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Case report

Fetal Niemann-Pick disease type C: ultrastructural and lipid findings in liver and spleen

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Abstract. We present the first ultrastructural study of liver and spleen from a 20-week fetus with Niemann-Pick disease type C in correlation with lipid studies of these tissues. The lipid storage pattern was characteristic of the disease and although the distribution of the lipid storage was similar to that of affected children, ultrastructural studies emphasized that many inclusions were qualitatively different. These are discussed. Concomitant with this complex lipid storage, ultrastructural evidence of cholestasis was observed and the early hyperplasia of pericanalicular microfilaments leads us to question the presence of a toxic metabolite which might induce cholestasis by acting upon microfilaments.

Key words: Niemann-Pick disease type C – Fetal liver – Fetal spleen – Ultrastructural pathology – Cholesterol

Introduction

Niemann-Pick disease type C (NP-C) is an autosomal-recessive neurovisceral lipid storage disease biochemically distinct from the primary sphingomyelin lipidoses (Niemann-Pick disease types A and B; NP-A and NP-B), with which it has traditionally been grouped (Spence and Callahan 1989; Vanier et al. 1991a). Recent studies have convincingly shown that the disorder is characterized by a unique error in cellular trafficking of exogenous cholesterol associated with lysosomal accumulation of cholesterol (Pentchev et al. 1987; Sokol et al. 1988; Liscum et al. 1989). This discovery has led to the development of reliable methods for postnatal and prenatal diagnosis of the disorder (Vanier et al. 1991b, 1992). Yet the primary molecular defect in NP-C remains elusive, and the pathophysiology of both the cerebral and the hepatic lesions is poorly understood. In liver and spleen of children in the terminal stage of the disease, several lipids are increased, with no compound predominating (Spence and Callahan 1989). Studies at the fetal stage are necessary for delineation of the disease. In this particular case they should also be helpful to differentiate changes directly related to the primary lesion from secondary changes. We present here the lipid storage patterns of liver and spleen associated with early cholestatic lesions in a 20-week fetus affected with NP-C.

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Materials and methods

Tissue samples from the affected fetus including liver, spleen, frontal and occipital brain, cerebellum and arm skin were taken. Pieces of every tissue were fixed separately in 2.5% cacodylate-buffered glutaraldehyde (pH 7.4), postfixed in 1% cacodylate-buffered osmium tetroxide (pH 7.4) and stained "en bloc" with 2% aqueous uranyle acetate. After washing, tissues were dehydrated in graded ethanols and embedded in araldite. Semithin sections of 1 μm prepared with a Reichert OMU3 ultramicrotome were stained with toluidine blue and 5% silver solution, which gives good contrast. Ultrathin sections were contrasted with lead citrate and examined on a Jeol JEM 1200 Ex electron microscope. The remainder of each tissue was fixed in formalin. Paraffin sections were stained with haematoxylin and eosin, periodic-acid Schiff and trichrome. On the liver, Perls staining was performed. Biopsies of brain and cerebellum were autolysed and could not be interpreted.

Tissue lipids were extracted, isolated and quantified following previously described procedures (Vanier et al. 1985).

Case report

Prenatal diagnosis was offered to a couple with a previous child affected with a severe infantile form of NP-C. The clinical history and the biochemical diagnosis of the index case have been described previously (case 11 in Vanier et al. 1988). Antenatal diagnosis performed on biochemical and ultrastructural studies of cultured amniotic cells (family I in Vanier et al. 1992) indicated an affected fetus. Termination of the pregnancy was induced at 20 gestational weeks by prostaglandin infusion. Autopsy of the fetus was performed upon delivery. Macroscopic studies of fetal organs revealed no evident enlarged liver or spleen. The diagnosis was confirmed by studies of low-density lipoprotein-cholesterol processing in fetal cultured fibroblasts.

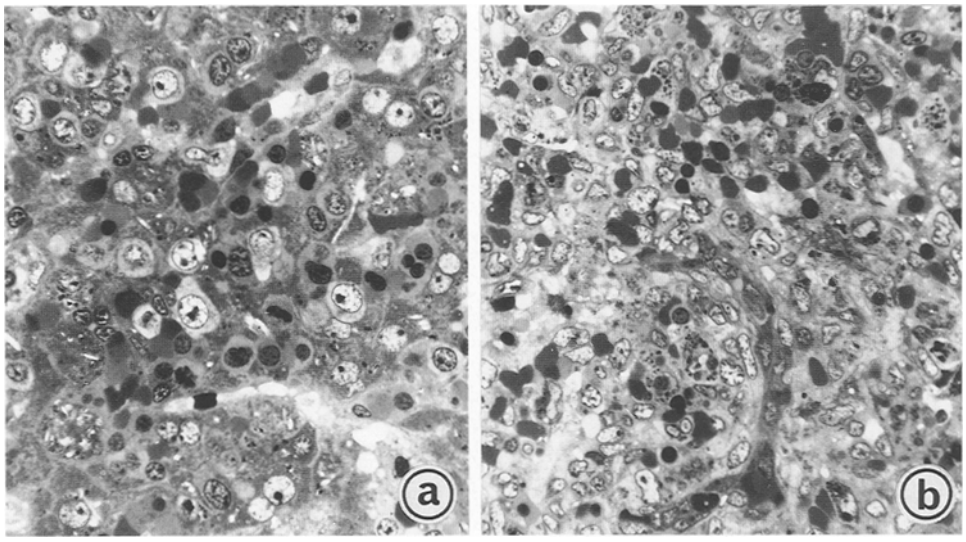

Fig. 1. a Fetal liver showing large cell cords and sinusoids sometimes obstructed by spumous macrophages. Note presence of clear clefts as an early sign of degeneration. **b** Red pulp of fetal spleen with sinus and extravascular spaces filled by macrophages with many granulations. Semithin sections stained with toluidine blue and silver solution (×1200)

Results

Changes in routine paraffin sections of liver tissue were restricted to sinusoids with scarce foamy histiocytes. No cholestasis was observed and Perls staining was negative. In semithin sections, wide blood sinusoids were obstructed by large macrophages containing many inclusions and a few clear clefts (Fig. 1a). Neither canalicular nor hepatic cholestasis or inflammation or fibrosis was noted. No spumous cells were seen in the spleen in paraffin sections. In semithin sections both sinus lumina and extravascular spaces of red pulp contained macrophages with numerous inclusions (Fig. 1b). White pulp, which was hardly developed, showed no storage.

Electron microscopy of the fetal liver showed that Kupffer cells and macrophages exhibited evident storage changes of varying degree. Some were foam cells with many pleomorphic lysosomes, mainly variously sized plurilobulated complex bodies (0.2–2 μm) formed by vacuoles, concentric lamellar profiles containing vesicles with osmiophilic granulations and amorphous electron-dense material present in different proportions from one inclusion to another (Fig. 2a). Some lysosomes were formed by translucent material with small lamellar inclusions (membrano-lucent lysosomes). Concentric lamellar bodies were numerous. Variously shaped crystalline structures, including crystal clefts with lucent material, were present in the cytoplasm (Fig. 2a, b). In particular, many crystals were bound together with complex bodies by the same membrane (Fig. 2a).

Hepatic cells were also seen to possess inclusions scattered throughout the cytoplasm but they were much fewer in number (three to ten inclusions per cell). They were made up of complex bodies with preponderant concentric lamellar profiles forming vesicles with osmiophilic granulations and a great number of them contained crystals (Fig. 2c–e). Concentric lamellar bodies were rare and lipofuscin granules were never seen. The cytoplasmic organelles and also glycogen and fat droplets were present as is normal for the stage of development but numerous mitochondria with irregular cristae were noted. At the biliary pole, microvilli of some canaliculi appeared to have a broader base or were longer or distorted and the axes of microfilaments were always dark. Moreover, widening of the microfilamentous rim could be seen around several bile canaliculi, nearly always restricted to the pericanalicular cytoplasm (Fig. 3). Occasionally some thin bundles of microfilaments were noted in the peribiliary zone. The lumen was clear, but sometimes a granular material different from bile pigment was present. Near some bile canaliculi, vacuoles with or without membrane containing bile pigments, typically seen in cholestasis, were present (Fig. 3). No dilated canaliculi without microvilli were noted.

Ito cells were seen with neither storage nor fat droplets. A few complex bodies were noted in the endothelium of portal vessels in the interlobular space, but the bile ductule epithelium appeared normal. No storage was seen in haematopoietic cells, which were essentially erythroblasts and megakaryocytes.

In the fetal spleen, complex lipid storage was also evident, particularly involving macrophages, only a few of which were foamy. Usually lysosomes were larger than in the liver. They were essentially large complex bodies with numerous vesicles (0.4–3 μm) and variously sized concentric lamellar bodies. Some dense inclusions with an entangled membranous appearance were scattered in the cytoplasm of some macrophages. Their pattern was irregular and their borders seemed continuous with the limiting membrane (Fig. 4d). In some other macrophages membrane-bound inclusions with the same membranous appearance contained large electron-lucent cavities (Fig. 4c). Crystals were also present bound together with complex bodies by the same membrane or scattered in the cytoplasm but they were less numerous than in the liver (Fig. 4a, b). Some reticular cells with clear hyaloplasm (clear reticular cells) and endothelial cells may contain rare small complex bodies. Dark reticular cells were never stored. Similarly, no storage was seen in haematopoietic cells, platelets or blood cells.

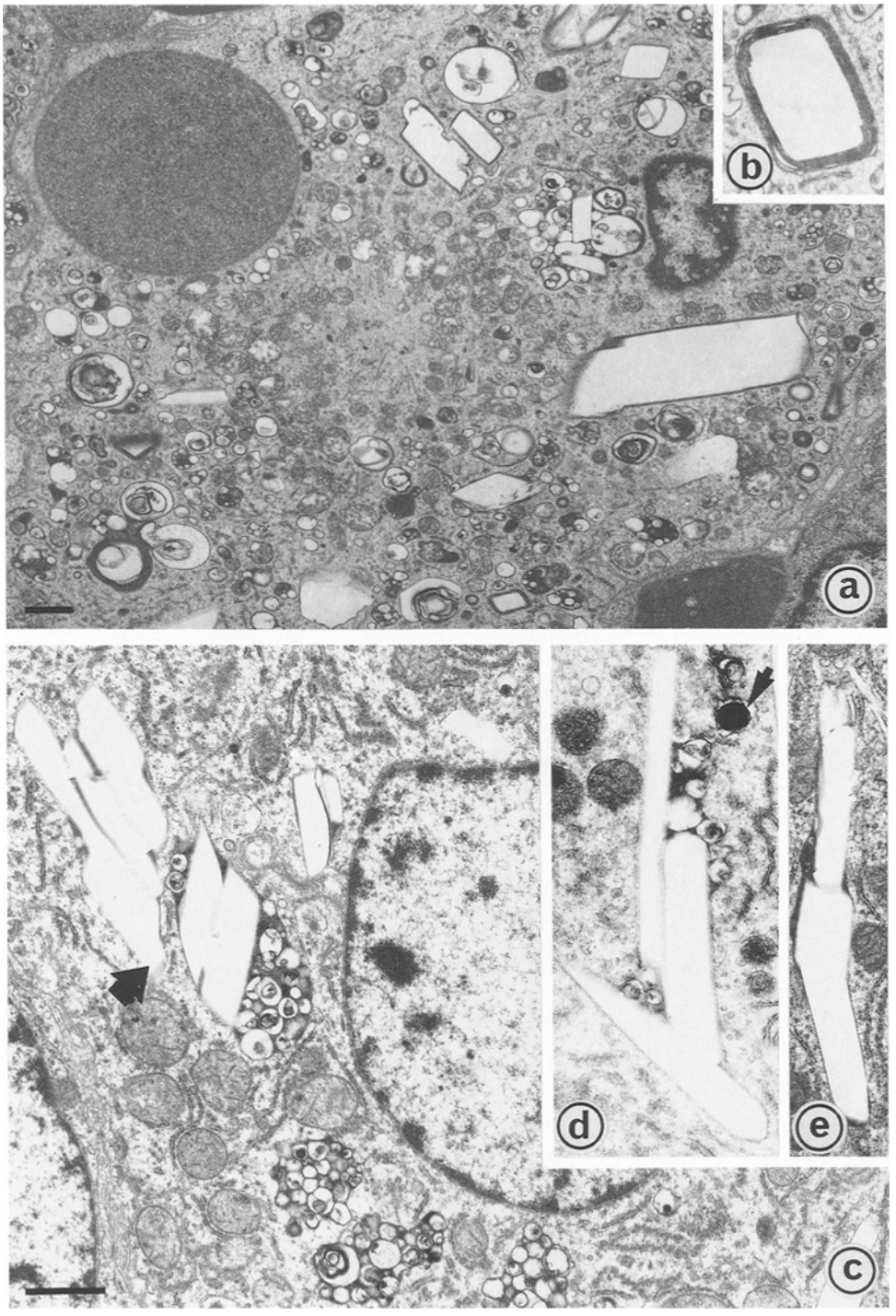

Fig. 2a–e. Ultrastructure of the fetal liver. **a** A Küpffer cell with numerous pleomorphic lysosomes and crystals. Note erythrophagocytosis. ×8500. **b** A crystalline structure in the cytoplasm of a macrophage. (×19500). **c** A hepatic cell with some complex bodies. Note numerous crystals in one of them (*arrowhead*). ×14000. **d, e** Other complex bodies with crystals. Note the cytoplasmic presence of bile pigment (*arrowhead*). **d** ×20000; **e** ×14000. Uranyl acetate and lead citrate. *Bar* = 1 μm

The concentrations of unesterified cholesterol, sphingomyelin and total phospholipids showed a two- to four-fold elevation both in liver and spleen in NP-C (Fig. 5a, b). There was no significant increase of cholesteryl esters. The profiles were very similar in both tissues, except for a larger sphingomyelin increase in the spleen. A striking feature was the elevation of the cholesterol to phospholipid molar ratio, from a normal value of approximately 0.5 to about 1.0. In NP-A fetal tissues, in spite of a larger increase in sphingomyelin and in total phospholipids, cholesterol only showed a moderate increase, and there was no elevation of the cholesterol to phospholipid ratio. Liver from a fetus with Gaucher's disease had values within the normal range for these lipids. Bis(monoacylglycero)phosphate, essentially undetectable in controls, was strikingly elevated in tissues from NP-C, and to a lesser extent in NP-A fetuses (data not shown). A paradoxical result was the eight-fold increase of glucosylceramide in NP-C fetal liver, while a value only slightly above the normal range was observed

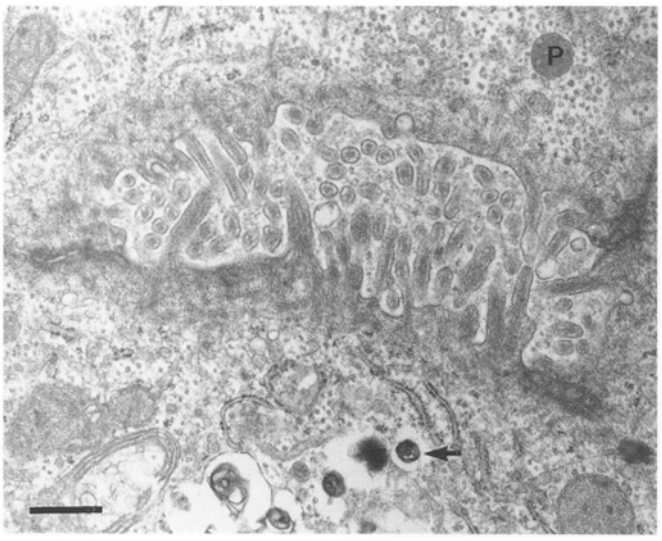

Fig. 3. Bile canaliculus bounded by three liver cells showing modified microvilli and accumulation of microfilaments in the pericanalicular cytoplasm and in the axes of microvilli. Note granular material in the lumen. *Arrowhead*, Bile pigment in a vacuole without membrane; *P*, peroxisome. Uranyl acetate and lead citrate. *Bar* = 0.5 μm. ×24000

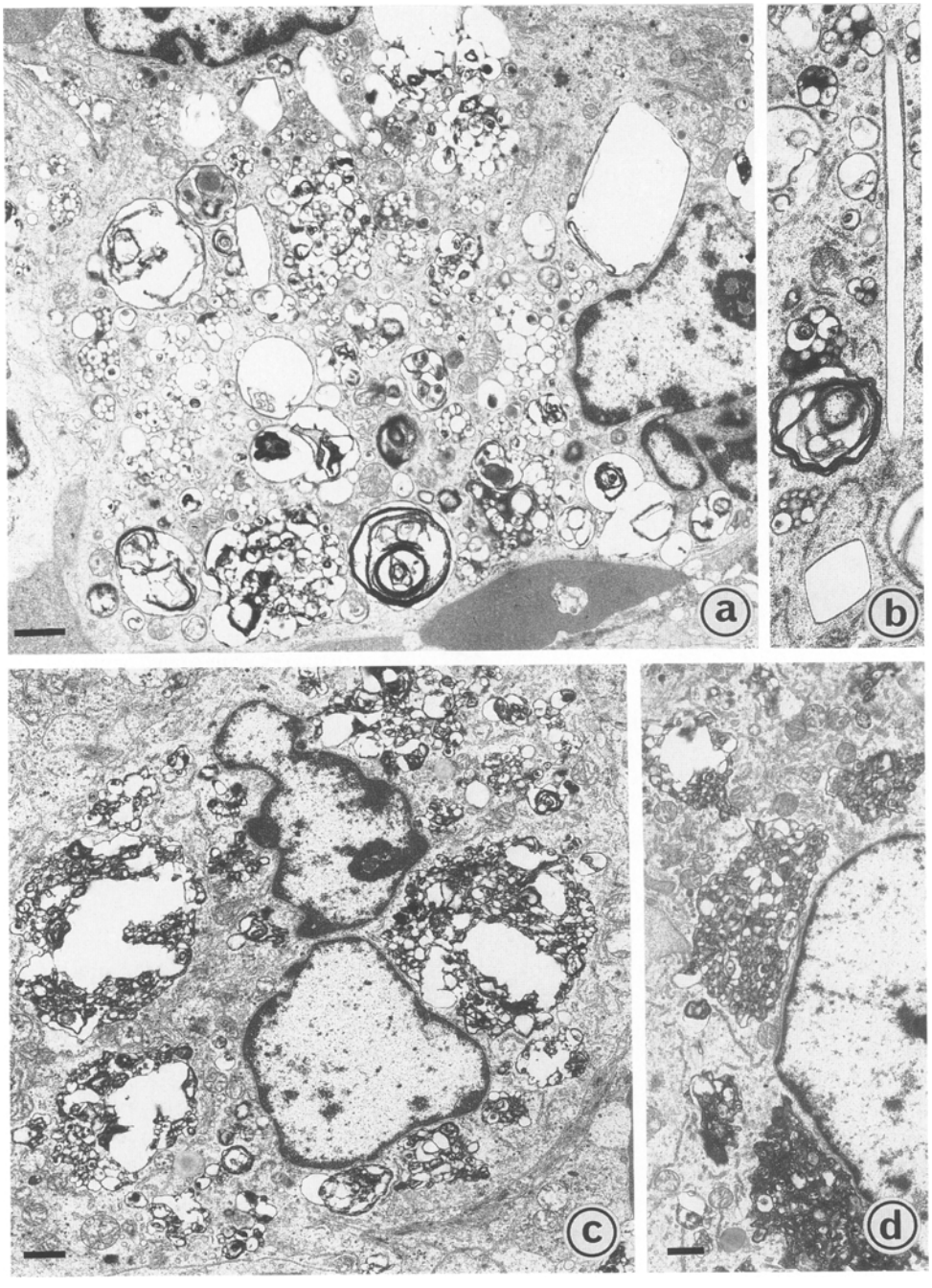

Fig. 4a–d. Ultrastructure of the red pulp of fetal spleen. **a** A free macrophage stored with large pleomorphic inclusions. ×8500. **b** Crystal cleft in the cytoplasm of a macrophage. ×14000. **c, d** Large characteristic inclusions in macrophages. **c** ×7000; **d** ×6000. Uranyl acetate and lead citrate. *Bar* = 1 μm

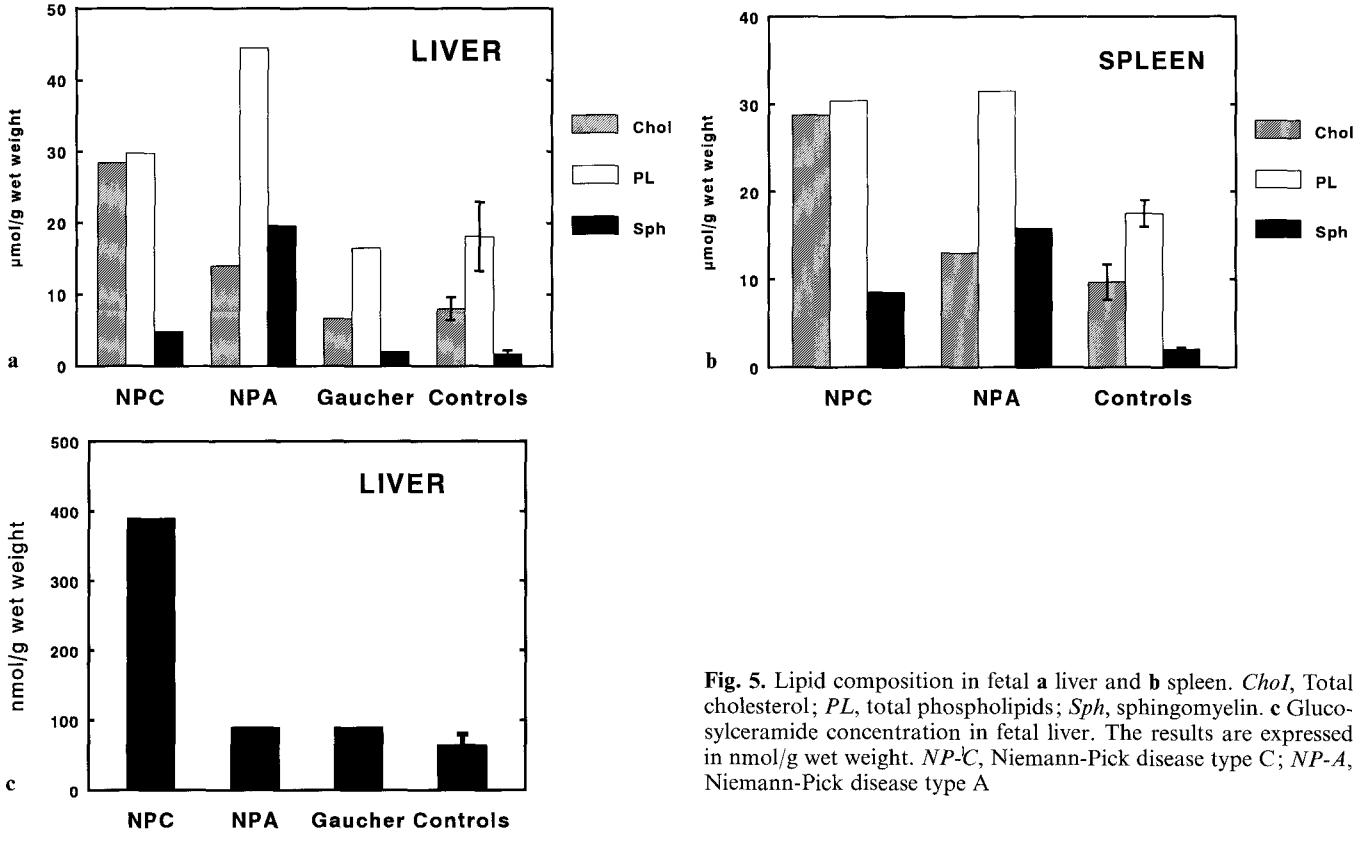

Fig. 5. Lipid composition in fetal **a** liver and **b** spleen. *ChoI*, Total cholesterol; *PL*, total phospholipids; *Sph*, sphingomyelin. **c** Glucosylceramide concentration in fetal liver. The results are expressed in nmol/g wet weight. *NP-C*, Niemann-Pick disease type C; *NP-A*, Niemann-Pick disease type A

in the liver from a fetus with Gaucher's disease, and in NP-A fetal liver (Fig. 5c).

Discussion

In the liver and spleen of this 20 week fetus affected with NP-C the complex lipid storage, characteristic of the disease, was already present. Other lipid studies of NP-C fetal liver, although less detailed, have indicated a similar trend (Harzer et al. 1978; De Winter et al. 1992; Vanier et al. 1992). The storage was almost as pronounced as at the final stage of the disease (Vanier 1983), a unique situation when compared with any sphingolipidosis suggesting a distinct mechanism of accumulation.

No other ultrastructural morphological study of fetal NP-C is known to us. The storage affected several cell types with a distribution similar to that described in liver and/or spleen (Wiedemann et al. 1972; Neville et al. 1973; Pellissier et al. 1976; Gilbert et al. 1981; Elleder et al. 1984; Witzleben et al. 1986; Adam et al. 1988; Chamlian et al. 1989). The reticulo-endothelial cells displayed the most pronounced storage, although this feature is not specific for the disease (Hers and Van Hoof 1973). More characteristic were the discrete hepatic cell changes, previously noted in several patients (Elleder et al. 1984; Chamlian et al. 1989). A normal appearance of hepatocytes was reported in one case (Pellissier et al. 1976), but in some cases the changes were quite prominent, often related to impairment of liver function associated with storage, particularly cholestasis (Elleder et al. 1984). An absence of lipid storage in Ito cells was also typical (Elleder 1984).

In contrast, in fetal liver and spleen, many lysosomes contained variously-sized and shaped crystalline structures, exceptionally described in hepatocytes of one postnatal case (Chamlian et al. 1989). These crystals were mainly observed in both reticulo-endothelial and parenchymal cells of the liver, and were found in smaller number in spleen macrophages. They occurred essentially within lysosomes but also in the cytoplasm. Most of them were similar to cholesterol crystals and its esters observed in some genetic metabolic disturbances and also in various localized troubles (Dustin and Dourov 1981). In our case, cholesteryl esters were hardly increased but there was a prominent storage of unesterified cholesterol.

These findings are very similar to those in a mutant Balb/c mouse shown to be an animal model of NP-C (Shio et al. 1982; Pentchev et al. 1984; Higashi et al. 1991). Higashi et al. (1991) suggested that the crystalline structures corresponded to the site of cholesterol deposition and the same interpretation seems logical for our observations. It is difficult to explain the absence of similar structures in postnatal human disease where cholesterol storage is also prominent. No crystalline inclusions were seen in lysosomes of fetal liver affected with NP-A (Schneider et al. 1972). Moreover, lysosomes in fetal NP-C were more electron-lucent and did not contain large membranous whorls.

Another type of inclusion observed in macrophages of the fetal spleen was made up of dark aggregates of smooth membranes (Fig. 4d). Such inclusions have never been observed in postnatal cases (Gilbert et al. 1981; Adam et al. 1988). A structural analogy may be found between them and drug-induced lipidosis inclusions, formed by membranes arranged in a reticular fashion (reticular inclusions) and observed in different tissues (Rawlins and Uzman 1970; Lüllmann-Rauch and Reil 1973; Jay-Jung and Suzuki 1978). The similarities between the lipid storage patterns of drug-induced lipidoses and NP-C have been underlined (Vanier et al. 1991 a).

In NP-C, initial and severe liver involvement, demonstrated by neonatal cholestatic jaundice with predominantly conjugated hyperbilirubinaemia and progressive hepatosplenomegaly, has been reported in almost half of the cases (Crocker and Farber 1958; Neville et al. 1973; Wenger et al. 1977; Vanier et al. 1988). Usually the jaundice regressed between the 2nd and 4th month of life and neurological impairment occurred later. In several cases, the clinical and pathological picture of "neonatal hepatitis" was always associated with lipid-laden foam cells and defined a rapidly fatal form where neurological degradation was absent or secondary (Ashkenazi et al. 1971; Guibaud et al. 1979; Jaeken et al. 1980; Semeraro et al. 1986). The pathogenesis of this cholestatic process is unknown.

In our case there was no microscopic evidence of cholestasis but subcellular changes regarding the bile canaliculi attested to intracellular cholestasis. The widening of the microfilamentous material in the pericanalicular cytoplasm may be considered to be an early feature of human cholestasis (Adler et al. 1980). In hepatic cells, pericanalicular microfilaments have been shown to contain actin (Oda et al. 1974; Gabbiani et al. 1975). They may be endowed with a contractile function, accounting for the tonicity of the canaliculi and thereby for the regulation of bile flow. Another role of microfilaments may be found in their apparent regulatory role of bile salt uptake (Reichen et al. 1981). The most convincing evidence for a role of the microfilament system in canalicular bile formation has been obtained with toxic drugs like cytochalasin B, a microfilament inhibitor, which induces cholestasis (Phillips et al. 1975). In the same manner, phalloidin, a mycotoxin, increases the microfilaments in hepatocytes by causing an irreversible polymerization of globular actin into filamentous actin. This induces cholestasis (Gabbiani et al. 1975; Dubin et al. 1978). A human model of neonatal severe cholestasis with giant cell transformation might be a model of microfilament dysfunction-induced cholestasis (Weber et al. 1981). In other cases of familial cholestasis, primary disturbance of bile acid secretion seemed to entail hypertrophy of pericanalicular microfilaments which play a role in the final step of bile secretion (De Vos et al. 1975; Kaplinsky et al. 1980). The frequent occurrence of neonatal cholestasis reflects a secondary response to the concomitant NP-C defect. It remains to be seen whether the hyperplasia of pericanalicular microfilaments represents a cause or an effect of the bile secretion disorders.

This investigation of the fetal liver and spleen in NP-C suggests variations in the ultrastructural configuration of the storage during the antenatal and postnatal stages of the disease. Morphological studies performed early in the natural history may help in the elucidation of the primary defect. Early cholestasis and the presence of a "toxic" metabolite acting upon pericanalicular microfilaments is an attractive pathogenesis for the production of these changes.

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