was stored for no more than 5 h at room temperature before use. For long-term storage, the samples were kept at −70 °C.

Materials

L(+)-Lactic acid, NAD⁺, iodonitrotetrazolium chloride (INT), and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co., St. Louis, MO 63178. Agarose Universal Electrophoresis Film (cat. no. 470100), diethylbarbituric buffer, pH 8.6 (cat. no. 470180), and the LDH staining kit (cat. no. 470014) were all from Corning Medical Co., Palo Alto, CA. The LDH kit (cat. no. 124885) was from

Table 1. Various Malignant Diseases of Patients with or without LDH-1ex Activity in Serum

<table>
<thead>
<tr>
<th>Malignant disease</th>
<th>With LDH-1ex</th>
<th>Without LDH-1ex</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant lymphomas</td>
<td>7</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Lung carcinoma</td>
<td>15</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>Colonic carcinoma</td>
<td>4</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>13</td>
<td>4</td>
<td>17</td>
</tr>
<tr>
<td>Bladder carcinoma</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Prostatic carcinoma</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Kidney carcinoma</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Stomach carcinoma</td>
<td>4</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Pancreatic carcinoma</td>
<td>5</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Liver carcinoma</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Uterus carcinoma</td>
<td>8</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Laryngeal carcinoma</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>59</td>
<td>127</td>
</tr>
</tbody>
</table>

χ², for a = 0.10 (P < 0.1) and 11 degrees of freedom, is 13.82. These data are compatible with the conclusion that LDH-1ex activity and anatomical location of malignancy are independent.

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Nonstandard abbreviations: LDH, lactate dehydrogenase; INT, iodonitrotetrazolium chloride; and PMS, phenazine methosulfate.

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Boehringer Mannheim GmbH, Mannheim, F.R.G. All additional reagents, generally of "AnalaR" grade, were from Sigma Chemical Co.

Analytical Procedures

Protein was determined by the method of Lowry et al. (30), with bovine serum albumin as standard. We assayed total LDH activity spectrophotometrically, determining the rate of oxidation of NADH at 25°C by measuring the change in absorbance at 340 nm with a Model 250 spectrophotometer (Gilford Instrument Lab. Inc., Oberlin, OH); for this, we used the reagents and methodology of the Boehringer kit.

Agarose electrophoresis. We electrophoresed 1-μL serum samples on commercially available 10 g/L agarose gels, with 65 mmol/L barbital buffer, pH 8.6, for 37 min at 90 V. To stain proteins, we fixed the gels and stained them with 200 mL of a 2 g/L (3.2 mmol/L) solution of Amido Black 10B in 50 mL/L acetic acid. The background was destained in 50 mL/L acetic acid solution.

The LDH isoenzymes in the serum samples were made visible with the use of an LDH staining kit (Corning), containing NADH, lactic acid, INT, and PMS. After electrophoresis and staining, we gelatinized the gels by heating at 60–70°C for 1 h, then scanned them at 570 nm with a Cliniscan automatic computing densitometer (Helena Labs., Beaumont, TX) to quantify each isoenzyme as a percentage of total LDH activity.

We tested the heat stability of LDH isoenzymes by heating the serum samples in a water bath at 60°C, then cooling them to 5°C just before electrophoresis.

Statistical analysis. For statistical analysis of our data we used the χ² test (31). The relationship between LDH-1x isoenzyme and anatomical location of the malignancy was analyzed with a contingency table of 12 rows and two columns (Table 1). The correlation between malignancy or metastasis and LDH-1x appearance was analyzed in a 2 × 2 contingency table (Table 2).

Results

Total LDH Activity

The mean (and SD) LDH activity in the serum from 33 normal donors was 210 (75) U/L. Only four of these apparently healthy individuals showed a total LDH activity >250 U/L.

The mean total LDH activity in the serum of our patients (304 U/L) was slightly above normal. Total LDH exceeded 250 U/L in 56 of 127 patients (44%). Samples with highly increased LDH activity (>1000 U/L) were observed in seven patients (5.5%).

LDH isoenzyme Electrophoretic Pattern

The electrophoretograms of LDH isoenzymes in the patients' sera showed a shift toward the M (muscle) type; the percentage of LDH-4 and LDH-5 isoenzymes was greater in the patients' pattern than in the pattern for the normal controls (Table 3).

In the electrophoretic analysis of LDH isoenzymes of the 127 patients, 68 had patterns characterized by the presence of an extra band (LDH-1x) migrating anodally to the LDH-1 band (Figure 1). This extra band was also present in three of 33 apparently healthy individuals examined; in all three the total LDH activity was much above normal (>300 U/L).

To confirm the nature of this band, we tried omitting different individual components of the staining kit (NADH, lactate, PMS, INT). The activity of LDH-1x was inhibited whenever any single component of this reaction was omitted. In addition, when we substituted ethanol for lactate, the abnormal band was not present. Thus, LDH-1x isoenzyme has a true LDH activity. Tests of myoglobin, hemoglobin, and albumin for possible staining interference or binding to LDH-1x gave negative results.

LDH-1x band is rarely found in normal serum. Furthermore, treatment of patients with anti-tumor agents and radiotherapy resulted in normalization of the LDH isoenzyme pattern and a gradual decrease or disappearance of the abnormal band. The same results were observed after surgical removal of the tumor. Moreover, the proportion of LDH-1x isoenzyme increased again during malignancy.

![Fig. 1. LDH isoenzyme pattern for serum from a patient with colonic carcinoma, after agarose gel electrophoresis and densitometric tracing of the electropherogram](image)

Electrophoresis was performed with 65 mmol/L barbital buffer, pH 8.6, for 37 min, at 90 V. The positions of LDH-1x isoenzyme and albumin are indicated by arrows. The albumin is visible only after staining with Amido Black.

<p>| Table 2. Correlation of LDH-1x Appearance and Presence of Malignancy |
|-------------------------|-----|-----|-----|-----|</p>
<table>
<thead>
<tr>
<th>LDH-1x isoenzyme</th>
<th>Yes</th>
<th>No</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignancy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>68</td>
<td>3</td>
<td>71</td>
</tr>
<tr>
<td>No</td>
<td>59</td>
<td>30</td>
<td>89</td>
</tr>
<tr>
<td>Total</td>
<td>127</td>
<td>33</td>
<td>160</td>
</tr>
</tbody>
</table>

χ² for a = 0.001 (P < 0.001) and 1 degree of freedom, is 20.96 (19.21 after Yates' correction) (31). This value indicates a correlation of LDH-1x isoenzyme appearance with malignancy at the 0.001 level of significance.

<p>| Table 3. Tendency to M-Type of LDH isoenzymes in Patients with Malignant Diseases |
|-------------------------|---------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>LDH isoenzyme</th>
<th>Mean (and SD) LDH isoenzyme activity, % of total LDH</th>
<th>No. (and %) of patients showing increased percent LDH isoenzyme activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH-1x</td>
<td>Control subjects</td>
<td>Patients</td>
</tr>
<tr>
<td>LDH-1x (H_1)</td>
<td>31.6 (6.2)</td>
<td>18.8 (5.6)</td>
</tr>
<tr>
<td>LDH-2 (H_2M)</td>
<td>37.4 (4.7)</td>
<td>24.1 (4.1)</td>
</tr>
<tr>
<td>LDH-3 (H_3M)</td>
<td>16.7 (4.4)</td>
<td>19.1 (3.2)</td>
</tr>
<tr>
<td>LDH-4 (H_4M)</td>
<td>7.2 (3.9)</td>
<td>13.9 (2.1)</td>
</tr>
<tr>
<td>LDH-5 (M_5)</td>
<td>5.5 (2.6)</td>
<td>18.8 (6.9)</td>
</tr>
</tbody>
</table>

* Calculated in comparison with the LDH isoenzyme pattern of the apparently healthy individuals (n = 33), shown in the second column.
relapse, as shown in follow-up studies on three of our patients.

Interestingly, LDH-1ex was essentially stable at room temperature for at least three days and at −70 °C for more than two months; it was only a little denatured by repeated freezing and thawing. Pre-incubating patients' sera at 60 °C for 30 min removed all but the LDH-1ex and LDH-1 bands. No bands were visible when we used temperatures higher than 60 °C.

Of those samples characterized by the presence of LDH-1ex isoenzyme, 37.9% had total LDH activity >250 U/L. However, this correlation was not significant. That samples with LDH-1ex activity also show an increase in the LDH-4 and LDH-5 isoenzymes is not critical, because such increase has been observed in all of our patients.

On the other hand, the presence of LDH-1ex activity at diagnosis did not show any clear relationship with the anatomical location of malignancy, as shown by statistical analysis (Table 1). Using standard statistical tables (31), we concluded that the presence of LDH-1ex isoenzyme in the serum of our patients is not related to a specific tissue abnormality. Furthermore, of the 68 patients with LDH-1ex isoenzyme, only 30 had metastases, as compared with 22 of 59 patients without LDH-1ex isoenzyme. These results, analyzed in a 2 × 2 contingency table (not shown) exclude any statistically significant dependence between the two criteria of classification ($\chi^2 = 0.360$ for $P < 0.1$, after Yates' correction) (31). In contrast, the data of Table 2 provided very strong evidence in favor of some kind of association between LDH-1ex and malignancy ($\chi^2 = 19.21, P < 0.001$).

Discussion

One of the best-characterized features of tumor growth is the associated alteration in the enzyme and isoenzyme pattern of tissues in the host organism. LDH is one of the enzyme systems preferentially produced and retained by cancer cells, being necessary to maintain tumor growth. When the LDH isoenzymes are released from neoplastic tissue in the serum, the LDH isoenzyme pattern of the serum changes. There have been several reports of anomalies in the synthesis and total LDH activity as well as in the pattern of LDH isoenzymes that correlate with cancers in humans (3–6, 8, 10, 12, 13, 15, 23–25, 28, 29).

The more interesting of our data refer to the occurrence of an extra band migrating anodally to LDH-1. Extra bands of a sixth LDH isoenzyme have been reported by several investigators, but the electrophoretic mobility of LDH-1ex suggests that this isoenzyme is not related to any abnormal isoenzyme previously reported.

Although the association between the appearance of LDH-1ex and the presence of malignancy was statistically significant, the variety of cancers in the patients we studied precludes the possibility of any specific cancer's being the cause or the source of LDH-1ex isoenzyme. However, because the abnormal band disappeared shortly after chemotherapy or radiotherapy was begun, or after surgical removal of the tumor, we believe this assay may have potential clinical usefulness, at least for follow-up studies of malignant diseases in which LDH-1ex isoenzyme appears. Moreover, the test is inexpensive, simple, rapid, and easily available.

The appearance of LDH-1ex in three of our healthy individuals (<10%) could have several interpretations. For example, the abnormal band may be the gene product of a genetic variant; such cases have also been published by other investigators (32). Alternatively, the extra band may originate from an early-stage tumor, as yet not detected (unknown to the donors), or may be caused by other diseases not diagnosed. The study of LDH isoenzymes in a larger group of normal individuals may in part solve this problem.

LDH–immunoglobulin complexes have been reported in sera from healthy individuals (15), and from patients with various diseases (16–23). However, in our study, we observed only an additional LDH band but no other abnormalities in the electrophoretic mobility of LDH isoenzymes. From the electrophoretic migration of LDH-1ex, we can exclude any resemblance to some of the LDH–immunoglobulin complexes presented in the literature. All of these complexes have been shown to be the result of an aggregation between one or more LDH isoenzymes and circulating proteins such as IgA (15, 20, 22, 23), IgG (17–19, 21, 23), IgM (21), or β-lipoproteins (33) and migrate between the normal location of LDH-1 and that of LDH-5. Immunofixation procedures and related techniques may resolve whether the LDH-1ex isoenzyme is a new immunoglobulin complex with some of the usual LDH isoenzymes. We are currently attempting to purify LDH-1ex isoenzyme, in order to elucidate its macromolecular nature and some of its biochemical properties.

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References