Description of two different patients with abetalipoproteinemia: synthesis of a normal-sized apolipoprotein B-48 in intestinal organ culture

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Abstract We describe here two patients, M. P. and S. L., with recessive abetalipoproteinemia. Analysis of restriction fragments of DNA from both patients using cDNA probes spanning the entire apolipoprotein B gene revealed no major insertions or deletions. Further, as defined by restriction fragment length polymorphism, abetalipoproteinemia, in these patients, did not appear associated with particular alleles of apolipoprotein B. Northern and dot blot analysis of intestinal mRNA of one patient (M. P.) revealed a normal-sized apolipoprotein B mRNA which was present in slightly reduced amounts. At the cellular level, apolipoprotein B was detected in both intestinal and hepatic biopsies, of one patient (S. L.), by immunoenzymatic techniques using polyclonal and monoclonal antibodies to apolipoprotein B-48 and/or B-100. The level of apolipoprotein B-48 appeared to increase in the intestine after a fatty meal. In the other patient (M. P.), although no apolipoprotein B was detected in the enterocytes using similar immunoenzymatic techniques, organ culture experiments using [^{35}S]methionine demonstrated the synthesis of a normal-sized apolipoprotein B-48 which appeared to be normally glycosylated. The glycosylation and processing of two intestinal membrane enzymes, sucrase-isomaltase and aminopeptidase N, were also normal. Although lipids and apolipoprotein B-48 were present intracellularly, no lipoprotein-like particles were observed by electron microscopy in the endoplasmic reticulum, the Golgi apparatus, or in the intercellular spaces of intestinal biopsies obtained in the fasted (M. P. and S. L.) or fed state (S. L.). The defect in theses cases of abetalipoproteinemia, therefore, does not appear to involve the apolipoprotein B gene nor the synthesis or the glycosylation of the apolipoprotein but instead appears to involve some aspect of lipoprotein assembly or secretion. —**Bouma, M. E., I. Beucler, M. Pessah, C. Heinzmann, A. J. Lusis, H. Y. Naim, T. Ducastelle, B. Leluyer, J. Schmitz, R. Infante, and L. P. Aggerbeck.** Description of two different patients with abetalipoproteinemia: synthesis of a normal-sized apolipoprotein B-48 in intestinal organ culture. *J. Lipid Res.* 1990. **31:** 1–15.

Supplementary key words hypobetalipoproteinemia • intestinal apoB mRNA • restriction fragment length polymorphisms • glycosylation • apoB gene • intestinal ultrastructure • lipoprotein assembly

A pivotal role for apolipoprotein (apo)B in the formation and secretion of triglyceride-rich lipoproteins has been deduced from two inherited disorders, abetalipoproteinemia and homozygous hypobetalipoproteinemia, in which apoB is absent from the plasma. Both abetalipoproteinemia and homozygous hypobetalipoproteinemia have virtually identical manifestations and are associated with acanthocytosis, retinitis pigmentosa, and a progressive neurological syndrome consisting of areflexia, ataxia, and the loss of proprioception. Although fatty acid absorption and esterification seem to be partially preserved, fat malabsorption results from the inability of the intestinal cells to export lipids as chylomicrons into the lymphatics. This leads to intracellular fat accumulation and to deficiencies of lipid-soluble vitamins, mainly vitamin E. The distinction between the disorders is made on the basis of the plasma lipid levels of the parents (1–5). Abetalipoproteinemia is an autosomal recessive disorder in which obligate heterozygotes are phenotypically normal. Familial hypobetalipoproteinemia is an autosomal dominant disorder in which heterozygotes have beta-lipoprotein levels of about 50% of normal. Recent studies have indicated that familial hypobetalipoproteinemia can be the result of mutations in the apolipoprotein B gene, and several kindreds have been described in which truncated apoBs have been demonstrated (6–12).

Abbreviations: apo, apolipoprotein; SI, sucrase-isomaltase; ApN, aminopeptidase N; endo H, endo-β-N-acetylglucosaminidase H; endo F, endo-β-N-acetylglucosaminidase F; IEF, isoelectric focusing; HDL, high density lipoproteins.

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In both abetalipoproteinemia and homozygous hypobetalipoproteinemia, the plasma is devoid of apoB-100, which derives mainly from the liver, and apoB-48, which appears to be derived solely from the intestine (13, 14). Both apoB-100 and apoB-48 are clearly derived from a single gene; the tissue-specific expression of these two forms apparently involves a novel form of RNA processing resulting in a translational stop codon at position 2153 in the intestine (15–17). Other lipoprotein deficiencies have been described in which the plasma lacks only apoB-100, normotriglyceridemic abetalipoproteinemia (18–20), or only apoB-48, Anderson's disease (21), and chylomicron retention disease (22, 23).

The molecular pathogenesis of abetalipoproteinemia is unclear. Although there have been several studies of the plasma from abetalipoproteinemic patients in which alterations in the lipid and protein compositions of all the lipoprotein classes have been noted (24–27), there have been few investigations of lipoprotein or apolipoprotein production at the cellular level (28–32). Glickman, Green, and Lees (29) were unable to demonstrate the presence of apoB in the intestinal mucosa of two patients by immunohistochemical techniques. Similarly, Levy et al. (30) failed to detect any synthesis of apoB-48- or apoB-100-containing lipoproteins in cultured jejunal explants from two other patients. In contrast, Dullaart et al. (31) detected reduced amounts of apoB in the liver and normal amounts in the intestine of one patient while Lackner et al. (32) found increased amounts of anti-apolipoprotein B immunoreactive material in liver sections associated with a sixfold increased hepatic apoB mRNA level in two other patients. Talmud et al. (33) suggested on the basis of linkage studies of two families that the apoB gene is not involved in cases of abetalipoproteinemia. Ross et al. (8) have suggested that abetalipoproteinemia is most likely secondary to defective cellular apoB secretion.

We describe here the concurrent evaluation of two patients with typical manifestations of abetalipoproteinemia. Restriction fragment analysis of DNA from both patients showed that there were no major insertions or deletions in the apoB gene and that no particular alleles of apoB were associated with abetalipoproteinemia. One patient had clearly evident intestinal and hepatic anti-apoB immunoreactive material and some lipoprotein abnormalities that have been described in other cases of abetalipoproteinemia. In contrast, although the other patient had no immuno-detectable apoB in the intestine, organ culture experiments demonstrated the synthesis of a normal-sized apolipoprotein B-48 which appeared to be normally glycosylated. Analysis of the intestinal apoB mRNA revealed, in this same patient, a slightly decreased amount of a normal-sized mRNA. In spite of the fact that both lipids and a normal apoB-48 were present, no chylomicron-like particles were detected intracellularly by electron microscopy. These results suggest that some cases of abetalipoproteinemia are not the consequences of abnormalities in the apoB gene or its product, apoB-48, or in the glycosylation of apoB-48.

METHODS

Description of the patients

M. P., a girl born in 1968, was the first of two children of unrelated healthy, French parents. The mother's values for plasma cholesterol, triglyceride, and apoB were normal. The father's lipid and protein levels could not be obtained.

S. L., a boy born in 1970, was one of two children of French parents having normal plasma cholesterol, triglyceride, and apoB levels. In 1983 he died from cardiomyopathy and heart failure.

Both M. P. and S. L. presented with a malabsorption syndrome followed by acanthocytosis, ataxia, and the loss of tendon reflexes at 5 and 10 years of age, respectively. Plasma cholesterol levels were very low (39 mg/dl for M. P. and 58 mg/dl for S. L.). Upon agarose gel electrophoresis, only traces of alpha-migrating lipoproteins and no beta or pre-beta lipoproteins were detectable in the plasma of either patient. Further, no apoB could be detected using sensitive ELISA assay (level of detection: 0.003 mg/dl); the amounts of apoA-I were 30 and 50% below the normal value (130 mg/dl $\pm$ 30) for M. P. and S. L., respectively. By preparative ultracentrifugation at densities less than 1.063 g/ml, very small amounts of lipoproteins were found, the protein content of which consisted of apolipoproteins A-I, A-II, and C. Upon negative staining electron microscopy, these particles formed square lattices (particle dimension of 13.3 nm) as has been observed previously in other cases of abetalipoproteinemia (25, 34). The HDL subclasses had an abnormal distribution: HDL_1 were present in slightly increased amounts. The HDL_2, which were markedly decreased in quantity, exhibited an abnormal chemical composition (increased cholesterol and phospholipid and decreased protein content) and were larger in size than normal HDL_2 (11.6 $\pm$ 1.8 nm vs 10.0 $\pm$ 2 nm for the normal) as determined by electron microscopy. The level of HDL_3 was also markedly diminished and the particle size was decreased (6.7 $\pm$ 1.1 nm vs 8.8 $\pm$ 1.6 nm for the normal). The clinical data for these two patients associated with the normal plasma cholesterol and apoB values of their parents are consistent with the diagnosis of abetalipoproteinemia. Both patients followed a low fat diet subsequent to their diagnosis.

Ultrastructure, histochemistry, and immunochemistry

Intestinal and hepatic biopsies from the patients and from normal subjects (having normal plasma cholesterol, triglyceride, phospholipid and apoB values) were obtained after informed consent was given, and intestinal epithelial

cells were isolated as previously described (21). Ultrathin sections of intestinal mucosal biopsies were studied by eletron microscopy (Phillips CM10 microscope) after fixation in 2.5% glutaraldehyde for 1 h, post-fixation in 2% osmium tetroxide, embedding in glycidether 100 (Merck), and staining with 1% aqueous uranyl acetate and Reynold's lead citrate. Lipid staining with Oil Red O was carried out on cells or cryo-sections without fixation. Immunohistochemistry was performed (21) on the intestine (isolated cells and mucosal sections) and on liver sections. Biopsy specimens were either placed into cold phosphate-buffered saline, pH 7.2, for the isolation of intestinal epithelial cells or were fixed in 10% formalin for 18 h (intestine or liver). Smears of intestinal cells were treated as previously described (21). The fixed tissue sections were deparaffinized by two washes in xylene and three washes in isopropanol before immunoenzymatic localization (35). Rabbit polyclonal antibodies to apoA-I, apoB, total apoC, apoC-II, and apoC-III (36) and monoclonal antibodies (Clin Midy Research Center, Montpellier, France) recognizing either apoB-100 (L3) or both apoB-100 and B-48 (L9) (37) were used.

Apolipoprotein analysis

Apolipoproteins prepared from lipoproteins (38) isolated by preparative ultracentrifugation (39) were analyzed by isoelectric focusing in a pH 4–6.5 ampholine gradient (40) and by sodium dodecylsulfate (SDS) polyacrylamide (15%) gel electrophoresis (41).

Biosynthetic labeling of intestinal biopsies in organ culture

Biopsy samples were metabolically labeled as previously described by Naim et al. (42–44). Briefly, biopsy specimens from M. P. and from normal control subjects were labeled with 150 μCi/ml [^{35}S]L-methionine RPMI medium for 30 min followed by incubation for 1 or 2 h with nonradioactive medium. The labeled intestinal tissue was then homogenized and solubilized. The solubilized material was incubated with protein A-Sepharose beads to which polyclonal anti-apoB or monoclonal anti-sucrase-isomaltase or anti-aminopeptidase N (45) had been absorbed. Immunoprecipitated proteins were eluted and divided into two identical aliquots, one of which was digested with endo-β-N-acetylglucosaminidase H (Endo H). Endo H hydrolyzes only Asn-linked carbohydrates of the high mannose type (46). The aliquots were then solubilized and electrophoresed on a 5–15% acrylamide gradient gel (41). The molecular weight standards included apoB thrombin fragments T4, T3, and T2, intact apoB, and combithek® from Boehringer (α-macroglobulin, 340000, phosphorylase b, 97400, glutamate dehydrogenase, 55400, lactate dehydrogenase, 36500, and trypsin inhibitor, 20100). Standards were electrophoresed with and without reduction with beta-mercaptoethanol. The gels were stained with Coomassie blue, destained, treated with "Enlightening" (New England Nuclear), dried, and autoradiographed with Kodak X-omat K films at −80°C.

Hybridization analysis of genomic DNA and RNA with apoB cDNA probes

DNA from the patient M. P. and from normolipidemic subjects was prepared from leukocytes (47). DNA from S. L. was obtained from cultured skin fibroblasts following addition of ribonuclease (1 mg/tube) to the cell lysate. The lysate was incubated at 42°C for 1 h before digestion with proteinase K.

Several cDNA or genomic probes spanning the apoB gene were used in Southern blotting experiments to examine apoB gene sequences in the DNA. These included: A6C, a 3.5 kb cDNA, ABF, a 6.5 kb cDNA and A7H, a 0.6 kb cDNA, all corresponding to the 5' region of the mRNA (48, 49); AB1, a 5.1 kb cDNA corresponding to the 3' end of the mRNA (49); RP2, a 0.3 kb cDNA corresponding to apoB residues 3029–3132 (48); and SB9, a 6 kb apoB genomic clone spanning most of exon 25 and overlapping the 5' clone ABF and the 3' clone AB1. The probes were labeled with [^{32}P]dCTP to specific activity of about 10^9 cpm/μg by random oligonucleotide priming or by nick translation. Southern blotting and hybridization analysis of genomic DNA were performed essentially as previously described (50). Ten μg of DNA was digested with restriction enzymes (XbaI, PvuII, PstI, EcoRI, HindIII, HinfI or TaqI), electrophoresed through agarose gels, and transferred to nylon membranes by blotting. Prehybridization and hybridization of filters with [^{32}P]dCTP labeled probes for apoB were performed as previously described. Total cellular RNA was isolated from an intestinal biopsy obtained from M. P. while fasting, as previously described (32). RNA were size-fractionated by 1% agarose gel electrophoresis in the presence of formaldehyde, transferred to "gene screen plus" membrane, and hybridized to the labeled A7H cDNA probe as described above. For dot blots, RNA pellets were dissolved in phosphate buffer, denatured 15 min at 65°C in the presence of formaldehyde, and immediately chilled on ice. RNA was spotted in serial dilutions on "gene screen plus" membrane. Filters, baked at 80°C for 2 h, were hybridized and autoradiographed and dot blots were scanned.

RESULTS

Analysis of the apoB gene and its intestinal mRNA

Restriction fragments of genomic DNA from three normal subjects and from both patients were analyzed with

several apoB cDNA or genomic probes that, together, spanned the entire apoB gene. DNA from the subjects was digested with various restriction enzymes (Xbal, PvuII, MspI, EcoRI, HindIII, HinfI, and TaqI) and the apoB gene fragments hybridizing to the individual labeled probes were examined. With the exception of restriction fragment length polymorphisms, the pattern of hybridizing fragments observed with all probes was similar for the patients and normal subjects (data not shown). This suggests that major deletions or rearrangements of the apoB gene are not found in these patients. The pattern of restriction fragment length polymorphisms between the two patients differed considerably, although precise haplotypes were not determined. Both patients were heterozygous for an EcoRI polymorphism that occurs at residue 4154 of the mature protein (51). Patient S. L. was homozygous for the 8.6 kb allele of the XbaI polymorphism that occurs at residue 2488 of the protein while patient M. P. was heterozygous (52). Both patients were heterozygous for polymorphisms that occur in the 3' hypervariable region of the apoB gene as detected with several enzymes (PvuII, MspI, HindIII, and TaqI) (53, 54) and all four alleles differed. Thus, abetalipoproteinemia does not appear to be associated with particular alleles of apoB as defined by restriction fragment length polymorphism.

Northern blot analysis of intestinal mRNA of M. P. revealed an apoB mRNA that was similar in size to the intestinal apoB mRNA isolated from normal subjects. The apoB mRNA level, as estimated by analysis of dot blot hybridization of total intestinal RNA, was slightly reduced (30%) as compared to that of control subjects (**Fig. 1**).

The lack of evidence indicating an abnormality of the apoB gene and the presence of a normal-sized intestinal mRNA suggest that apoB might be synthesized in spite of its absence from the plasma. This possibility was investigated by immunohistochemistry and organ culture of intestinal and/or hepatic biopsies from our patients and from normal individuals.

Immunoenzymatic staining of the intestine

Enterocytes from fasted normal individuals had a diffuse brown cytoplasmic immunoperoxidase staining using polyclonal antibodies to apoB (**Fig. 2A**), total apoC (Fig. 2D) and apoC-III (Fig. 2E). There was also a weak but conspicuous supranuclear reaction with monoclonal antibodies, L9, to both apoB-100 and apoB-48 (Fig. 2B). Staining was minimal or absent with a monoclonal antibody, L3, to only apoB-100 (Fig. 2C). Control reactions were all negative as exemplified by incubation of peroxidase labeled anti-rabbit (Fig. 2F) or anti-mouse (Fig. 2G) serum followed by the DAB reaction.

Intestinal tissue sections obtained from S. L. while fasting or enterocytes isolated from S. L., either fasted or fed, showed marked diffuse cytoplasmic immunoperoxidase staining with polyclonal antibodies to apoB (**Fig. 3, E and I**), total apoC (Fig. 3, F and J), apoC-III (Fig. 3G) and apoA-I (Fig. 3K). Control reactions (Fig. 3, H, and L) were all negative. With monoclonal antibodies, L3, to only apoB-100 (**Fig. 4A**) or L9, to both apoB-48 and B-100 (Fig. 4B), there was brown immunoperoxidase staining around large lipid droplets in the supranuclear areas of the enterocytes in intestinal tissue obtained from S.L. while fasting. After a fatty meal, however, only monoclonal antibody L9 (anti apoB-48 and apoB-100) gave a much stronger immunoperoxidase staining in the same area (Fig. 4C) suggesting an increase in apoB-48. All control reactions were negative (Fig. 4D).

In contrast, enterocytes isolated from M. P., in the fasting state, showed no cytoplasmic immunoperoxidase staining with anti apoB polyclonal antibodies (Fig. 3A) and only mild staining with anti-total apoC (Fig. 3B) or anti-apoC-III (Fig. 3C) antibodies. As illustrated by the peroxidase-labeled anti-rabbit serum alone followed by the DAB reaction (Fig. 3D), all the controls were negative. The weak brown staining on the brush border of isolated enterocytes was considered as a nonspecific reaction.

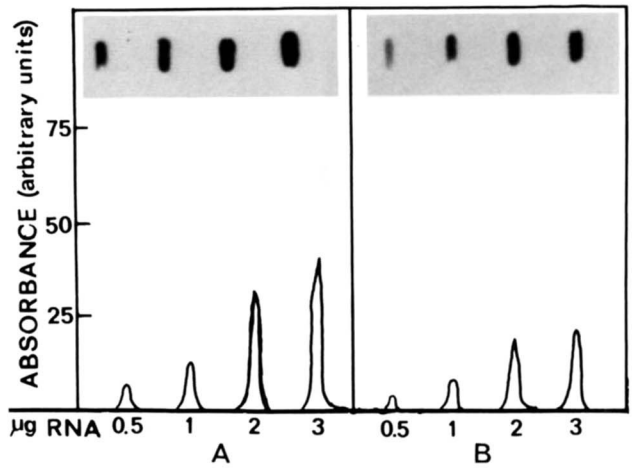


Fig. 1. Dot blots of normal (A) and abetalipoproteinemic (B) intestinal RNA. Different amounts of total RNA were applied to gene screen membranes and hybridized to the A7H probe. The autoradiographs were quantitated by densitometry.

Immunoenzymatic staining of the liver

Immunoperoxidase staining, with polyclonal antibodies to apoB, of liver sections from a fasted normal subject gave a marked brown cytoplasmic reaction localized in the perinuclear area and around lipid droplets (Fig. 2H). Monoclonal antibody L3 to apoB-100 gave a weak diffuse cytoplasmic staining along with a dark brown granular staining in the perinuclear areas of the hepatocyte (Fig. 2J). Using monoclonal antibody, L9 (anti-apoB-48 and B-100), the diffuse cytoplasmic as well as the brown granular

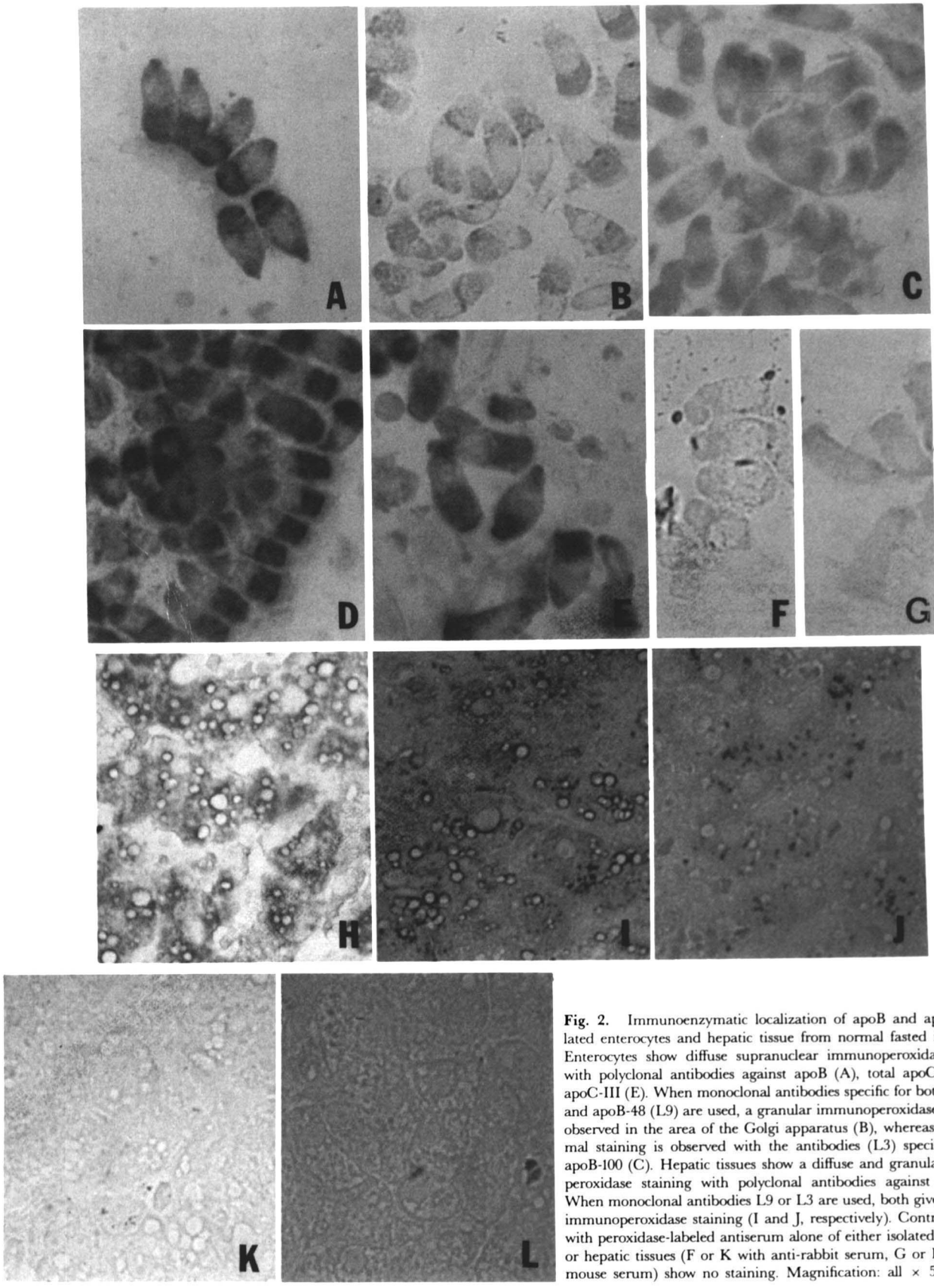


Fig. 2. Immunoenzymatic localization of apoB and apoC in isolated enterocytes and hepatic tissue from normal fasted individuals. Enterocytes show diffuse supranuclear immunoperoxidase staining with polyclonal antibodies against apoB (A), total apoCs (D), and apoC-III (E). When monoclonal antibodies specific for both apoB-100 and apoB-48 (L9) are used, a granular immunoperoxidase staining is observed in the area of the Golgi apparatus (B), whereas only minimal staining is observed with the antibodies (L3) specific for only apoB-100 (C). Hepatic tissues show a diffuse and granular immunoperoxidase staining with polyclonal antibodies against apoB (H). When monoclonal antibodies L9 or L3 are used, both give a granular immunoperoxidase staining (I and J, respectively). Control reactions with peroxidase-labeled antiserum alone of either isolated enterocytes or hepatic tissues (F or K with anti-rabbit serum, G or L with anti-mouse serum) show no staining. Magnification: all × 500.

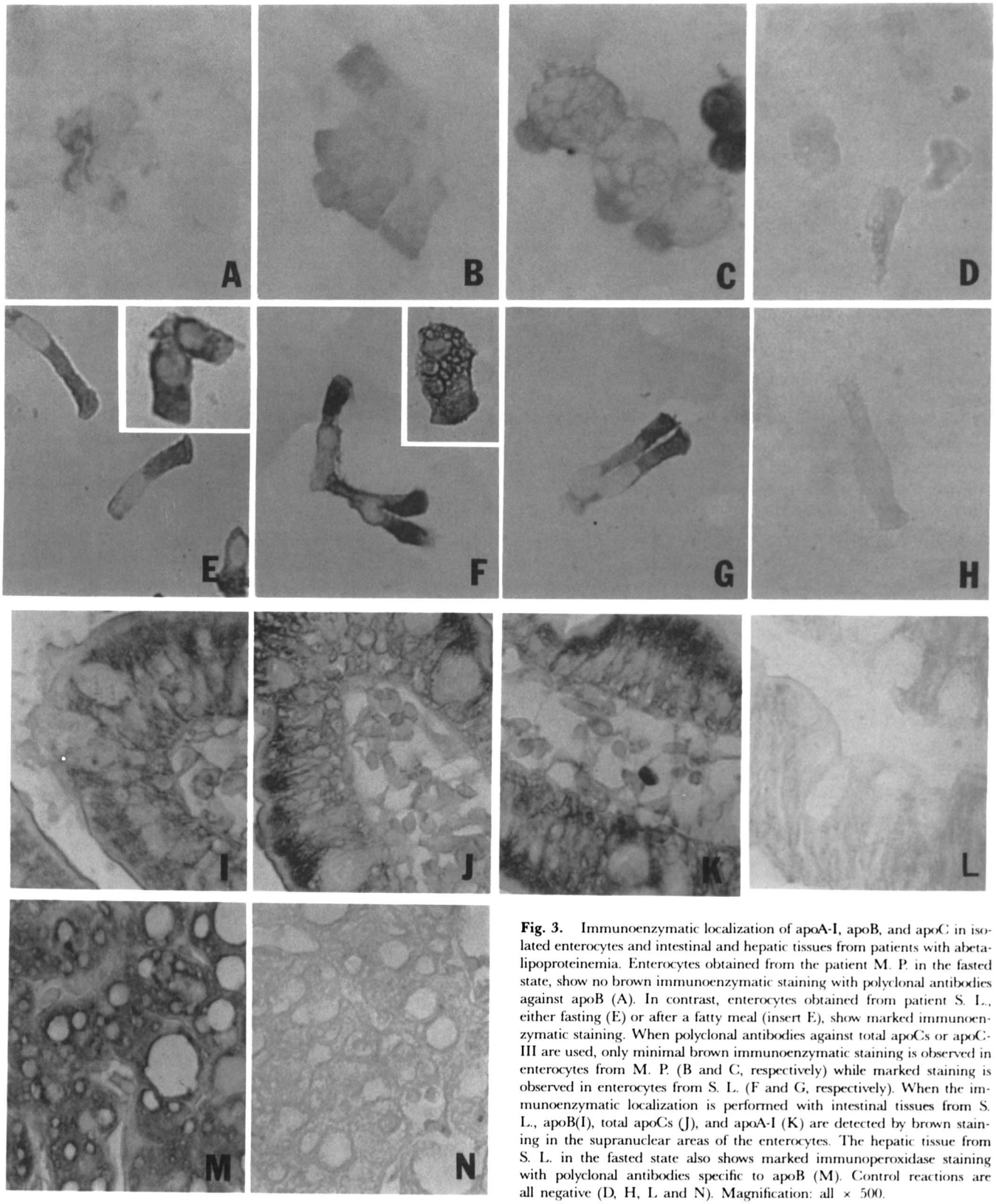


Fig. 3. Immunoenzymatic localization of apoA-I, apoB, and apoC in isolated enterocytes and intestinal and hepatic tissues from patients with abetalipoproteinemia. Enterocytes obtained from the patient M. P. in the fasted state, show no brown immunoenzymatic staining with polyclonal antibodies against apoB (A). In contrast, enterocytes obtained from patient S. L., either fasting (E) or after a fatty meal (insert E), show marked immunoenzymatic staining. When polyclonal antibodies against total apoCs or apoC-III are used, only minimal brown immunoenzymatic staining is observed in enterocytes from M. P. (B and C, respectively) while marked staining is observed in enterocytes from S. L. (F and G, respectively). When the immunoenzymatic localization is performed with intestinal tissues from S. L., apoB(I), total apoCs (J), and apoA-I (K) are detected by brown staining in the supranuclear areas of the enterocytes. The hepatic tissue from S. L. in the fasted state also shows marked immunoperoxidase staining with polyclonal antibodies specific to apoB (M). Control reactions are all negative (D, H, L and N). Magnification: all × 500.

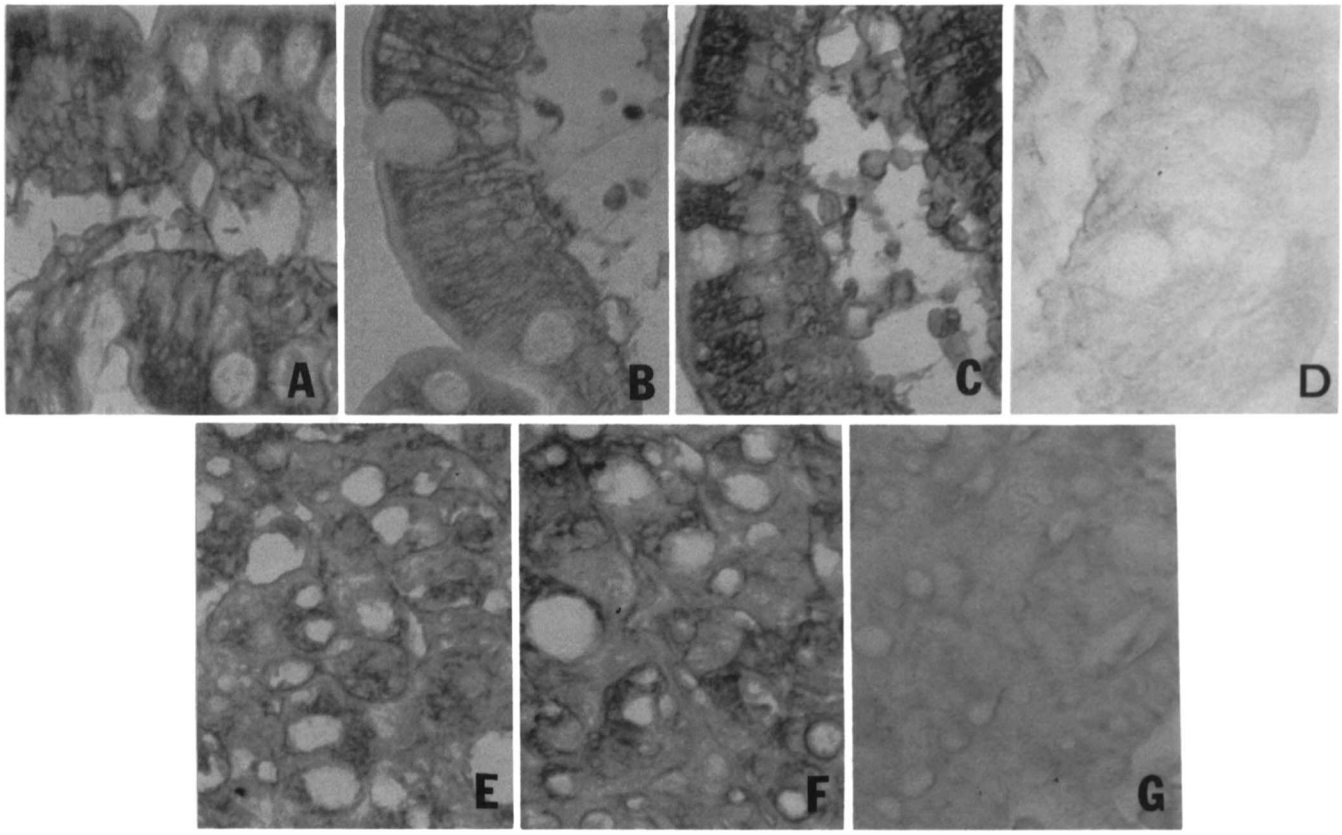


Fig. 4. Immunoenzymatic staining of intestinal and hepatic tissues of patient S. L. using monoclonal antibodies against LDL. Enterocytes obtained from the patient in the fasted state show a brown immunoenzymatic staining using monoclonal antibodies (L3) specific for only apoB-100 (A) and antibodies (L9) specific for both apoB-100 and apoB-48 (B). Enterocytes obtained from S. L. following a fatty meal show increased brown immunoenzymatic staining in the supranuclear area of the cytoplasm (C) when L9 was used. Immunoenzymatic localization of apoB in hepatic tissue using monoclonal antibodies L3 (E) and L9 (F) shows a brown cytoplasmic staining surrounding the lipid droplets. Control reactions using peroxidase labeled antisera alone show no staining (D, G). Magnification: all × 500.

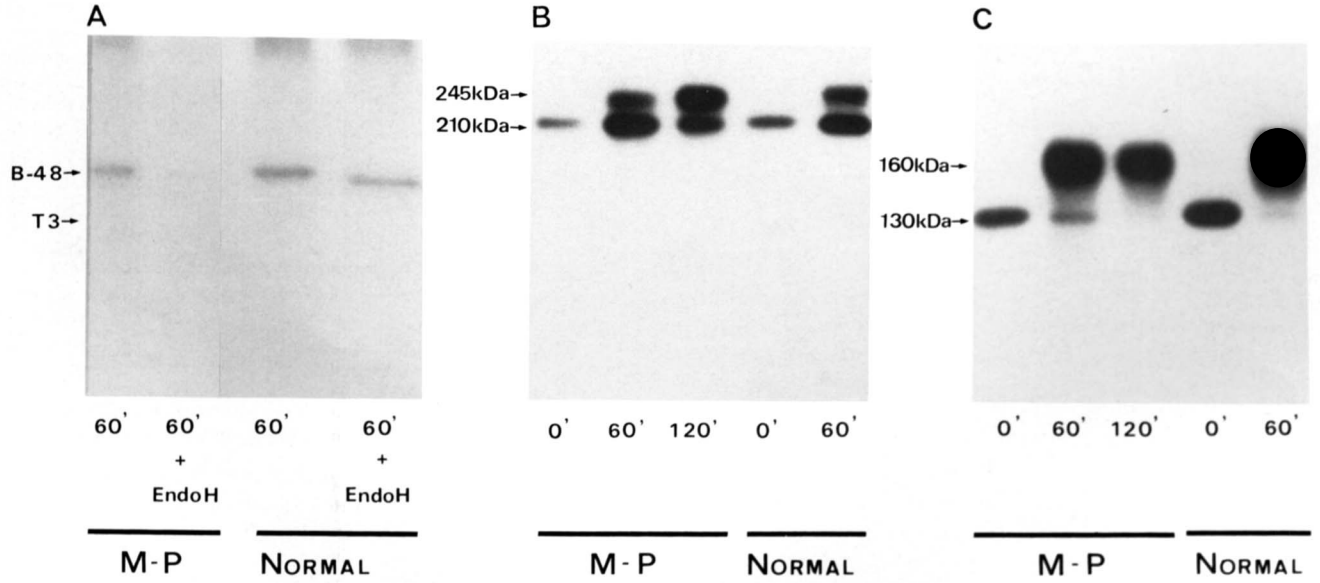


Fig. 5. Molecular forms of apoB, SI, and ApN in biosynthetically labeled biopsy specimens of the patient M. P. and a normal individual. Biopsy specimens were labeled for 30 min with [^{35}S]methionine followed by a cold chase of the indicated interval and then the samples were homogenized. ApoB (A), SI (B) and ApN (C) were immunoprecipitated and subjected to SDS gel electrophoresis followed by autoradiography. An apoB was present in M. P. which had the same apparent molecular weight as the apoB-48 produced in the normal control and both of which exhibited similar slight reductions in molecular weight upon treatment with Endo H. The biosynthesis and processing of SI and ApN were similar in the patient and the control.

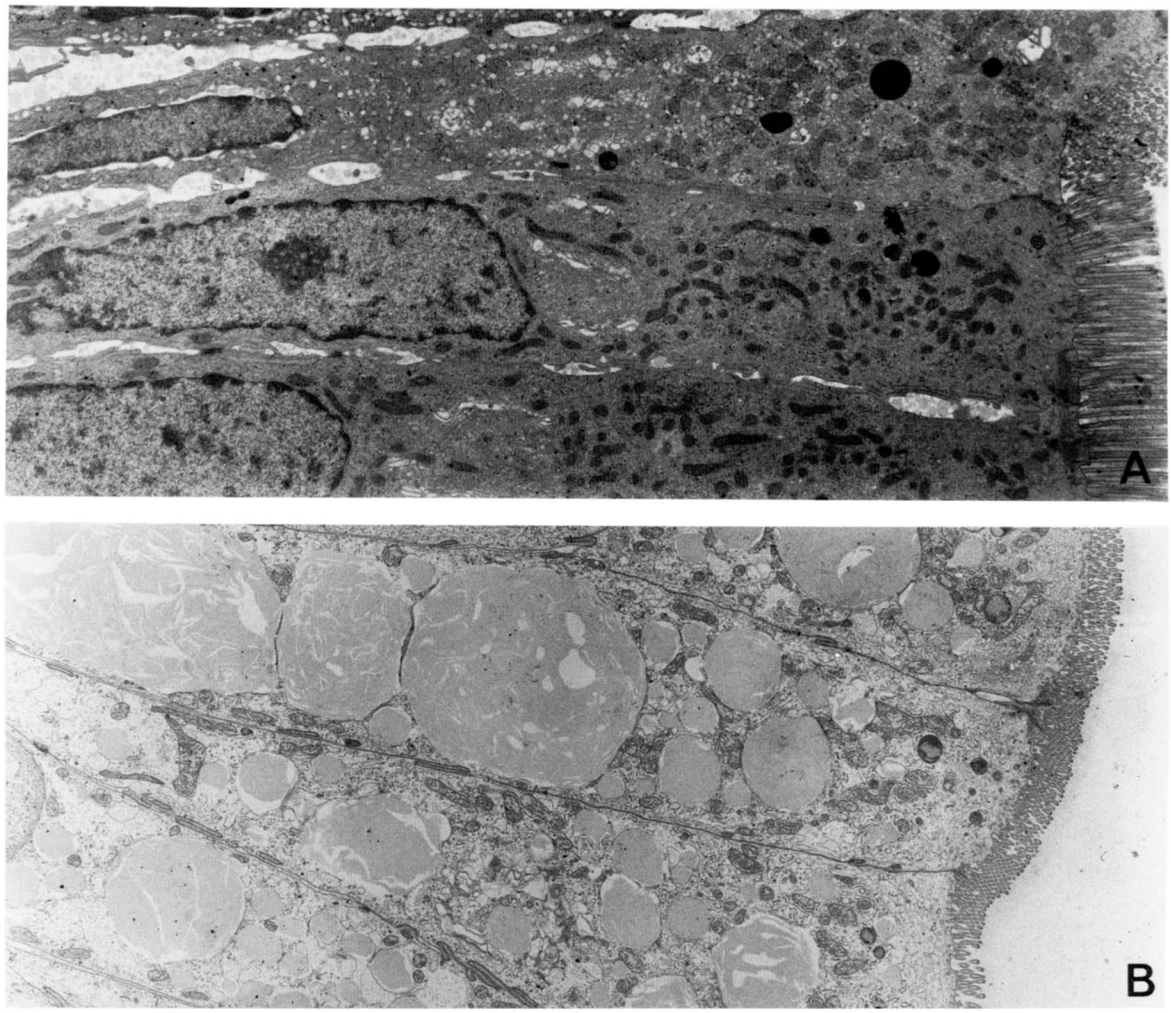


Fig. 6. Ultrastructure of intestinal biopsies from normal individuals and from patients with abetalipoproteinemia. Enterocytes from normal fasted individuals (A) contain only a few lipid droplets while all the enterocytes from the patient M. P., fasted, (B), and some of the enterocytes from the patient S. L., fasted, (C), show large lipid droplets scattered at the apical area of the cytoplasm. In the enterocytes obtained from normal individuals postprandially, lipoprotein-like particles are clearly apparent in the Golgi apparatus and the basolateral spaces (D). In contrast, enterocytes obtained from S. L. after a fatty meal are filled with lipid droplets spreading to the basal area of the cytoplasm. The vacuoles of the Golgi apparatus are empty (without lipoprotein-like particles) and the lipid droplets do not appear to be membrane bound (E). Magnification: A (× 4000); B (× 2500); C, (× 2900); D, (× 16,000); E (× 14,000).

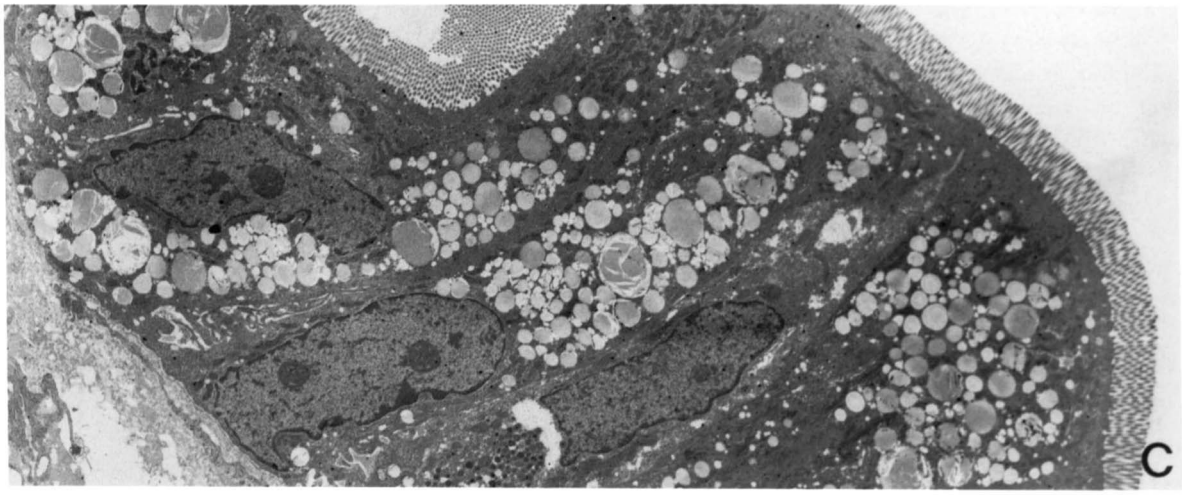
staining was intense (Fig. 2I). All the control reactions were negative (Fig. 2, K and L).

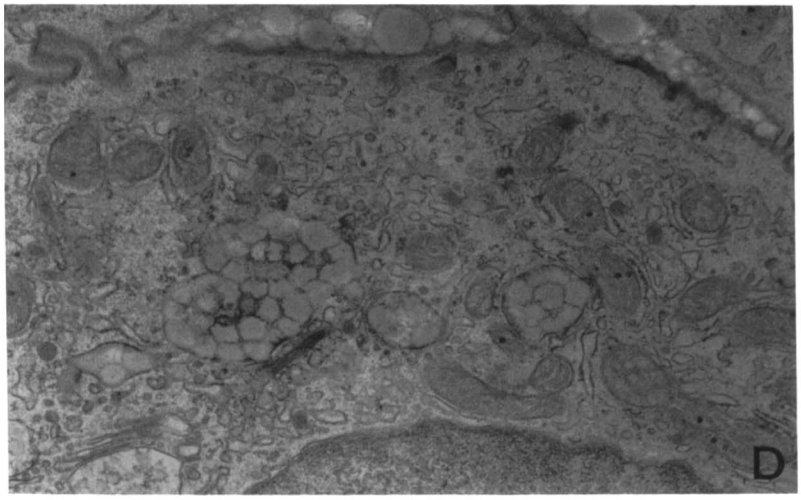
Immunoperoxidase staining of the liver sections from S. L. with anti apoB polyclonal antibodies (Fig. 3M) was more intense than that of the normal liver (Fig. 2H), particularly around lipid droplets. Monoclonal antibodies L3 and L9 gave similar immunoperoxidase staining (Fig. 4, E and F). As compared to normal liver (Fig. 2, J and I), the hepatic sections from S. L. showed an increased cytoplasmic staining localized around the lipid droplets.

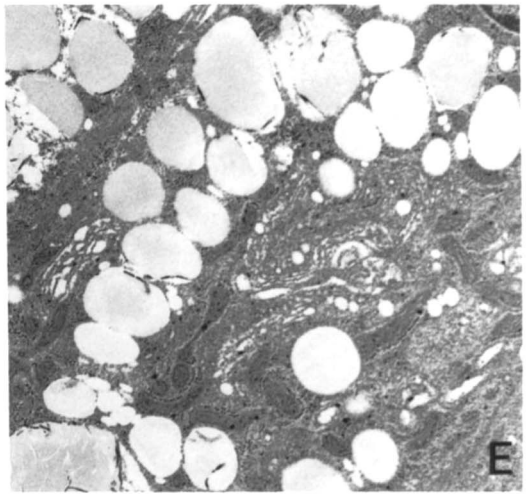
The results of the immunohistochemical studies of the biopsies from S. L. strongly suggest that apoB-48 is present intracellularly. The biosynthesis of apoB in M. P. was further investigated by organ culture of intestinal biopsies.

Production and processing of apoB and two brush border membrane hydrolases in organ culture

After incubation with [^{35}S]methionine-containing medium, intestinal biopsies from both normal individuals and from M. P. were homogenized and immunoprecipitated with an antiserum against both apoB-100 and B-48. After a 30-min pulse and a 1-h chase, a protein was immunoprecipitated from the intestinal mucosa of M. P. which co-migrated on an SDS gel with the apoB-48 synthesized by the intestinal mucosa of normal individuals (**Fig. 5A**). The treatment with endo H led to a slight decrease in the apparent molecular weight which was identical to that observed with the apoB-48 from controls. Further, despite







comparable [^{35}S]methionine incorporation into TCA-precipitable proteins in biopsies from M. P. and the controls, there was less apoB-48 produced by the biopsy from M. P. as compared to the normal subject.

To further study intracellular protein processing, we also investigated, in biopsies of M. P. and control subjects, the biosynthesis of two major brush border membrane hydrolases, sucrase-isomaltase (SI) and aminopeptidase N (ApN). Within 30 min of pulse labeling, a single polypeptide of apparent molecular weight of 210,000 was immunoprecipitated with monoclonal anti-SI antibodies (Fig. 5B, lane 1). This species corresponds to the high mannose precursor of pro-SI (43). A similar protein was present in the control biopsy (Fig. 5B, lane 4). After 1 h of chase, the mature, complex glycosylated form of pro-SI, apparent molecular weight of 245,000, was present in both the patient's and the control biopsies (Fig. 5B, lanes 2 and 5). The ratios of the labeling intensities of the mature form as compared to the high mannose precursor were similar (as assessed by densitometric scanning of the autoradiogram) in the patient's and the control biopsies. After 2 h of chase the intensity of the mature form increased with a concomitant decrease in that of the high mannose precursor (Fig. 5B, lane 3). The kinetics of processing of SI in the patient's tissue were comparable to those found in normal explants (42).

Similarly, the biosynthesis and processing of ApN in the patient's and control biopsy samples proceeded essentially as shown previously in normal human small intestinal epithelial cells (43, 44). After 30 min of pulse, a band of Mapp equal to 130,000 was identified which corresponds to the high mannose precursor of ApN (Fig. 5C, lanes 1 and 4). The labeling intensity of this species was similar in the patient's and the control biopsy. After 1 h of chase, most of the high mannose precursor had been converted

to a species of Mapp equal to 160,000 (Fig. 5C, lanes 2 and 5) which represents the complex glycosylated, mature form of ApN. A complete conversion to this final form was obtained in the patient's biopsy within 2 h of chase, which is consistent with the processing kinetics of ApN in normal intestinal tissue (42–45). Collectively, these data show that the processing of two control brush border glycoproteins in the patient's biopsy sample was not affected, indicating that there is no general cellular glycosylation defect.

Because of the evidence suggesting that an apparently normal apoB-48 is synthesized, electron microscopy was used to search for evidence that apoB-containing lipoproteins were assembled and present intracellularly.

Intestinal histochemistry and ultrastructure

While enterocytes from fasted normal subjects showed only a few Oil Red O-positive lipid droplets in the cytoplasm, enterocytes obtained from M. P., in the fasting state, were filled with lipid droplets. In contrast, the enterocytes from S. L. while fasting showed only some lipid droplets at the apical and basal cell areas. After a fatty meal, the quantity of lipid droplets increased and filled the entire cell. Hepatic biopsies from normal fasted subjects showed little Oil Red O staining while that from S. L. showed marked accumulation of lipid droplets throughout the tissue (data not shown). These results indicate that neutral lipids that might potentially be used in the assembly of lipoproteins are abundant intracellularly in both patients.

Electron microscopic examination of the intestinal absorptive cells obtained from M. P. and S. L., while fasting, revealed normal appearing microvilli, cell organelles, and nuclei (**Fig. 6, B and C** as compared to A). However, while enterocytes from fasted normal subjects contained only a few small lipid droplets along with some lipoprotein particles intercellularly (Fig. 6A), all the enterocytes from M. P. (Fig. 6B) but only some of those from S. L. (shown in a selected area in Fig. 6C) had large lipid droplets, 0.45–5 μm in diameter. No lipid droplets or lipoprotein-like particles were observed in the intercellular spaces of the enterocytes of either patient or in the extracellular spaces of the lamina propria. After a fatty meal, lipoprotein particles were clearly apparent in the Golgi apparatus as well as the intercellular spaces in biopsies from normal individuals (Fig. 6 D). Biopsies obtained from S. L., postprandially, had large lipid droplets with diameters of 0.33–3.6 μm scattered throughout the cytoplasm. Particular attention was given to the appearance of the lipid droplets; they were not membrane bound and did not appear as dense particles. The Golgi apparatus was flat and no chylomicron-like structures were observed within the enterocyte or in the basolateral spaces (Fig. 6E).

Characterization of the apolipoproteins

The apolipoprotein content of the plasma was also studied, in part to ascertain whether any apoB was detectable in a density range where it is not normally present. No apoB was detected using a sensitive ELISA assay. Isoelectric focusing (IEF) showed that lipoproteins from S. L. and M. P. of density less than 1.063 g/ml contained apoA-I as the major component along with apoE and apoCs as minor components (**Fig. 7C**). The HDL subfractions from M. P. appeared similar to those from normal subjects when studied by IEF (Fig. 7, A and B) and by SDS polyacrylamide gel electrophoresis (not shown). In contrast, the same fractions from S. L. were markedly different: by IEF, the distribution of the apoA-I isoforms was normal (A-I 1, 2, 3, 4), but an increased amount of proapo-A-I was present in all HDL subfractions (Fig. 7, D–F). Further, an unusual isomorphism of apoa-II (+ 1 and − 2s) in addition to the normally occurring forms (0 and − 1s) (55) was present in HDL_2 and HDL_3 but not in HDL_1. Other abnormalities in the HDL protein composition of S. L. were detected by SDS polyacrylamide gels (**Fig. 8**). There was an increased amount of the apoE–A-II complex, and there were some additional proteins of Mapp 45,000–90,000 (not reactive with anti-apoB antisera) and Mapp between 18,000 and 27,000 which are not normally present in HDL_2 or HDL_3. This unusual protein pattern resembled those described in other cases of abetalipoproteinemia (25). Analysis of the density greater than 1.21 g/ml "bottom fraction" did not reveal any apoB immunoreactive material.

DISCUSSION

Both patients described here exhibited clinical and biochemical features typical of classical abetalipoproteinemia: absence of apoB-containing lipoproteins in the plasma along with typical alterations of plasma lipids, lipoproteins and apolipoproteins, steatorrhea, growth retardation, retinitis pigmentosa, acanthocytosis, and a neurological syndrome consisting of areflexia, ataxia, and the loss of proprioception. Evaluation of apoB synthesis at the cellular level in abetalipoproteinemic patients has provided contradictory results: in some cases (31, 32) the presence of apoB has been demonstrated by immunohistochemical techniques while in other cases similar techniques (29) as well as organ culture experiments (30) have detected no apoB. In our patients, although normal apolipoprotein B-100 and B-48 were absent from the plasma, apoB was clearly demonstrated in the hepatocytes and enterocytes by immunohistochemical and organ culture techniques. Further, as opposed to previous studies in

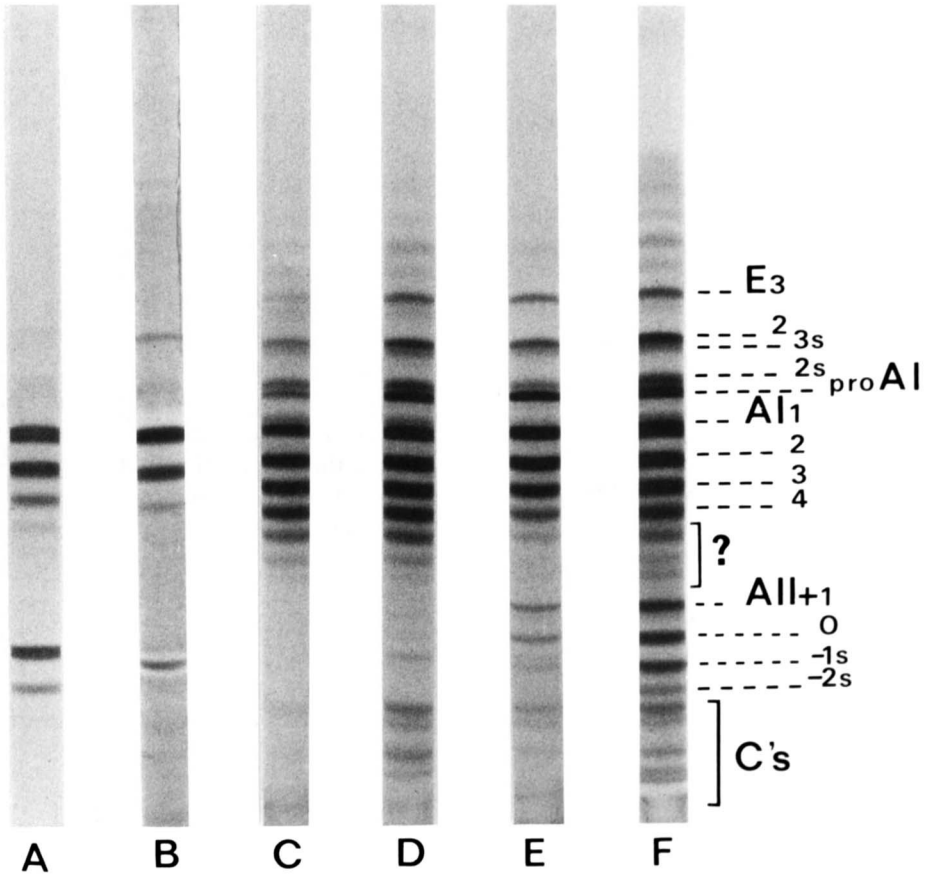


Fig. 7. Isoelectric focusing of apolipoproteins from a normal individual and from the patients S.L. and M.P. HDL_3 from a normal individual (A) and from M. P. (B) and "LDL" (C), HDL_1 (D), HDL_2 (E), and HDL_3 (F) from S. L. were delipidated and isoelectric focusing was performed over a pH 4–6.5 gradient. Note the presence of some additional protein bands in the fractions of S. L. which do not appear in the apoproteins from the normal individual.

which only the presence of apoB was demonstrated by immunological techniques, we show here that the apoB produced in the intestinal organ cultures of one of our patients had an apparent molecular weight (B-48) and glycosylation similar to that produced by the intestinal cells from normal individuals. The presence of a normal-sized apoB-48 in abetalipoproteinemia is in marked contrast to the truncated apolipoprotein Bs which have been described in cases of familial hypobetalipoproteinemia (6, 7, 9, 11, 12).

To examine whether the synthesis and processing of other mucosal epithelial cell glycoproteins were affected, we investigated the biosynthesis of sucrase-isomaltase and aminopeptidase N, two major brush border membrane hydrolases. Our data clearly show that the biosynthesis and processing of these two brush border glycoproteins in the patient's biopsy sample were normal, indicating that there is not a general cellular glycosylation defect in this abetalipoproteinemic patient.

Because of his untimely death, the investigation of in vitro apoB synthesis could not be performed in S. L. When the isolated enterocytes or intact intestinal and liver tissue of S. L. were examined, extensive staining was obtained with polyclonal antibodies directed against apoB. The nature of the apoB immunoreactive material in the enterocytes and hepatocytes of S. L. was investigated using two different monoclonal antibodies: one specific for both apoB-100 and apoB-48 and the other specific for only apoB-100. Enterocytes as well as hepatocytes possessed material recognized by both antibodies. After a fatty meal, there was increased immunoreactivity of the enterocytes towards monoclonal antibodies recognizing both apoB-100 and B-48 (L9) but not towards antibodies recognizing only apoB-100 (L3), suggesting an augmentation of only apoB-48. Other studies (29, 56, 57) involving normal individuals have also indicated an increased intracellular apoB content postprandially. Whether or not the increased immunoperoxidase staining in S. L. postprandially is due to an intra-

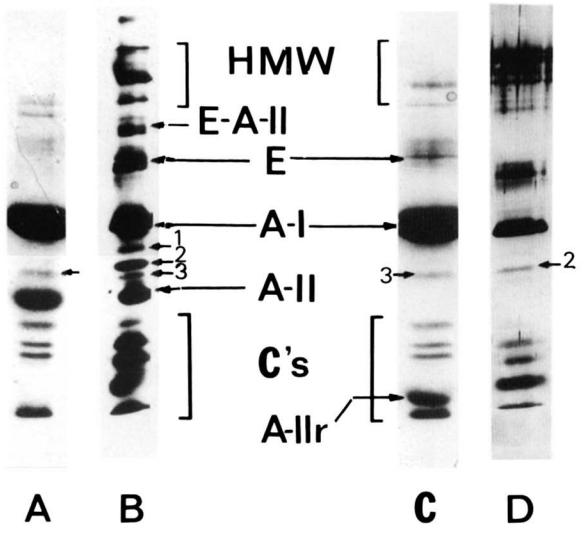


Fig. 8. Polyacrylamide gel electrophoresis in SDS of HDL_3 apoproteins from a normal individual (A, C) and S. L. (B, D). Lipoproteins were delipidated and the apoproteins were separated on a 15% polyacrylamide gel in SDS, and the proteins were stained with Coomassie Brilliant Blue, (A and C) or silver (B and D). Gels A and B are non-reduced whereas gels C and D have been reduced with beta-mercaptoethanol. The gel patterns are characterized by the presence of A-I, A-II, reduced A-II (A-IIr), C, E, E-A-II, and some higher molecular weight proteins (HMW) which are denoted by arrows. There are a number of additional protein bands, also denoted by arrows and numbered 1, 2 and 3, that have electrophoretic mobilities between A-I and A-II.

cellular redistribution of the apoB, as suggested by Christensen et al. (58), is not clear. The difference between the two patients with respect to the amount of intracellular apoB was striking. As compared to normal individuals, apoB was clearly present intracellularly in the case of S. L., whereas in M. P. the amount of apoB present was markedly decreased. Thus, as exemplified by these patients and by reports in the literature (29–32), the amounts of apoB that can be detected intracellularly appear to vary widely. The origin of this difference is unclear at present.

The presence of a normal-sized apoB-48 in these patients is consistent with the results of our analysis of the patient's RNA and DNA. The restriction fragment analysis of the apoB genes in these patients, using cDNA clones spanning the entire gene, revealed no major insertions or deletions. Futher, the pattern of restriction fragment length polymorphisms differed considerably, suggesting that abetalipoproteinemia in these patients is not associated with particular alleles of apoB as defined by restriction fragment length polymorphisms. These results are consistent with those of Talmud et al. (33) who have suggested, on the basis of linkage studies of two families with abetalipoproteinemia, that the apoB gene is not involved in this disease. Further, we have shown that the intestinal apoB mRNA in the patient M. P. was of normal size. However, as opposed to the results of Lackner et al. (32) in which hepatic apoB mRNA levels were increased five- to six-fold in some abetalipoproteinemic patients, the intestinal apoB mRNA levels in M. P. were slightly decreased. The reason for this difference is not clear.

Taken together, these results suggest that in S. L. as well as in M. P. the B apolipoproteins normally involved in the formation of chylomicrons and VLDL are synthesized since they are recognized by our polyclonal and/or monoclonal antibodies. With respect to apoB-48, these patients recall several cases of Anderson's disease (21) or chylomicron retention disease (22, 23) in which, although apoB-48 and chylomicrons are absent from the plasma even after a fatty meal, the protein is readily demonstrated in intestinal cells. However, in Anderson's disease the defect seems to involve primarily intestinal cells since apoB-100 is present, although in decreased amounts, in the plasma.

Although apoB is clearly present in the intestinal cells of our patients, the large (0.4–0.5 μm) lipid particles observed do not appear to represent chylomicrons but rather free fat in the cytoplasm. According to Palay and Karlin (59), after a fatty meal, chylomicrons normally appear in the enterocytes as particles of about 0.1–0.3 μm having a dense rim on their periphery. The lipid droplets in the enterocytes of our patients were not membrane bound and appeared to be free in the cytoplasmic matrix. Further, in our patients, the Golgi apparatus was empty, free of vacuoles containing lipoprotein-like structures, and no lipoproteins were observed in the intercellular spaces, either in the fed (S. L) or fasted state (M. P., S. L.). The observation that lipid droplets in the enterocytes of S. L. increase throughout the cytoplasm after a fatty meal suggests that the absorption of fatty acids into the intestinal cells occurs with subsequent esterification into triglycerides but without the formation of chylomicrons. The exit of postprandial lipid droplets might proceed by a reverse pinocytosis mechanism involving membrane fusion as suggested by Dobbins (28). Alternatively, there might be exit of lipids via intestinal HDL synthesis and secretion.

Since a normal apoB-48 is synthesized and triglycerides are apparent intracellularly, some other factor necessary for the assembly and secretion of apoB-containing lipoproteins is apparently missing. With respect to other apolipoproteins, the presence of apoA-I and apoC-III was demonstrated in our patients by immunohistochemistry. Schwartz et al. (60) also detected apoA-I in the enterocytes in two cases of abetalipoproteinemia whereas Green et al. (56) in three other cases did not. A generalized defect in glycosylation seems to be ruled out in our patients since apoB glycosylation and the glycosylation and processing of two enterocyte membrane hydrolases were apparently normal. Studies with rat and chick hepatocytes in culture (61, 62) also suggest that glycosylation is not ne-

cessary for lipoprotein secretion. Acylation, phosphorylation, or some other factor in the assembly process might be implicated. Clearly, further studies of patients such as these should help broaden our understanding of the factors normally involved in the formation and secretion of macromolecular assemblies such as lipoproteins.

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