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Diagnosis of Type III Hyperlipoproteinemia by Chromatography of Plasma Lipoproteins on Columns Containing Agarose

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Agarose column chromatography has been used to separate plasma lipoproteins into very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Applied to the diagnosis of primary type III hyperlipoproteinemia, the procedure is capable of demonstrating three characteristic and specific changes from normality in the elution pattern of lipoproteins from patients with this condition. In the type III profile there is (*a*) incomplete separation of VLDL from putative LDL material, (*b*) early elution of the type III LDL with respect to a normal LDL marker, and (*c*) relative deficiency of type III LDL with elution characteristics of normal LDL. We advocate the use of this method in the diagnosis of type III hyperlipoproteinemia.

Primary type III hyperlipoproteinemia (also called dysbetalipoproteinemia, floating beta disease, and xanthoma tuberosum) is an uncommon condition that nevertheless is of considerable clinical interest owing to its association with a high incidence of severe and premature atherosclerosis of the coronary or peripheral arteries, or both. It is characterized by accumulation, in the plasma, of heterogeneous lipoprotein particles with electrophoretic mobility and chemical composition intermediate between that of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) (*1, 2*). Present evidence suggests that these particles may arise either from overproduction of VLDL, causing saturation of the normal pathway of VLDL metabolism to LDL (*3*), or may accumulate because of a defect in the catabolism of VLDL (*4*). The condition can be diagnosed by a variety of electrophoretic (*5, 6*) and chemical analytical procedures (*7, 8*).

Difficulties involved in deriving and interpreting these data prompted us to search for a simpler test for the abnormality. We believed that the altered chemical composition of the intermediate particles would be likely to produce in them a change either in mass or conformation (or both) that would be detectable by gel-exclusion chromatography. To test this hypothesis, lipoproteins separated from the plasma of fasting type III hyperlipoproteinemic patients by ultracentrifugation were fractionated by chromatography on a column containing agarose. The elution profiles we obtained were characteristic of type III hyperlipoproteinemia and were distinctly different from the patterns of normal subjects or of patients suffering from hyperlipoproteinemia of types IIa, IIb, IV, or V.

We offer this method as a direct diagnostic index of type III hyperlipoproteinemia, and believe that it possesses definite advantages over other procedures.

Materials and Methods

Samples

Venous blood, collected into disodium ethylenediaminetetraacetic acid (840 g/liter) after a 12- to 14-h fast, was obtained from the following subjects:

(*a*) Eight healthy volunteers (four men, four women).

(*b*) Four patients known to have type III hyperlipoproteinemia. These patients met recently defined criteria for the condition (*8*). One of these patients was the subject of a previous report (*9*), and another was tested before and after treatment of the condition.

(*c*) Five type IIa hyperlipoproteinemics.

(*d*) Five type IIb hyperlipoproteinemics.

(*e*) Five type IV hyperlipoproteinemics.

(*f*) Two type V hyperlipoproteinemics.

All subjects ate a regular diet and, except for the type III patient who was examined while on therapy, none had been prescribed drug therapy known to affect plasma lipoprotein values during the previous two weeks.

Ultracentrifugation

The solvent density of 6.0 ml of fasting blood plasma from each patient was increased to 1.225 kg/liter by addition of 2.11 g of solid KBr. The samples were then placed in 10-ml Oak Ridge tubes (Measuring

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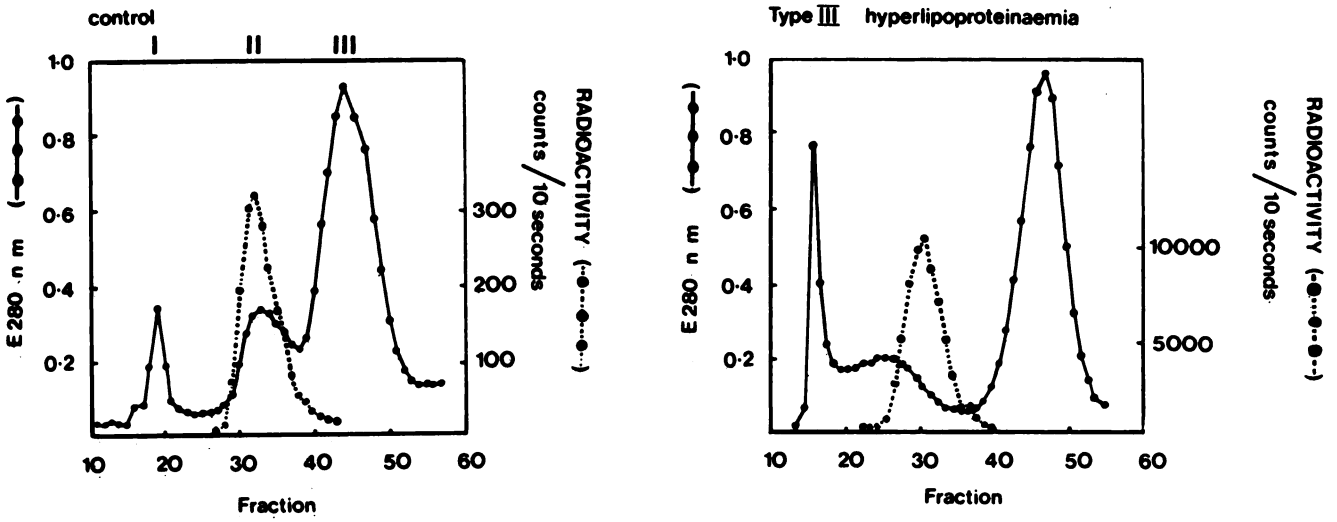

Fig. 1. Agarose column elution profile of lipoproteins from control and type III hyperlipoproteinemic plasma

Fasting plasma from patient A.M. and a control subject were separated according to the procedure described in *Methods*. The agarose column elution profile with superimposed LDL marker peak is shown. The plasma from the control subject (age 21 y) contained 2360 mg/liter cholesterol and 973 mg/liter triglyceride

and Scientific Equipment, Crawley, England) and overlayered with tris(hydroxymethyl)aminomethane buffer (0.1 mol/liter, pH 7.0) containing NaCl (150 mmol/liter), disodium ethylenediaminetetraacetate (100 mg/liter), and KBr to a density of 1.225 kg/liter. Ultracentrifugation for 24 h (307 000 × *g*, 15 °C) in the Ti 72 rotor of an MSE 75 superspeed centrifuge (Measuring and Scientific Equipment, Crawley, England) separated the bouyant lipoproteins—as an orange-colored surface layer—from the remainder of the plasma proteins, which sedimented to the bottom of the tube. The surface layer was removed carefully with a Pasteur pipette and reduced in volume to about 0.15 ml in a Minicon B15 concentrating cell (Amicon Corp., Lexington, Mass. 02173). Approximately 20 μg of marker ^{125}I-labeled LDL, purified from pooled normolipemic plasma by agarose column chromatography followed by ultracentrifugation between densities 1.030 and 1.050 kg/liter and labeled with ^{125}I by the McFarlane procedure (*10, 11*), was added to the isolated plasma lipoproteins before their separation on agarose columns. This marker material was chemically and immunologically indistinguishable from native LDL and its biological decay in rats was monoexponential, as measured by whole body counting (manuscript in press). The material therefore is a suitable marker for native LDL.

Agarose Column Chromatography

The chromatography procedure was adapted from the method described by Rudel et al. (*12*). The total lipoprotein fraction from each 6.0-ml sample of plasma was applied to a 0.9 × 50 cm column containing Bio-Gel A5m, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif. 94804) and separated by descending chromatography at room temperature with the eluent, tris(hydroxymethyl)aminomethane buffer (10 mmol/liter, pH 7.0) containing disodium ethylenediaminetetraacetate (100 mg/liter) and NaCl (150 mmol/liter). Fractions of 0.5 ml were collected at a rate of 15 fractions per hour. The radioactivity of each fraction was determined on a Wilj Gamma Counter (Wilj Electronics Ltd., Ashford, Kent, England) and its absorption at 280 nm was measured with a Model 240 spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio 44074).

Results

The selection of patients for this investigation of type III hyperlipoproteinemia was based on the identification of two diagnostic variables in the condition: the presence of floating beta lipoproteins (*1, 8, 13*) and chemical analysis of plasma lipoproteins of density less than 1.006 kg/liter (*7, 8, 13*). Table 1 shows that each patient had a diagnostic floating beta band on electrophoresis of his plasma supernatant fraction after preliminary ultracentrifugation at 1.006 kg/liter. Furthermore, the VLDL cholesterol/total triglyceride ratio was in three cases greater than 0.3, this value being reputedly diagnostic of type III hyperlipoproteinemia (*8*). The ratio, in regard to patient J.W., was 0.28, strongly suggestive of type III hyperlipoproteinemia (*8*). The other subjects of the investigation were classified as normal or suffering from dyslipoproteinemias other than type III according to the criteria established for the Lipid Research Clinics Program of the NIH, Bethesda, Md. (*13*). None of these subjects demonstrated either of the variables described above as diagnostic of the type III condition.

Figure 1 shows the agarose column elution profiles of plasma lipoproteins from a representative control and from a type III subject. Peaks I, II, and III from the normal profile correspond to VLDL, LDL, and HDL, respectively (*12*).

In the control subject, use of A5m Bio-Gel permitted complete separation of VLDL and LDL and provided a profile in which exogenously added ^{125}I-la-

Table 1. Chemical Analysis of Type III Hyperlipoproteinemic Plasma

Patient (sex)	Age y	Clin. stigmata of hyperlipoproteinemia	Total triglyceride	Total cholesterol	VLDL cholesterol	LDL cholesterol	HDL cholesterol	VLDL cholesterol/ total triglyceride	Agarose gel electrophoresis of $d < 1.006$ kg/liter fraction[a]
			mg/liter						
M.S. (F)	52	Peripheral vascular disease. Angina	2610	2710	930	1160	620	0.35	β-lipoproteins present
M.Y. (F)	48	Planar palmar xanthomata	5310	4260	2240	1160	850	0.42	β-lipoproteins present
A.M. (M)	42	Planar palmar xanthomata	3200[b]	3700	1550	1310	840	0.48	β-lipoproteins present
			1990[c]	2170	460	1080	620	0.23	
J.W. (M)	36	NIL	4780	2670	1360	710	390	0.28	β-lipoproteins present

[a] Lipoproteins were measured in plasma specimens from each (fasting) type III hyperlipoproteinemic patient as described in the Lipid Research Clinics Program "Manual of Laboratory Operations" (*13*). Commercially available pre-prepared agarose gels (Corning-EEL, Halstead, Essex, England) were used to obtain electrophoretograms of the VLDL fraction isolated by ultracentrifugation. The definition of "floating" beta lipoproteins given by Fredrickson et al. (*8*) was adopted in this study.

[b] Untreated.

[c] After four months of dietary (<300 mg cholesterol/day; restricted carbohydrate) and drug (clofibrate, 2 g/day) therapy.

beled LDL was coincident in elution with the endogenous LDL peak. This finding was not a feature of the type III profile, which consistently showed incomplete separation of VLDL from putative LDL material, early elution of this material with respect to the marker, and relative poverty of endogenous LDL at the elution volume of the marker. Repeat chromatographic analysis of lipoproteins from patient A.M. after four months of effective dietary and drug therapy (Table 1) demonstrated that the type III profile was qualitatively unaffected by this treatment. The identity of the LDL-like material in the plasma of type III patients was investigated as described in the legend of Figure 2. Alternate samples from the fractions derived from a Bio-Gel A5m column between the "VLDL" and "LDL" peaks were electrophoresed on pre-prepared agarose plates. The VLDL peak material (fraction No. 11) migrated with a mobility consistent with its identification as normal VLDL. The lipoproteins of the column fraction (No. 23) coincident with the LDL marker, however, moved ahead of an LDL standard separated contiguously on the agarose electrophoresis plate. Fraction 21, corresponding to the peak of the putative endogenous LDL, contained lipoproteins that electrophoresed with a mobility intermediate between VLDL and LDL and undoubtedly contributed to the broad beta band of type III hyperlipoproteinemia (*14*). The plasma lipids of patients J.W. and M.Y. were very labile, and on a few occasions the total plasma triglyceride values exceeded 10 g/liter. Under these circumstances, the grossly exaggerated VLDL peak on A5m gel chromatography overlapped the LDL-like material and obscured the diagnostic features described above. This problem was circumvented by determining the cholesterol content of the eluted fractions. The profile of that

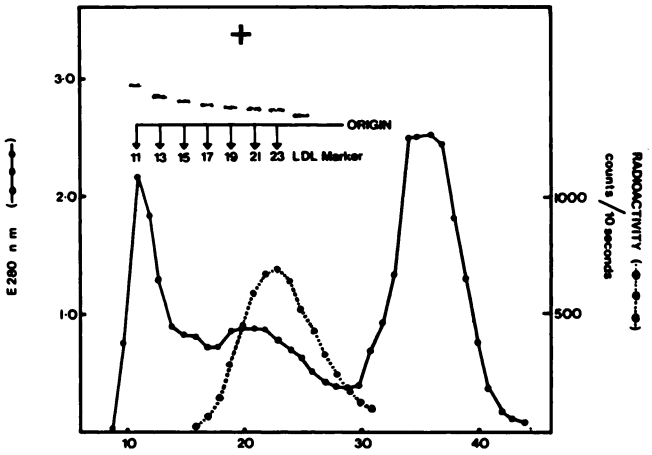

Fig. 2. Agarose column elution profile and gel-electrophoresis pattern of lipoproteins from type III hyperlipoproteinemic plasma

After separation of plasma lipoproteins from patient M.S. according to the procedure described in *Methods*, alternate fractions were subjected to agarose gel electrophoresis

lipid permitted the location of the putative LDL peak, unobscured by VLDL.

Column-chromatographic profiles from patients with hyperlipoproteinemia types IIa, IIb, IV, and V (Figure 3) are characteristic of these conditions and differ from the elution pattern of a normal subject.

Several points are noteworthy. First, the VLDL and LDL peaks from these dyslipoproteinemias are well resolved from each other on chromatography and do not show the typical features of the type III pattern (Figure 1).

Secondly, the LDL peak from type IIa, IIb, and IV plasma elutes coincidentally with the ^{125}I-labeled LDL marker. In the case of type V hyperlipoproteinemia the marker precedes the endogenous LDL peak

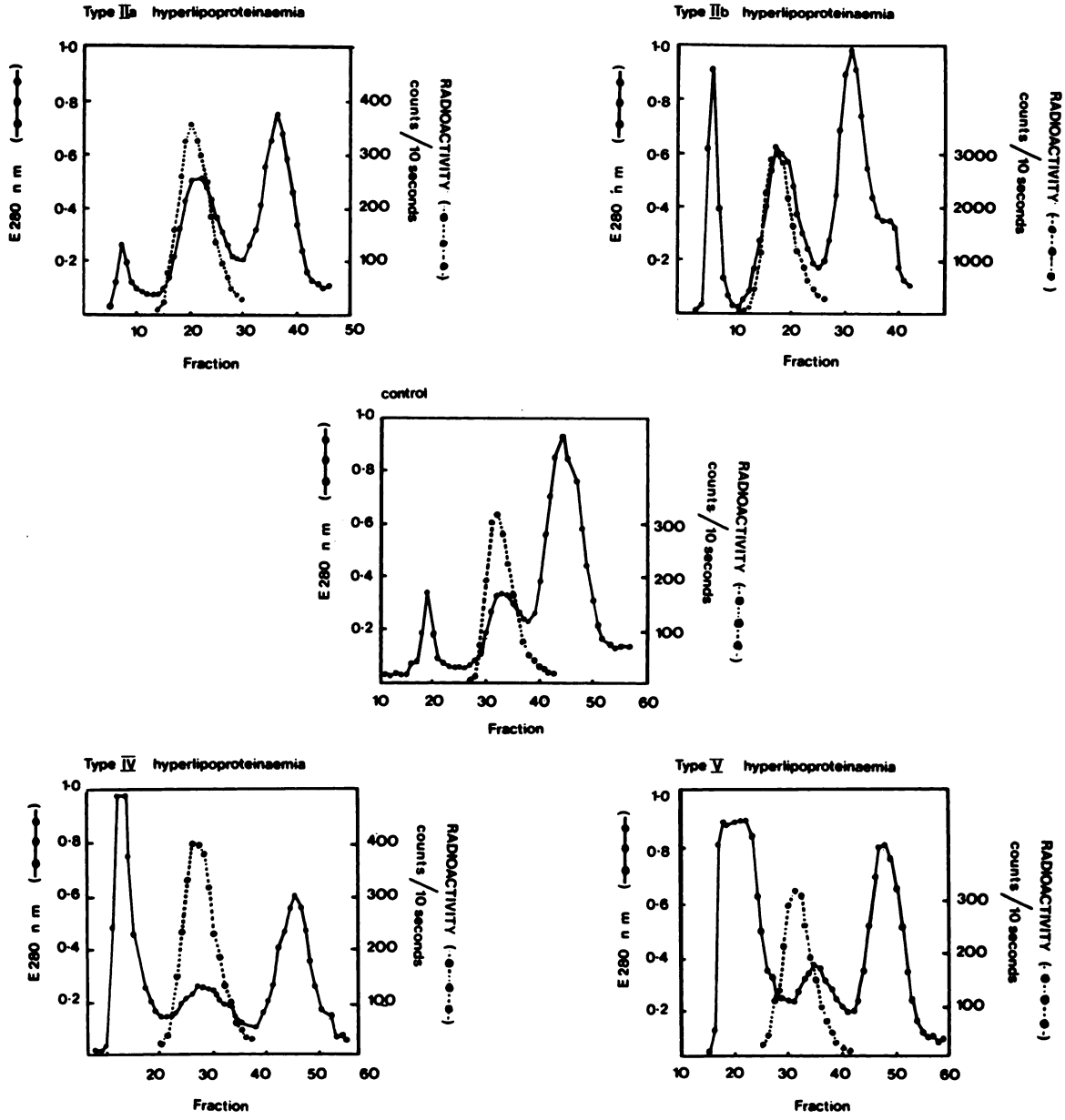

Fig. 3. Lipoprotein elution profiles from control and four types of hyperlipoproteinemic plasma

Fasting blood lipoproteins from a control subject and various dyslipoproteinemic patients were separated by ultracentrifugation/agarose column chromatography

The plasma lipid profiles for the subjects were

Subject type	Age (y)	Plasma cholesterol (mg/liter)	Plasma triglyceride (mg/liter)
Control	21	2360	973
IIa	40	3750	1500
IIb	32	3250	2430
IV	45	2590	3110
V	37	2630	11500

in elution from the column (*cf.*, type III pattern of Figure 1). This phenomenon will be discussed in detail in a subsequent publication.

Thirdly, the relative area subtended by each peak on the elution profile provides a useful diagnostic indication of the lipoprotein status of the plasma donor. Details of this chromatographic lipoprotein typing system will be presented elsewhere.

Discussion

The multiplicity of tests offered as diagnostic indices of type III hyperlipoproteinemia is a good indication of the difficulties involved in establishing this diagnosis. At present, the two most acceptable diagnostic criteria (*13*) of the condition are (*a*) the presence of lipoproteins with beta mobility on paper or agarose gel electrophoresis of that fraction of blood plasma of density less than 1.006 kg/liter, and (*b*) a gravimetric ratio of "VLDL cholesterol" to plasma triglycerides exceeding 0.3 when the plasma triglyceride concentration is in the range 1.5–10 g/liter.

Objections may be raised to the use of these criteria. Iammarino (*15*) has remarked on difficulties in

interpreting electrophoretograms from the plasma of type III patients, and does not consider that "we have seen the end of possible approaches to the laboratory diagnosis of type III hyperlipoproteinemia."

Furthermore, recent re-evaluation of 35 type III dyslipoproteinemics has shown that, as a diagnostic marker of the condition, the identification of a "floating beta band" is superceded in specificity by the chemical index of VLDL composition mentioned above (*8, 16*). Even this criterion, however, is unreliable when the plasma triglyceride is less than 1.5 or more than 10 g/liter; and some patients with normal VLDL cholesterol/plasma triglyceride ratios according to the criteria of Fredrickson et al. (*8*) have been shown by these authors to be classifiable as type III hyperlipoproteinemic on the basis of the presence of "floating beta" lipoprotein.

This study reports a new approach to the specific diagnosis of type III hyperlipoproteinemia based on the appearance in the plasma of a lipoprotein species of density and chemical composition intermediate between VLDL and LDL (*2*). This species, we have shown, produces a characteristic alteration in the A5m Bio-Gel exclusion chromatography profile of human lipoproteins. Its intermediate size causes it to appear on the gel profile midway between VLDL and LDL, and appears to prevent the normally complete separation of these two species.

Moreover, its preponderance over normal LDL (Figure 1), a feature of the type III lipoprotein distribution noted by others (*2*), causes the putative LDL peak to elute earlier from the column than does a marker of normal LDL. The elution pattern was qualitatively unaltered by dietary and drug therapy (clofibrate) that was effective in restoring the lipid profile to normal.

We have found that, in this small survey of dyslipoproteinemias, the chromatographic profile described above and seen in Figure 1 is peculiar to type III hyperlipoproteinemia. We offer the test as a specific index of the condition and predict that it may have application in the hitherto impossible detection of the occult disease.

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