Characterization of Apparent Lactate Dehydrogenase Isoenzyme 6: A Lactate-Independent Dehydrogenase

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We found in a prospective study of lactate dehydrogenase isoenzyme 6 (LD6) in human tissues obtained at autopsy that LD6 was usually present in liver when death was preceded by prolonged hypotension or impaired ventilation but not in cases of sudden death. Other organs containing LD6 were kidney and spleen. LD6 is heat stable, differs from H-subunit-containing LD isoenzymes by pyruvate resistance, differs from M-subunit-containing LD isoenzymes by immunoprecipitation, and is distinct from spermatic LDX. LD6 from liver extracts acted without lactate as substrate and could be enhanced by ethanol added to the substrate. These results indicate that LD6 is not a true lactate dehydrogenase, and that it frequently appears in severe liver injury.

Additional Keyphrases: liver injury • hypotension • hypoxia • ethanol-enhanced activity

Isoenzymes of lactate dehydrogenase (l-lactate: NAD⁺ oxidoreductase, EC 1.1.1.27; LD)² released into serum are widely evaluated for diagnosis of specific organ injury because of the characteristic isoenzyme content of those organs (1). LD has a tetrameric structure including all five possible combinations of two subunits, H (heart) and M (skeletal muscle), which are encoded by different genetic loci (2). In addition, a third genetic locus encodes for another LD subunit that is expressed as a single tetramer, designated LDX (3, 4). LDX is a mitochondrial enzyme (5).

Recently, an additional LD isoenzyme, migrating cathodally to LD6 and designated LD6, has been reported (6, 7). It has been detected on occasion in the serum of patients who have experienced major medical crises involving severe hypotension or impaired ventilation. The reported mortality of those who had LD6 detected in their serum was very high. Results of studies of autopsy material have suggested that liver is the source of LD6 (7).

We have examined human livers and some other organs obtained at autopsy for their LD6 content, and we found LD6 to be present in a large proportion of cases. In addition, we further characterized LD6 and found it to be distinct from LDX of sperm and to be different in several ways from both H-subunit- and M-subunit-containing forms of LD. LD6 did not require lactate as substrate, but its activity was strongly enhanced by ethanol as previously described for a similar LD variant (8, 9).

Materials and Methods

Samples. Tissue specimens, obtained from cadavers stored for 2 to 24 h at 4 °C prior to autopsy, were kept at -20 °C until examination. Serum from hospitalized patients was available from routine testing for LD isoenzymes.

Reagents. NAD⁺ (Grade III-β, NADH (Grade III-β), p-nitroblue tetrazolium chloride (NBT), phenazine methosulfate (PMS), sodium pyruvate (type II), antimony A, neumaminidase (EC 3.2.1.18) (type VIII), equine heart cytochrome c, porcine heart cytochrome c reductase (EC 1.6.99.3), and bovine heart cytochrome oxidase (EC 1.9.3.1) were from Sigma Chemical Co., St. Louis, MO 63178. Sodium cyanide and ascorbic acid were from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Lithium lactate was from Eastman Kodak Co., Rochester, NY 14650. "Isomune-LD" kit (Roche Diagnostics, Nutley, NJ 07110) contains a goat primary antibody against LD6 (or the M subunit) and a donkey second antibody directed against goat immunoglobulins and linked to an inert polymer so as to immunoprecipitate the LD isoenzymes that contain M subunit. Phenylmethyl sulfonyl fluoride, used as a protease inhibitor during neuraminidase digestion, was from Pierce Chemical Co., Rockford, IL 61105.

Methods. To separate the lactate dehydrogenase isoenzymes, we used agarose gels and the "Paragon" electrophoresis system (Beckman Instruments, Inc., Brea, CA 92621). Electrophoresis in pH 8.2 barbitral buffer was at 100 V for either 20 or 28 min. After electrophoretic separation, gels were incubated in contact with substrate containing 206 mmol of lithium lactate and 5.58 mmol of NAD⁺ per liter. In principle, the NADH generated by the conversion of lactate to pyruvate then reduces NBT (1.86 g/L solution) in the presence of PMS (0.33 mmol/L) to form a blue formazan indicator pigment at enzyme sites. Developed gels were fixed in 50 mL/L acetic acid solution and heat dried.

Tissue extracts were prepared as follows. After bluntercerebrocerebral maceration (500 compressions) in equal volumes of isotonic saline until no solid macroscopic tissue fragments remained, the resulting suspensions were centrifuged (5 min, 2000 × g). The supernates were either analyzed immediately or stored at -20 °C until analysis, usually within two days.

Results

LD6 in Autopsy Tissues

LD6 migrates as a distinct band, cathodal to LD5 (Figure 1). The organ with the greatest concentration of LD6 activity was liver, but small amounts were also detectable in kidney and spleen. Tissues in which we could detect no LD6 included brain (Figure 1), heart, erythrocytes, testis, and skeletal muscle (psoas). In a series of 15 consecutive autopsies, LD6 was detected in the livers of 11 cases (Table 1), all of whom had undergone more than 12 h of severe and sustained hypotension or hypoxia just before death. Detectable LD6 in liver did not appear to correlate with either atherosclerosis or long-term renal disease. Serum collected postmortem contained LD6 in only three of these cases (first, fourth, and ninth). Four cases (third, fourth, fifth, and sixth) had antemortem determination of LD isoenzymes in serum, and none showed LD6. LD6 was not detected in the livers of four patients who died relatively suddenly (Table 2).
Column Elution, Heat Stability, and Neuraminidase Digestion

Liver extract was dialyzed against tris(hydroxymethyl)-aminomethane hydrochloride buffer (20 mmol/L, pH 8.2) and a 0.1-mL aliquot applied to a diethylaminoethyl-Sephadex anion-exchange column (bed volume 0.8 mL) equilibrated and eluted with the same buffer. We collected three-drop fractions. LD6 and LD5 eluted together in fractions 8–11, followed by LD4 in fractions 11–15, while LD1, -2, and -3 remained on the column. With similar preparation and application to a carboxymethyl cellulose anion-exchange column (bed volume 1.5 mL), both LD6 and LD5 were retained while LD1, -2, -3, and -4 passed through the column with the eluent front. Washing the carboxymethyl cellulose column with 80 mmol/L NaCl in the same buffer (0.5-mL fractions collected) eluted LD5 primarily in fractions 7–11 and LD6 in fractions 9–11.

Liver extracts heated at 56 °C for as long as 1 h showed no loss of LD6 activity. In contrast, LD isoenzymes 1 through 5 were gradually inactivated, the M-subunit-containing forms being the most heat-labile.

We incubated liver extract not containing LD6 at 37 °C for 46 min in the presence of 5 U of neuraminidase per milliliter, in an attempt to remove sialic acid residues from LD isoenzymes 1 through 5 and generate a more cationic form. Although parallel neuraminidase digestion of serum did alter the migration of glycoproteins in the alpha-1, alpha-2, and beta regions, as demonstrated by staining for protein, the migration behavior of the LD isoenzymes was unchanged and LD6 did not appear in the electrophoretic pattern.

Comparison of LD6 with Isoenzymes Containing H, M, or Spermatic LDX Subunit

Testicular extracts contained the spermatic isoenzyme LDX, which migrated anodally to LD4 (Figure 2), thus establishing that LD6 is distinct from LDX.

Immunoprecipitation with antibody to the M subunit removed LD2, -3, -4, and -5, both from testicular and liver extracts, but did not remove LD1, LDX, or LD6 (Figure 2). Evidently LD6 is antigenically distinct from M-subunit-containing forms of LD.

When pyruvate (125 mmol/L final concentration) was present in the substrate during development of enzyme activity, there was complete inhibition of those isoenzymes containing the H subunit (LD1, -2, -3, and -4) and of LDX (Figure 3, lanes A, B, and C). This concentration of pyruvate also inhibited almost all the activity of LD5. In contrast, LD6 activity was uninhibited, indicating it to be different in product-inhibition properties from other LD isoenzymes, particularly those containing the H subunit.

During storage at −20 °C for one month, the spermatic LD isoenzyme in the testicular extract spontaneously reassorted into some new tetramers by combination of its monomers with H and M monomers (Figure 3, lane F). This generation of isoenzymes, some of which are not found in vivo, demonstrates the molecular equivalence of LD subunits. A similarly stored liver extract containing LD6 did not show any such reassortment (Figure 3, lane D), indicating that LD6 cannot exchange subunits with LD1 through LD5.

Substrate Requirements, Metabolic Inhibitors, and Protein Markers

We investigated the requirement of LD6 for each component of the substrate mixture. After electrophoresis of liver

<table>
<thead>
<tr>
<th>Age/ Sex</th>
<th>Cause of Death</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>60/F</td>
<td>Pulmonary embolus</td>
<td>Carcinoma in tail and body of pancreas, with metastasis to liver and focal biliary stasis; abdominal carcinomatosis; portal vein thrombosis</td>
</tr>
<tr>
<td>55/M</td>
<td>Bilateral bronchopneumonia</td>
<td>Acute myelogenous leukemia, three months; severe cellulitis of thigh; respiratory insufficiency from interstitial fibrosis and acute pneumonitis</td>
</tr>
<tr>
<td>76/M</td>
<td>Cardiogenic shock</td>
<td>Unstable angina; hypotension for 34 h after percutaneous transluminal coronary angioplasty and acute myocardial infarction</td>
</tr>
<tr>
<td>70/M</td>
<td>Septic shock</td>
<td>Alcohol abuse, emesis, and hypovolemia leading to non-occlusive complete large-bowel infarction, perforation, and Gram-negative sepsis</td>
</tr>
<tr>
<td>49/F</td>
<td>Cardiogenic shock</td>
<td>Rheumatic heart disease; mitral commissurotomy, 18 years; hypotension for 72 h; hepatic failure</td>
</tr>
<tr>
<td>76/F</td>
<td>Septic shock</td>
<td>Arteriarlem nephrosclerosis, renal failure, peritonitis while on peritoneal dialysis, and hypotension poorly responsive to vasopressors for two weeks</td>
</tr>
<tr>
<td>63/M</td>
<td>Small bowel obstruction, myocardial infarction</td>
<td>Pancreatic carcinoma, obstruction to small bowel, dehydration, myocardial infarction 24–36 h, and episodic hypotension for one week</td>
</tr>
<tr>
<td>65/M</td>
<td>Ruptured aortic aneurysm</td>
<td>Sudden drop in blood pressure for 8 h, surgical repair of aneurysm, adult respiratory distress syndrome, and exanguination into abdomen with terminal hypotension for 5 h</td>
</tr>
<tr>
<td>60/M</td>
<td>Pulmonary embolus</td>
<td>Astrocytoma; syncope and hemiparesis; shift from hypertension to blood pressure of 100/60 mmHg terminally for three weeks</td>
</tr>
<tr>
<td>9/M</td>
<td>Respiratory distress</td>
<td>Neurovascular deficit due to meningitis at nine months of age; impaired ventilation, atelectasis, aspiration; terminal respiratory insufficiency for &gt; 24 h</td>
</tr>
<tr>
<td>35/M</td>
<td>Asphyxiation</td>
<td>Epiglottitis (Hemophilus influenzae) with partial to complete obstruction of trachea for 12 h</td>
</tr>
</tbody>
</table>
Table 2. Patients with No LD6 in Liver

<table>
<thead>
<tr>
<th>Age/ Sex</th>
<th>Cause of death</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>74/F</td>
<td>Sudden-onset cardiac arrhythmia (asystole)</td>
<td>Acute pancreatitis; disseminated intravascular coagulation</td>
</tr>
<tr>
<td>58/M</td>
<td>Sudden-onset cardiac arrhythmia</td>
<td>Chronic lymphocytic leukemia, autoimmune anemia; aortic stenosis</td>
</tr>
<tr>
<td>78/F</td>
<td>Cardiovascular insufficiency</td>
<td>Dilated cardiomyopathy of unknown etiology</td>
</tr>
<tr>
<td>65/F</td>
<td>Cardiovascular insufficiency</td>
<td>Diabetes mellitus, post-cerebrovascular accident, decubitus ulcers, rapid demise with drop in blood pressure 7 h before death</td>
</tr>
</tbody>
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![Fig. 2. Comparison of LD6 with testicular LDX and immunoprecipitation of M-subunit-containing forms](image)

After electrophoresis of two liver extracts (sample 1, lanes A and D; sample 2, lanes B and E) and a testicular extract (lanes C and F), LD6 activity was developed in the usual manner (D, E, and F) or with the addition of 125 mmol/L sodium pyruvate (A, B, and C). Pyruvate inhibited the activity of H-subunit-containing isoenzymes (LD1, -2, -3, and -4) and of LDX. It also inhibited almost all the activity of LD6, but none of the LD6 activity, thus distinguishing LD6 from H-subunit-containing isoenzymes of LD.

![Fig. 3. Pyruvate inhibition of LD isoenzymes](image)

indicating that cellulose acetate may also serve as a substrate for LD6.

In an attempt specifically to inactivate LD6 activity, we mixed aliquots of a liver extract with potassium cyanide, ascorbic acid, or antimycin A before electrophoresis. Both cyanide and ascorbic acid completely inhibited LD6 activity without inhibiting activities of LD1 through LD6 (Figure 4b). Antimycin A had no effect. Ascorbic acid and NADH themselves produced color changes during the incubation, but their electrophoretic migrations were anodally beyond LD1 (electrophoretic mobilities relative to LD1 were: NADH, 2.67; ascorbic acid, 2.46; LD6, -1.77; LD6, -2.0). Purified equine-heart cytochrome c, purified porcine-heart cytochrome c reductase, and purified bovine-heart cytochrome oxidase were also electrophoresed, but none duplicated the lactate-independent activity of LD6. Their only active component was apparently true LD isoenzymes of these animal species, present as contaminants. Other human heme-containing proteins (hemoglobin and myoglobin) were also different from LD6, because they migrated anodally and because LD6 activity was not detectable in erythrocytes or muscle.

Ethanol Enhancement of LD6 Activity

Because neither lactate (Figure 4a) nor pyruvate (Figure 3) served as a substrate for LD6, we evaluated ethanol as a substrate, prompted by earlier reports of similar activity (8, 9). The samples we examined were: serum from a patient with skeletal muscle release of LD following a grand mal seizure, LD6-containing serum obtained from a patient during terminal cardiogenic shock precipitated by a bradyarrhythmia, and an extract of autopic liver containing LD6. After electrophoresis, isoenzymes were developed with the usual mixture of LD substrate and, in parallel, with 17 mmol of ethanol added to that substrate per liter. The sample with the high LD6 activity as a result of skeletal muscle injury showed no LD6, and no additional bands developed with ethanol in the substrate (Figure 5, lanes A and D). The serum containing LD6 showed no enhancement of LD6 activity by ethanol (Figure 5, lanes B and E). However, the liver extract showed substantially greater LD6 activity in the presence of ethanol (Figure 5, lanes C and F).
Fig. 4a. Lactate independence of LD6
Extract of liver obtained at autopsy was electrophoresed and then developed for LD activity with complete substrate mixture (lane A) and for other (non-LD) activity by using the mixture with lactate deleted (lane B). This liver extract had lactate-dependent activity in LD isoenzymes 3, 4, and 5 as well as at the origin (ori) from non-migrating material. However, LD6 activity (marker bar) was equally intense with or without lactate in the substrate.

4b. Inhibition of LD6 activity
Aliquots of liver extract were mixed with (A) electrophoresis buffer (control), (B) 167 mmol/L potassium cyanide, (C) 57 mmol/L ascorbic acid, or (D) 10 mg/mL antimycin A for 30 min before electrophoresis and development for LD activity.

Fig. 5. Enhancement of LD6 activity by added ethanol (+EtOH) to substrate
Lanes A and D: Serum containing high LD5 activity of skeletal origin; no LD6 detected. Lanes B and E: serum with LD6 and high LD5 activity, collected from a patient during terminal cardiogenic shock. Lanes C and F: extract of liver, obtained at autopsy, with strong band of LD6. After electrophoresis, Lanes D, E, and F were developed for LD activity with the usual substrate mixture, while lanes A, B, and C were developed in parallel but with 17 mmol/L ethanol added to the same substrate mixture. LD6 activity from liver was enhanced by ethanol (lane C vs lane F) while LD6 in serum showed no change (lane B vs lane E). Ethanol had no effect on LD1 through LD5 and F), and when lactate was omitted from the substrate mixture. LD6 activity was lactate independent in the antemortem serum of another patient with terminal hypotension due to a leaking aortic aneurysm. In that case, too, there was no enhancement of serum LD6 activity by ethanol.

Lability and Reconstitution of LD6
The serum of a patient with chronic pulmonary disease transiently showed LD6 during an episode of acute respiratory insufficiency; it persisted for about 8 h after successful mechanical assistance to ventilation. The disappearance of LD6 from the patient's circulation coincided with its disappearance from previously collected samples of his serum stored at room temperature, although LD1 through LD5 remained active. Thus the apparent clearance from the circulation may in fact simply be spontaneous inactivation.

LD6 remained active for at least several days in serum stored at -20 °C. Refrigerated liver extracts gradually lost LD6 activity over periods of several days. However, we could reconstitute more than one-half of the original LD6 activity in such instances by adding the reducing agent dithiothreitol, at 50 mmol/L final concentration.

Discussion
LD6 activity has been reported in serum of patients who had catastrophic and often fatal medical crises, largely involving severe shock and passive congestion of the liver, resulting in above-normal activities of LD5 and total LD in serum (6, 7). Examination of those patient's livers at autopsy revealed LD6 in some instances, a finding that suggested liver to be the source of LD6 in the serum (7). Accordingly, we studied livers from a series of 15 consecutive autopsies, finding LD6 in 11. LD6 thus appears more commonly than previously was suspected on the basis of its frequency of occurrence in serum. All of the 11 patients had in common a sustained period of circulatory or ventilatory impairment for 12 h or longer before death. These same patients also had detectable LD6 activity in their kidney and spleen, albeit small in comparison with that in their liver. The remaining four patients, those showing no LD6 in their livers at autopsy, had died relatively suddenly.

LD6 has been assumed to derive from true LD because of its electrophoretic migration close to LD5 and its clinical occurrence only in association with increased LD5. However, we have found it to be far more heat stable than LD5 or any of the other LD isoenzymes, including LD1. LDX, the other naturally occurring form of LD, is distinguished from
LD6 by differences in electrophoretic migration. By resistance to pyruvate inhibition LD6 differs from LDX and H-subunit-containing forms of LD. By immunoprecipitation LD6 differs from M-subunit-containing forms of LD. These results lead us to conclude that LD6 differs substantially from the true LD isoenzymes and probably is not derived from any of them. In fact, LD6 retained full activity in this assay regardless of whether lactate was present or not, indicating that it is not a true lactate dehydrogenase. Our finding that LD6 activity in serum is labile at room temperature may account for the failure to detect it more often in serum—it may also lose activity rapidly in vivo following release from hypoxic tissue sources.

This activity is similar to a previously described lactate-independent “nothing dehydrogenase” observed in polyacrylamide separation of LD isoenzymes and that had alcohol dehydrogenase (EC 1.1.1.1) activity (8, 9). We also found that LD6 activity in liver extracts was strongly enhanced by addition of ethanol to the substrate with or without lactate. Alcohol dehydrogenase is found in the cytosol of hepatocytes (10) and therefore should be readily extractable from all livers. Given that we did not detect LD6 in cases of sudden death, the mechanism by which its activity increases in the liver may be enzyme induction, de novo synthesis in response to hypoxia.

LD6 activity in serum was apparently not affected by ethanol; it may therefore be a wholly different lactate-independent enzyme, which simply comigrates with liver LD6 activity. In support of this possibility, higher-resolution electrophoresis has shown as many as three additional cathodal bands of supposed LD activity in the serum of some patients (11, 12). Another possibility is that a cofactor may be necessary for LD6 activity to have alcohol dehydrogenase activity and that the cofactor is lost when LD6 enters the circulation. Whatever its true identity, LD6 may be useful as a serum marker for severe liver injury and as a tissue marker at autopsy for extended antemortem liver damage.

References