Immunochemical Measurement of Lipoprotein-X

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Lipoprotein-X, an abnormal lipoprotein that is specific for cholestasis, was quantitated by immunochemical methods. Interfering lipoproteins also present in normal serum and sharing antigenic determinants with lipoprotein-X were removed before the sample was applied, by precipitation with purified anti-lipoprotein B or a γ-globulin fraction of specific lipoprotein B antiserum. On Laurell electrophoresis, peak height was linearly related to lipoprotein-X concentration in the range 0.20–10 g/liter of serum. Sensitivity could be increased further by staining the plates. The coefficient of variation was less than 5%.

Single radial immunodiffusion (Mancini et al. technique) was somewhat less sensitive and accurate. Results were available after 3 h by Laurell’s electroimmunodiffusion technique, and after 72 h by the technique of Mancini et al. Equivalent results were obtained for samples of lipoprotein-X of extrahepatic or intrahepatic origin.

Additional Keyphrases: "rocket" electroimmunodiffusion
- diagnostic aid
- radial immunodiffusion
- hepatic disease
- cholestasis
- liver-function test

Currently, diagnosis of cholestasis is based on a number of biochemical tests, including the determination of serum bilirubin or of the activities of alkaline phosphatase (EC 3.1.3.1), n-glutamyltransferase (EC 2.3.2.1), or certain other serum aminotransferases (EC 2.6.1.1, 2.6.1.2, or 2.6.1.6) (1, 2). The alteration of serum lipid values, especially the increase in phospholipids and unesterified cholesterol observed in patients suffering from cholestasis, is attributable to an abnormal lipoprotein (3), called lipoprotein-X (LP-X) (4), "cholestasis specific lipoprotein" (5), or "abnormal lipoprotein." According to Seidel et al. (4), LP-X is composed of 6% protein, 66% phospholipids, 25% cholesterol, and 3% triglycerides. The protein part of this abnormal lipoprotein consists of albumin (40%) and apolipoprotein C (60%) (4), the major constituent of very-low-density lipoproteins and chylomicrons of normal serum and chyle (6).

The introduction of an immunochemical test for LP-X (4) added a new, simple analytical method for diagnosis of cholestasis. Only one other disease has been described thus far in the literature in which LP-X can be demonstrated in the serum in the absence of cholestasis (7). This disease is the result of a deficiency of lecithin:cholesterol acyltransferase (EC 2.3.1.43), and is a rare inborn error of metabolism. In all other cases a positive LP-X test almost completely coincides with the presence of cholestasis (8). Although the LP-X test alone does not distinguish extrahepatic and intrahepatic obstruction, such a differentiation might be possible if activity of the transferase and serum free and esterified cholesterol were also determined at the same time (9).

At the onset of liver disease, the cholestatic phase does not always coincide with the appearance of LP-X. Therefore, it seems to be less important to qualitatively demonstrate LP-X than to observe fluctuations in the LP-X concentrations before and after medical treatment.

Seidel et al. (10) described a semiquantitative LP-X test, in which serum is continuously diluted to a point where no LP-X can be detected by the regular qualitative immunoelectrophoresis. The only truly quantitative determination of LP-X, described by Magnani and Alapovic (11), is based on the observation that LP-X migrates cathodally on certain agar gels. The cathodal region where LP-X could be expected is punched out of the gel and phosphorus determined in it. By multiplying by a factor based on the assumption that most of the LP-X preparations contain 60% phospholipids by weight, the LP-X content in the serum is estimated.

To avoid some problems arising during the latter quantitation method (see Discussion), we describe here a new, easier immunochemical method for quantitation of LP-X. LP-X could not be accurately measured in the past because it shares antigenic determinants with other serum lipoproteins that are present, both normally and in disease. With our method, non-LP-X lipoproteins are removed by immunoprecipitation, and LP-X in the supernate is assayed by single radial immunodiffusion (Mancini technique), electroimmunodiffusion (Laurell technique), or both, by using antisera monospecific for LP-X.

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2 This paper is dedicated to Prof. Dr. Otto Hoffman-Ostenhof, President of the Austrian Society of Clinical Chemistry, on the occasion of his sixtieth birthday.

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Materials and Methods

Samples

Sera from patients with different forms of liver disease of extra- or intrahepatic origin showing the presence of LP-X by the conventional immunochemical test (1) or by the “Rapidophor LP-X test system” (Immuno AG, Vienna) were used for quantitation. Blood samples were taken after the patient had fasted for 12–14 h, and were investigated within 24 h. In some cases, samples were stored at 4 °C for several days after addition of 1 g of sodium azide and 0.5 g of disodium ethylenediaminetetraacetate per liter.

Purification of LP-X

Pure LP-X necessary as references for standardization of the assay as well as for immunization was isolated individually from strongly positive sera of patients with extrahepatic or intrahepatic obstruction, as follows (G. Kostner, in preparation). Total very-low-density lipoproteins and low-density lipoproteins were first precipitated with sodium phosphotungstate according to Burstein and Morfin (12). The precipitate was dissolved in NaCl solution (0.1 mol/liter) and ultracentrifuged at 120,000 × g for 18 h at 16 °C. The upper third of each tube was removed by slicing the tube. The density of the lower layer, which consisted of low-density lipoproteins and some serum contaminants, was adjusted to 1.065 kg/liter by adding solid sodium chloride, and it was centrifuged at 120,000 × g for 24 h at 16 °C. The supernatant lipoproteins (lipoprotein B plus LP-X) were dialyzed vs. sodium phosphate buffer (0.1 mol/liter, pH 6.8) and passed over a 1.2 × 15 cm column packed with hydroxyapatite (Biogel HT; Bio-Rad, Richmond, Calif. 94804). Elution with the sodium phosphate buffer yielded a single peak, representing most of the LP-X present in the original mixture. Lipoprotein B, adsorbed under these conditions, could be eluted with a more concentrated sodium phosphate buffer (0.65 mol/liter, pH 6.8). The fraction containing LP-X was concentrated by dialysis under reduced pressure and rechromatographed on Biogel A-5m. This step removed small amounts of impurities of lipoprotein A. The main peak in this chromatographic procedure represented pure LP-X.

This material reacted in immunodiffusion experiments only with antibodies to LP-X or lipoprotein C, and gave no reaction with antisera to albumin, lipoprotein B, lipoprotein A, or anti-human serum. In agar gel electrophoresis the purified LP-X preparation migrated cathodally and in agarose it migrated like the β-fraction of serum. The concentrations of purified LP-X preparations were assayed by determining the residual weight after equilibrium dialysis against NaCl (0.15 mol/liter) and evaporation in a dessicator, and drying at 50 °C under reduced pressure.

Preparation of antisera

A solution of 10 mg of purified LP-X in 2 ml of NaCl solution (0.15 mol/liter) was emulsified with an equal volume of complete Freund’s adjuvant and injected intraperitoneally into rabbits (3–4 kg body weight) at 10-day intervals. After three to four consecutive injections the antibody titer was usually high enough. Most of these antisera showed an antibody activity to LP-X and a slight activity against albumin. Occasionally, antibodies to γ-globulins or lipoprotein B could be detected as well. All antibody activity directed against antigens other than LP-X was adsorbed by adding an appropriate amount of normal human serum that was free of very-low-density lipoprotein.

Antisera of several animals were pooled in order to get enough antiserum for this research. The antiserum titer, as determined with purified LP-X, was 13 g/liter of antiserum (i.e., one liter of antiserum would precipitate 13 g of LP-X). Other antisera—to normal lipoproteins, apolipoproteins or serum proteins—used in this investigation were the same as described elsewhere (1, 6, 13, 14). Pure antibodies to lipoprotein B were isolated from horse antiserum as described previously (15). The antiserum was concentrated to the extent that 40 μl in NaCl solution (0.15 mol/liter) precipitated all the lipoproteins containing apolipoprotein B from 1 ml of human serum. In some cases the γ-globulin fraction of horse antiserum was used instead of pure antibodies, prepared by ammonium sulfate precipitation and chromatography on a column of diethylaminoethyl-cellulose (15). However, the antibody titer of these solutions was 10-fold less than that for the solution of pure antibodies.

Quantitation of LP-X

“Rocket” electrophoresis. LP-X was measured by Laurell “rocket” electrophoresis (16) or by one-dimensional radial immunodiffusion, in principle as described earlier (14). For Laurell electrophoresis, we used agarose gels (Bio-Rad), 10 g/liter of barbital buffer (0.02 mol/liter sodium barbital, adjusted to pH 8.2 by adding concd HCl). The glass plates used for this purpose were 5 × 5, 7 × 7, or 6 × 11 cm in size. The volume of agarose gel containing 25 μl of antiserum to LP-X per milliliter of gel was 4, 8, and 10 ml, respectively. In some cases the “intermediate gel technique” (17) was applied. The intermediate gel containing pure antibodies to lipoprotein B (10 μl/ml of agarose gel) was 1 cm wide. Electrophoresis was carried out in a water-cooled chamber (Behringwerke A.G., Marburg/Lahn BRD) at constant voltage (8 V/cm for 3 h or 3 V/cm for 10 h). The height of the rocket-shaped electrophoresis peaks were measured, either immediately or after soaking the agar plates in NaCl solution (0.15 mol/liter) for a few hours. In some cases, the plates were dried and stained with Sudan Black. The LP-X concentration of the individual sera was calculated from the peak
heights extrapolated on a standard curve, a plot of concentration of purified LP-X vs. heights of the rocket-shaped peaks formed on electrophoresis. Controls, sera with known concentrations of LP-X, were also run on each plate.

Radial immunodiffusion. Quantitation of LP-X by one-dimensional radial immunodiffusion (Mancini et al. technique, 18) was performed in agarose gels. 10 g/liter of sodium barbital buffer (0.1 mol/liter, pH 7.8), containing 17 μl of anti-LP-X serum per milliliter of agarose gel. At end-point diffusion, the concentration of LP-X was calculated from a standard curve, prepared by plotting the squares of the diameters of precipitate rings of pure LP-X vs. concentration. The end point of the diffusion at room temperature (22 °C) was reached after 72 h. Before LP-X was measured by both methods (Laurell and Mancini et al.) it was necessary to remove apolipoprotein C containing non-LP-X lipoproteins interfering in the described immunochemical assays. Because most of the apolipoprotein C polypeptides are complexed to lipoprotein B, forming very-low-density lipoproteins, interfering lipoproteins could be removed with pure anti-lipoprotein B. For this purpose, sample serum was mixed with purified antibodies to lipoprotein B (40 μl/ml of sample serum) and incubated for 15 min at 37 °C, followed by 1 h of incubation at 4 °C. The immunoprecipitate that formed was removed by low-speed centrifugation, and 5 μl of sample was applied to the plates with a 10-μl Hamilton syringe. The dilution of samples with antibody solution was taken into consideration in calculating of LP-X concentrations. In the intermediate gel technique the precipitation step has been omitted. All chemicals used during this investigation were "pro analysi" reagents from E. Merck, Darmstadt, BRD.

Results

In the first experiments, normal fasting serum was investigated by the quantitative immunochemical assay. Each serum gave a weak but relatively high peak of precipitated very-low-density lipoproteins. Since it was known from earlier experiments that anti-lipoprotein B precipitates all the lipoproteins from normal serum of density <1.063 kg/liter (19), we used this technique to remove interfering non-LP-X lipoproteins. In using complete antisera to lipoprotein B to remove very-low-density lipoproteins from LP-X positive sera, we observed that peak heights for LP-X in Laurell electrophoresis varied with pre-incubation time. If pure antibodies to lipoprotein B or γ-globulin fraction of antisera were used, this was not the case. In the following experiments we used a stock solution of antibodies to lipoprotein B. We found that 40 μl of pure antibodies or 400 μl of γ-globulin fraction, added to 1 ml of human serum, removed all very-low-density lipoproteins and lipoprotein B from at least 50 normal or from more than 20 LP-X-positive sera. If normal sera were tested after precipitation, either by Laurell electrophoresis or radial immunodiffusion, no precipitate was formed by incorporating anti-LP-X or anti-lipoprotein B into the gel. LP-X positive sera gave only immunochemical reaction with anti-LP-X.

Figure 1 shows a typical pattern for Laurell electrophoresis, as obtained in the system with purified LP-X. The amount of sample applied was in all cases 5 μl, and the LP-X concentration varied from 0.5 to 6 g/liter. LP-X concentration was linearly related to peak heights from 0.2 to 10 g/liter (Figure 2). Concentrations of 0.2 g down to 0.1 g/liter gave a small peak, but accurate quantitation was not possible. If the anti-LP-X content of the gel was decreased, peak heights increased so much that linearity was regained, but the peaks were less intense, and at lowest antibody concentrations (10 μl/ml of gel) they were too weak to be measured accurately unless stained with Sudan Black. Samples containing more than 10 g of LP-X per liter had to be diluted. To show the general applicability of this method, we assayed several sera containing various amounts of LP-X from patients suffering from obstructive jaundice. Lipoprotein B-containing lipoproteins were removed by precipitation with specific antibodies and diluted in distinct steps, either with NaCl (0.15 mol/liter, adjusted to pH 7.15 with NaOH) or normal human serum from which the lipoproteins had been removed totally by ultracentrifugation at a density of 1.23 kg/liter. On quantitation of LP-X by the Laurell technique, we found a linear relation between peak heights and dilution in all cases. The absolute LP-X concentration of these samples was ascertained from the peak heights, by extrapolating from the standard curve (Figure 2). Dilutions of LP-X positive sera with normal human serum were not linear in LP-X concentration vs. peak heights.

Because the absolute LP-X content of none of these sera was known, it was necessary to prove the validity of the LP-X calculation, which was done with purified LP-X, through extrapolation on the standard curve. Several different dilutions of a strong LP-X-positive serum were mixed with various amounts of purified LP-X, and assayed by both sys-
tems. In all cases, the total amount of added LP-X was proportional to peak height or precipitin-ring diameter.

LP-X may lose its cathodal migration on agar upon storage, owing to denaturation. Therefore several LP-X positive sera were stored at 4 °C and quantitated at two-day intervals. We saw no alteration in peak heights on Laurell electrophoresis on storage for as long as two weeks.

From the variable analytical data published on "purified LP-X" one is tempted to postulate that there are various kinds of LP-X in different forms of liver disease. To test this, we isolated pure LP-X from sera of five patients. The peak heights obtained by electrophoresis were the same in all cases at identical concentrations, when the same LP-X antiserum was used.

By using the "intermediate gel technique," it was unnecessary to precipitate lipoproteins containing lipoprotein B before the sample was applied, by addition of purified antibodies to lipoprotein B. Antibodies were incorporated on a gel strip located between sample gel and gel containing anti-LP-X. An experiment in which this technique is used is shown in Figure 3. It shortens the procedure by 75 min, the time necessary for incubation of the sample serum with pure anti-lipoprotein B. The intermediate strip of gel containing lipoprotein B did not influence the linear relation between LP-X concentration and peak heights. To test the reproducibility of the LP-X quantitation method, we ran 12 samples of different LP-X sera simultaneously on one plate (6 × 11 cm) and compared their peak heights. The coefficient of variation was less than 5%.

Although the quantitation of LP-X by Laurell electrophoresis gave good results quickly, this method requires equipment that is not available in every clinical laboratory. Therefore we tested the validity of quantitation of LP-X by radial immunodiffusion and investigated its clinical application. Here again, it was necessary to remove interfering lipoproteins by precipitation with anti-lipoprotein B before the sample was introduced into the agar wells. There was a linear relation between the square of the diameter of the precipitin ring and LP-X concentration, in the range 0.5 to 10 g/liter (Figure 4). Figure 5 shows a characteristic pattern obtained by testing several dilutions of an LP-X positive serum. Samples with LP-X concentrations greater than 10 g/liter could only be assayed after dilution. The coefficient of variation for the Mancini technique for quantitation of LP-X was about 7%.

Discussion

LP-X is present in experimental animals during experimental cholestasis (20). As early as 24–48 h after ligation of the common bile duct, LP-X could
be demonstrated in the serum of dogs (21) and mice (22), with a rapid decrease in concentration after prolonged obstruction. The activities of enzymes of hepatic origin in the serum, such as serum amino-
transferases, alkaline phosphatase, and others seems to be much less useful for following the course of cholestasis in the early stage than is observation of fluctuations of LP-X concentrations. In addition, in-
crease of hepatic enzyme activity in the serum as well as other biochemical tests (except the qualita-
tive LP-X test) are not specific for cholestatic liver disease (6, 10). Thus, determination of LP-X concen-
tration in the serum is one more addition to the many other liver-function tests, one which may allow a clearer differentiation between extra- and intrahe-
patic cholestasis.

The quantitative LP-X test reported earlier (11) may have some disadvantages over the immuno-
chemical methods described in this paper. First, our method requires less work and time. Secondly, LP-X variants exhibiting no or only slow cathodal migra-
tion have been demonstrated in the serum of differ-
ent patients (2). In addition, the extent of cathodal migration depends on age, the conditions under which the sample is stored, batch of agar used, and the concentration of LP-X itself. Therefore, it some-
times may be hard to decide which gel compartment has to be punched out for phosphorus determination. In addition, some lipoproteins of normal serum may also show slow cathodal migration in agar gels, thus contributing to the phosphorus content of the cat-
thodal region. Quantitation of LP-X by immuno-
chemical methods makes it necessary to remove in-
terfering non-LP-X lipoproteins before the sample is applied. The LP-X antiserum used during this study showed a major activity against apolipoprotein CI and a minor one against other apolipoprotein C-pol-
lypeptides. Because apolipoprotein CI peptides are constituents of very-low-density lipoproteins of nor-
mal and LP-X positive sera, these lipoproteins were removed by precipitation with antiapoprotein B. In a recent investigation, a very-low-density lipoprotein subspecies was isolated from human serum that gave no immunochemical reaction with anti-lipoprotein B and apparently was free of apolipoprotein B (23). These very-low-density lipoprotein species would disturb the quantitative LP-X test because they would not be removed by precipitation with anti-li-
poprotein B. In addition, lipoprotein C has been demonstrated in high-density lipoproteins as well, not linked to apolipoprotein B (13). After precipita-
tion of all lipoprotein B-containing lipoproteins with pure antibodies, more than 50 normal sera tested in this study gave no immunochemically detectable reaction with the anti-LP-X applied. We conclude that the amount of lipoprotein C not linked with apolipoprotein B in fresh human serum is too small to interfere with the described LP-X test. Instead of purified antibodies, the γ-globulin fraction of strong antisera to lipoprotein B had been used with the same success. This was without influence on samples with LP-X concentrations >0.35 g/liter, but makes the test somewhat less sensitive at lower concentra-
tions because of the sample dilution. Because serum very-low-density lipoprotein and lipoprotein B con-
centrations vary from patient to patient, we precipi-
tated these lipoproteins in routine tests with a slight excess of antibody, which did not interfere with the LP-X quantitation and made it unnecessary to de-
termine the equivalence point for each serum sample separately (40 μl of pure antibody solution per millili-
ter of sample proved to be enough for almost all sera tested, but in some cases of hyperlipoproteinemia it was necessary to increase this volume to achieve a complete precipitation of all very-low-density lipo-
proteins). Instead of pure antibodies or γ-globulin fraction, complete antisera—even those with high ti-
ters—could not be used to remove lipoprotein B. We observed that added normal lipoproteins from hu-
mans or animals, especially high-density lipopro-
teins, reacted with LP-X, changing the antigenic be-
havior in Laurell electrophoresis and in the tech-
nique of Mancini et al. The peak heights in the Laurell electrophoresis and the diameters of circles in the Mancini technique increased proportionally with time. For the same reasons LP-X samples could not be diluted with normal serum containing high-
density lipoproteins. If the intermediate gel tech-
nique was used, the precipitation step could be omit-
ted. In addition it was possible to quantitate in this step the total amount of very-low-density lipopro-
teins and lipoprotein B from the peak heights formed in the gel containing anti-lipoprotein B.

Although it is possible that LP-X variants with different antigenicity may be formed in various kinds of liver diseases, in testing several purified LP-X samples from different patients, we always found the same peak heights and precipitin diameters at identi-
cal concentrations. In addition it could be demon-
strated that the apolipoprotein CI content of at least eight different LP-X preparations was practically identical (G. Kostner, in preparation). Thus, we found no evidence in support of this possibility.

**Fig. 5.** One-dimensional radial immunodiffusion of various dilutions of an LP-X-positive serum

Concentration of anti-LP-X in the gel was 17 μl/ml. 5 μl of sample were applied. The concentration of LP-X ranged from 0.2 to 4 g/liter.
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References