

## Modified (Desialylated) Low-Density Lipoprotein Measured in Serum by Lectin–Sorbent Assay

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Modified low-density lipoprotein (LDL) with a low sialic acid content was found in the blood of patients with coronary atherosclerosis. This desialylated lipoprotein causes lipid accumulation in arterial smooth-muscle cells and stimulates cell proliferation and production of the extracellular matrix, i.e., induces all atherogenic manifestations at the cellular level. We have developed a lectin–sorbent assay for the determination of desialylated LDL in sera. The assay is based on the binding of desialylated LDL by immobilized *Ricinus communis* agglutinin with subsequent measurement of lipoprotein through use of anti-apolipoprotein (apo) B antibody. The assay is sensitive to desialylated apo B concentrations as low as 5  $\mu\text{g/L}$ . The intraassay and interassay CVs were 4.8% and 11.3%, respectively. Comparison between the lectin–sorbent assay and a lectin chromatographic technique showed a good correlation. This determination of modified desialylated LDL in human serum with high accuracy and reproducibility may help establish the diagnostic value of this lipoprotein as a risk factor of atherosclerosis.

**Indexing Terms:** atherosclerosis/sialic acid/immunoassay/atherogenesis/risk factors

Several years ago we showed that serum of patients with coronary atherosclerosis induces lipid accumulation in cells cultured from unaffected intima of human aorta (1). This phenomenon was termed atherogenicity. Modified low-density lipoprotein (LDL) and anti-LDL antibodies were found to be the atherogenic components of patients' sera (2–5).<sup>2</sup> Subsequent investigations of modified LDL have shown that this LDL differs dramatically from native LDL in biochemical composition, physical characteristics, and interaction with cell receptors (6, 7). The major difference is the low content of sialic acid, a terminal saccharide of the biantennary chains of apolipoprotein (apo) B (3, 8). Given that galactose becomes the terminal saccharide after the removal of sialic acid, we have developed a method for the isolation of modified LDL on a sorbent with immobilized agglutinin from *Ricinus communis* (RCA<sub>120</sub>), which has a high affinity for galactose (9). Here we present a method for determining the content of modified LDL. This method is based on the binding

of desialylated LDL to RCA<sub>120</sub> immobilized on plastic with subsequent measurement of bound apo B with peroxidase-conjugated polyclonal antibody.

### Materials and Methods

**Blood sample collection.** Blood samples of 22 men (28–56 years) were used in the study. Serum total cholesterol and triglyceride concentrations were <5.2 mmol/L and 1.7 mmol/L, respectively. Blood for investigations was taken from the ulnar vein after a 12-h overnight fast. Serum or plasma (1 g/L EDTA) were prepared by centrifugation and sterilized by filtration (filter pore diameter 0.45  $\mu\text{m}$ ).

**Chemicals.** All reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

**Lipoprotein preparation.** LDL (density 1.019–1.063 kg/L) was isolated from blood plasma by sequential ultracentrifugation in a preparative ultracentrifuge according to Lindgren (10). Lectin chromatography on RCA<sub>120</sub>-agarose was used to separate the sialylated and desialylated LDL (9). Sialic acid-poor LDL was prepared by treatment with agarose-linked *Clostridium perfringens* neuraminidase as described earlier (6). The sialic acid content in LDL preparations was determined by the colorimetric method of Svennerholm (11).

**Desialylated LDL determination by lectin–sorbent assay (LSA).** Microtiter plates (96-well) (Nunc, Roskilde, Denmark) were coated for 2 h at 37 °C with 100  $\mu\text{L}$  of 30 mg/L RCA<sub>120</sub> in phosphate-buffered saline (PBS), pH 7.5. The wells were washed four times with PBS containing 2 g/L bovine serum albumin (BSA; PBS/BSA), and 100  $\mu\text{L}$  of 20 g/L BSA in PBS was added and left at room temperature for 1 h. The wells were then washed with PBS/BSA, and 100  $\mu\text{L}$  of specimen diluted in PBS/BSA was added to each well. The plates were incubated for an additional 2 h at 20 °C, after which they were washed again, and 100  $\mu\text{L}$  of peroxidase-conjugated polyclonal anti-apo B antibodies was added and incubated for 60 min at 20 °C. After another wash with PBS/BSA, 100  $\mu\text{L}$  of substrate mixture (0.1 g/L *o*-phenylenediamine in sodium citrate, pH 4.5, with 30 mg/L H<sub>2</sub>O<sub>2</sub>) was added and incubated for 30 min at 20 °C. The reaction was stopped with 20  $\mu\text{L}$  of 500 g/L sulfuric acid, and the absorbance was read on a microplate reader (Multiscan Bichromatic; Labsystems, Helsinki, Finland) at 492 nm.

**Apo B determination.** Apo B content in LDL and serum samples was determined by an ELISA according to Koren et al. (12). Control human serum preparation Precipath L (cat. no. 1 285 874; Boehringer Mannheim, Mannheim, Germany; assigned value for IFCC method

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<sup>2</sup> Nonstandard abbreviations: LDL, IDL, and VLDL, low-, intermediate-, and very-low-density lipoproteins; RCA, *Ricinus communis* agglutinin; LSA, lectin–sorbent assay; PBS, phosphate-buffered saline; BSA, bovine serum albumin; apo, apolipoprotein; and Lp(a), lipoprotein(a).

Received October 17, 1994; accepted April 20, 1995.

1.77 g/L) was used as calibrator. The intraassay (12 replicates) and interassay (6 assays in triplicate) CVs were 3.0% and 4.4%, respectively.

**Statistical analysis.** The four parameter logistic function analysis from the Sigmaplot 4.01 package was used for data calculation. Significance of the correlation coefficient was evaluated with the use of Fisher's Z-transformation (13).

## Results

**Optimization of lectin-sorbent assay conditions.** Fig. 1 shows data on optimization of RCA<sub>120</sub> concentration used for well coating. The amount of neuraminidase-treated sialic acid-poor LDL bound to RCA<sub>120</sub>-coated LSA wells remained constant at 30–50 mg/L RCA<sub>120</sub>. Therefore, the concentration of 30 mg/L was chosen as optimum for subsequent experiments.

Five dilutions were used to determine the optimal concentration of anti-apo B polyclonal antibody. As shown in Fig. 2, the amount of apo B detected remained unchanged at the antibody concentration range of 5–20 mg/L; thus, 10 mg/L was chosen as optimum.

Figure 3 shows a typical titration curve for neuraminidase-treated sialic acid-poor LDL obtained with the use of optimal concentrations of the lectin and anti-apo B antibody.

The calibration curves for neuraminidase-treated sialic acid-poor LDL and desialylated LDL isolated from patients' blood by lectin chromatography (Fig. 4) almost coincide. As shown, desialylated LDL can be measured at a concentration range of 20–800 µg/L; sialylated LDL isolated by lectin chromatography does not bind to RCA<sub>120</sub> (up to a concentration of 1 mg/L).

**Specificity of lectin-sorbent assay.** To study the specificity of LSA for desialylated LDL, we treated LDL samples with galactosidase to remove carbohydrates as described by Nagai et al. (14). This procedure has been shown to remove >95% of lipoprotein galactose. Galactosidase-treated desialylated LDL tested by LSA showed no detectable LDL compared with nontreated lipoprotein (data not shown).

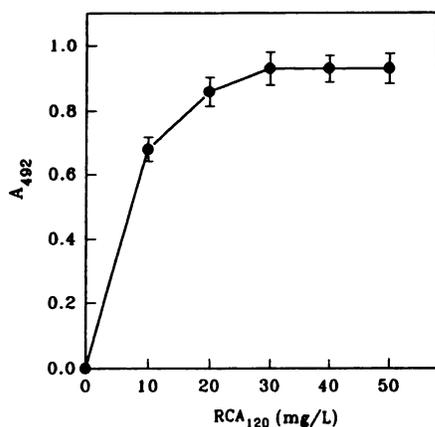


Fig. 1. Calibration curve for RCA<sub>120</sub> coating solution. Bars indicate SEM (n = 3).

**Comparison of desialylated LDL determination in lipoprotein preparations with that in human sera.** Fig. 5 demonstrates the titration curves for sera and LDL preparations isolated from human blood. The fact that these curves coincide indicates that serum components do not affect the determination of desialylated LDL in

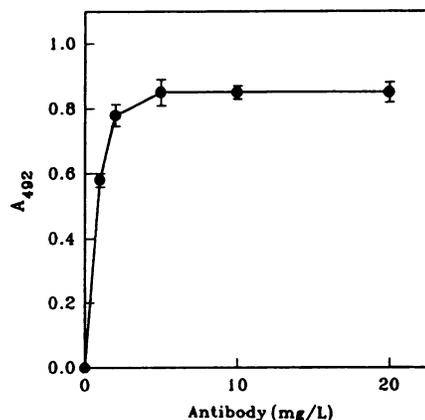


Fig. 2. Calibration curve for anti-apo B polyclonal antibody. Bars indicate SEM (n = 4).

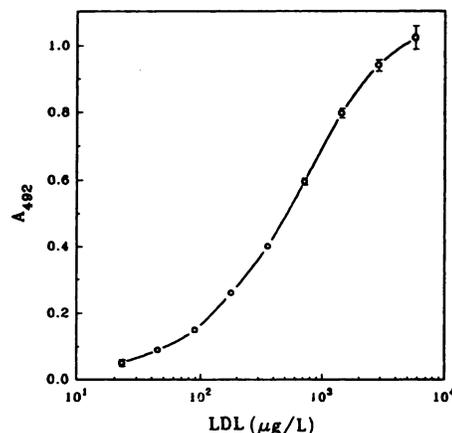


Fig. 3. Calibration curve of LSA for neuraminidase-treated LDL. Bars indicate SEM (n = 3).

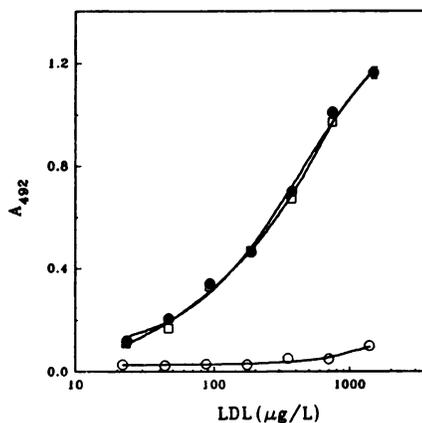


Fig. 4. Representative calibration curves for neuraminidase-treated (□), desialylated (●), and sialylated (○) LDL. Bars indicate SEM (n = 3).

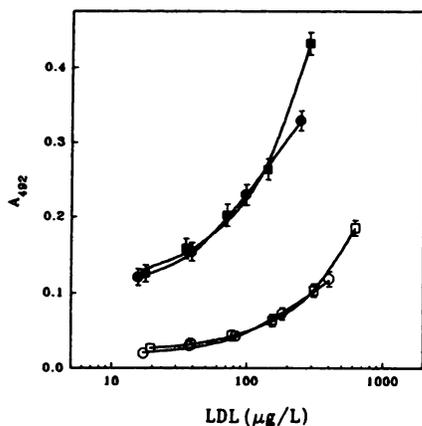


Fig. 5. LSA for healthy donor's (O, □) and patient's (●, ■) serum (O, ●) and LDL (□, ■).

Bars indicate SEM (n = 3).

the concentration range of 20–200  $\mu\text{g/L}$ . Fig. 6 shows the relation between the concentration of desialylated LDL in serum and in total LDL preparations isolated from the blood of the same donors. The correlation coefficient is 0.90 (n = 12,  $P < 0.001$ ).

**Comparison of determination of desialylated LDL by LSA and by lectin chromatography.** Fig. 7 shows the strong correlation between the content of desialylated LDL determined by LSA and apo B concentration in preparations of desialylated LDL isolated by affinity chromatography on  $\text{RCA}_{120}$  ( $r = 0.95$ , n = 53,  $P < 0.005$ ).

**Performance characteristics of LSA technique.** The precision of the LSA was estimated by measuring the same serum sample 12 times in duplicate in one run. The intraassay CV of desialylated LDL in four sera was 4.8%.

The desialylated LDL concentration in three serum samples (35.6, 87.8, 154.3 mg/L) was measured in triplicate in each LSA run, repeated on six different days. The interassay CVs were 13.3%, 11.7%, and 8.8%, respectively.

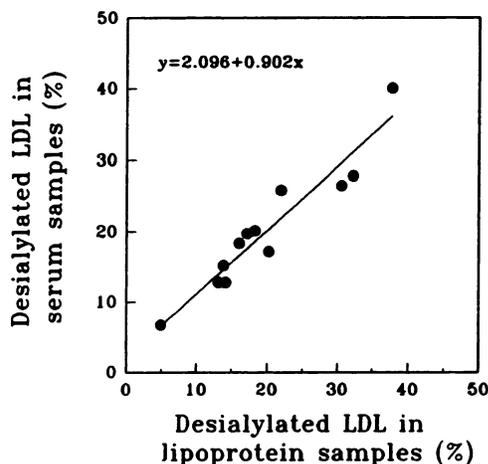


Fig. 6. Relation between desialylated LDL concentration determined by LSA in whole serum and lipoprotein samples.

Each point represents the mean of duplicate measurements.

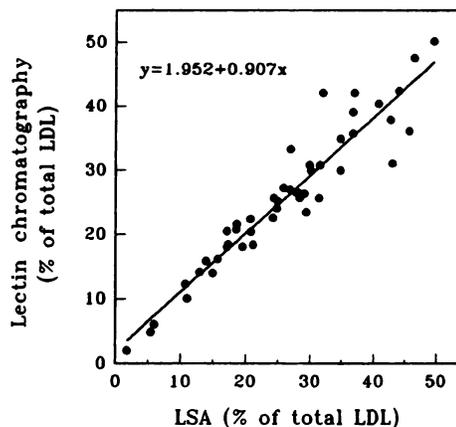


Fig. 7. Comparison of LSA and lectin chromatography in detecting desialylated LDL in human sera.

Each point represents the mean of duplicate measurements by each method.

The recovery, defined as the increase measured when 50 and 100 mg/L of desialylated LDL were added to two serum samples containing 28.8 and 60.7 mg/L desialylated LDL, respectively, ranged from 90.7% to 105.4% (mean  $94.5\% \pm 3.4\%$  for the first serum and  $98.9\% \pm 2.3\%$  for the second serum).

The detection limit of LSA was calculated as the concentration 2 SD from the mean zero standard result when 24 replicates were determined. The detection limit of desialylated LDL determination was typically  $< 5 \mu\text{g/L}$  (five sera).

## Discussion

The LSA procedure described displays high sensitivity and reproducibility. Comparison of the values obtained with LSA and chromatography on  $\text{RCA}_{120}$  columns showed good correlation. Thus, this method allows accurate and reliable determination of the concentration of desialylated LDL without isolation of the lipoprotein fraction.

In addition to blood serum LDL, very-low-density lipoprotein (VLDL) and intermediate-density lipoproteins (IDL) contain apo B. However, the apo B content of VLDL and IDL in normolipidemics is  $< 10\%$  of the LDL apo B content (15). Moreover, the amount of desialylated apo B in VLDL and IDL is usually  $\sim 1\%$  of plasma apo B (data not shown). Therefore, when apo B of desialylated LDL is measured in the serum of individuals with no appreciable changes in lipid metabolism, practically apo B of LDL is determined.

The plasma concentration of apo B-containing lipoprotein(a) [Lp(a)] is usually 300–500 mg/L, and apo B constitutes  $\sim 10\text{--}12\%$  of Lp(a) (by mass). Therefore, plasma concentration of apo B from Lp(a) accounts for 30–50 mg/L. The percentage of desialylated Lp(a) in a total Lp(a) preparation is usually lower than that of desialylated LDL in a total LDL preparation obtained from the same patient (data not shown). This means that the plasma concentration of desialylated apo B from Lp(a) cannot be  $> 20\text{--}30 \text{ mg/L}$ . Thus, we conclude that desialylated Lp(a) usually has minimal effect on desialylated LDL determination.

Accordingly, desialylated LDL content can be measured in sera of normolipidemics in the presence of other apo B-containing lipoproteins. Additional study is needed to evaluate the influence of apo B-containing particles on desialylated LDL determination in hyperlipidemic subjects. In case of any limitation, desialylated LDL content should be measured in isolated LDL preparations.

We have established a strong correlation between the degree of coronary atherosclerosis and atherogenicity of patients' sera in the presence of desialylated LDL and anti-LDL autoantibodies (1, 2); circulating immune complexes containing desialylated LDL are markers of coronary atherosclerosis (16, 17). The method for determining desialylated LDL described here allows assessment not only of the pathogenetic role of desialylated LDL in atherogenesis but also of the diagnostic and prognostic significance of this lipoprotein as a risk factor of atherosclerosis.

The research described was made possible in part by grant 94-04-12013 from the Russian Foundation for Basic Research.

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