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Metabolic abnormalities in feline Niemann–Pick type C heterozygotes

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Summary: Niemann–Pick disease type C (NPC) is an autosomal recessive neurovisceral lysosomal storage disorder in which cholesterol lipidosis results from defective intracellular transport of unesterified cholesterol. The primary molecular defect of NPC is unknown; regulatory mechanisms of cholesterol metabolism are impaired, resulting in retarded esterification of exogenous cholesterol with accumulation of unesterified cholesterol in lysosomes and secondary storage of glycolipids and sphingomyelin. In obligate heterozygotes from a feline NPC model, cultured skin fibroblasts challenged with exogenously derived cholesterol exhibited intermediate rates of cholesterol esterification and accumulation of unesterified cholesterol. Liver lipid analyses of obligate heterozygote cats demonstrated intermediate cholesterol and sphingomyelin concentrations. Vacuolated skin fibroblasts were found in 2 of 3 heterozygote cats, and occasional cortical neurons exhibited intracellular inclusions immunoreactive for GM2-ganglioside. Ultrastructural studies provided evidence of storage in liver and brain. We believe these morphological and biochemical findings are the first example of manifestations of CNS abnormalities in a genetic carrier for a neuronal storage disease.

Niemann–Pick disease type C (NPC) is an autosomal recessive neurovisceral lysosomal storage disorder in which cholesterol lipidosis results from defective intracellular transport of unesterified cholesterol (Liscum et al 1989; Roff et al 1992). The primary molecular defect of NPC is unknown; however, NPC in humans has been linked to chromosome 18 (Carstea et al 1993). Unlike Niemann–Pick types A and B, NPC is not a primary sphingomyelinase deficiency (Pentchev et al 1995). The regulatory mechanisms of cholesterol metabolism are impaired, which results in retarded esterification of exogenous cholesterol with accumulation of unesterified cholesterol in lysosomes and secondary storage of

glycolipids and phospholipids, including sphingomyelin (Vanier 1983). This disease is clinically heterogenous but most often becomes evident at an early age and results in delayed mental and motor development and premature death. There is currently no effective treatment for NPC.

Heterozygotes of storage diseases due to lysosomal hydrolase defects generally have enzyme activity sufficient to prevent metabolic abnormalities (Beaudet et al 1995). This is in contrast to human NPC heterozygotes in whom bone marrow macrophages with storage (foam cells) and inclusions in fibroblasts of skin biopsies have been reported rarely (Frank and Lasson 1985; Ceuterick and Martin 1986). Cholesterol esterification and cholesterol accumulation in cultured skin fibroblasts are intermediate between those of normal and affected individuals in approximately half of human NPC obligate heterozygotes tested (Kruth et al 1986). Lymphocytes of some heterozygotes in Batten disease reportedly have tubular cytoplasmic inclusions (Markesbery et al 1976); Batten disease and NPC are both classed as neuronal storage disorders which lack known primary lysosomal enzyme deficiencies.

We have identified a feline model of NPC that has the characteristics of the human disease. We now describe metabolic abnormalities in clinically normal feline NPC heterozygotes not previously described in human NPC heterozygotes.

ANIMAL MODEL

A feline model of NPC has been established and characterized (Lowenthal and Walkley 1989; Lowenthal et al 1990; Brown et al 1994a,b; Muñana et al 1994). Numerous features of this feline lipid storage disorder and human NPC are remarkably similar, and include onset and progression of clinical neurological signs and autosomal recessive inheritance. Neurovisceral storage is evident by light microscopy, with progressive neuronal cell loss, axonal spheroids, and ectopic neurites demonstrated (Lowenthal et al 1990; Brown et al 1994a,b). Biochemical analysis of storage material consists of unesterified cholesterol, glycolipids, sphingomyelin and other phospholipids in viscera, and gangliosides and glycolipids in the central nervous system (Lowenthal et al 1990; Brown et al 1994a,b). Cultured skin fibro-blasts have impaired ability to esterify exogenous cholesterol with perinuclear accumulation of unesterified cholesterol demonstrated by filipin staining. The age of onset and progression of clinical disease of this animal model are consistent with juvenile-onset human NPC.

MATERIALS AND METHODS

Animals

Seven NPC obligate heterozygote domestic shorthair cats (ages 3–10 years; 2 males, 5 females), and a 10-week-old putative heterozygote from a research colony at Colorado State University were evaluated. Cats were definitively diagnosed as obligate NPC heterozygotes by breeding trial. Homozygous NPC cats ($n=22$, ages 0–10 months; 13 males, 9 females), and normal control cats ($n=15$, ages 0–6 years; 8 males, 7 females) were similarly evaluated. The animals were fed a standard commercial diet and housed in a central animal facility which is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Biochemical analyses:

Cholesterol esterification assay: Fibroblast cultures were established from skin biopsies obtained from anaesthetized cats. Briefly, the skin was shaved and surgically prepared, a 0.5-cm^2 full-thickness section was taken and placed in Hank's buffered saline solution (HBSS) containing 2% penicillin–streptomycin. The tissue was minced and washed with fresh HBSS, transferred to flasks, and maintained in Eagle's minimum essential medium supplemented with 2 mmol/L L-glutamine, gentamicin (10 mg/ml) and 10% (v/v) fetal calf serum. Cholesterol depletion and subsequent challenge with exogenous cholesterol in the form of low-density lipoprotein as previously described (Kruth et al 1986) was used to measure the ability of cultured skin fibroblasts to esterify exogenous cholesterol at 6 and 24 h post-challenge. Cholesterol esterification in fibroblasts was measured by [^{3}H]oleate (New England Nuclear) incorporation and compared to values for normal control cats and NPC homozygous cats. Unesterified cholesterol accumulation in challenged fibroblasts was demonstrated by staining with filipin (Sigma), which binds specifically to unesterified cholesterol (Kruth et al 1986).

Liver lipid analysis: Liver biopsies were taken through a mid-ventral laparotomy as described previously (Breznock 1983) under isoflurane general anaesthesia, or collected at necropsy; a portion was frozen at −70°C for lipid analysis. Lipid extraction of fresh frozen liver was performed by the method of Folch (Folch et al 1957). Briefly, a known weight of tissue was extracted with chloroform–methanol (2:1 v/v) and filtered; 1/5 volume of water was added to the filtrate. The phases were separated by centrifugation and the lower phase was used for quantification of individual lipids. Total cholesterol and total phospholipid were quantified from an aliquot of the lower phase as previously described (Wenger et al 1980). Additional aliquots were spotted on silica gel thin-layer chromatography (TLC) plates for separation and quantification of individual lipids using standard methods (Wenger et al 1980). Standards for all lipids measured were plated simultaneously. Specifically, for cholesterol separation, the solvent system used was hexane–ether–glacial acetic acid (90:10:1 by vol). Bands were visualized with iodine vapour, unesterified cholesterol and cholesterol ester regions were scraped, and the lipid was eluted with chloroform–methanol (2:1 v/v) for quantitative analysis. The solvent system used for individual phospholipid separation was chloroform–methanol–glacial acetic acid–water (75:45:12:6 by vol). Bands were visualized with iodine vapor, regions of sphingomyelin, phosphatidylinositol and phosphatidylcholine were scraped, and lipid was eluted and quantified as described (Wenger et al 1980). Unesterified cholesterol, cholesterol ester, sphingomyelin, phosphatidylinositol and phosphatidylcholine were expressed as nmol lipid/g wet weight of tissue.

Serum biochemical analysis: Blood was collected by jugular venepuncture following a 12-h fast. Serum was separated immediately following clot formation. Analyses were performed using a discrete automated analyser (Hitachi 704 or 911; Boehringer-Mannheim Corporation, Indianapolis, IN, USA) on serum from NPC obligate heterozygote cats, and compared to NPC homozygous and age-matched normal control cats. Analytes included serum glucose, urea nitrogen, creatinine, phosphorus, calcium, total protein, albumin, globulin, cholesterol, total bilirubin, alkaline phosphatase, alanine aminotransferase,

aspartate aminotransferase, gamma-glutamyl transpeptidase, sodium, potassium, chloride, total carbon dioxide, and bile acids.

Ganglioside analysis: Cerebral cortex was collected from two obligate heterozygote NPC cats at necropsy and frozen at −70°C. Gangliosides were extracted and purified by a modified Suzuki method (Ledeen and Yu 1982). Total sialic acid was quantified by the method of Svennerholm (1957); gangliosides were separated by high-performance thin-layer chromatography (HPTLC); plates were scanned by densitometry (Quick-Scan Flur-Vis densitometer; Helena Laboratories, Beaumont, TX, USA); and GM1, GM2, and GM3 gangliosides were quantified. Results were compared to normal control cats and NPC homozygous cats.

Morphological analyses

Bone marrow: Bone marrow aspirates were collected from tibia of anaesthetized cats and preparations were made as previously described (Tyler and Cowell 1989). Marrow films were allowed to air-dry; three were stained with Wright–Giemsa stain, the remainder were fixed for 30 min in formalin vapour and stained with a 0.5 mg/ml filipin solution for 30 min. Filipin-stained slides were washed with phosphate-buffered saline, coverslipped using Vectashield (Vector Laboratories) mounting media, and viewed by fluorescence microscopy with a Zeiss fluorescent microscope, excitation wavelength 360 nm.

Liver and skin: Portions of liver and skin obtained from biopsy or necropsy were placed in 4% paraformaldehyde–1% glutaraldehyde for electron microscopy and in 10% neutral buffered formalin for light microscopy. Paraffin sections were cut at 6 μm and stained with haematoxylin and eosin (H&E); plastic sections were cut at 2 μm and stained with toluidine blue.

Liver imprints were made on biopsied specimens from heterozygotes and normal controls. Unesterified cholesterol was demonstrated using the fluorescent polyene antibiotic filipin (Sigma). Samples were air-dried and fixed for 30 min with formalin vapour followed by staining with a 0.5 mg/ml filipin solution for 30 min, with further processing as described above.

Immunocytochemistry and Golgi: Cerebral cortex, obtained at necropsy of two obligate heterozygote cats, was fixed in 4% paraformaldehyde–0.1% glutaraldehyde, sectioned on a vibratome (30–40 μm), and incubated overnight in a monoclonal antibody (mAb) against GM2 ganglioside. The mAb was labelled with a biotinylated secondary antibody, followed by peroxidase-conjugated avidin as described elsewhere (Goodman et al 1991). In some cases, sections containing cells immunoreactive for GM2 ganglioside were flat embedded in epon and subsequently examined in 2-μm-thick sections stained with toluidine blue, or at the electron-microscopic level. Rapid Golgi and Golgi–chloral hydrate methods were used on cerebral cortical sections (Walkley 1987) to visualize individual neurons.

Electron microscopy: brain, skin and liver: Sections of liver (obtained by biopsy or at necropsy), cerebral cortex and cerebellum (collected at necropsy of two obligate heterozygote cats), and skin (obtained by biopsy) were fixed in buffered 4% paraformaldehyde–0.1%

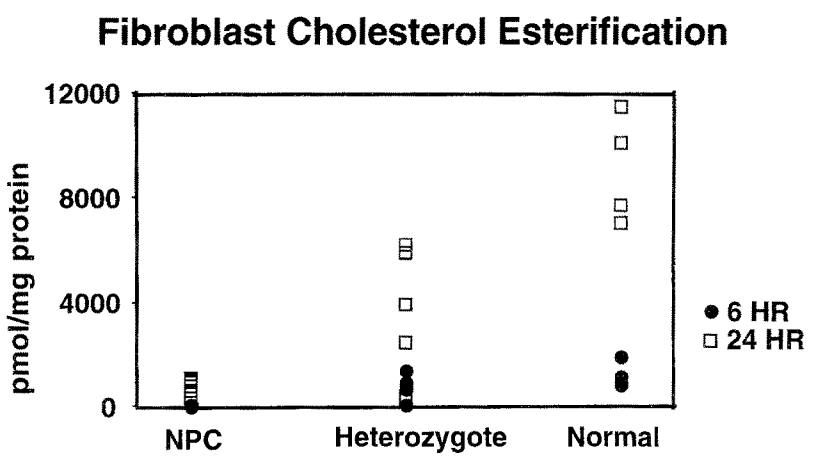

Figure 1 Cholesteryl ester synthesis after 6 and 24 h of LDL uptake in cultured skin fibroblasts. Results are expressed as the difference between levels of cholesteryl [^{3}H]oleate formed in the presence and absence of lipoproteins. Six cats were tested in each group

glutaraldehyde. Tissues were post-fixed in 2% osmium tetroxide, stained *en bloc* with 1% uranyl acetate, and dehydrated through a series of alcohols. Tissues were embedded in Spurr plastic, sectioned at 2 μm and stained with toluidine blue, and sectioned at approximately 100 nm and placed on copper grids. Grids were stained with lead citrate for 2 min, then washed through a series of water and methanol. Sections were viewed and photographed by transmission electron microscopy (Jeol JEM-2000EX-II transmission electron microscope).

Statistical analysis

Non-parametric statistical comparison between 3 groups was performed using the Kruskal–Wallis test and the Mann–Whitney *U*-test for post-testing, accepting a p-value of <0.05 and <0.01, respectively, as statistically significant. One-way ANOVA with repeated measurements was used to statistically evaluate serum biochemical data over time.

RESULTS

Biochemistry

The quantity of cholesterol ester produced in NPC heterozygote fibroblasts is intermediate between the concentration in normal control cat cells and the low concentration in NPC homozygotes (Figure 1). Filipin staining for unesterified cholesterol is increased and intermediate in fluorescent intensity in the challenged heterozygote fibroblasts when compared simultaneously between normal control and NPC-affected cats (Figure 2).

Liver lipid analyses of NPC obligate heterozygote cats demonstrated total cholesterol, unesterified cholesterol, total phospholipids and sphingomyelin which were intermediate in concentration and significantly different between normal control and homozygous NPC cats (Table 1). On repeated liver lipid analysis, recovery of cholesterol fractions from NPC cats with high cholesterol concentrations was better (average 80%) than that from normal

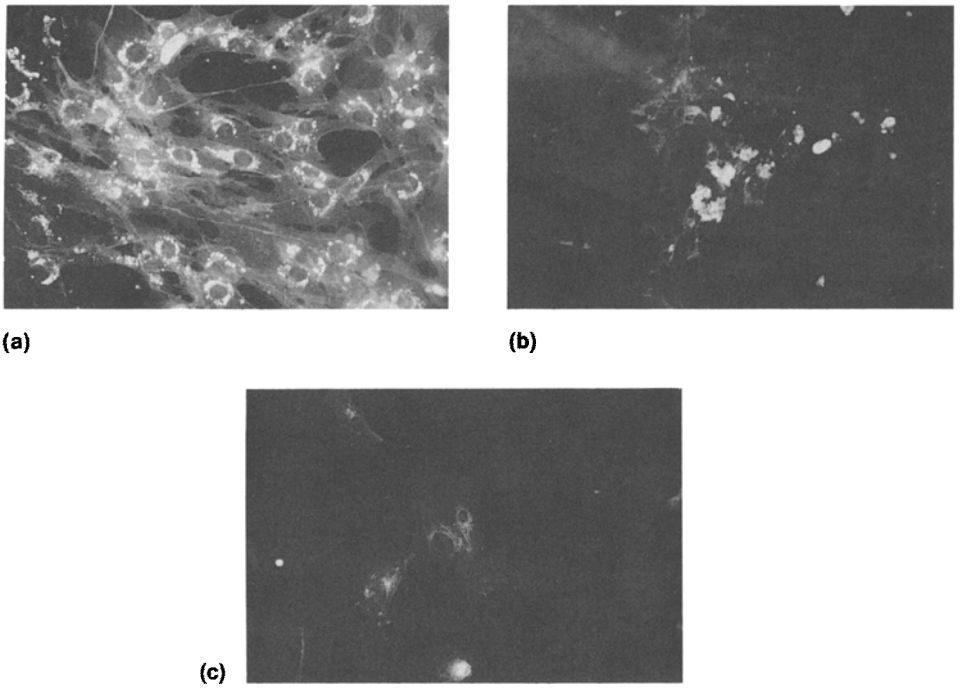

Figure 2 Feline cultured skin fibroblasts grown in lipoprotein-deficient medium, incubated with low-density lipoprotein (50 μg/ml) for 24 h, then stained for unesterified cholesterol with filipin. The NPC heterozygote (b) has intermediate filipin reactivity between NPC homozygote (a) and normal cat (c). ×95

Table 1 Liver lipid concentrations (nmol/g wet weight tissue; mean ± SD)

	NPC ($n=10$)	*Heterozygote* ($n=7$)	*Normal* ($n=8$)
Total cholesterol	62500±18900	25500±11200	12800±4400
Unesterified cholesterol	45800±14700	7800±3700	3900±1500
Cholesterol ester	760±700	2500±1200	2000±1500
Total phospholipid	46100±9900	34500±4100	25400±7700
Sphingomyelin	5790±2700	2790±1740	850±680
Phosphatidylcholine	10600±3480	11300±800	7970±2530

control and heterozygote cats with lower liver cholesterol concentrations (average 50% recovery). Subtle increase of GM2-ganglioside was demonstrated in cerebral cortex of NPC obligate heterozygotes by HPTLC analysis. Median GM2 concentration in NPC-affected cats was 35 μg/g (range 20–83), in normal controls 8 μg/g (range 0–35), and in NPC obligate heterozygotes 25 μg/g (range 8–41). While the GM2-ganglioside differences are not statistically significant, they do reflect a trend and are supported by morphological data.

There was no statistically significant difference between serum biochemical parameters of NPC obligate heterozygote and normal control cats. Homozygous NPC cats have serum

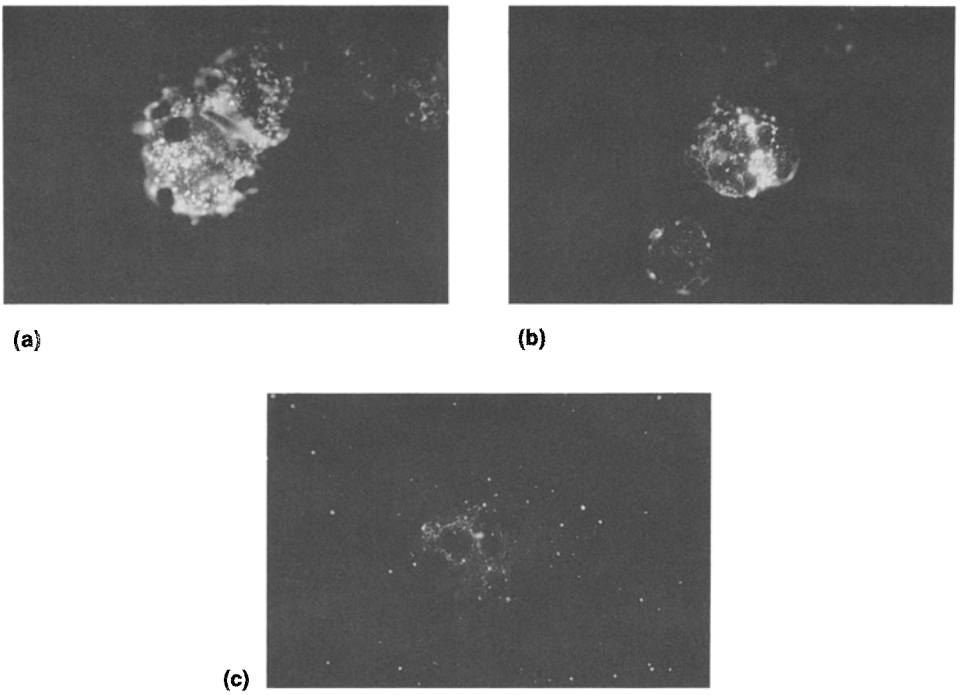

Figure 3 Liver imprints of (a) NPC homozygote, (b) NPC heterozygote and (c) normal cat; filipin stain. Note the intermediate accumulation of unesterified cholesterol demonstrated by the fluorescence of the NPC heterozygous cat's hepatocytes. ×15

biochemical abnormalities which include significantly increased serum alkaline phosphatase and alanine aminotransferase activity throughout the course of disease. In addition, serum cholesterol, total bilirubin, and bile acids increase as the disease progresses. Serum albumin decreases significantly as the disease progresses. These changes are all referable to progressive hepatic disease in homozygous NPC cats.

Morphology

No increase in foamy macrophages was identified on examination of bone marrow aspirate cytology of NPC heterozygotes compared to normal controls using Wright–Giemsa stain. No increased cholesterol in macrophages, as determined by filipin staining, was seen in heterozygote or normal cat bone marrow aspirates. Foamy cholesterol-laden macrophages, which are filipin reactive, are numerous in NPC-affected bone marrow aspirates.

Liver imprints from obligate heterozygotes stained with filipin had increased reactivity compared to normal controls (Figure 3). Occasional hepatocytes, biliary epithelial cells and Kupffer cells with polymorphic membranous cytoplasmic inclusions were seen on ultrastructural examination of the liver (Figure 4).

In brain of a 10-year-old heterozygote, rare cells scattered through neocortex, many appearing to be pyramidal neurons, exhibited marked accumulation of intracellular inclusions which were immunoreactive for GM2 ganglioside (Figure 5). These inclusions contained

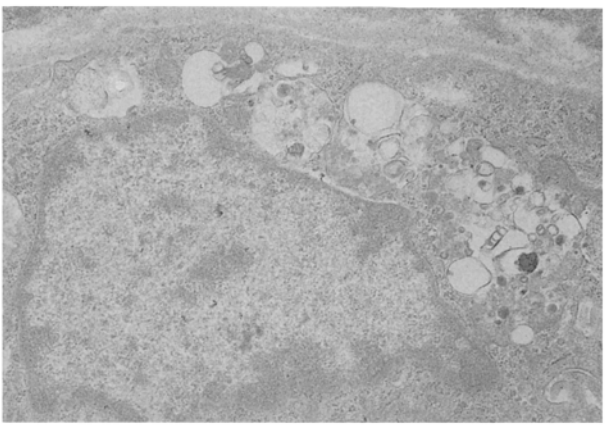

Figure 4 Electron micrograph demonstrating polymorphic membranous cytoplasmic inclusions in biliary epithelial cell of a 10-year-old NPC obligate heterozygote. ×7500

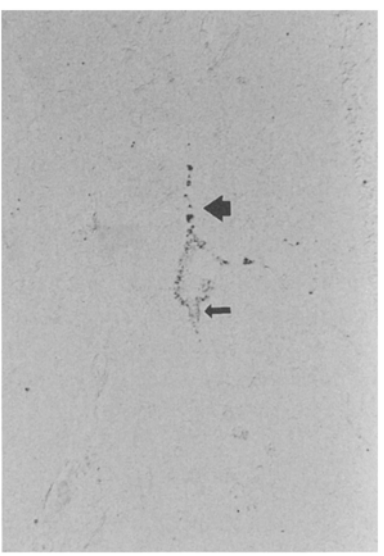

Figure 5 Positive reactivity for GM2 ganglioside in cortical pyramidal neuron of a 10-year-old NPC obligate heterozygote queen; 2-μm plastic section, GM2 immunocytochemistry, apical dendrite (large arrow) and axon hillock (small arrow). ×180

membranous material ultrastructurally (Figure 6). A 10-week-old putative heterozygote (based on intermediate cholesterol metabolic defects in cultured fibroblasts) was found to have ectopic dendrites on occasional cortical pyramidal neurons (Figure 7).

Vacuolated skin fibroblasts were evident by light-microscopic examination of H&E-stained sections of NPC-affected cats. Examination of 2-μm-thick sections demonstrated vacuolated skin fibroblasts in NPC-affected cats and 2 of 3 obligate heterozygote cats.

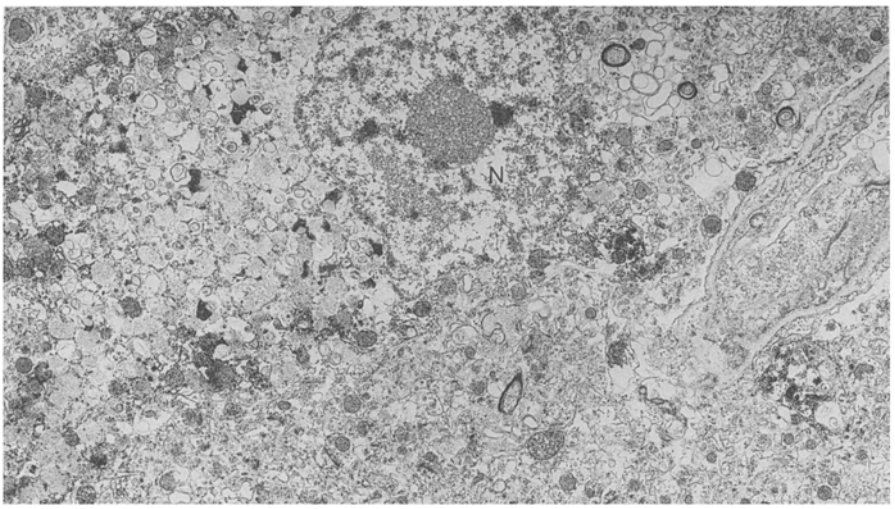

Figure 6 Electron micrograph of cortical pyramidal neuron from a 10-year-old NPC obligate heterozygote demonstrating membranous cytoplasmic inclusions. N = nucleus. ×4300

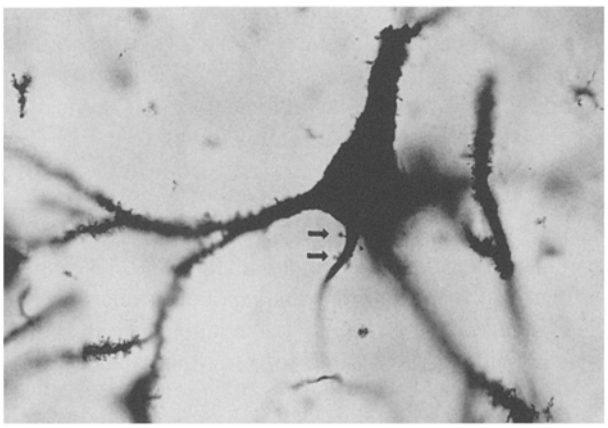

Figure 7 Golgi stain of layer III cortical pyramidal neuron from a 10-week-old NPC putative heterozygote demonstrating aberrant spines on soma and axon hillock (arrows). ×200

On electron-microscopic examination, membranous cytoplasmic inclusions containing flocculent material were present (Figure 8).

DISCUSSION

Unlike heterozygotes of storage diseases due to lysosomal hydrolase defects, in which enzyme activity is sufficient to prevent metabolic abnormalities, feline NPC heterozygotes have biochemical and morphological evidence of intermediate storage of unesterified

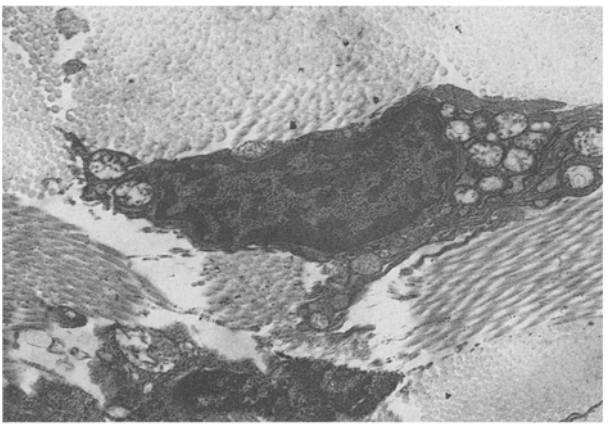

Figure 8 Electron micrograph demonstrating membranous cytoplasmic inclusions in skin fibroblast (uncultured) obtained by skin biopsy of NPC obligate heterozygote. ×3500

cholesterol and sphingomyelin in liver, as well as storage in occasional neurons of the cerebral cortex. Vacuolated skin fibroblasts are also demonstrated in some heterozygote cats. It is possible that the defect in NPC is that of a cholesterol transport or regulatory protein (Pentchev et al 1995). Heterozygotes probably have intermediate levels of that putative protein, which results in the metabolic abnormalities described in this animal model. Niemann–Pick type C obligate heterozygote cats have no apparent clinical signs, and they demonstrate no abnormalities on clinical neurological examination.

The presence of occasional neurons exhibiting storage in the cerebral cortex of NPC heterozygotes suggests that these cells are particularly vulnerable to the metabolic defect. They may represent a unique class of neurons with specific differences in cholesterol metabolism. Whether they are all pyramidal neurons or represent both pyramidal and non-pyramidal cells is yet to be determined. The accumulation of GM2-ganglioside and presence of ectopic axon hillock neurite growth are findings consistent with studies in a host of neuronal storage disorders (Walkley 1995). For the latter there is evidence that abnormal increase of GM2-ganglioside is responsible for re-initiating the growth of dendrites on pyramidal neurons.

There is currently no screening test for carriers of human NPC (Pentchev et al 1995), as only approximately half of human heterozygotes tested demonstrate an intermediate level of cholesterol esterification and cholesterol accumulation in cultured skin fibroblasts (Kruth et al 1986; Roff et al 1992). We have demonstrated abnormalities in feline NPC heterozygotes including liver lipid accumulation, increased filipin positivity in liver aspirates and membranous cytoplasmic inclusions in fibroblasts from skin biopsies; these tools may be useful to identify carriers prior to identification of the defective gene. These findings may also be useful for understanding the pathophysiology of NPC. Further investigation of other neuronal storage diseases may reveal metabolic abnormalities in heterozygotes. We believe these morphological and biochemical findings in NPC heterozygotes are the first example of manifestations of CNS abnormalities in a heterozygote for a neuronal storage disease.

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