Influence of Oleic Acid on Serum Lipoprotein-X in vitro

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Lipoprotein-X is no longer detectable in serum by either the agar gel-electrophoresis/polyanion precipitation technique or immunoelectrophoresis with specific antisera after in vitro addition of oleic acid. Evidence is presented which indicates that this ostensible loss is due not to its destruction, but rather to altered electrophoretic mobility. The findings suggest an explanation for the well known post-heparin “disappearance” of lipoprotein-X, and indicate that caution may be needed in the interpretation of such lipoprotein-X testing of sera from subjects with increased concentrations of lysolipids in their blood.

Additional Keyphrases: electrophoresis/polyanion precipitation technique • immunoelectrophoresis • effects of heparin administration • source of analytical error

Lipoprotein-X (LP-X) is an abnormal lipoprotein that is present in the blood of subjects with cholestasis (1–5) or lecithin:cholesterol acyltransferase (EC 2.3.1.43, LCAT) deficiency (6, 7). It is no longer demonstrable by the electrophoresis/polyanion precipitation technique (8, 9) shortly after intravenous administration of heparin, and post-heparin plasma will induce apparent clearing in vitro (10–15).

Attempts to elucidate the mechanism of this post-heparin disappearance of LP-X have led to consideration of several possibilities. A direct action of heparin itself has been excluded, since its addition to LP-X positive sera in vitro in concentrations of up to 1000 int. units per milliliter, vastly more than is needed in vivo, does not affect LP-X (14). The facts that (a) LP-X consists mostly of cholesterol and lecithin (16) and (b) the lecithin/lysolecithin ratio decreases after heparin administration suggested that heparin might have or induce an LCAT-like action (10). However, LCAT itself is rapidly inhibited in post-heparin blood (17) due to blocking by excess nonesterified fatty acids and lysolecithin (18) released by the post-heparin lipases (19); absence of any LCAT-like activity (14) but normal hepatic and peripheral lipoprotein lipase activity (20) have been demonstrated accompanying the post-heparin phenomenon in subjects with genetically determined LCAT deficiency; and, finally, it later was found that the clearance of LP-X after heparin does not correlate with LCAT activity (12). Degradation of LP-X by direct action of heparin-induced phospholipases (11, 14, 15) or lipoprotein lipases (10, 13) has also been proposed as a possible mechanism, and no evidence to controvert this has been yet published.

An alternative and simpler explanation is supported by the results of experiments described here, which were suggested by (a) previous observations regarding post-heparin electrophoretic mobility changes of normal lipoproteins (21) and (b) the fact that detection of LP-X by the electrophoresis/polyanion precipitation technique depends upon (besides other features) its cathodal electrophoretic mobility under the test conditions. The data obtained indicate that the ostensible disappearance of LP-X after heparin administration may be due simply to increased anodal electrophoretic mobility as a consequence of binding of nonesterified fatty acids or other negatively charged lysolipids, so that the abnormal lipoprotein no longer appears in the expected location.

Materials and Methods

We examined fasting sera from a panel of normal adults and from 11 patients with extrahepatic cholestasis for the presence of LP-X by (a) a modified (9) electrophoresis/polyanion precipitation technique (8), using 1% Difco Agar (Difco Laboratories, Detroit, Mich. 48232), and (b) microimmunoelectrophoresis (22), using 1% Ionagar No. 2 (Oxoid Ltd., London, U.K.) in aqueous barbital buffer of ionic strength 0.05 and pH 8.5 ± 0.1 containing, per litre, 0.5 mol of sodium diethyl barbiturate, 0.01 mole of barbituric acid, and 500 mg of sodium azide as preservative. Details of minor modifications of technique and staining have been published previously (23). We found it necessary to “age” the

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Difco agar slides overnight after pouring; freshly poured preparations gave inconsistent or even false-negative results. Aliquots of serum specimens were subjected to analysis both in the native state and after addition of oleic acid to final concentrations in serum of 0.5, 1.0, 2.0, 4.0, and 6.0 ml/litre. Some samples of LP-X protein sera containing 2 ml of oleic acid/litre were run for comparison beside aliquots to which had been subsequently added as well 400 ml of human albumin per litre (250 g/litre, “for injection”; Connaught Laboratories Ltd., Willowdale, Toronto, Canada). These additions were mixed by gentle inversion six times, with care to avoid shaking or frothing. Samples were subjected to analysis either immediately or after incubation in a water bath at 37 °C for 5, 10, 20, 30, 60, 90, or 120 min. Other aliquots were diluted with normal serum to final concentrations of 80, 60, 40, 20, 10, and 5 ml of LP-X-positive serum per decilitre before analysis. Immunoelectrophoretic preparations were developed with rabbit antisera to LP-X (a gift from Behringwerke, Marburg/Lahn, West Germany). All chemicals used were of analytical grade.

Results

All cholestatic sera examined by the electrophoresis/polyanion precipitation technique showed a typical (8, 9) positive result, which appeared as a narrow creamy-white arc situated a short distance cathodal to the origin and exhibiting marked anodal concavity. The appearances differed somewhat between sera, ranging from a delicate, faint arc to a very dense, thick arc connected from its concavity to the cathodal margin of its well of origin by a narrow, creamy-white trail. Even in the same electrophoretic run some specimens showed arcs located more cathodally than others, and the differences in this distance showed no evident correlation with the density of the arc. No specimen of normal serum showed a cathodal arc. All specimens of serum, whether normal or LP-X positive, showed a virtually identical creamy-white trace extending some distance anodal from the well in the form of a narrow “V” with its open end directed anodally.

Dilution of LP-X positive sera resulted in lightening of the cathodal arc and weakening of the cathodal trail, but did not alter the distance between the arc and the well (Figure 1). Addition of oleic acid to LP-X positive serum, by contrast, did not notably affect the arc density but resulted in decreased cathodal mobility. The magnitude of this mobility change varied directly with the concentration of oleic acid, so that at the highest concentrations mentioned (4 and 6 ml/litre) a cathodal arc was no longer visible. At these concentrations a new component was evident anodal to the origin, and the degree of its anodal mobility also varied directly with the oleic acid concentration. This new component was

Fig. 1. Electropherogram of LP-X-positive serum
Difco agar; anode left. Wells contain dilutions of LP-X-positive serum in normal serum to final concentrations (ml/dl) of LP-X serum as shown by the digits. Note no change in cathodal migration distance with dilution, but decrease in intensity of LP-X arc and trail

Fig. 2. Electropherogram of LP-X-positive serum with added oleic acid
Difco agar; anode left. All wells contain the same LP-X-positive serum, either native (top well) or with oleic acid at final concentrations (ml/litre of serum) as indicated by the digits. Note the progressively more anodal location of the LP-X arc with increasing concentration of oleic acid until disappearance and, thereafter, the appearance of a new anodal component showing increasing anodal mobility with increasing oleic acid concentration
no longer arc-shaped but in the form of a laterally slightly doubled spot (Figure 2).

The amount of oleic acid necessary to abolish cathodal mobility of the LP-X in the various sera varied in proportion to the apparent quantity of LP-X present, as judged by the arc and trail characteristics, so that those showing the weakest positive were converted to "negative" at a concentration of as low as 1.0 ml (approximately 4 µEq or µmol) of oleic acid per litre of serum. Addition of albumin to a concentration of 100 g/litre of LP-X-positive serum largely or completely abolished the oleic acid effect, at least at the 2 ml/litre level.

Characterization of the Behringwerke rabbit LP-X antiserum with normal serum and its components indicated immunoprecipitin activity only against α₂-lipoprotein, very similar to that of an antiserum specific for apolipoprotein-C (kindly provided by Prof. Dr. Gerhard Kostner, Graz, Austria), with no trace of reaction to albumin. Use of the LP-X antiserum in immunoelectrophoresis with sera that were LP-X-positive in the electrophoresis/precipitation technique showed, in addition to the α₂-lipoprotein immunoprecipitin arc as in normal serum, a faint and delicate immunoprecipitin arc cathodal to the origin in a position corresponding to (but at right angles to) the particular location of the electrophoresis-technique arc in each specimen. Dilution of the such LP-X-positive sera merely weakened the immunoprecipitin arc, but the presence of oleic acid resulted in more anodal location of the arc in parallel to the altered location of the electrophoresis arc as described above. Thus, with the greatest concentration of oleic acid, the only detectable change was accentuation of the anodal end of the α₂-lipoprotein arc, presumably representing the presence of the new electrophoretic component described above.

Incubation of the mixtures produced only minimal accentuation of the oleic acid effect, which therefore must have gone to completion within the 3 or 4 min at room temperature required to set up the gel plate in the electrophoresis apparatus.

Discussion

These findings indicate that addition of sufficient of the negatively charged nonesterified fatty acid, oleic acid, to cholestatic sera causes increased electrophoretic mobility of LP-X towards the anode, to a degree that readily yields a false negative result by both the electrophoretic and immunoelectrophoretic techniques. The concentration of oleic acid needed to "convert" the less strongly positive LP-X sera in vitro was well within the range reported for post-heparin nonesterified fatty acids in vivo (10, 12), but somewhat higher for the most strongly positive (up to about 15 mmol/litre). This apparent deficit may be explained by a potentiating effect of the also negatively charged product of heparin-induced phospholipase, lysolecithin (10, 12, 14, 15), not examined here.

The new anodal electrophoresis/polyanion precipitation component observed with the highest concentrations of oleic acid in LP-X-positive sera was not in the shape of an arc, but of a spot. It was never observed in LP-X-negative sera, or in LP-X-positive sera in the absence of enough oleic acid to abolish cathodal mobility of the abnormal lipoprotein. It therefore seems likely to represent LP-X that is sufficiently unchanged to retain its particular characteristics of polyanion-dependent (and maybe immunological) precipitability, but sufficiently altered with respect to net charge and, apparently, either molecular size or some other factor related to interaction with the supporting medium for this morphological variation to ensue.

The fact that the presence of charged lysolipid at concentrations that commonly obtain after administration of heparin is associated with an artefact of the electrophoretic and immunoelectrophoretic tests to this extent may explain the phenomenon of apparent dissipation of LP-X after heparin administration. None of the evidence presented here excludes the possibility of a direct action of post-heparin lipases or phospholipases in either destroying the LP-X or causing electrophoretic mobility change so as to render it undetectable by the techniques employed. However, the more simple if indirect influence via charged lysolipids released by such enzymes from other substrates would seem to be sufficient to explain the phenomenon, and the ready reversal of the effect by added extra albumin may be considered to support this view.

Our findings indicate a possible source of error in the examination of sera for the presence of LP-X, in that increased concentrations of negatively-charged lysolipids, from any cause, may provide falsely negative results of testing by both the electrophoresis/polyanion precipitation and immunoelectrophoretic techniques.

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References