Apolipoprotein B detected in the plasma of a patient with homozygous hypobetalipoproteinaemia: implications for aetiology

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SUMMARY A hypobetalipoproteinaemic kindred is described in which the proband manifested the clinical and biochemical features of the homozygous state. Unlike the apparent complete absence of apolipoprotein B in the plasma of the five cases of homozygous hypobetalipoproteinaemia reported so far, we were able to demonstrate minute quantities of this protein (approximately 0.025% of normal) in the plasma of the proband. This finding suggests that the disorder may not result from a structural gene defect but may rather reflect a failure of secretion.

The homozygous state of the autosomal recessive condition abetalipoproteinaemia (ABLP_R), and the autosomal dominant disorder hypobetalipoproteinaemia (HBLP), are both characterised by the apparent complete absence of apolipoprotein B (apoB) from the plasma.^{1,2} Homozygous HBLP is an extremely rare condition having been described in only five patients from four separate kindreds.³⁻⁶ Both ABLP_R and homozygous HBLP manifest with similar plasma lipoprotein and lipid phenotypes and present with steatorrhoea, growth retardation, reduced plasma levels of the fat soluble vitamins, and anaemia characterised by abnormal red blood cell morphology, notably acanthocytosis.^{1,2,7} The two disorders are principally distinguished by the presence of normal levels of apoB and the apoB containing lipoproteins in the plasma of obligate heterozygotes for ABLP_R, in contradistinction to the reduced levels present in heterozygotes for HBLP, and by the relative absence of severe ocular and neuromuscular pathology in homozygous HBLP.

The distinctive modes of inheritance and the differing clinical presentation of the two syndromes imply that a different molecular defect underlies each disorder. In a recent report on a normotriglyceridaemic variant of ABLP_R, in which the liver form of apoB (apoB-100) was absent, Malloy *et al*⁸ suggested that HBLP is probably the result of a structural gene defect. In this paper we report a fifth hypobetalipoproteinaemic kindred in which the proband presented with the clinical and biochemical

features of homozygous HBLP. The presence of minute amounts of immunoreactive and electrophoretically normal apoB detected in the plasma of this patient suggests that hypobetalipoproteinaemia may be the result of a regulatory or secretory abnormality rather than a structural gene defect.

Case report

The proband, a female weighing 3 kg at birth, was of mixed Malay-Coloured parentage. Pregnancy and labour were uneventful and she was breast fed initially. Symptoms of recurrent vomiting, diarrhoea, and failure to thrive were present within the first few months and frequent episodes of urticaria, bronchospasm, and respiratory infections also occurred. Despite dietary advice and supplementation, the above features persisted and were associated with intermittent abdominal distension. The patient's haemoglobin level varied from 8.6 to 13.1 g/dl. Reticulocyte counts were generally raised (2 to 10%), and anisocytotic, poikilocytotic, hypochromic, and crenated red blood cells were reported. Leucocytosis and eosinophilia were also frequently present but repeated investigations for intestinal parasites were negative, except for the finding of *Giardia* ova in the stool on one occasion. At the age of 1.4 years general osteopenia, distension of the bowel, and thickening of the bowel wall were noted on radiographs. Despite continued ill health and failure to thrive, with height and weight well below the 5th centile, the patient's albumin and immunoglobulin levels were essentially normal throughout. At the age of 3.5 years a routine

blood chemistry yielded a cholesterol value of 0.57 mmol/l (22 mg/dl). Further investigations were instituted to establish the precise diagnosis in the proband and to define the pattern of inheritance in the kindred.

Materials and methods

Lipid and lipoprotein studies were carried out on venous blood collected, after an overnight fast, into tubes containing trisodium-EDTA (1 mg/ml). Plasma cholesterol was measured by a single step enzymatic method (Boehringer Mannheim GmbH, catalogue number 1483 93) and plasma triglyceride was also determined using a commercial kit (Boehringer Mannheim GmbH, catalogue number 124032). High density lipoprotein-cholesterol (HDL-C) was determined after heparin-manganese precipitation of the apoB containing lipoproteins,^{9 10} and low density lipoprotein-cholesterol (LDL-C) was calculated according to the formula proposed by Friedewald *et al.*¹¹ Plasma phospholipids were assayed according to Kates.¹² Lipoproteins were subjected to electrophoresis on cellulose acetate membranes and stained with Oil Red 7B.

ApoB and apoA in plasma from the proband and her mother were sought using standard methods of immunoelectrophoresis and double immunodiffusion. Antibodies were obtained from Behringwerke AG Marburg and plasma or lipoprotein fractions were concentrated four- to five-fold to improve detection. These experiments failed to reveal the presence of apoB in the proband and efforts were made to concentrate the $d < 1.063$ g/ml lipoprotein fraction further before polyacrylamide gel electrophoresis¹³ or electroimmunophoresis using the Laurell technique.¹⁴ Specifically, 50 ml of blood was collected into tubes containing EDTA (1 mg/ml blood) and centrifuged immediately to obtain the plasma to which the following preservatives were then added: penicillin 30 mg/100 ml, streptomycin 5 mg/100 ml, dithiothreitol 50 mg/100 ml, sodium azide 20 mg/100 ml, phenylmethylsulfonyl fluoride 0.6 mmol/l, and ϵ -aminocaproic acid 130 mg/100 ml. A total of 25 ml of plasma was over-layered with KBr ($d = 1.063$ g/ml) containing the above preservatives and 15 mg/100 ml EDTA and centrifuged at 27 000 rpm for 26 hours in a Beckman L5-65 ultracentrifuge using a SW27 rotor. The supernatant $d < 1.063$ g/ml lipoproteins were recovered by tube slicing, then resuspended in KBr, and washed through the same solution at 42 000 rpm for 16 hours in an SW50.1 rotor. A final volume of 0.7 ml was collected by tube slicing and dialysed against 0.019 mol/l sodium chloride containing a diluted mixture of the above preservatives.

Before polyacrylamide electrophoresis, delipidation was carried out by shaking 0.5 ml of the dialysed lipoprotein fraction with 5 mg sodium dodecyl sulfate and 1 ml of a 40 : 60 butanol/diisopropyl ether (DIPE) mixture. After centrifugation the aqueous layer was washed with 1 ml DIPE to remove the butanol and 0.3 ml of the mixture was further concentrated to 60 μ l under a gentle stream of dry nitrogen gas. To the 60 μ l of sample was added 50 μ l of buffer (50 mmol/l Tris HCl, pH 6.8, containing 20 g/100 ml glycerol and 5 g/100 ml B-mercaptoethanol); 80 μ l of the mixture was applied to a polyacrylamide gradient gel and electrophoresed at 70 V for 16 hours. The above procedure was performed on two separate samples of plasma with an intervening interval of 6 months. Great care was taken to ensure that no extraneous apoB contamination occurred, especially on the second occasion. The final aliquot applied thus represented the total apolipoprotein content of the $d < 1.063$ g/ml LP fraction from 11 ml plasma, ignoring non-specific losses incurred during the procedure. The same lipoprotein fraction obtained, after previous removal of chylomicrons, from 4 ml plasma from non-fasting, normolipemic control subjects was isolated using essentially similar procedures. The amount of apolipoprotein, however, finally applied to the gel represented that derived from only 5.6 μ l of the original plasma. For purposes of immunochemical detection the $d < 1.063$ fraction was isolated by ultracentrifugation as above, but without washing. The six times concentrated ultracentrifugation fraction was dialysed overnight against Tris-HCl, pH 7.4, and an aliquot was concentrated a further 34-fold by means of ultrafiltration and dialysis (MicroProDiCon, Bio-Molecular Dynamics, Oregon). Of this, 3 μ l were applied directly to the well of an agarose gel plate (108 μ l of apoB antiserum in 15 ml of 0.75% agarose gel). Since we usually dilute whole plasma 15-fold for apoB assay by electroimmunophoresis, the $d < 1.063$ fraction from the proband was therefore concentrated 3060 times ($6 \times 34 \times 15$) relative to normal plasma. To check specificity $1.080 < d < 1.25$ lipoprotein fractions from the proband and from a normal control were also run. The sensitivity was ascertained by diluting a $d < 1.063$ lipoprotein fraction of normal plasma 1200 times and 2400 times before being processed in the same way as plasma from the proband. Small 'rockets' were seen with both these dilutions whereas the $1.080 < d < 1.25$ fraction from a normal subject failed to react with antiserum.

The viscosity of whole blood and plasma was determined by means of a core/plate viscometer (Wells—Brookfield Microviscometer, Stoughton, Mass) according to the manufacturer's instructions.

FIG 1 Pedigree of kindred.

Values on whole blood were corrected for haematocrit and were expressed in centipoise (cps). Other laboratory investigations were carried out using standard procedures.

Results

FAMILY STUDIES

Plasma lipid and lipoprotein concentrations were measured in most of the available family members (table, fig 1). The mother of the proband was not married to the alleged biological father who denied parenthood and refused investigation. There was no history of consanguinity. The mother, maternal grandmother, a maternal half-aunt, and maternal half-uncle showed reduced LDL-C and low plasma cholesterol levels. Phospholipid levels were low in the mother and maternal grandmother. Plasma triglyceride values were occasionally low-normal in affected family members but could not be obviously distinguished from the unaffected relatives. Plasma HDL-C concentrations were unremarkable.

LIPID AND LIPOPROTEIN STUDIES ON THE PROBAND

The proband manifested total absence of LDL-C

and markedly diminished plasma cholesterol, triglyceride, and phospholipid concentrations which did not overlap with those of her affected relatives (table 1). Her plasma HDL-C levels were low to low-normal. A breakfast loaded with liberal quantities of cream failed to elicit any significant increase in her post-prandial lipid values: basal levels, 0.85 mmol/l (33 mg/100 ml) cholesterol and 0.27 mmol/l (24 mg/100 ml) triglyceride; 2 hour levels, 0.72 mmol/l (28 mg/100 ml) cholesterol and 0.31 mmol/l (27 mg/100 ml) triglyceride; 4 hour levels, 0.72 mmol/l (28 mg/100 ml) cholesterol and 0.19 mmol/l (17 mg/100 ml) triglyceride.

Lipoprotein electrophoresis performed at the age of 4 years showed an alpha migrating lipid fraction only. On centrifuging the patient's fasting plasma at 19 000 rpm for 2.5 hours in the 40.3 rotor of a Beckman model L5-65 ultracentrifuge, traces of a creamy substance were obtained in the supernatant. ApoB was undetectable in a plasma sample from the proband concentrated five-fold by ultrafiltration (fig 2a), but was clearly present in her mother (fig 2b). On cellulose acetate electrophoresis of plasma obtained from the proband at the age of 8 years a small amount of lipid staining material was observed migrating in the prebeta region (fig 3), and, on another occasion, a distinct band was present at the origin. Once again immunoelectrophoresis and double immunodiffusion failed to reveal the presence of apoB in the plasma, even when concentrated approximately four-fold by ultracentrifugation. ApoA was immunochemically detectable in approximately normal quantities in the plasma of the proband and her mother.

On two separate occasions, at the age of 8 and 9 years, polyacrylamide gel electrophoresis of the $d < 1.063$ lipoprotein fraction of the proband, carried out as described under Methods, revealed the presence of a protein fraction co-migrating with

TABLE Lipid and lipoprotein levels in the kindred.

Case	Age (yr)	Total plasma cholesterol (mmol/l)	Plasma triglyceride (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	Plasma phospholipid (mmol/l)
Proband	3.5-8.0	0.83* (0.62-0.99)	0.27* (0.23-0.32)	0.82 (0.72-0.91)	0* (0-0.02)	0.62* (0.61-0.63)
Half-brother	3	3.48	1.03	0.91	2.10	2.74
Half-sister	5	3.52	0.40	1.35	1.99	2.26
Mother	32-35	2.29* (2.20-2.40)	0.53 (0.29-0.76)	1.27 (0.93-1.22)	0.77* (0.43-1.14)	1.56* (1.52;1.61)
Maternal grandmother	48	3.18* (3.08;3.28)	1.58 (1.55;1.62)	1.14 (1.05;1.23)	1.33* (1.29;1.37)	1.18*
half-aunt	7	4.83	1.00	1.30	3.07	—
half-aunt	12	2.51*	0.46	1.55	0.75*	—
half-aunt	13	3.24	0.45	1.29	1.74	—
half-aunt	15	3.78	0.71	1.33	2.12	—
half-uncle	20	2.52*	0.86	1.16	0.97*	—

*Values below 5th centile of age adjusted normal range. SI conversion factors from mmol/l to mg/dl: cholesterol $\times 38.7$; triglyceride $\times 88.5$; phospholipid $\times 77.5$.

FIG 2 (a) Immunoelectrophoresis of plasma from proband and healthy subject (cont) against anti-apoB antibody. Plasma from proband was concentrated 5-fold by ultrafiltration before application. (b) Immunoelectrophoresis of plasma from mother of proband and healthy subject (cont) against anti-apoB antibody. Plasma from mother was concentrated 5-fold by ultrafiltration.

normal apoB (fig 4). A number of other bands were also apparent on the strip. One heavy band was in the same position as apoE while two further bands were roughly co-migratory with the apoC peptides. Resolution was poor in that region of the gel and no attempt was made to identify the C peptides further. The presence of immunoreactive apoB in plasma from the proband was confirmed by means

FIG 3 Densitometric tracing of lipoproteins from the proband and normal control separated by electrophoresis on cellulose acetate.

FIG 4 Polyacrylamide gel electrophoresis of plasma lipoprotein fraction from proband and control subject. Lanes A, B, and D: molecular weight standards, Ferritin subunit, M_r 220 000; phosphorylase b, M_r 94 000; albumin, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000 (Pharmacia Fine Chemicals AB, Uppsala, Sweden). Lane C: apolipoproteins of plasma fractions $d < 1.063$ from proband prepared as given under Methods. Lanes E and F: apolipoproteins of plasma fraction $d = 1.006-1.063$ g/ml from control subject. Lane G: apolipoproteins of chylomicron fraction (plasma centrifuged for 30 min at 14 500 rpm in SW 27 rotor) from a control subject.

B-100, apoB $M_r = 549$ 000; B-48, apoB $M_r = 264$ 000. Terminology according to Kane *et al.*²⁰

FIG 5 Electroimmunophoresis of lipoprotein fractions from proband and control subjects. See Methods for technical details.

No 8, 9, 10: frozen low density lipoprotein pool appropriately diluted. No 11, 12, 15, 16: $d < 1.063$ g/ml lipoprotein fraction from proband. No 13, 14: diluted (1200x and 2400x) $d < 1.063$ g/ml lipoprotein fraction from control subject. No 17: normal whole plasma diluted 1/15.

of electroimmunophoresis (fig 5) of the $d < 1.063$ g/ml fraction concentrated 3060-fold. Interestingly, a small amount of apoB immunoreactive material was also found in the concentrated $1.080 < d < 1.25$ fraction from the proband but was absent, as expected, from the same fraction obtained from normal plasma.

RESPONSE TO TREATMENT

The proband was placed on a low fat intake supplemented with medium chain triglyceride, vitamin A (5000 mg/day), vitamin K (10 mg/day), and vitamin E (400 to 1200 mg/day). Compliance has been variable but the vomiting and diarrhoea ceased. Faecal fat excretion, which was high when measured initially (35 mmol/day, normal ≤ 19 mmol/day), was normal when measured on three subsequent occasions. Plasma vitamin E concentration measured during a period of non-compliance was $2.8 \mu\text{g/ml}$. After supplementation for 1 week the plasma level rose to $6.0 \mu\text{g/ml}$ (normal range 8.3 to $15.4 \mu\text{g/ml}$). Plasma alkaline phosphatase, which had been low initially (44 U/l, normal range 70 to 150 U/l), also increased to 129 U/l by the age of 6.5 years. The plasma carotene level was low (33% of normal mean) shortly after diagnosis and has not been reassayed. Other routine biochemical investigations were normal except for a slightly reduced plasma magnesium on one occasion (0.64 mmol/l , normal range 0.70 to 0.95 mmol/l).

The patient's haemoglobin levels have varied between 8.2 and 10.8 g/dl . Acanthocytosis has been repeatedly demonstrable on both wet and dry preparations. Reticulocytosis also persisted but was slightly diminished relative to values before the institution of treatment. Osmotic fragility and autohaemolysis, carried out in saline with and without added glucose and ATP, were normal. The viscosity

of whole blood, corrected for haematocrit, was 7.35 cps at the age of 8 years. A reference range derived from 15 healthy adult subjects was 4.25 to 6.75 cps . Increased microviscosity of red cell membranes in abetalipoproteinaemic subjects has previously been reported.¹⁵ Whole blood viscosity in the mother of the proband was normal (5.66 cps).

The patient's neurological and ophthalmological status was completely normal on careful clinical examination at the time of diagnosis and when last determined at the age of 9 years. Her peroneal nerve conduction rate (53 m/s) and terminal latency period (2.05 m/s) were also within normal levels at the age of 5 years, and at the age of 6.3 years her electroretinogram, visual evoked responses, and visual acuity were completely normal. The recurrent respiratory infections have ceased but growth has remained severely retarded ($< 3\text{rd}$ centile).

Discussion

The trait for hypobetalipoproteinaemia in this kindred is vertically transmitted through three maternal generations (fig 1), culminating in apparent abetalipoproteinaemia in the proband. All family members examined, apart from the proband, were in good health and acquired causes of hypobetalipoproteinaemia can be excluded. The frequency of familial hypobetalipoproteinaemia has been variously put at between 1 per 1000¹⁶ and 1 per 3000¹⁷ in unselected populations. These figures imply a general population incidence of one case per 4 to 36×10^6 for homozygous HBLP. Even in the absence of biochemical information on her biological father, it is reasonable to conclude that the proband represents the homozygous state in view of the repeated absence of apoB from her plasma by the usual methods, the markedly reduced total plasma cholesterol and phospholipid levels compared with her hypobetalipoproteinaemic maternal relatives, and the characteristic clinical presentation. It is not possible, however, to exclude the more remote chance of genetic heterogeneity, including double heterozygosity for ABLP_R and HBLP.

The biochemical phenotype in homozygous HBLP is virtually indistinguishable from ABLP_R. Both conditions are characterised by an apparent complete absence of apoB and the apoB containing lipoprotein fractions of $d < 1.063 \text{ g/ml}$. In the patients with homozygous HBLP reported so far, apoB has been undetectable by means of immunodiffusion and immunoelectrophoresis against both laboratory prepared^{3 4 6} and commercial⁵ antibodies and by polyacrylamide gel electrophoresis. Cottrill *et al*⁴ used four- to six-fold concentrated plasma fractions to enhance the sensitivity of their procedures and Salt *et al*³ claimed that the (unspecified) immuno-

chemical technique employed by them could detect apoB at 1/1000 of its normal plasma concentration. We failed to find apoB immunoreactivity in the proband's plasma on immunoelectrophoresis or double immunodiffusion capable of detecting apoB at a concentration of about 2% of normal.

Despite our negative findings, using relatively insensitive methods, and the failure of previous workers to detect apoB using comparable or more sensitive techniques, on polyacrylamide electrophoresis of a highly concentrated plasma fraction from the proband, we were able to visualise clearly a protein band co-migrating with normal apoB. In the procedure we finally adopted, the apolipoproteins derived from an approximately 2000-fold greater volume of plasma from the proband than from the control subject were applied to the gel (see Methods). Despite this discrepancy, visualisation of the stained bands (fig 4) revealed a two-fold greater intensity of the apoB band in the control strips. These observations imply that the concentration of apoB in the proband's plasma was about 0.025% of normal, or 0.02 mg/100 ml assuming a normal plasma apoB concentration of 80 mg/100 ml.¹⁸ The electrophoretic results were corroborated by detecting small, but definite, amounts of apoB immunoreactivity in a highly concentrated $d < 1.063$ lipoprotein fraction from the proband (fig 5). The presence of some apoB in the HDL ($1.080 < d < 1.25$) fraction obtained from the proband was surprising, but could not be attributed to non-specificity since no reaction was seen using concentrated HDL from a healthy control. Since, by the time this analysis was performed, the $d > 1.063$ g/ml fraction from the proband had stood at $+4^\circ$ for more than 4 weeks, the possibility of contamination cannot be ruled out.

Recently a number of distinct apoB isoproteins have been found in plasma from rats¹⁹ and humans.²⁰ Kane *et al*²⁰ have demonstrated the presence in human plasma of liver derived apoB, with an apparent molecular weight of 549 000 (apoB-100), and a gut derived species with a molecular weight of 264 000 (apoB-48) secreted in association with chylomicrons. In addition, the cleavage products of apoB-100, apoB-74, and apoB-26 may often be detected in plasma from normal subjects. Polyacrylamide electrophoresis of plasma from the proband failed to reveal any apoB species other than apoB-100. Considering the minute amounts of apoB present, however, it is not possible to draw firm conclusions concerning the secretion of apoB-48. Evidence that apoB-48 is more rapidly cleared from the plasma than is apoB-100,²¹ and the temporal association of apoB-48 with food intake, makes its detection inherently less likely.

Our data suggest that the patients with homozygous HBLP are capable of synthesising normal apoB. While firm conclusions will ultimately depend upon more detailed structural studies, it is attractive to regard HBLP as being the result of failure of secretion or of a regulatory abnormality. This proposal is in accord with the report of Sigurdsson *et al*²² that the apparent apoB synthetic rate is markedly reduced in hypobetalipoproteinemic subjects, which they attributed to impaired secretion of very low density lipoprotein. A failure of secretion could, theoretically, be confirmed by demonstrating the presence of apoB specific immunofluorescence in the intestinal mucosal cells or hepatocytes of the proband; the absence of immunoreactive apoB in intestinal biopsy material has been described in patients with ABLP_R.²³ Unfortunately permission to biopsy the proband was refused and thus the possibility of a failure of synthesis could not be excluded.

It is appropriate to speculate whether the presence of minute quantities of apoB in the plasma of patients with homozygous HBLP can account for the differences in clinical presentation when compared with ABLP_R. It is possible that the actual rate of apoB secretion or fat absorption in the former condition is greater than suggested by the plasma levels, owing to rapid removal by the presumably high receptor content of cells in contact with apoB depleted plasma. This possibility may account in part for the discrepancy, noted by Illingworth *et al*⁶ in a 16-year-old girl with homozygous HBLP, between the negligible (0.5%) absorption of [³H]-cholesterol determined by blood radioactivity levels and the 24 to 30% difference between ingested and faecal [³H]-cholesterol. Even reduced secretion coupled with rapid turnover could allow for the greater transport of putatively protective factors, such as vitamin E, in amounts sufficient to retard the development of the characteristic ocular and neurological pathology associated with ABLP_R.

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