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Case report

Subclinical course of cholesterol ester storage disease (CESD) diagnosed in adulthood

Report on two cases with remarks on the nature of the liver storage process

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Summary. An extremely benign variant of cholesterol ester storage disease (CESD) was diagnosed in two female patients aged 43 and 56 years. In one of them the course was entirely subclinical until a stroke at the age of 47, most probably a complication of secondary hyperlipoproteinaemia. The diagnosis was made accidentally in vivo during extensive examination for concomitant monoclonal gammapathy. The other patient (aged 56), still displays a fairly stable course with minor dyspeptic symptoms. The clinical findings in both patients were confined to moderate well tolerated hepatomegaly, hyperlipoproteinaemia of IIb type and xanthelasmata. Acid lipase activity was markedly deficient in peripheral leukocytes and cultured fibroblasts. These cases represent a rare adult variant the existence of which should be borne in mind in the differential diagnosis of chronic liver disease in advanced age and of hyperlipoporteinaemic states. The diagnostic criteria for the routine clinicopathological steps are summarized with emphasis on a special lipopigment deposition pattern, encompassing inhibition and modification of lipofuscin generation in hepatocytes and an excess of ceroid production in both portal and intralobular histiocytes. The varied ultrastructural appearance of the lysosomal limiting membrane complex is described.

Key words: Acid lipase deficiency – Adult variant – Hepatocytic lipofuscin – Ceroid

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Introduction

The protracted form of acid lipase deficiency, originally called cholesterol ester storage disease (CESD; Lageron et al. 1967), is the second main allelic variant in the acid lipase genetic locus mutation (Hoeg et al. 1984), the first being the classical fatal Wolman's disease (Wolman et al. 1961). For unknown reasons and in contradistinction to current practice the eponym Wolman is used only for the fatal variant first recognized.

CESD is usually diagnosed much earlier in life, during the first or second decade (Assman and Fredrickson 1983). The oldest case (aged 57) was diagnosed postmortem by Dincsoy et al. (1984); patients aged 43 (Lageron et al. 1967) and 46 (Lageron et al. 1975) have been diagnosed during life. We would like to add two more patients whose condition was diagnosed in advanced adulthood during protracted subclinical course.

Case reports

Patient No 1. V.P., female, born 1938. Aged 15 she was hospitalized for infectious hepatitis which resolved completely. At age 30 she observed the first eruption of eyelid xanthelasmata, which were surgically removed but recurred. At that time hypercholesterolaemia (around 10 nmol/l) and increased triglycerides (2.82 nm/l) were found. This, with an increase in both beta- and prebetalipoproteins led to a diagnosis of IIb hyperlipoproteinaemia. The blood lipid levels were resistent to dietary restriction of fat, despite a substantial weight reduction (from 82 to 65 kg). In 1981 there was a brief spell of uncomplicated upper respiratory infection followed by persistently increased ESR (75 mm/h; normal 3–8) for which she was hospitalized. On admission she reported no subjective complaints. There was moderate hepatomegaly (3 cm below the costal margin). The spleen was not palpable. Corneas and ocular fundi were normal.

There were eyelid xanthelasmata and moderate varicosities on the lower extremities. Laboratory findings were normal (blood sugar, liver function tests ALT, AST, ALP, GMT, bilirubin): although thymol was increased to 26 units (normal 5–7 units). There was slight eosinophilia 0.19×10^9/l. Renal function and urinalysis were also within normal range as was the blood pressure (110/80 mmHg). Serum protein electrophoresis showed an increase in gammaglobulins which was further specified as due to monoclonal gammapathy of the IgM type (IgM > 268 milliunits). The bone marrow examination did not show any quantitative abnormalities in any haemopoietic component including plasmocytes. There was discrete uniform vacuolization of plasmocytes and monocytes and occasionally of lymphocytes, accompanied by increase in acid phosphatase activity. Typical foam cells and "sea-blue" histiocytes were rare but raised the suspicion of a storage disease. The subsequent liver biopsy and fibroblast culture led to the diagnosis of acid lipase deficiency. The patient was discharged with the following diagnosis: Acid lipase deficiency, hyperlipoproteinaemia of IIb type without signs of vascular disease, monoclonal gammapathy of the IgM type (benign paraproteinaemia). There was no further contact. She died at 47 after a cerebral stroke.

She had no children, and two stepbrothers. Her mother is reported to have had eyelid xanthelasmata.

Patient No 2. B.C. was born in 1933 and was female. She sought medical advice at 53 years of age when she presented with abdominal discomfort classified first as biliary dyspepsia. The liver was found to be slightly enlarged without focal lesions. The spleen was of normal size. There were skin lesions of xanthelasma type with fresh psoriasiform eruptions. X ray of the lung and of the abdominal region did not show any abnormalities. The following tests gave normal results: ECG, blood pressure, urinalysis, blood sugar, haemogram, bilirubin, alkaline phosphatase, liver function tests (ALT was in the upper normal range), urea nitrogen, and total protein. Australian antigen was negative. The first liver biopsy (October 1986) was interpreted as showing chronic persistent hepatitis. The ESR was moderately increased on repeated examinations (around 30–40 mm/h; normal 3–8). There was constant hypercholesterolaemia (values oscillated around 12 nmol/l). The serum lipoprotein pattern corresponded to type IIb hyperlipoproteinaemia. Alpha-2 globulins were slightly increased, gamma globulins were in the upper normal range. There was slight but distinct and uniform vacuolization of plasmocytes and monocytes, occasionally of lymphocytes with increased acid phosphatase activity in the bone marrow smears. Neither typical storage cells nor "sea-blue" histiocytes were observed. The final diagnosis (February 1988) was: chronic persistent (agressive?) hepatitis with biliary dyspeptic and neurastenic syndrome, hyperlipoproteinaemia IIb. The second liver biopsy (February 1988) was taken for reassessment of the liver changes, because of the suspicion of storage disease raised by revision of the first biopsy. Her two siblings and three children are reported healthy. Her parents died at an advanced age, the cause of death being unknown.

Material and methods

The liver biopsy specimens were examined using standard sections with PAS, Sudan Black B, aldehydefuchsin after peroxidation with acidified permanganate and with Gomori's hot methenamine solution (90–120 min at 56° C, without preoxidation step). Lipid histochemistry carried out in cryostat sections of unfixed tissue encompassed: autofluoresence (unstained sections mounted in glycerin), birefringence (unstained sections mounted either in glycerin-gelatine, glycerine alone, or into Apáthy's syrup either unpretreated or after extraction with anhydrous acetone), Schultze's method for cholesterol, Sudan Black B (in 70% ethanol). Extractions: anhydrous acetone (Elleder and Lojda 1971), total lipid extraction with chloroform methanol (2:1 v/v) for 30–60 min at room temperature.

The enyzme activities examined were: acid phosphatase (Naphthol ASBI phosphate, Lachema, CSSR) with aqueous and semipermeable membrane techniques, beta-glucuronidase (Naphtol ASBI-beta-D-glucuronide, Koch and Light) and E600 resistent acid esterase (1-naphthylacetate, Lachema, CSSR), both using the semipermeable membrane technique. For further details of the methods see Lojda et al. (1979). Incubation time: overnight at 37° C, E600 resistent acid esterase also for 1 h.

Ultrastructural examination was done after double fixation with phosphate buffered 10% paraformaldehyde and 1% osmium tetroxide, dehydration with ethanol and embedding in an Araldite-Epon mixture. The sections were contrasted with uranyl acetate and lead citrate. Part of the aldehyde fixed tissue were examined after delipidization with chloroform-methanol (2:1 v/v) (Elleder and Šmíd 1977) and compared with ultrastructure in fragments of paraffin embedded liver tissue which were deparaffinized in xylene and osmicated with 1% OsO_4 in CCl_4 (overnight at room temperature).

Lipid chromatography of apolar lipids, extracted twice with chloroform-methanol (2:1 v/v), was carried out on Silicagel precoated plates (Merck, Darmstadt, FRG). Sovlent mixture: hexane:ether:acetic acid 90:10:1. Detection: iodine vapours. Acid lipase activity was measured with 4-nitrophenyl laurate (Sigma) using a slightly modified technique of Beaudet et al. (1974) in samples of peripheral leukocytes and cultured skin fibroblasts. The assay contained in a final volume of 0.2 ml: 100 mM sodium acetate buffer (pH 4.5), 1 mM EDTA, 1.5% Triton X-100, 0.05% bovine serum albumin, 4 mMol 4-nitrophenyl laurate, 100–130 µg of leucocyte protein (40–60 µg of fibroblast protein) and was incubated for 60 min at 37° C. The reaction was stopped by addition of 1.3 ml of 0.2 M Tris-HCl buffer pH 9.0; tubes were centrifuged 10 min at $1000 \times g$ and absorbances of liberated 4-nitrophenol were measured immediately at 410 nm. Control enzymes were beta-galactosidase and beta-hexosaminidase, measured with MU derivatives (Sigma) according to Suzuki (1978).

Results

The majority of the liver biopsy findings were identical in both cases and will be reported together. The architecture of the tissue was almost normal with slight periportal fibrosis and accumulation of storage macrophages mostly with granular cytoplasm. These were dispersed individually or in small clusters throughout the lobules, and infiltrated also the portal spaces. There were also portal lymphocytic infiltrates, scarce in the first case but focally prominent in the second case (Fig. 1 A). The cytoplasm of hepatocytes was finely vacuolated, but not typically foamy (Fig. 1 B) and was light due to a slight excess of glycogen. The degree of vacuolation was less in the second case. In both there was a remarkable paucity of lipofuscin (see Fig. 1 B) the remnants of which were best revealed with Gomori's hot methenamine solution (Fig. 1 C–E).

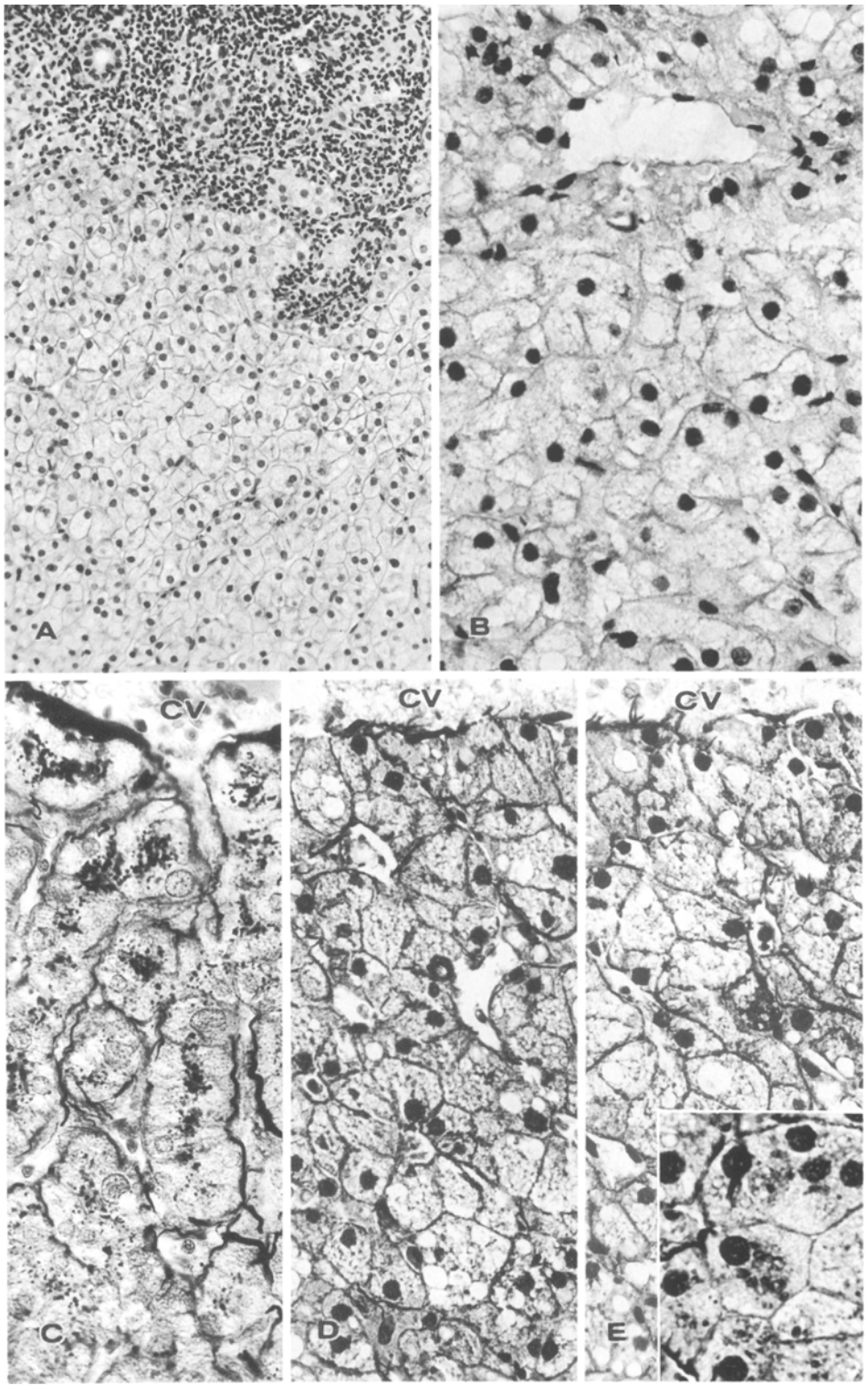


Fig. 1. A Persistent hepatitis with "innocent" appearance of hepatocytes H & E, ×160. **B** Detail of the cetrizonal area showing microvacuolation of hepatocytes and absence of lipofuscin H & E, ×400. **C–E** Detection of lipofuscin with hot silver methenamine. **C** Control (female aged 53) with normal gradient of lipofuscin accumulation toward the central vein (CV). **D** case 1, **E** case 2, both with absence of detectable solid granules of lipofuscin and with rudimentary ring-like lysosomal accretions stained positively (cf. with Fig. 3A). Mag. **C–E** ×400. *Inset* shows details of exceptionaly advanced deposits in a hepatocyte focus with markedly decreased intensity of lipid storage ×600

Lipid histochemistry (Fig. 2) showed a mass of highly anisotropic acetone-soluble apolar lipid both in hepatocytes, less so in histocytes. The anisotropy of the lipid droplets was of Maltese-cross type, which, in cryostat sections, changed relatively rapidly to solid crystals when glycerin-gelatine was used for mounting, or totally disappeared after mounting into Apathy's syrup. They were most stable in glycerin mounted sections. Histiocytes contained a large amount of ceroid with a high

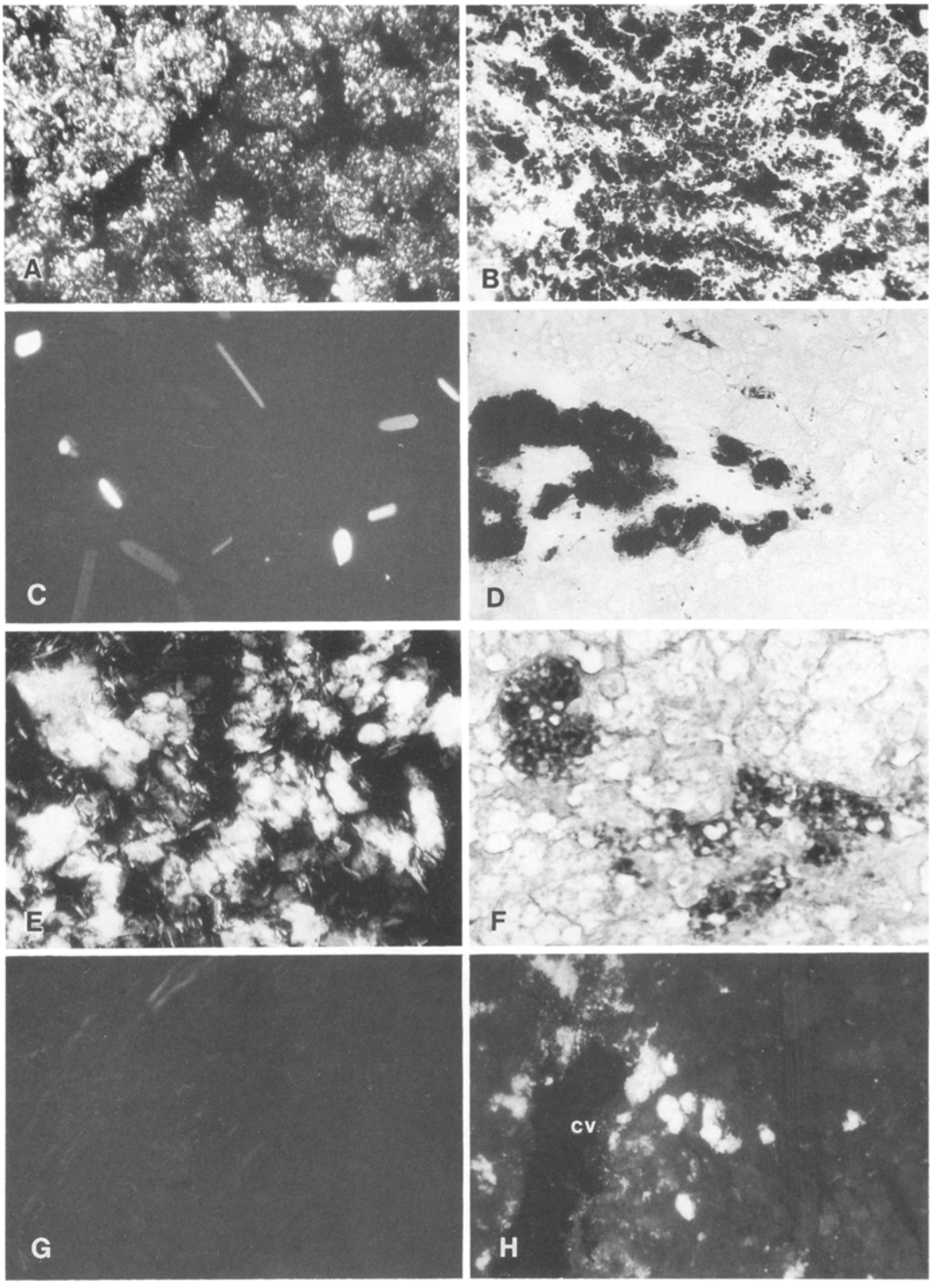


Fig. 2. A, C, E, G Birefringence patterns caused by different mounting media. **A** Cryostat section of the liver freshly mounted into Apathy's syrup. Prevalence of small cholesterol ester liquid crystals with Maltese-cross type birefringence. **C** the same section next day. Birefringence persisting only in scarce solid crystals. **E** liquid-solid transformation after several hours in glycerin-gelatine. **G** abolition of the birefringence by anhydrous acetone (section mounted in glycerin-gelatine; cf. with **E**). **B, D, F, H** lipid – lipopigment differentiation in the storing liver sample. **B** massive staining of all deposits with Sudan Black B. **D** only ceroid in histiocytes stains strongly after preextraction with acetone. Ocasionally there are individual fine lipofuscin granules in the sinusoidal endothelium stained positively. **F** vacuolized or ring – like staining of histiocytic ceroid in paraffin section which provides better detail. **H** intense autofluorescence of histiocytic ceroid in UV. Note the absence of autofluorescence over hapatocytes with the exception of slight autofluorescence in pericentral hepatocytes (CV-central vein). **A–H** ×160

Table 1. Enzyme activities in diagnostic samples (expressed in nmol/mg/h; values in parentheses represent percents of control) mean

	Peripheral leukocytes			Cultured fibroblasts		
	acid lipase	beta-gal	beta-hex	acid lipase +	beta-gal	beta-hex
Case 1	5 (<1%)	175	1250	84	ND	ND
Case 2	20 (3%)	238	1708	18 (<2%)	657	1400
controls	668±34 x̄±SEM n=10	183±9 x̄±SEM n=30	1231±52 x̄±SEM n=30	1242 850	566 640	8200 12090

ND, not done; + meassured in Prof. A.D. Patrick's laboratory (control range was 1800–7200 nmol/mg/h). Activity of b-galactosidase was within normal range

degree autofluorescence and sudanophilia. In cryostat sections the ceroid was partly soluble in chloroform methanol (see also Elleder 1981). It was present either in the form of solid granules or in a ring-like form on the lysosome periphery causing the rather typical vacuolated appearance of ceroid-bearing histiocytes (Fig. 2F). Only rudimentary deposition of lipofuscin could be proved hepatocytes as occasional rims around the lysosomal vacuoles best revealed with Gomori's hot methenamine (see insert in Fig. 1). Occasional lipofuscin granules were present in the sinusoidal endothelium (Fig. 2D).

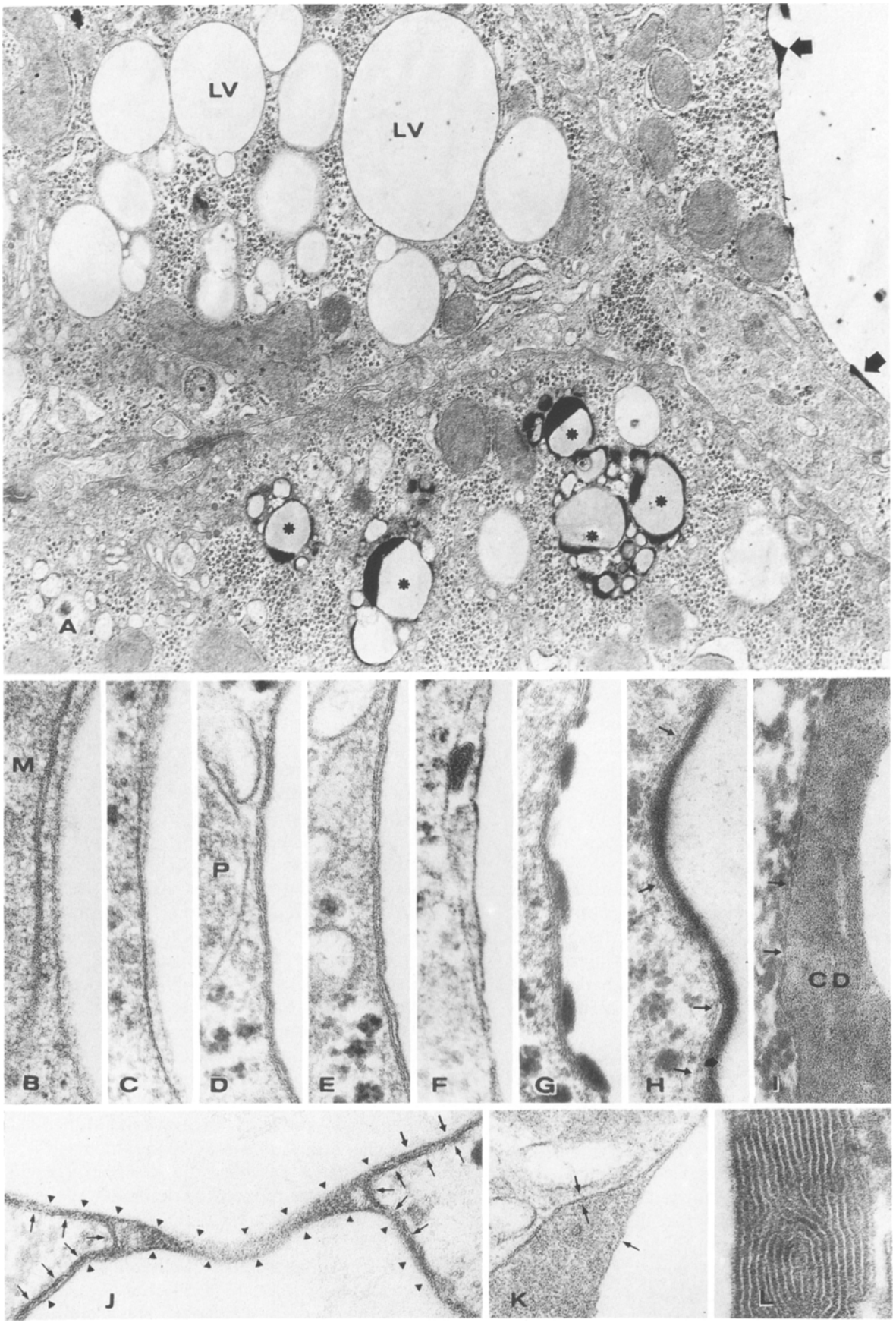
Acid phosphatase in an aqueous medium was moderately increased in hepatocytes and markedly so in histiocytes where it was tartarate resistent in part. Using the semipermeable membrane technique the activity was generally stronger and was localized in most of the hepatocytic cytoplasm. Beta-glucuronidase activity was increased, that of beta-galactosidase practically unchanged. Acid esterase activity (E600 resistent) in aqueous medium was entirely negative as it was in the controls due to enormous solubility of the enzyme (Lake, personal communication). With the semipermeable membrane technique the activity in hepatocytes was greatly decreased or absent but was relatively strong in small sinusoidal cells.

Lipid chromatography carried out only in the first case showed a large increase of cholesterol esters. Triglycerides and cholesterol were not increased.

The ultrastructure was dominated by a number of hepatocytic lipid vacuoles which possesed a relatively thick (about 20 nm) conspicuous boundary, and a lucent homogeneous low-density content sometimes concentrated on the periphery. The border region consisted of a classical single unit membrane 7.7–7.8 nm wide. There was, on its internal side, another layer heterogeneous in appearance, consisting either of a membrane of varying thickness (6.4–12.8 nm) or of a narrow blurred band of moderate density (see Fig 3). The two layers were occasionally separated (Fig. 3F) or the space between them was often progressively distended by deposition of hemidense (Fig. 3K) or dense almost homogeneous material in a crescent or rim-like fashion. In larger deposits the inner layer ceased to be discernible (Fig. 3H). The process, when advanced was associated with vacuoles coalescing into larger aglomerations, giving rise to lipofuscin-resembling structures. This corresponded well with similar structures revealed by lipofuscin staining methods in paraffin sections (see Fig 1). Moreover, the process was more intense in the second case and was more expressed in centrizonal hepatocytes. The deposits were very much denser then the residual stored lipid. Typical lipofuscin was absent. Very often, there were small dense humps attached to the inner layer of the lysosomal membrane complex (Fig. 3G).

Histiocytes showed deposition of the ceroid mass which occurred mostly on the lysosomal periphery, the deposits being homogeneous with the focal membranous trilaminar structure (12.8 nm width). Occasionally stacks of these membranes fused into a crystaloid structure with a periodicity of 8.9 nm. Individual membranes were hapzardly orientated throughout the homogenous interior of the ceroid mass; sometimes they were parellel to the lysosomal limiting membrane (8–9 nm thick). Simple lipid storage with the above mentioned features (see hepatocytes) was infrequent.

In delipidized samples the peripheral membrane complex was reduced to remnants of the external unit membrane. The contents of the vacuoles content disappeared completely. The dense or hemidense lysosomal deposits in hepatocytes were significantly less dense (Fig. 3I) but remained in situ, and infrequently a lamellar substructure ap-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peared (Fig. 3L) not appearent in unextracted specimens. The histiocytic ceroid deposits were resistant to extraction. Their overall density was possibility decreased.

Acid lipase was found to be deficient (see Table 1).

Discussion

Two items are worth discussing: firstly, the atypical clinical picture of CESD, and, secondly, some liver changes which besides being diagnostically useful are also interesting from the biological point of view.

Eight families with ten cases of CESD have been diagnosed in Czechoslovakia so far. One of them, in the lower age group has been published (Hanák and Elleder 1984). This contrasts with the infantile phenotype which has not been diagnosed so far in this country. The frequency of the CESD subvariant diagnosed in advanced adulthood is surprisingly high in our series (20%), and our second patient is probably the oldest living case at the time of diagnosis. This contrasts with only three adult cases (mentioned above) described so far who, morover, were manifestly ill. The 67 year old case of Lageron et al. (1984) is diagnostically uncertain as the atypical liver changes would need biochemical, rather than histochemical, enzyme verification.

The clinical picture in our two cases represents a remarkable difference from the disease pattern of CESD which classically is dominated by relatively pronounced liver symptomatology with earlier progression toward cirrhosis (Assmann and Fredrickson 1983), and probably represents a new subvariation of this condition. The discrepancy between the subclinical course and very low values of the residual enzyme activity (proved independently in two laboratories, see Table 1) is not surprising and is in accord with other reports (Besley et al. 1984; Hoeg et al. 1984). A secondary increase in neutral lipase activity in CESD may explain the protracted course of this variant (Hoeg et al. 1984).

The diagnosis of CESD in our first case was reached accidentaly during extensive haematological examination for monoclonal gammapathy, probably unrelated to the genetic storage disorder. The metabolic disorder was entirely subclinical and even laboratory tests gave no reason for alarm. Nevertheless, the rudimentary bone marrow storage together with a history of an episode af jaundice led the clinician to undertake liver biopsy which was diagnostic. The cause of death may have been related to cerebral atherosclerosis accelerated by hyperlipoproteinaemia which is considered to be a risk factor (Assmann and Fredrickson 1983). Diagnosis in the second case was initiated by a routinely taken liver biopsy for suspicion of chronic persistent hepatitis. The significance of the focal chronic inflammatory changes in the liver is unclear. They have been described in CESD (Assmann and Fredrickson 1983) and are probably secondary in nature. Even the bout of "hepatitis" (case 1) might have been secondary to the lysosomal disorder and not necessarily of viral origin (see also D'Agostino et al. 1988; Lageron et al. 1985).

The close association of CESD with type II hyperlipoproteinaemia is well known (Assmann and Fredrickson 1983). It appears that each patient with this disorder should be screened by the leucocyte enzyme assay for the oligosymptomatic homozygous form of CESD or even for the carrier status of the disease (see also Wolf et al. 1974). Whether or not CESD might cause a disturbance in Ig production is a moot point and requires further study.

The storage pattern in the liver in CESD has been throughly described (Künnert et al. 1979; Pfeifer and Jeschke 1980; Lageron et al. 1985). Still there are several point worth adding, especially the extreme difference in lipopigment production between hepatocytes and histocytes under identical conditions of cholesterol ester storage. While the lysosomal compartment of the latter

Fig. 3. Survey of ultrastructural pathology of hepatocytes. **A** Lipid vacuoles (LV) with conspicuous limiting membranes and abortive lipofuscin generation (*asterisks, arrows*) here of exceptional intensity (*asterisks*) ×20000. **B–H** Variations of the limiting membrane complex. Unchanged appearance of the external membrane of the unit type identical with peroxisomal (P) membrane and with each one of the mitochondrial (M) membran pair. Variable appearance of the inner layer which is absent or in form of disorganized densities (**B,C**), or as a thick closely apposed (**D**) or slightly detached thick line (**E**). **F** separation of both layers. **G** dense humps attached to the inner layer. **H** continuous depositions of dense material substituting the inner layer. Outer membrane marked by *arrows*. **I** *delipidized* sample (paraffin embedded) showing decreased density of extraction resistant crescentic deposists (CD) on the outer limiting membrane (*arrows*). **J** two coalescing lipid lysosomes with continuous external unit membrane (*arrows*) and still discontinuous internal layers (*arrowheads*). **K** deposition of hemidense amorphous material between the external unit membrane (double arrow) and the internal layer (*single arrow*). **L** deplipidized sample (paraffin embedded originally) showing demasked lamellar structure and decreased density of the originally homogeneous dense crescentic deposit. Magnifications: **B, C, D, E, F, H, L** 76000; **G, J** 90000; **I** 44000

is highly stimulated to produce an excess of the ceroid type variant, the same compartment in the former cell type does not produce any type of lipopigment. On the contrary, current lipofuscinogenesis is suppressed. This clearly points ot fundamental pathogenetic differences between the two main lipopigment types. Suppressed lipofuscin generation in hepatocytes has been also described in other lysosomal storage disorders (Elleder 1987) and might thus be related to the storage mediated activation of lysosomal function interfering with the spontaneous lipofuscinogenesis.

Rudimentary pigment production may be represented by dense focal or circumferential deposits on the inner aspect of the lysosomal membrane. An argument in favour of this is that the dense structures were partly extraction resistent and displayed a masked lamellar structure. Extractable free lipid (stored?) may have been responsible for the original high density as it has been described previously (Elleder et al. 1984). The crescentic-shaped dense accretions correspond well with similar structures in the lysosomal periphery in paraffin section stained with methods for lipofuscin (Fig. 1C, E, and 2H). Their constant relationship to the lysosomal unit membrane suggests they might be seen as its product (Wolfe et al. 1987).

The structure of abortive lipofuscinogenesis strongly resemble the so-called lipolysosomes described by Hayashi et al. (1977) and considered to be a consequence of an influx of apolar lipids in liver steatosis into the lysosomal compartment as described experimentally (Nenemiah and Novikoff 1974). The picture published (Hayashi et al. 1977) suggest that the influx passes into a small fraction of lipofuscin harbouring lysosomes. Theoretically, this process, when sufficiently intensive, might negatively influence the lipofuscin deposition rate in hepatocytes. However in a series of steatotic livers we were unable to prove any such effect at the light microscopy level (unpublished observation).

As for the organization of the lysosomal boundary region our results differ slightly from those of Pfeifer and Jeschke (1980) regarding the inner layer. Its heterogeneity and occasional absence correspond well to the findings of Ferrans and Fredrickson (1975) who also described the varied appearance of the edge of the cytosol cholesterol ester droplets in Tangier's disease. This suggest that the arrangement of the inner layer of the peripheral membrane complex in acid lipase deficiency may be caused by pure physical phenomena of uneven intensity which are probably heterogeneous (including lipid peroxidation).

The findings of lipid histochemistry point to the hitherto undescribed effect of various mounting media on the birefringence of the cholesterol ester liquid crystals. This suggests that the birefringence is of the "form" type (Wolman 1975) as distinct from the spherocrystals of the spingolipids (Elleder 1988).

Due to the equivocal clinical presentation of the CESD adult variant, liver biopsy may provide a clue to diagnosis. It should be stressed that the intensity of storage in these atypically benign cases may be low, and especially when combined with an excess of glycogen, the histology may be quite unimpressive. Concommitant lymphocytic infiltration can further blur the picture so that even an experienced pathologist may miss the diagnosis of lysosomal storage in routine sections.

We recommend, therefore, careful assesment of the lipopigment deposition pattern. The absence or paucity of lipofuscin in hepatocytes displaying microvacuolation in a middle aged patient should always raise a strong suspicion of lysosomal lipid storage, especially of CESD which is unique in its tendency to affect hepatocytes even in very slowly advancing variants. This in combination with an excess of ring-like ceroid in clustered histiocytes is highly suggestive of CESD. The liver changes in adults with a protracted form of sphingomyelinase deficiency rarely attain the intensity of CESD in the hepatocytes (Elleder 1989) and histiocytic ceroid is in the form of solid granules mostly.

Bone marrow is not so reliable for diagnosis. Nevertheless we would stress the new finding of vacuolation of plasmocytes seen in both of our cases and in the previously published early-onset case (Hanák and Elleder 1984). Even low-grade bone marrow storage should rise a suspicicion of CESD and, if there is an indication for liver biopsy, the specimen should be examined by a multi approach study, including lipid histochemistry which is capable, using a battery of suitable methods and control steps, of providing a diagnosis within one hour after the delivery of the specimen.

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