A Lactate Dehydrogenase–Immunoglobulin G1 Complex, Not Blocked by Anti-Idiotype Antibody, in a Patient with IgG1-λ Type M-Proteinemia

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The serum of a patient with IgG1-λ type M-proteinemia showed an abnormal isoenzyme pattern for lactate dehydrogenase (LDH, EC 1.1.1.27). By affinity chromatography, we showed that four isoenzymes (LDH2, LDH3, LDH4, and LDH5) were bound to the M-protein. This complex formation was not blocked by anti-idiotype antibody, even though the binding capacity of IgG was exclusively located in the Fab region of the molecule. Moreover, heavy and light chains of the patient’s IgG, obtained by reduction, separately had affinities for each of the LDH isoenzymes. LDH–IgG complex was easily dissociated by affinity chromatography on 5%-AMP-Sepharose 4B or by added NADH. We propose the following hypothesis for the LDH–IgG complex formation: LDH can recognize the γ-Fab region of IgG at the NAD+ binding site of the molecule, but the affinity of the LDH molecule for immunoglobulin is much weaker than that for NADH or 5’-AMP.

There are several reports on binding of lactate dehydrogenase (LDH, EC 1.1.1.27) by IgA, IgG, and IgM immunoglobulins to form complexes that produce unusual electrophoretic patterns for LDH isoenzymes (1–8). IgG–LDH complexes involve IgG3 (2), but IgG1, IgG2, and IgG4 may also form complexes (3, 5, 6). IgG–LDH complexes characterized to date appear to involve all five LDH isoenzymes (2, 6–8). Although some IgA–LDH complexes appear to involve all LDH isoenzymes (1), most of them involve only the specific LDH isoenzyme (4). However, it is not clear whether this is the result of a specific antigen–antibody reaction or is simply a nonspecific protein–immunoglobulin complex. Here we describe a patient with IgG1-λ type M-proteinemia whose LDH is shown to be bound to the M-protein, the structurally homogeneous protein found in high concentration in plasma cell dyscrasias. The complex formation was not blocked by anti-idiotype antibody, even though the binding capacity of IgG was exclusively located in the Fab region of the molecule. We have attempted to elucidate the mechanism of complex formation.

Case Report

The patient, a 80-year-old man, was hospitalized for anemia and demyelinating encephalopathy.

He had slight normochromic normocytic anemia (erythrocytes 375 × 106/L, hematocrit 36%, Hb 119 g/L). Total LDH activity in serum was 271 U/L, which is within our normal range (190–360 U/L), and the LDH electrophoretogram was abnormal. The electrophoretic distribution of serum proteins (g/L, and percent of total) was: albumin 41.9 (65.5%), α1-globulin 2.4 (3.7%), α2-globulin 5.6 (8.7%), β-globulin 5.1 (7.9%), and γ-globulin (the fraction containing the M-protein) 9.1 (14.2%). Serum immunoglobulin concentrations, as determined by single radial immunodiffusion (normal reference intervals in parentheses), were: IgG 14 750 mg/L (9500–15 500 mg/L), IgA 2000 mg/L (1500–2700 mg/L), and IgM 1000 mg/L (750–1750 mg/L).

Materials and Methods

Reagents: NADH (specially purified, Grade II) was from Boehringer Mannheim GmbH, Mannheim, F.R.G. Anti-LDH5 (M4) antiserum was from Roche Diagnostic Systems, Nutley, NJ. Monoclonal antibodies to the subclasses of IgG were a gift.

Enzyme activity: LDH activity was determined at 37 °C in an automated analyzer (JCA-VX 1000 Clinalyzer; Japan Electron Optics Laboratory, Ltd., Tokyo, Japan) with use of a LDH test kit (LDH-NA Test; Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Electrophoresis: LDH isoenzymes were separated on agarose gel (Universal film; Ciba–Corning, Palo Alto, CA) with use of a barbiturate buffer (60 mM/L, pH 8.6). To reveal LDH activity, we used an LDH reagent (Iatron LDH-LQ; Iatron Laboratories, Ltd., Tokyo, Japan) that contains lactic acid, NAD+, and Nitro Blue Tetrazolium chloride, incubating the agarose gel with the reagent for 30 min at 37 °C. For isoelectric focusing we used agarose gel (IEF film, pH 3–10; Ciba–Corning) according to the manufacturer’s instructions. The pl markers were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. For immunoelectrophoresis on gel plates prepared from a mixture of agar (8 g/L) and agarose (4 g/L) we used the same buffer as described above and a procedure based on the method of Scheidegger (9).

Determination of relative molecular mass: For gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals) we used a 96 × 2.6 cm column; the eluent was Tris HCl buffer (0.1 mol/L, pH 8.0) containing 0.2 mol of NaCl per liter. We applied 2 mL of the patient’s serum to the column and collected 4-mL fractions with a fraction collector, monitoring the elution at 280 nm. We measured the LDH activity in each fraction, using fivefold the usual sample volume.

Purification of LDH isoenzymes: LDH isoenzymes were purified from pooled serum and human liver. Liver tissue obtained at autopsy was homogenized in sodium phosphate buffer (0.1 mol/L, pH 7.2) containing 150 mmol of NaCl per liter, then centrifuged (2750 × g, 15 min). The supernatant

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liquid was further centrifuged at 3000 x g for 20 min. The supernatant was mixed with two volumes of pooled serum and filtered through a 15 x 9.0 cm column of 5'-AMP-Sepharose 4B (Pharmacia Fine Chemicals). The column was washed with the sodium phosphate buffer, and the LDH that had been bound to the 5'-AMP ligand was eluted with 1 mmol/L NADH (in the buffer). The LDH fraction was purified by chromatography on a 55 x 16 cm column of diethylaminoethyl-Sepharose (Pharmacia Fine Chemicals) with use of a NaCl gradient, 0 to 0.5 mol/L.

**Purification of IgG**: We isolated the IgG from the LDH-IgG complex in the patient's serum as follows. The patient's serum was fractionated by affinity chromatography on a 15 x 9.0 cm column of Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) according to the modified method of Chenais et al. (10). We applied 5 mL of the patient's serum to the column and eluted the bound proteins with 1.0 mol/L acetic acid (pH 2.2) at a flow rate of 16 mL/h. All acid-eluted fractions (IgG fraction) were pooled and promptly dialyzed against pH 8.0 Tris HCl buffer, then concentrated by ultrafiltration. We then applied 2.5 mL of the concentrated serum to a column of diethylaminoethyl-Sepharose and eluted the fraction with a NaCl gradient of 0 to 0.2 mol/L.

**Purification of γ-Fab and γ-Fc fragments**: γ-Fab and γ-Fc fragments were prepared by digestion with papain (EC 3.4.22.2; Merck, Darmstadt, F.R.G.) under the conditions described by Porter (11) at an enzyme/protein ratio of 2 g/100 g for 5 h at 37 °C. To separate the intact IgG from the fragments, the reaction mixture was then filtered through a 70 x 16 cm column of Sephadex G-100 (Pharmacia Fine Chemicals) in the sodium phosphate buffer. The γ-Fab and γ-Fc fragments, in effluent corresponding to the second peak, were subsequently purified by affinity chromatography on a column of Protein A-Sepharose CL-4B with use of the sodium phosphate buffer. Unbound proteins (γ-Fab fragment) were eluted with more of the same buffer, until the absorbance at 280 nm was <0.02. We then eluted bound proteins (γ-Fc fragment) with 1.0 mol/L acetic acid.

**Reduction and alkylation**: Heavy and light chains were separated according to Fleischman et al. (12). We reduced 3 mL portions of 6.5 g/L solutions of the purified IgG with a 3.75 mol/L solution of 2-mercaptoethanol, and after 1 h at room temperature alkylated them with iodoacetamide (final concentration 0.75 mol/L). After dialysis, the sample was fractionated by affinity chromatography on Protein A-Sepharose CL-4B. The unbound (light chain) and bound (heavy chain) fractions were separately isolated by gel filtration on Sephadex G-200.

**Preparation of anti-idiotypic antiserum**: Antiserum was raised in a rabbit after primary immunization and two booster injections with 2 mg of the patient's IgG in Freund's complete adjuvant. Antiserum was absorbed by normal pooled serum and another patient's M-protein sera and purified by affinity chromatography with use of the patient's IgG coupled to CNBr-Sepharose 4B (Pharmacia Fine Chemicals). Specificity was checked by double immunodiffusion and immunoelectrophoresis.

**Results**

Characterization of the LDH-IgG1 Complex

Agarose gel electrophoresis of the patient's serum disclosed an M-component in the slow gamma region. Immuno-electrophoresis showed this M-protein to be of the IgG-λ type. Mixing the patient's serum with monoclonal antibod-ies to the IgG subclasses removed the M-protein in the case of monoclonal anti-IgG1, but not in the case of anti-IgG2, -IgG3, or -IgG4.

The LDH electrophoretogram of the patient's serum was abnormal: the LDH2, LDH3, and LDH4 bands were too cathodically migrated, and each of them (and also LDH5) were broad. The macromolecular nature of the patient's LDH was not demonstrated by gel filtration on Sephadex G-200 (data not shown). Figure 1 shows the elution profile for the patient's IgG on diethylaminoethyl-Sepharose ion-exchange chromatography. The patient's IgG was fractionated into three peaks, which we designated as fractions 1, 2, and 3 (3a and 3b, respectively). Each fraction was mixed with normal serum, and the LDH isoenzymes of the mixture were separated by agarose gel electrophoresis. As Figure 2 indicates, the same abnormal LDH isoenzyme pattern as seen with the patient's serum was observed in fractions 1, 2, and 3a, which contained M-protein, but not in fraction 3b, which did not. We used isoelectric focusing to demonstrate the five-band heterogeneity (Figure 3) of the patient's M-protein. Fraction 1 contained M-protein with pI values of 8.4 and 8.5; fraction 2 contained M-protein with pIs of 8.2, 8.3, and 8.4; and fraction 3a contained M-protein with pIs of 8.0 and 8.2.
To see whether LDH isoenzymes were inactivated by the patient's M-protein, we mixed the purified LDH isoenzyme with an equal volume of the patient's IgG. Inhibition was 0% for LDH1, 27.4% for LDH2, 23.1% for LDH3, 14.4% for LDH4, and 16.3% for LDH5.

To see whether or not the patient's LDH2 and LDH3 isoenzymes showing an abnormally broad band were modified by his patient's M-protein, we did the following study. After collecting the agarose gel block of LDH2 and LDH3 isoenzyme fractions corresponding to an abnormal broad band, we extracted them with phosphate-buffered (pH 7.2) isotonic saline, concentrated the extracts by ultrafiltration, and repeated the electrophoretic analysis for LDH isoenzymes. Rather than the broad bands seen before, the bands for these LDH2 and LDH3 isoenzyme fractions were normal. This demonstrates that the patient's IgG bound to LDH molecules was no longer present in the position of the LDH2 and LDH3 bands, the LDH molecule having become dissociated from the patient's IgG.

When normal serum was filtered through the patient's IgG-coupled CNBr-Sepharose 4B, analytical recovery of LDH activity was 103%, and the percentage of LDH isoenzymes (the pre-treatment value in parentheses) were: LDH1 21.3 (21.7), LDH2 38.0 (36.9), LDH3 24.9 (26.9), LDH4 8.3 (8.9), and LDH5 7.5 (7.2). Evidently the affinity of the patient's IgG for LDH molecules is very weak.

Immunological Properties of the LDH–IgG1 Complex

To demonstrate the association of LDH with γ Fab fragment, we digested the purified IgG (containing M-protein) with papain and separated γ Fab and γ Fc fragments by affinity chromatography on Protein A–Sepharose CL-4B. We separately mixed γ Fab and γ Fc fragments with normal serum in the ratio of 2:1 (by vol) and incubated at 37 °C for 30 min. After incubation, the LDH isoenzymes of the mixture were separated by electrophoresis on agarose gel. The same abnormal LDH isoenzyme pattern as for the patient's serum was observed in the case of the γ Fab fragment, but not in the case of the γ Fc fragment (Figure 4).

Having demonstrated the association of LDH with heavy and light chains, we attempted to confirm the binding between LDH and heavy or light chains. After reduction and alkylation, the heavy and light chains from the patient's IgG were isolated by column chromatography on
agarose gel. Bands corresponding to LDH2, LDH3, LDH4, and LDH5 were not seen. Instead, they formed a single broad band in the fast gamma region. When 10- or 20-fold Tris-diluted antiserum to LDH5 was mixed with normal serum before electrophoresis, the bands of LDH3 and LDH4 were diffuse (Figure 8), but the mobility of LDH did not completely change cathodically as compared with that for the patient’s serum.

(2) To investigate the possibility that LDH–IgG complexes were dissociated in the presence of NADH, we incubated the patient’s serum or the mixture of anti-LDH5 antiserum and normal serum with NADH in concentrations of 25, 50, 100, 150, or 200 mmol/L. The mixtures were then gel-electrophoresed. The electrophoretic pattern for the patient’s serum with NADH showed a normal LDH isoenzyme pattern with NADH concentration as low as 25 mmol/L. In contrast, the corresponding immune complexes with normal serum were not dissociated even by a NADH concentration of 200 mmol/L.

(3) The patient’s serum, applied to the 5’-AMP–Sepharose 4B column, was eluted with 1 mmol/L NADH added to the elution buffer.Likewise, a mixture of anti-LDH5 antiserum and normal serum was applied and eluted. In the patient’s serum, the LDH–IgG complexes were dissociated by passage through the column—e.g., the electrophoretic LDH isoenzyme pattern for concentrated bound fraction was

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**Fig. 7.** Electrophoretic pattern of the patient’s IgG with anti-idiotype antiserum (left) and LDH isoenzyme pattern (right)

left: NS, rabbit serum; A-Id, anti-idiotype antiserum; P-lgG, patient’s IgG; A-Id + P-lgG, the patient’s IgG mixed with anti-idiotype antiserum; right: NS, normal serum; P-lgG, patient’s IgG mixed with normal serum; A-Id + P-lgG, the mixture of the patient’s IgG and anti-idiotype antiserum mixed with normal serum

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**Fig. 8.** LDH isoenzyme pattern in the mixture of anti-LDH5 antiserum and normal serum

NS, normal serum; A-LDH5, anti-LDH5 antiserum mixed with normal serum; A-LDH5(10x), anti-LDH5 antiserum diluted 10-fold with Tris HCl buffer (pH 8.0) mixed with normal serum; A-LDH5(20x), anti-LDH5 antiserum diluted 20-fold with Tris HCl buffer (pH 8.0) mixed with normal serum

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**Fig. 6.** Specificity of anti-idiotype antiserum on double immunodiffusion (lcp) and immuno-electrophoresis (bottom)

NS, normal serum; PS, patient’s serum; P-lgG, patient’s IgG; wells 1–4, another IgG M-protein sera; A-, anti-idiotype antiserum; A, anti-γ-chain antiserum; AHS, anti-human whole serum; A-lgG, anti-γ-chain antiserum; A-Id, anti-idiotype antiserum; A-L, anti-lambda light chain antiserum

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normal. Although the unbound fraction was devoid of LDH activity, a mixture of this fraction and normal serum had the same abnormal pattern as the patient's serum alone. However, this was not true for a mixture of anti-LDH5 antiserum and normal serum.

Discussion

The presence of LDH–immunoglobulin complexes in serum has been previously reported (1–8). However, the clinical significance of these complexes is still poorly defined. The complexes have been observed in serum from both healthy individuals and patients with various apparently unrelated diseases. The LDH isoenzyme patterns for sera containing such complexes generally display one or more of the following features: an abnormal number of LDH isoenzyme bands, altered electrophoretic mobility, or broadening of one or more bands. It is not yet clear whether this complex formation reflects specific binding of a nonprecipitating antibody to the enzyme via its antigen binding site or nonspecific aggregation of immunoglobulin and enzyme molecule.

In the present case, LDH–IgG complexes were present in the serum of a patient with IgG1-α type M-proteinemia. By affinity-chromatography techniques, the IgG1-α type M-protein was demonstrated to have affinity for LDH2, 3, 4, and 5. However, no macromolecular complex was detectable in the patient’s serum by gel filtration. Conceivably, the LDH molecule separated from IgG during gel filtration; indeed, the LDH binding capacity for the patient’s IgG is very weak, and the LDH–IgG complexes in his serum are easily dissociated. The evidence for this is: (a) when normal serum was filtered through the patient’s IgG–Sepharose 4B column, we saw no significant change in LDH activity or in the proportions of LDH isoenzymes; (b) the broad bands in LDH2 and LDH3 isoenzyme fractions were not seen, but instead normal bands, when we electrophoretically analyzed LDH isoenzymes extracted from the agarose gel block corresponding to LDH2 and LDH3 isoenzyme fractions composing an abnormal broad band.

Imoto et al. (6) claimed that the complex formation of the LDH–IgG complexes in a case of oxphenestin-induced lupoid hepatitis was a specific antigen–antibody reaction because the LDH was attached to the γ Fab region, but not to the γ Fc region. The same results have been described for some other enzymes (13–15). Similarly, the M-protein in our patient apparently was suggested to be an anti-LDH autoantibody, because the binding capacity was exclusively located in the Fab region of the molecule. However, the major discrepancy arises from the fact that heavy and light chains of the patient’s IgG, obtained by deoxidation, separately had affinities for each of the LDH molecules. Although the variable domains of heavy and light chains are responsible for the antigen-binding specificity of the antibody molecule, the light chains per se have little or no antigen-binding capacity (16). This fact indicates that the complex formation in our case is due to nonspecific binding—a suggestion supported by the fact that binding of the patient’s IgG to LDH molecules was not blocked by anidiotype antibody that was associated with hypervariable regions of an antibody molecule (16) and that we saw many differences between the LDH–IgG complex in the patient’s serum and anti-LDH5 antibody. However, the anti-idiotype antibody data may be not strong enough to support the conclusion; i.e., the possibility exists that the binding could take place at the antigen-binding site but with low affinity since the patient’s M-protein is heterogeneous and the anti-idiotype antibody is not necessarily directed to all hypervariable regions of the M-protein.

Gorus et al. (3) succeeded in dissociating LDH–IgG complexes by adding NAD+, which presumably bound to LDH and altered its antigenicity. With regard to the mechanism of complex formation, it is interesting that the LDH–IgG complexes in our patient also were dissociated either by added NADH or by passing the patient’s serum through a column containing 5’-AMP–Sepharose 4B, which can be considered as a fragment of NAD+. The dinucleotide-fold region, the function of which is to bind NAD+, has been found in LDH (17, 18). This region is composed of two very similar parts: one binds the adenosine phosphate portion of the coenzyme, the other binds the nicotinamide mononucleotide portion, and thus the region may be recognized as a molecule that can mimic the conformation of NAD+. The binding constant for the enzyme–"pseudo" ligand complex is usually much weaker than the binding constant of the corresponding enzyme–cofactor complex (18, 19).

From these facts, we propose the following hypothesis rather than a specific antigen–antibody reaction for the LDH–IgG complex formation: LDH can recognize the γ-Fab region of IgG at the NAD+ binding site of the molecule, but the affinity of the LDH molecule for immunoglobulin is very weak as compared with its affinity for NADH or 5’-AMP. Although other LDH–IgG complexes that are not dissociated by NADH have been reported (2), the findings that the LDH–IgG3 complexes, not dissociated by NADH, were easily dissociated by affinity chromatography on 5’-AMP–Sepharose 4B (20) support the possibility that these LDH–IgG complexes also become dissociated on the column.

Why was LDH1 not involved in binding to the IgG, in spite of the fact that LDLH also has an NAD+ binding site? It should be considered that the affinity of each LDH isoenzyme for immunoglobulin is different because the LDH M4 and H4 isoenzymes have different affinities for both NAD+ and NADH (21). Therefore we suggest that LDH1 is uninfluenced by the IgG molecule because the affinity of LDH1 for the patient’s IgG is very weak.

Enzyme activity of LDH was partly inhibited by IgG because the patient’s IgG bound to the dinucleotide-fold region of LDH. But it is not clear whether the mechanism of action of the patient’s IgG is via occupying an NAD+ binding site or via both occupying and steric hindrance to substrate.

Why did the LDH isoenzyme pattern display abnormally broad bands? We suggest that each LDH molecule is slowly dissociated from IgG during electrophoresis, thus not all of it could move completely to the usual electrophoretic position.

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References


