Partial Characterization, Properties, and Clinical Significance of a Lactate Dehydrogenase–Immunoglobulin A$_K$ Complex in Serum

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The existence of a lactate dehydrogenase–immunoglobulin A$_K$ complex was demonstrated as a marked increase of lactate dehydrogenase activity at the lactate dehydrogenase-3 band in the agar–agarose gel-electrophoresis pattern of sera from seven patients from an unselected group of 21 800 patients. The complex was isolated, in an almost pure form, by gel filtration and affinity chromatography, from the serum of a patient with circulating hepatitis B surface antigen. The complex had a relative molecular mass ($M_r$) of approximately 445 000 as determined by gel filtration. Electrophoresis in sodium dodecyl sulfate in the absence of reducing agents showed the presence of two subunit bands with $M_r$ 170 000 and 34 000. We therefore propose that the native complex consists of one monomeric immunoglobulin A$_K$ linked to two tetrameric lactate dehydrogenase molecules. The enzymic activity of the complex is probably from lactate dehydrogenase isoenzyme-5.

Additional Keyphrases: hepatitis B · isoenzymes · determination of relative molecular mass

Several studies on the isoenzyme pattern of lactate dehydrogenase (LD; 1-lactate:NAD$^+$ oxidoreductase, EC 1.1.1.27) in serum from patients with different diseases and from clinically asymptomatic individuals indicate the presence of LD isoenzymes linked to immunoglobulin A of the kappa or lambda type [IgA$_K$, IgA$_\lambda$; (1–11)]. Regardless of the electrophoretic techniques used—i.e., agarose gel electrophoresis (1), agar gel electrophoresis (2–4, 6–8, 10, 11), or cellulose acetate electrophoresis (5, 9)—the complexes are associated with abnormal LD isoenzyme patterns characterized by the absence of the LD-2 isoenzyme (2–6, 8, 9); the presence of one or more additional LD isoenzymes (2–9); an alteration of the electrophoretic mobility of the LD isoenzymes (1); the presence of a broad, diffuse zone of LD activity (4, 11); or a marked increase of activity at the LD-3 band (3, 4, 8, 9).

LD isoenzyme–IgA complexes from patients with different diseases and from clinically asymptomatic individuals have also been studied with use of gel-filtration chromatography (1–4, 7–9). These studies demonstrate only LD isoenzymes of small molecular size but also LD isoenzyme–IgA complexes of large molecular size. From these studies one also may conclude that one LD isoenzyme (3, 8), two LD isoenzymes (3, 4, 8, 9), or all five LD isoenzymes (1, 8) are involved in the formation of the complex.

The published literature through 1981 records six patients whose serum LD isoenzyme pattern showed a marked increase of activity at the LD-3 band, due to a LD–IgA complex, but with all five electrophoretically normal LD isoenzyme bands also present (1, 2). This group of patients is characterized as follows: a large molecular form of LD, having the electrophoretic mobility of the LD-3 isoenzyme in agar gel, circulates in patient's serum. The LD activity in serum is normal to moderately increased. One of the serum proteins is a LD–IgA complex, which is not dissociated in the presence of added NAD$^+$.

In serum from a patient with hepatitis B surface (HBs) antigens we observed, by use of agar–agarose gel LD isoenzyme electrophoresis, a marked increase of LD activity at the LD-3 band. Immunoelctrophoresis followed by assay of LD activity demonstrated the presence of an LD–IgA complex. We report here our purification of this complex, and its composition. Also, we examine the clinical data for patients having serum with the above-mentioned features and evaluate the complex in terms of its clinical chemistry and its diagnostic significance.

Materials and Methods

Subjects

We studied seven subjects, selected because their serum showed markedly increased activity at the LD-3 band in the agar–agarose LD isoenzyme pattern. The five men and two women, ages 20 to 64 years (median: 35 years), were admitted to the Departments of Internal Medicine and Cardiology of the Onze Lieve Vrouwe Gasthuis. The subject from whose serum we purified the LD–IgA complex was a 20-year-old woman with HBs antigens in her serum.

Chemicals

Routine chemicals of analytical grade were supplied (except where noted) by Merck, Darmstadt, F.R.G. The biochemicals were generally of the highest purity available and were obtained from the following sources: LC-Partigen-IgA radial immunodiffusion plates and human protein standard serum LC-V (Behring Diagnostics, Marburg, F.R.G.); bovine albumin standard and Coomasie Brilliant Blue G-250 (Bio-Rad Laboratories, Richmond, CA 94804); NAD$^+$ and NADH (Boehringer Mannheim GmbH, Mannheim, F.R.G.); phenazine methosulfate (PMS; Calbiochem–Behring Co., La Jolla, CA 92037); monospecific rabbit antiserum (anti-γ, α, μ, κ, and λ-chains); Dako, Copenhagen, Denmark, or Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands; agar Noble (Difco Laboratories, Detroit, MI 481412); agarose (Litex, Denmark); Ultrogel AcA 22 and Ultrogel AcA 44 (LKB, Stock-
holm, Sweden); 2-(p-iodophenyl)-3-(p-nitropheryl)-5-phenyl-2H-tetrazolium chloride (INT; Merck, Darmstadt, F.R.G.); 5'-AMP-Sepharose 4B (N²-(6-aminohexyl)-5'-AMP bound to Sepharose 4B), carboxylic anhydride (EC 4.2.1.1), ovalbumin, phosphorylase kinase (EC 2.7.1.38), aldolase (EC 4.1.2.13), catalase (EC 1.11.1.6), ferritin, and thyroglobulin (Pharmacia, Uppsala, Sweden); and a-chymotrypsinogen, IgG, and transferrin (Sigma Chemical Co., St. Louis, MO 63178).

All solutions were prepared in de-ionized water.

**Procedures**

**Preliminary assays.** Total LD activity was assayed at 30 °C with the "PRISMA" analysis system (Boehringer Mannheim–Clinicon, Bromma, Sweden) according to the method recommended by the Netherlands Society for Clinical Chemistry (12). The 95th percentile for serum LD activity in normal persons by this method in our hands is 275 U/L.

Serum immunoglobulins were quantified by single radial immunodiffusion (13).

Protein concentrations were measured by the biuret assay (14) or by the Coomassie Brilliant Blue G-250 assay (15).

To determine whether free IgA was associated with LD, we incubated, at 37 °C for 15 min, test mixtures of a patient's serum mixed with an equal volume of a control serum and a patient's serum mixed with an equal volume of a serum having a relatively high LD-5 content. These were then subjected to agar-agarose gel electrophoresis.

Tests for hepatitis antigens and antibodies were performed by radioimmunossay (Abbott Laboratories, North Chicago, IL 60064) at the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

**Ultrafiltration.** Samples were concentrated with ultrafiltration equipment (Model 402 and Model 8MC) and ultrafiltration membranes [PM-10 (10 000 Mₗ, cutoff) and PM-30 (30 000 Mₗ, cutoff)] from Amicon Corp., Lexington, MA 02173.

**Electrophoresis.** For agar–agarose (7/3, by wt; 9.6 g/L total) gel electrophoresis we used gels containing 36 mg of disodium EDTA and 196 mg of human albumin per liter of barbital buffer (50 mmol/L, pH 8.4), essentially according to Wieme (16), with the following modifications. On each half of the 76 × 26 × 2.5 mm slides we applied two 2-μL serum samples 30 mm apart, then electrophoresed at 5 °C and 25 mA per slide (constant current), for 15 min. To stain for LD activity, we covered the agar–agarose gel with a 2.5-mm thickness of 8 g/L agar gel in barbital buffer (54 mmol/L, pH 8.4) containing 90 mmol of sodium lactate, 40 mg of disodium EDTA, 900 mg of NAD⁺, 240 mg of INT, and 24 mg of phenazine methosulfate per liter and incubated for 2 h at 37 °C.

Polyacrylamide disc gel electrophoresis was done by the "Quick-Disk QDA" specific reagent-kit instructions (no. 3510, revised 10/74; Miles Labs., Elkhart, IN 46514). Detection of LD activity was based on its conversion of INT, for which we used the LD isoenzyme substrate set of Corning, Palo Alto, CA 94306. A duplicate gel loaded with the same sample was stained for proteins with Coomassie Brilliant Blue G-250.

After immunoelectrophoresis as described by Scheidegger (17), LD activity was detected according to Brendel et al. (18). A duplicate gel loaded with the same sample was stained for proteins with Coomassie Brilliant Blue G-250.

**Determination of relative molecular mass.** Gel electrophoresis in sodium dodecyl sulfate was done according to Weber and Osborn (19). Before applying the sample to the gels, we boiled them for 2 min in 10 g/L sodium dodecyl sulfate solution without 2-mercaptoethanol.

We used a log plot of the Mᵣ values of reference proteins vs their respective gel migration to establish the Mᵣ of the complex. To determine the Mᵣ of the purified protein under native conditions, we applied it to an Ultrigel AcA 22 column (1.4 × 100 cm) equilibrated with Tris HCl buffer (100 mmol/L, pH 8.0) containing, per liter, 500 mmol of NaCl and 0.2 g of NaN₃ (buffer A). Fractions of 2.35 mL were collected at a flow rate of 4.5 mL/h.

**Purification.** Blood (100 mL total) from the median cubital vein was collected into 10 separate glass tubes and allowed to clot by standing at 37 °C for 30 min. The clot was removed by centrifuging (3360 × g, 15 min). After centrifuging (3360 × g, 15 min) the 50-mL of combined supernate, we added 0.2 g of NaN₃ per liter of serum (Pool X₀) and passed 8.0 mL samples of this pool through an Ultrigel AcA 44 column (5.0 × 90 cm) with buffer A.

Fractions of the effluent were assayed for LD activity, and those corresponding to the two peaks with LD activity were pooled separately (Pool X₁ and Pool Y₁) and were respectively concentrated to about 30 mL each by ultrafiltration through a PM-30 membrane. We then assayed the concentrated Pools X₁ and Y₁ for LD activity and examined them by electrophoresis and immunoelectrophoresis. Because Pool Y₁ showed no evidence of the LD–IgA complex, we discarded it from further study. To remove most of the Tris HCl buffer, we diluted the concentrated Pool X₁ fivefold with phosphate buffer (100 mmol/L, pH 7.0), then reconstituted it to about 40 mL. After centrifugation at 22 000 × g for 60 min, we applied the Pool X₁ supernate to a 26 × 70 mm column containing 10 g of 5'-AMP–Sepharose 4B gel, perfusing the sample through the gel according to the method of Pettit et al. (20). After assaying the eluted fractions for LD activity, we combined those that had LD activity (which eluted with 0.4 mmol/L NADH solution) into Pool X₂.

Pool X₂ was concentrated to about 3 mL by ultrafiltration with a PM-10 membrane and analyzed by electrophoresis. To remove NADH from the concentrated pool X₂, we passed the pool through an Ultrigel AcA 44 column (1.4 × 100 cm) with buffer A at a flow rate of 4.7 mL/h, collecting 2.35-mL fractions. The fractions were assayed for LD activity and those with LD activity were combined into Pool X₃. Pool X₃ was concentrated to about 2.7 mL by ultrafiltration through a PM-10 membrane.

**Results**

**Purification of the LD–IgAₐ Complex**

Given the presence of an abnormal agar–agarose LD isoenzyme pattern, i.e., a marked increase of LD activity at the LD-3 band (Figure 1A), in the serum of a 20-year-old woman with HBs antigens, we assumed the presence of a LD–immunoglobulin complex. Immunoelectrophoresis, followed by assay for LD activity, demonstrated the presence of a LD–IgAₐ complex (Figure 2).

The complex was purified about 10 000-fold from the serum to apparent homogeneity (Table 1). When Pool X₀ (8 mL per run, total LD activity = 650 U/L) was applied to an Ultrigel AcA 44 column, the LD activity was eluted in two peaks (Figure 3A). In all, 48 mL of Pool X₀ was passed through the AcA 44 column. After concentration, 40 mL of Pool X₁ contained 17.6 g of protein and 430 U of LD activity per liter. The calculation of LD activity recovered was based on the LD–IgAₐ complex containing 66% of the total LD activity of the original serum sample, as estimated by peak area measurements (Figure 3A). Agar–agarose gel electrophoresis of concentrated Pool X₁ demonstrated LD activity...
Table 1. Purification of LD-IgA complex from Human Serum

<table>
<thead>
<tr>
<th>Step</th>
<th>Vol, mL</th>
<th>Total LD-IgA, mg</th>
<th>Total protein, mg</th>
<th>Spec. acty., U/g</th>
<th>Recovery, %</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original serum (Pool X₀)</td>
<td>48</td>
<td>20 280</td>
<td>3835</td>
<td>5.3</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ultrogel AcA 44 chromatography, followed by concentration (Pool X₁)</td>
<td>40</td>
<td>17 200</td>
<td>705</td>
<td>24.4</td>
<td>84.8</td>
<td>4.6</td>
</tr>
<tr>
<td>5'-AMP-Sepharose 4B chromatography, followed by concentration (Pool X₂)</td>
<td>3</td>
<td>16 800</td>
<td></td>
<td></td>
<td>82.8</td>
<td></td>
</tr>
<tr>
<td>Ultrogel AcA 44 chromatography, followed by concentration (Pool X₃)</td>
<td>2.7</td>
<td>5 700</td>
<td>0.1</td>
<td>57 000</td>
<td>28.1</td>
<td>10 775</td>
</tr>
</tbody>
</table>

**Fig. 1.** Agar-agarose LD patterns after the first stage of purification on serum LD-IgA complex

A, Original serum sample; B, pooled fractions of low-M, LD activity obtained after Ultrogel AcA 44 column chromatography; C, pooled fractions of high-M, LD activity obtained after Ultrogel AcA 44 column chromatography. LD activity applied (at points indicated by arrow) to gels A, B, and C was 1250, 375, and 475 μU, respectively.

only at the LD-3 band (Figure 1C). Immunoelectrophoresis, followed by assay for LD activity, demonstrated the presence of the LD-IgA complex. Agar-agarose electrophoresis of the concentrated Pool Y₁ material showed a normal distribution of LD activity among the five characteristic LD isoenzymes (Figure 1B); immunoelectrophoresis followed by assay of LD activity demonstrated the complete absence of the LD-IgA complex. Therefore, we no longer studied this pool.

The concentrated Pool X₁ was diluted fivefold with the affinity chromatography buffer, applied to the 5'-AMP-Sepharose 4B column, and then eluted with the washes shown in Figure 2B. LD activity was eluted as a single peak by the 0.4 mmol/L NADH wash. The fractions containing LD activity were pooled (Pool X₂; 40 mL) and concentrated to 3 mL; total LD activity was 5600 U/L.

To remove the NADH, the concentrated Pool X₂ was passed through an Ultrogel AcA 44 column (Figure 3C). After concentration of the fractions having LD activity (42 mL concentrated to 2.7 mL, Pool X₃), total LD activity was 2110 U/L. Concentrated Pool X₃ contained 100 μg of protein, including 22 μg of IgA protein; immunoelectrophoresis followed by assay for LD activity indicated the presence of the LD-IgA complex. Electrophoresis of 3.7 μg of concentrated Pool X₃ standard polyacrylamide disc gel followed by assay for LD activity demonstrated a single, densely staining band with LD activity (Figure 4A). A second gel, stained with Coomassie Brilliant Blue G-250, revealed a single band with the same Rf as the densely staining band with LD activity (Figure 4B).

**Characterization of the LD-IgA Complex**

We estimated the relative molecular mass of the purified
Fig. 3. LD activity (○ and broken line) and absorbance (solid line) of 125-μL aliquots of fractions eluted from the patient's serum
A, elution pattern obtained with Ultrogel AcA 44 filtration; B, elution pattern obtained by affinity chromatography on 5'-AMP-Sepharose 4B; C, elution pattern obtained with Ultrogel AcA 44 filtration. For details, see Materials and Methods.

Fig. 4. Disc gel electrophoresis of purified LD-IgA<sub>1</sub> complex followed by assay for LD activity (A) or by staining for protein with Coomassie Brilliant Blue G-250 (B).

IgA<sub>1</sub> complex by electrophoresis of concentrated Pool X<sub>3</sub> under non-reducing circumstances on polyacrylamide gels (100 g/L) containing sodium dodecyl sulfate. Migration distances of two subunits, i.e., monomeric IgA and monomeric LD, indicated M<sub>r</sub>'s of 170 000 and 34 000, respectively, by comparison with proteins of known M<sub>r</sub> (Figure 6).

Fig. 5. Determination of the M<sub>r</sub> of purified native LD-IgA<sub>1</sub> complex (A) by AcA 22 chromatography
For details, see Materials and Methods. The M<sub>r</sub>'s of the comparison proteins are: rabbit muscle aldolase, 158 000; bovine liver catalase, 232 000; horse spleen ferritin, 440 000; and bovine thyroid thyroglobulin, 968 000.

Fig. 6. Estimation of the M<sub>r</sub>'s of the subunits (A, B) of purified LD-IgA<sub>1</sub> complex by sodium dodecyl sulfate polyacrylamide gel electrophoresis in the absence of reducing agents
For details, see Materials and Methods. The M<sub>r</sub>'s of the comparison proteins are: IgG, 180 000; phosphorylase b (phosphorylase B), 94 000; transferrin, 78 000; bovine serum albumin, 68 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; and α-chymotrypsinogen, 25 000.

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Table 2. Clinical and Clinical Chemical Data for Individuals Whose Serum Showed the Presence of an LD-IgA Complex

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex and age, yr</th>
<th>Diagnosis</th>
<th>Mean serum LD acy., U/L</th>
<th>Months elapsed after first observation</th>
<th>LD-3 acy., % of total serum LD acy.</th>
<th>Relative mobility</th>
<th>Large-M, K,R</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>♂, 23</td>
<td>HB antigen present</td>
<td>370</td>
<td>25</td>
<td>44</td>
<td>0.34</td>
<td>0.0260</td>
</tr>
<tr>
<td>B</td>
<td>♂, 28</td>
<td>Apparently healthy</td>
<td>365</td>
<td>12</td>
<td>58</td>
<td>0.35</td>
<td>n.d.</td>
</tr>
<tr>
<td>C</td>
<td>♂, 50</td>
<td>Myopathy</td>
<td>280</td>
<td>41</td>
<td>33*</td>
<td>0.34</td>
<td>n.d.</td>
</tr>
<tr>
<td>D</td>
<td>♂, 39</td>
<td>Apparently healthy</td>
<td>410</td>
<td>60</td>
<td>48</td>
<td>0.35</td>
<td>0.0269</td>
</tr>
<tr>
<td>E</td>
<td>♀, 35</td>
<td>Apparently healthy</td>
<td>375</td>
<td>29</td>
<td>51</td>
<td>0.35</td>
<td>0.0271</td>
</tr>
<tr>
<td>F</td>
<td>♂, 20</td>
<td>HB antigen present</td>
<td>625</td>
<td>9</td>
<td>61</td>
<td>0.35</td>
<td>0.0265</td>
</tr>
<tr>
<td>G</td>
<td>♂, 64</td>
<td>Cardiac asthma</td>
<td>600</td>
<td>7</td>
<td>68</td>
<td>0.34</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* As defined in text, with increased LD activity at the LD-3 band after electrophoresis on agar–agarose gel.

a Reference value: 95th percentile: 275 U/L.
b Reference value (%) of total serum LD activity, mean (±SD): LD-3 = 19.1 (1.6%).
c If the distances migrated by the LD-IgA complex and the most basic IgA are 1, and 1p, respectively, the relative mobility R1,2 is 1/1p.
d n.d. not done.

Therefore, we conclude that one molecule of monomeric IgA, kappa type, is linked with two molecules of tetrameric LD.

Clinical Observations

During a six-year study the agar–agarose LD isoenzyme patterns for serum of seven subjects from an unslected population of 21 800 outpatients demonstrated a marked increase of LD activity at the LD-3 band in the presence of all five electrophoretically normal LD isoenzyme bands. Table 2 summarizes the clinical data for these subjects. Although subjects C and G suffered from myopathy and cardiac asthma, respectively, the remaining subjects were apparently healthy. Subject B was referred to our outpatient clinic for evaluation of epigastric pain; and A, D, E, and F were referred for evaluation of a long-standing complaint of over-tiredness.

For all of these subjects the increased total serum LD activity (Figure 7) and the marked increase of LD activity at the LD-3 band in the agar–agarose pattern (Table 2) were present from the beginning and have persisted so far without interruption. In all seven the activities of serum alkaline phosphatase (EC 3.1.3.1), alanine aminotransferase (EC 2.6.1.2), and γ-glutamyltransferase (EC 2.3.2.2) were less than their respective 95th percentile values. Immunelectrophoresis, followed by assay for LD activity, revealed in all cases the presence of an LD-IgA complex.

As in previous reports (3, 8), in all cases the LD activity was present on a small and constant part of the precipitation line of IgA, cathodic to the application point. The agar–agarose serum LD pattern of the patients did not change on addition of as much as 10 g of NAD⁺ per liter of serum. The LD isoenzyme patterns of the test mixtures containing patient’s serum and control serum, and of the test mixtures containing patient’s serum and serum with a relatively high amount of LD-5 did not demonstrate a relative increase of LD activity at the LD-3 band.

To investigate a correlation between an infection with hepatitis virus and the existence of LD–IgA complex (21), we performed serological tests for HBe antigen, hepatitis B core antigen, hepatitis B "e" antigen, and their respective antibodies (anti-HBs, anti-HBc, and anti-HBe), and for antihepatitis A antibody (see Figure 7); the results are summarized in Table 3. One or more of the test results was

Table 3. Occurrence of Hepatitis B Antigens, Anti-Hepatitis B Antibodies, and Anti-Hepatitis A Antibody in Sera Having an LD–IgA Complex

<table>
<thead>
<tr>
<th>Subject</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBsAg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anti-HBs</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anti-HBc</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HBeAg</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
<td>-</td>
<td>n.d.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Anti-HBe</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
<td>-</td>
<td>n.d.</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Anti-HA</td>
<td>+</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+, antigen/antibody demonstrable; -, antigen/antibody not demonstrable; n.d. not done
positive in five cases, whereas in the other two cases neither antigens nor antibodies were demonstrable.

For a more detailed study of the LD-IgA<sub>x</sub> complex, we collected, with the subjects' permission, 40-mL blood samples from A, D, E, and F. For each, the elution profile from the Ultrogel AcA 44 column (Figure 3A) clearly indicated both a large (peak X) and a small (peak Y) molecular size LD. The agar–agarose LD pattern for peak X material revealed LD activity only at the LD-3 band, whereas the peak Y material had a normal distribution of LD activity among the five characteristic LD isoenzymes (Figure 8; for the results for subject F, see Figure 1). Immunoelectrophoresis followed by assay for LD activity demonstrated that the LD-IgA<sub>x</sub> complex was present only in peak X material and was completely absent from peak Y material.

Finally, the serum concentrations of IgG, IgA, and IgM were all within the reference intervals for normal subjects.

**Discussion**

The LD-IgA<sub>x</sub> complex (which has the same electrophoretic mobility as the LD-3 isoenzyme) and the low-M<sub>r</sub> LD isoenzymes account for all the LD activity present in the original serum specimen, because the yield of LD activity after the first passage through the Ultrogel AcA 44 column was almost 100%. Our results indicate that the 100 μg of the final preparation, X<sub>y</sub>, was almost homogeneous.

The estimated M<sub>r</sub> of the native complex (approximately 445 000) and of the dissociated subunits (170 000 and 34 000, representing monomeric IgA and monomeric LD, respectively) rules out the presence of secretory IgA (M<sub>r</sub> = 390 000) (22) or of polymers of IgA. Although one can conclude from these data that the complex contains one molecule of monomeric IgA associated with two molecules of tetrameric LD, the estimated protein content ratio of IgA to IgA–2LD based on our results is 0.22 instead of the theoretical value of 0.38. Two factors may be responsible for this discrepancy. Firstly, we used a lyophilized standardized protein preparation of bovine plasma albumin to estimate the protein content of preparation X<sub>y</sub>, which contained both IgA and LD; however, from Wilgenburg et al. (23) it appears that the sensitivity of Coomassie Brilliant Blue G-250 differs significantly for different proteins. Secondly, the immunological estimation by single radial immunodiffusion of the IgA content of preparation X<sub>y</sub> is uncertain because some of the antigenic determinants of IgA may be shielded by the presence of LD (24).

Relative molecular masses of various complexes in which an Ig molecule is associated with an enzyme molecule have been reported on several occasions: amylase–IgA (25), creatine kinase–IgG (26, 27), creatine kinase–IgA (28), and alkaline phosphatase–IgG (29). To our knowledge, however, no data on M<sub>r</sub> values of immunoglobulin–LD complexes have been reported. We assigned 445 000 ± 17 000 for the M<sub>r</sub> of a native complex consisting of one molecule IgA<sub>x</sub> associated with two LD molecules. Because IgA<sub>x</sub> contains two antigen-binding sites per molecule, an antibody–antigen nature of the binding between IgA and LD in the LD-IgA<sub>x</sub> complex is strongly suggested. These results extend the previous observations (25–29) of macro-enzymes involving an immunoglobulin molecule associated with two enzyme molecules.

By electrophoretic separation of LD isoenzymes on a medium exerting little or no retarding effect related to molecular size, i.e., agar or agar–agarose gel, one may obtain a pattern characterized by a marked increase of LD activity at the LD-3 band in the presence of all five LD isoenzyme bands. The increase of LD activity at the LD-3 band may even exceed the activity of the LD-2 band. To our knowledge, this well-defined LD pattern does not correlate with any particular disease. However, the data for our seven cases that had this characteristic LD pattern confirm the data of a case reported by Nagamine (30), and of five cases reported by Biewenga and Feltkamp (31). Therefore, we combined the results for the 13 cases and summarize the features of this group as follows: Of all 13 subjects (eight men and five women, ages 20 to 73 years), five were younger than 29, indicating that the onset of the phenomenon might occur at an early age. Four of the 13 were apparently healthy, apart from a long-standing complaint of overtiredness, and the diagnosis of the remaining nine failed to explain the biochemical abnormalities. Thus, the pathogenetic mechanism resulting in the above LD pattern is so far unknown. The pl of the LD-IgA<sub>x</sub> complex was, within narrow limits, constant; the LD activity was localized on a small part of the serum IgA precipitation line, cathodic to the application point. Furthermore, in all seven of our subjects the relative mobility of the LD-IgA<sub>x</sub> complex was constant (Table 2). The relative molecular mass also was constant, as reflected by the constant partition coefficients we estimated by Ultrogel AcA 44 chromatography (Table 2). On diagnostic grounds, in four of our seven subjects we could expect the LD-5 activity to be increased: in A and F because of the presence of hepatitis B antigen (30, 31), in C because of myopathy (32), and in G because of hepatic lesion associated with cardiac asthma (33). Although we cannot at this time provide experimental evidence concerning the nature of the LD isoenzyme of the LD-IgA<sub>x</sub> complex, we believe the clinical data on these four patients favor a complex of IgA<sub>x</sub> with the LD-5 isoenzyme. The results of the test-mixture experiments led to the conclusion that either the concentration of free IgA<sub>x</sub> associating with LD was below the detection limit of our procedures or the free IgA<sub>x</sub> was unable to bind the LD from other patients. Our findings show that the serum LD-IgA<sub>x</sub> complex was present during a period ranging from seven to 60 months (mean = 26 months). This result contrasts with other reports on the biological half-lives of LD (52 h (34)) and IgA (six to seven months).
days (35, 36). Given the results of this study and of a previous study (21), the initiating trigger mechanism may well be an infection with a virus, probably hepatitis B. In our study, three subjects showed serological evidence of infection with hepatitis B. Hepatitis B antigens or antibodies were undetectable in the other four, either because there were none present or because of low titers as a result of the time elapsed between the onset of the infection and the performance of the tests. We expect that further studies will lead to a more detailed insight into the binding site on the IgA molecule for the LD molecule and into the pathogenic mechanism that produces this well-defined LD isoenzyme pattern.

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