Lactate Dehydrogenase Isoenzymes in Tissues of Normal and Ehrlich-Lettré Ascites Tumor-bearing Swiss Mice

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SUMMARY

This study characterizes the lactate dehydrogenase (LDH) isoenzyme electrophoretic patterns and total activities of brain, heart, kidney, liver, lung, skeletal muscle, small intestine, spleen, testes, and ventral abdominal wall in healthy Swiss mice and in mice with advanced Ehrlich-Lettré ascites tumor. In addition, the total LDH activity and isoenzyme patterns of the ascites tumor cell itself and of its subcellular fractions were investigated. The LDH isoenzyme patterns of mouse tissues are quite different and migrate more anodally at pH 8.6 than do the LDH isoenzymes of other animal species. Several of the tissues from mice, especially liver, spleen, and erythrocytes, have more LDH5 (subscripts represent isoenzymes, with negativity increasing with decreasing value of subscript) than do these tissues from other animals. Erythrocytes have LDH5 almost exclusively. The testes have a high percentage of LDH5. Liver, spleen, and testes from tumorous mice have more total LDH and abdominal wall has less LDH than do the same tissues from control mice. The tumor cells have a large complement of LDH, mostly LDH5, with small amounts of LDH4 in the mitochondria. Liver cell mitochondria and microsomes also have mostly LDH4, with small amounts of LDH5 and even less of LDH3 and LDH2. Relative thermal stability determinations on the isoenzymes in kidney and heart showed LDH5 and LDH1 to be the most stable, with LDH4, LDH2, and LDH3 being somewhat less stable.

INTRODUCTION

Characteristic LDH2 isoenzyme patterns are consistently noted for each tissue of an organism, and these patterns remain remarkably constant among mature, healthy individuals of the same species (17). However, among the tissues of a given animal, the complement and quantities of the 5 isoenzymes will vary strikingly, from primarily 1 isoenzyme to nearly equal amounts of all 5 (18). Testicular tissue has a 6th isoenzyme (LDH5). Furthermore, the pattern will vary widely in response to developmental (9), pathological (15), and environmental (5) changes. Also, from one animal species to another, there are striking differences in the patterns of the isoenzymes and in the electrophoretic mobilities of the corresponding isoenzymes. For example, the LDH isoenzymes of mouse heart migrate more rapidly toward the anode than do the LDH isoenzymes of ox heart (8). Although large numbers of studies have been made on tissues of human, rabbit, rat, and monkey (18), much fewer have been made on mouse tissues. The most pertinent of these references to the mouse is that of Allen (2).

In the experiments reported here, tissues from normal mice and from mice growing Ehrlich-Lettré carcinoma were analyzed for total LDH activity and for the patterns of the various isoenzymes. In addition, the isoenzyme patterns of the components of the cancer cell and of mouse liver cells were determined. Also heat stability tests were made on the LDH isoenzymes of heart and kidney.

MATERIALS AND METHODS

Chemicals and Media. The INT was Sigma Grade I (Sigma Chemical Co., St. Louis, Mo.) and PMS was a product of Calbiochem (Los Angeles, Calif.). The NAD and NADH were Sigma Grade III. The sodium lactate, Sigma 60% D-lactic acid (sodium salt), was adjusted to pH 8.5 and diluted to 40%. The protein standard was crystallized bovine plasma albumin (Armour Pharmaceutical Co., Kankakee, Ill.). The distilled water was redistilled from a Pyrex glass still.

An isotonic, 54 mM sodium phosphate-Locke’s solution (10) was used in washing tissues and suspending cells. A 0.05 M potassium phosphate buffer, pH 7.5, was used in extracting the tissues and diluting the extracts when necessary. For grinding the cancer cells and separating the cell particulates, a 30 mM potassium phosphate buffer, pH 7.4, was used. This was preceded by washing the cells with a sucrose-EDTA-Tris buffer, pH 7.45 (11).

A phosphate-pyruvate solution was utilized for determining total LDH activity. This solution contained 3.1 x 10^{-4} M pyruvate in the 0.05 M potassium phosphate buffer, pH 7.5. The NADH solution used was prepared fresh each day by adding 5 mg NADH to 0.75 ml of the phosphate-pyruvate solution.
A barbital-barbiturate buffer, pH 8.6 and ionic strength 0.05, was used in the electrophoresis. The solution contained 10.8 g sodium diethyl barbiturate and 1.5 g diethylbarbituric acid per 1000 ml water solution. For the staining procedure, several solutions were necessary. The Tris solution was 0.1 M and was adjusted to pH 9.0 with HCl. The substrate solution was prepared by mixing 3.6 ml of 40% sodium DL-lactate solution, pH 8.5, with 46.4 ml of the 0.1 M Tris-HCl buffer. This solution is stable for several weeks when kept refrigerated. The color-developing stain consists of 24 mg INT, 10 mg NAD, 3 ml of the above substrate solution, and 7 ml H2O. The PMS solution contains 2 mg PMS in 10 ml H2O.

Normal and Cancerous Mice and Tumor Cells. Webster White Swiss male mice, 7 to 10 weeks old, were used in these studies. The cancerous mice were produced by injecting i.p. 0.2 ml Ehrlich-Letter hyperdiploid carcinoma ascites fluid into mice and allowing the tumor to grow for 20 to 22 days. The animals were then sacrificed and the tissues were used for LDH analyses. When tumor cells only were analyzed, they were obtained after 8 to 12 days of growth in the mice.

Tissue Extraction and Cell Fractionation Procedures. The mice were anesthetized with ether and killed by rapid exsanguination from a neck artery. Every effort was made to remove the blood as completely as possible. The tissues to be analyzed were excised immediately and placed in ice-cold 54 mM phosphate-Locke’s buffer solution until extracted by the following procedure.

Each tissue was blotted free of buffer solution and weighed to the nearest 5 mg. The masses of tissues utilized varied from about 100 to 500 mg, depending on the amounts of tissue available and the LDH activity present. Each tissue was ground for 5 min in an ice bath with the use of a motorized Ten-Broek tissue grinder with 2.0 ml 0.05 M phosphate buffer. The brei was then transferred to a centrifuge tube containing two 1.0-ml portions of buffer. The material was centrifuged in the cold at 39,000 x g for 15 min. The supernatant was filtered through medium fine filter paper, and the filtrate was used in the various LDH activity determinations and electrophoretic procedures.

The tumor cells were washed and ground, and particulates (mitochondria, microsomes, and cell sap) were separated and treated as described in a previous publication (11). The liver cell particulates were separated in a similar way following homogenization of the liver tissue by means of a Ten-Broek tissue grinder in an ice bath with the use of sucrose-EDTA-Tris buffer (see “Media and Chemicals”).

Protein Analysis. Protein was determined in aliquots of the tissue extracts and of the tumor cells and cell particulates by the quantitative biuret method (11).

Total LDH Activity Determinations. Total LDH activity was measured spectrophotometrically at 340 nm by determining the rate of oxidation of NADH in the enzymatic conversion of pyruvate to lactate (3). The reaction was carried out in the potassium-pyruvate solution, pH 7.5, a final volume of 3 ml with a pyruvate concentration of 2.95 x 10^(-4) M and 0.5 μmole NADH. The absorbance changes, determined every 30 sec, were quite constant for 3 or 4 min. The mean decrease in absorbance per min for the first 2 min was used for calculating the LDH activity. Dilutions of the tissue extracts with 0.05 M phosphate buffer were made such that the absorbance changes were between 0.02 and 0.08 unit/min. Duplicate determinations were made in every case. Total LDH activities are given in i.u. (3), defined as the amount of LDH activity to reduce 1.0 μmole NADH in 1 min. The units of activity in the various tissues are calculated and presented as units/mg protein. Under the conditions used in the determinations, there was no significant oxidation of NADH in the absence of added pyruvate.

Electrophoresis and Determination of Isoenzymes. Electrophoresis of the LDH isoenzymes was carried out by means of a Gelman electrophoresis chamber and equipment and with cellulose acetate paper (Sepraphore III). The procedure of Opfer et al. (13) was followed. The amount of tissue extract to be placed on the paper was determined by first measuring the total amount of LDH activity. The optimum total LDH activity to be applied to a paper strip was between 0.005 and 0.01 unit. Therefore, dilutions of the extracts were made with the 0.05 M phosphate buffer so that the volumes used were from 2 to 10 μl. The smaller volumes were applied with the Gelman serum applicator while the larger volumes were applied with the large Beckman paper electrophoresis sample applicator. Because all of the LDH isoenzymes of the mouse migrate anodally in the system used, the samples were placed somewhat on the cathodal side of the center of the strip. Great care was taken to align the areas of application (origins) on all the strips, so that direct comparisons of migration rates of the various isoenzymes could be made. A direct current of 360 V (not initially exceeding 2.5 ma/strip) was applied for 90 min. The full capacity of 8 strips was usually used.

The staining method used for visualizing the positions and determining the relative activities of the isoenzymes was that of Mager et al. (7), with INT as the dye indicator. Because the PMS, which acts as the hydrogen transporter between NADH and INT, is extremely sensitive to light, it is made up in a dark glass container just prior to use. The developmental stain solution consisted of 3 ml substrate solution containing the INT and NAD, 1 ml PMS solution, and 7 ml H2O (see “Media and Chemicals”). This developmental stain solution was poured into a Petri dish, and the substrate strips were placed on the solution and allowed to become saturated. The stain was kept covered when not in use because it is sensitive to light. These substrate strips are then removed, placed on the electrophoretically treated strips, and incubated at 37° in the dark for 20 min. The paired substrate and electrophoretically treated strips were then separated, the dye was fixed by immersing the strips in 5% acetic acid for 5 min, the strips were thoroughly washed in running water for 5 min, excess water was blotted off, and the strips were allowed to dry.

The relative amounts of each of the isoenzymes were determined with the aid of a Gelman manual scanner and Beckman Analytrol with Microzone scanning attachment. The 2 machines were checked against each other on a number of different isoenzyme patterns and found to give comparable values. The formazan deposits on both the substrate and electrophoretically treated strips were added together and utilized for calculating the percentage of each isoenzyme.
Thermal Stability Techniques. The relative thermal stabilities of the various LDH isoenzymes were determined by measuring total enzyme activities and relative isoenzyme activities on electrophoresis strips before and after varying periods of heating at temperatures of 56°, 60°, and 65°. A Haake Model F circulator and small insulated bath were used for maintaining constant temperatures.

RESULTS

Table 1 shows the percentages of the various isoenzymes in the tissues of normal mice. Table 2 gives values for the same tissues from mice growing the tumor for 20 to 22 days. Table 3 gives total LDH values for the same tissues from both normal and tumor-bearing mice.

Liver, spleen, testes, and ventral abdominal wall have the largest differences in total LDH and no overlap in the ranges of values between normal and tumor-bearing animals. Abdominal wall tissue from a cancerous animal has less total LDH than from a normal animal, while the other 3 tissues from cancerous animals have more LDH. The reasons for this are not apparent, except that the cancerous animals at 20 to 22 days have grossly distended abdominal cavities, are somewhat edematous, and are perhaps less active metabolically. On the other hand, the liver and spleen enlarge, apparently are placed under extra stress, and undoubtedly are more metabolically active. The reason for an increase in LDH activity of the testes is not apparent. However, this tissue shows a much greater change in quantities of the various LDH isoenzymes than any of the other tissues. The shift in distribution is primarily toward LDH5 and away from the others, especially LDH1, LDH2, and LDHX. Shifts in the isoenzyme patterns of the tissues, other than testes, is minimal. The only other tissue that may have a significant change is spleen. Here, there is a shift away from LDH5 and toward LDH4 and LDH3.

Ascites fluid, free of cells, contains 2, 9, and 89% of LDH3, LDH4, and LDH5, respectively, but no measurable amounts of LDH1 or LDH2. Extracts of the whole cell contain, as near as can be determined, only LDH5; however, the isolated...
Table 3

Total LDH of tissues from normal and Ehrlich-Lettré tumor-bearing mice.

Values are expressed i.u./mg protein in tissue extract.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Normal No. of animals analyzed</th>
<th>Mean ± S.D.</th>
<th>Tumor No. of animals analyzed</th>
<th>Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>6</td>
<td>4.27 ± 0.08</td>
<td>2</td>
<td>4.25 ± 0.05</td>
</tr>
<tr>
<td>Lung</td>
<td>4</td>
<td>0.58 ± 0.03</td>
<td>3</td>
<td>0.67 ± 0.08</td>
</tr>
<tr>
<td>Liver</td>
<td>8</td>
<td>1.65 ± 0.14</td>
<td>4</td>
<td>2.64 ± 0.31</td>
</tr>
<tr>
<td>Spleen</td>
<td>7</td>
<td>0.68 ± 0.05</td>
<td>2</td>
<td>0.99 ± 0.15</td>
</tr>
<tr>
<td>Small intestine</td>
<td>7</td>
<td>3.51 ± 0.08</td>
<td>4</td>
<td>3.25 ± 0.03</td>
</tr>
<tr>
<td>Testes</td>
<td>4</td>
<td>1.57 ± 0.03</td>
<td>3</td>
<td>2.35 ± 0.07</td>
</tr>
<tr>
<td>Ventral abdominal wall</td>
<td>4</td>
<td>7.23 ± 0.16</td>
<td>4</td>
<td>4.03 ± 0.31</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>12</td>
<td>6.76 ± 0.05</td>
<td>4</td>
<td>6.65 ± 0.06</td>
</tr>
<tr>
<td>Kidney</td>
<td>11</td>
<td>2.95 ± 0.25</td>
<td>2</td>
<td>3.66 ± 0.50</td>
</tr>
<tr>
<td>Brain</td>
<td>4</td>
<td>2.10 ± 0.14</td>
<td>3</td>
<td>2.19 ± 0.16</td>
</tr>
<tr>
<td>Ehrlich-Lettré tumor cells</td>
<td></td>
<td></td>
<td>9</td>
<td>3.52 ± 0.41</td>
</tr>
<tr>
<td>Ascites fluid</td>
<td></td>
<td></td>
<td>9</td>
<td>0.46 ± 0.18</td>
</tr>
</tbody>
</table>

mitochondria contain 7% LDH4 and 93% LDH5. Microsomes did not contain detectable amounts of isoenzymes other than LDH2. As shown in Table 3, the total LDH in the tumor cells was 3.52 units/mg protein, with a wide range. The variations had no correlation with age of the tumor. The total amount of LDH in the ascites fluid, as would be expected, ranged widely from 0.15 to 0.77 unit/mg protein. A comparison of the isoenzyme distribution was made between tumor cell particulates and liver cell particulates. Here too, the liver cells contain, as nearly as can be determined, only LDH5, but the mitochondria contain 5 and 9% of LDH3 and LDH4 and microsomes contain 3 and 10% of LDH3 and LDH4, respectively.

Thermal stability studies were made on the isoenzyme components of extracts of heart and kidney from normal mice. Table 4 shows the data for temperatures of 56°, 60°, and 65°.

**DISCUSSION**

The LDH isoenzyme patterns of the various tissues of mouse are very different from those of other animals, especially heart, brain, and erythrocytes of the rabbit, and to a lesser extent the same tissues in man, monkey, and even the rat (18). In the mouse heart and brain, there is fairly even distribution of the 5 isoenzymes, with somewhat more of LDH3 and LDH2, while in the other animals there is mostly LDH1 and LDH2. The differences are even more striking with mouse erythrocytes, where almost all the isoenzyme is LDH5. In most other animals, there is a predominance of LDH1 and LDH2. The rat also has mostly LDH4 in its erythrocytes (16). These findings of mouse heart, brain, and kidney having lesser amounts of LDH1 do not particularly fit with the theory that tissues which metabolize under aerobic conditions have a predominance of LDH1. The high LDH5 content of mouse erythrocytes, however, does fit the theory.

The duplication of total LDH values on a given tissue extract was excellent, usually ±2%. However, the electrophoretic separations of the isoenzymes and determination of percentage of composition was much less precise, particularly when the amount of a component was less than 5% of the total. Duplicate or triplicate electrophoresis strips were always run, and the larger isoenzyme components would usually check
within ±5% and the smaller ones would check within ±10 to 20%.

The larger variations in total LDH values for abdominal wall of tumor mice may be due to rather large differences in the amounts of ascites tumor in the various animals, and thus differences in abdominal distention. The peritoneal walls of cancerous mice were observed to be thinner and more reddish, indicating hemorrhaging and possibly cellular disruption. These changes may explain the lower LDH values. The mice growing carcinoma cells have greatly enlarged spleens (up to 100%) and slightly enlarged livers (up to 20%). This increase in size and activity of these organs may well explain their increased LDH activities. The striking increase in total LDH and slight shift toward LDH₅ of the testicular tissues of cancerous mice is not explainable on any basis or theory of which we are aware. This interesting tissue deserves further study, not only from this standpoint, but because it is the only tissue we studied that shows the LDH₅ component. This is in contrast to the work of Stambaugh and Buckley (14), which showed the X isoenzyme to be present in extracts of rabbit heart, kidney, and ampulla, as well as in seminal fluid, testes, and spermatozoa. Preliminary studies show the mouse X isoenzyme to have unusual heat stability.

The cancer cells showed rather wide variations in total LDH activity from one mouse to another. This did not seem to depend on the amount of homogenization as this was checked by repeated homogenization and extraction. The ascites fluid from different mice varied even more. Heart, lung, small intestine, skeletal muscle, kidney, and brain do not appear to show appreciable differences in total LDH between normal and cancerous mice.

The Ehrlich-Lettre tumor cells and the tissues most affected by growth of the tumor in mice (except testes) have an overwhelming proportion of LDH₅. The intracellular distributions of LDH isoenzymes in liver and Ehrlich-Lettre cells were considered in this study. Different parts of these cells show different patterns. The liver cells have considerable quantities of LDH₃ and LDH₄ in the mitochondria and microsomes, with little or none of these isoenzymes in cell sap. The tumor cells have an appreciable amount of LDH₄ in their mitochondria but no detectable amounts in the microsomes or cell sap. Agostini et al. (1) have reported mostly LDH₅ in whole liver cells with only a trace of LDH₄, while fractionation of the cell showed appreciable amounts of LDH₄ in the mitochondria. These observations fit the theory that the more anodic LDH isoenzymes are geared for maximum activity under aerobic conditions and tend to shunt pyruvate into oxidative pathways. Conversely, the cathodic isoenzymes function optimally in anaerobic metabolism. The ascites fluid contains significant amounts of LDH₅, while fractionation of the cell showed appreciable amounts of LDH₄ in the mitochondria. The thermal stability studies reported here show results in direct opposition to the data for other mammals where thermal stability increases with increasing anodic mobility (4, 12). With LDH₁ and LDH₂ of the mouse mouse having the maximum thermostability one is tempted to consider this to be due to the homogeneity of the subunits of LDH₅ and LDH₁ and their ability to bind more strongly. In fact, Kaplan and Goodfriend (6) have suggested that the reason that many reptiles, birds, and other lower animals demonstrate only 2 isoenzymes of LDH is because the subunits produced are so heterogenous as to have lost the ability to hybridize. Further experiments with such protein-denaturing agents as urea might allow a more definitive idea into what order the stability of mouse LDH isoenzymes fall.

REFERENCES

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