Virchows Arch [Pathol Anat] (1984) 402:307–317

Virchows Archiv A

Niemann-Pick disease type C with enhanced glycolipid storage

Report on further case of so-called lactosylceramidosis

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Summary. A case of infantile neurovisceral disease was classified according to the morphological and chemical analysis of fixed tissue as a chemically different type of Niemann-Pick disease (NPD) type C, with glycolipids dominating the storage process. The diagnosis was reached on the basis of massive accumulation of neutral glycolipids in visceral storage elements (hepatocytes and macrophages) as an outstanding feature of lipid histochemistry. Chemical lipid analysis corroborated the findings by detecting a manyfold increase of glucosyl ceramide, lactosyl ceramide, ceramide trihexoside and GM_3 ganglioside. In addition, macrophages contained variable quantities of sphingomyelin. The brain showed slightly increased quantities of lactosylceramide (Slower fraction) and glucosyl ceramide. Apart from the classical neuronal storage changes there was also marked neuroaxonal dystrophy. In terms of quality, the glycolipid spectrum was comparable to that of NPD type C, in terms of quantity, the changes were consistent with those in so-called lactosylceramidosis, which, however, was reclassified as NPD type C only recently. In our view, the present case is the second published observation of lactosylceramidosis classifiable as a glycolipid (GL) variety of NPD type C in which the normally considerable tendency to glycolipid storage is further enhanced while the storage of sphingomyelin is less expressed.

Key words: Niemann-Pick disease type C – Glycolipid storage – Sphingomyelin – Neuroaxonal dystrophy

Introduction

In Crocker's original work, group C of Niemann-Pick disease was defined as a slow progressing infantile-juvenile form with neurovisceral symptoma-

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tology (Crocker 1961). Subsequently, sphingomyelinase activity was found to be retained in this group (Schneider and Kennedy 1967). Still later a reduction or even absence of its cathodic component in cultured fibroblasts was demonstrated though this appears to be a secondary manifestation of the disease (Besley 1977; Besley and Moss 1983a and b; Vanier et al. 1983). Several extreme varieties of the disease have been described, particularly as regards the clinical picture (Guibaud et al. 1979; Wherret and Rewcastle 1969; others in Elleder and Jirásek 1983). There is also an extreme chemical variety known as lactosylceramidosis. This was originally shown to have deficiency of lactocerebrosidase activity and involvement of glycolipid metabolism (Dawson and Stein 1970). The chemical picture showed an absolute predominance of neutral glycolipids particularly glucosyl- and lactosyl-ceramides (Dawson 1972) but only moderate visceral increase of sphingomyelin (Dawson, correspondence). The case was reclassified as belonging to the NPD complex by Wenger et al. (1975), who found pronounced SMase deficiency in fibroblast cultures. Besley and Moss (1983a) examining the same cells, found suppression of the SMase cathodic isoenzyme, thus making its classification as type C even less ambiguous. The atypical stored lipid pattern of this disorder appears to be an extreme manifestation of the tendency to neutral glycolipid storage in NPD type C, a feature given particular attention recently (Vanier 1983; Vanier et al. 1983a). The case we wish to present here is identical with Dawson's in terms of chemical pathology and represents, to the best of our knowledge, the second published case of this type.

Case report

M.Š., a girl, was born to a 17-year old primipare in the 37th week of pregnancy. Birth weight 2,670 g, length 47 cm. There were signs of moderate prematurity, hypotonia and hyporeflexia of moderate degree, tremor of upper extremities, the lower extremities were in the calcaneovalgous position. There was also a nonconstant major tremor or even convulsions of upper extremities. The liver extended below the costal margin by 2 cm, the spleen by 1 cm. There was physiological jaundice. Laboratory tests for blood sugar, cholesterol, urea, calcium, sodium, potassium, and magnesium where within normal values, and so was the blood count. The baby was kept in an incubator for suspected hypothermia. She died at 4 weeks displaying signs of bilateral bronchopneumonia. The clinical diagnosis was vague – degenerative brain disease.

Post mortem examination revealed failure to grow (2,700 g, 51 cm), a mildly activated and enlarged spleen (20 g for 11.44 g) and a pale orange-tinged liver with no visible enlargement (135 g for 139 g). The rest of the viscera exhibited no visible changes except for the lungs which were affected by bilateral suppurative bronchopneumonia. Thrombosis of the sagittal and transverse sinuses was found in the cranial cavity. Macroscopically, the brain was consistent with the patient's age with no visible pathological changes. Several samples of the brain tissue and the essential viscera were taken for routine histology and fixed in formaldehyde (liver, spleen, lungs, intestines, stomach). Histology revealed the presence of a storage process which was subsequently analyzed with other methods.

Methods

All the following methods were performed in formol-fixed tissues as no unfixed tissues were available. In addition to standard histological examination, the liver and spleen were examined

electronoptically following double post fixation with paraformaldehyde and osmium tetroxide. Stored lipids were analyzed by the following histochemical tests in frozen free – floating sections: the iron haematoxylin method for the detection of phospholipids and spingomyelin (Elleder and Lojda 1973a and b), the PAS reaction for the detection of glycolipids, and cresyl violet in a Hirsch-Pfeiffer test for the detection of acidic lipids (for details see Adams 1965). Pre-extraction with chloroform methanol 2:1 v/v (1 h, room temperature) was used to differentiate lipids from non-lipid substances. Sudan black was used for general lipid staining and for demonstration of lipopigments. Autofluorescence and birefringence of lipid deposits were also studied.

Specimens of the liver, spleen and brain were examined with chromatographic techniques. Spingomyelin was assayed after two-dimensional phospholipid TLC and after sphingomyelin zone phosphorus determination (Rouser et al. 1970). The neutral glycosphingolipids were isolated by peracetylation and chromatography on florisil (Saito et al. 1971) and separated on TLC plates of silicagel 60 (E. Merck, Darmstadt, FRG) with chloroform-methanol-water 65:25:4 as solvent system. The amount of individual neutral glycosphingolipids was measured by quantitative densitometry of spots and standards of each glycolipid in the range 1,25–10 nmols separated on the same TLC plate. The plate was visualized with orcinol reagent (Robert and Rebel 1975) and scanned with TLD-100 Scancer Vitatron in reflectance mode at 546 nM.

Gangliosides from the brain were isolated using a DEAE Sephadex column, and chromatographed on HPTLC silica gel plates (Merck, Darmstadt, FRG) in a 55:45:10 C:M:0.02% $CaCl_2$. Detection: Resorcinol – HCl (Ueno et al. 1978). Gluco- and galacto-cerebrosides were separated on a layer of silica gel impregnated with tetraborate (Kean 1966).

Skin fibroblasts from the mother and controls were cultured in Ham's F10 medium containing 20% newborn calf serum and antibiotics. Cells were removed by trypsinization (2.5 g/l trypsin and 0.4 g/l EDTA), washed in phosphate buffered isotonic saline and stored at −70° C (up to 1 month) prior to assay. Enzyme assays were carried out on total cell extracts prepared by sonication (2 × 15 s bursts) in water, or, for sphingomyelinase assay, on supernatant extracts preparated by four cycles of freeze – thawing in sodium cholate (5 mg/ml) followed by centrifugation at 8,500 × g for 2 min. Lysosomal enzyme assays were carried out using the following substrates [14C]-sphingomyelin (Besley 1977), [3H] galactocerebroside (Besley and Bain 1976), p-nitrocatechol sulphate (Baum et al. 1959) and appropriate conjugates of 4-methylumbelliferone (Koch-Light Ltd. Colnbrook, England). The activity of β-glucosidase was measured at pH 5.5 in the presence of taurocholate (Peters et al. 1976).

Results

Histology. The most salient feature was the infiltration of some of the organs with foam cells of different size and sometimes even with multiple nuclei. In the spleen there were larger groups of foam cells in the red pulp (Fig. 1a). In the sinus endothelia, there was no detectable storage, the white pulp being reduced. There were small foci of haematopoiesis. The liver exhibited considerable quantities of storage cells in the sinuses along with a small amount of haematopoiesis. There was generalized microvacuolization of hepatocytes (Fig. 1b). Apart from the suppurative inflammatory changes in the lungs there were also major infiltrates of foam cells. The peripheral nervous system (gastrointestinal tract) exhibited storage changes.

All we had at our disposal for the neuropathological examination were parts of the frontal and insular cortex, the adjacent segments of the semioval centre, claustrum, striatum, pallidum, thalamus, hypothalamus and internal capsule. The cerebellum, the brain stem, and the spinal cord could not be examined. Neuronal ballooning was found throughout, being less pronounced in the cortex. Stainings for lipopigment were all negative. Another

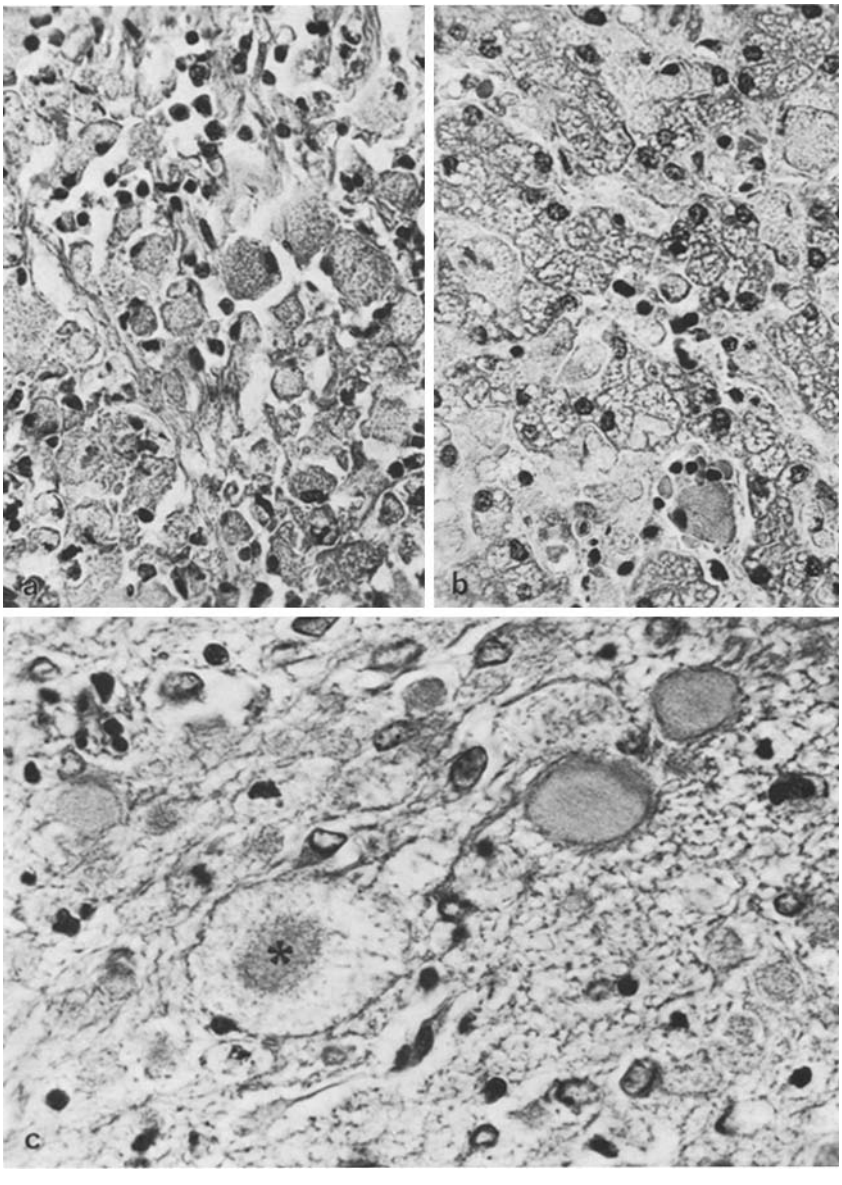

Fig. 1a–c. Spleen with a collection of foam cells of various size. **a** HE staining ×400. **b** liver with a number of foam cells in the sinusoids. Hepatocytes are finely vacuolated. HE staining ×400. **c** Spheroids in axons of the internal capsule. One of them (asterisk) is surrounded by a finely vacuolated storage halo. HE staining ×600

highly prominent finding was the massive neuroaxonal dystrophy (Fig. 1c) bound to the processes and to the perikarya. Granular oxyphil spheroids were mostly involved. Most of those changes were found in the striatum, and internal capsule, less so in the thalamus and the centrum semiovale. Pronounced plasmatic gliosis was discernible mainly in the nonmyelinized

Table 1. Results of lipid histochemistry

Method	Central neurons	Hepato-cytes	Foam cells (liver, spleen)	
Fe haematoxylin	−	+	−/+	(++/+++)[a]
after alkal. hydrolysis	−	−	−/+	(++)
after chlorof. methanol	−	−	−	(−)
P A S	±	+/++	++++	(+/++)
after alkal. hydrolysis		+/++	++++	(+/++)
after chlorof. methanol	−	−	−/+	(−/+)
Cresyl violet	−	−	−	(−/+)
after chlorof. methanol	−	−	−	(−/+)
Sudan black B	±	±	+	(+/++)
after chlorof. methanol	−	−	−/+	(−/+)
Birefringence	−	−	+[b]	(+)
Autofluorescence	−	−	−/+[c]	(−/+)

− negative, ± faintly positive, + mild, ++ intermediate, +++ strong, ++++ very strong

[a] values in parentheses are results in foam cells of the reference case of NPD type C given here for comparison. Results in hepatocytes will be published separately (manuscript in preparation)

[b] slightly unstable Maltese crosses with partial solid crystal transformation

[c] orange-green

white matter of the subcortex. Also present was a fairly marked resorptive reaction of dispersed gitter cells containing a mixture of non-polar lipid and lipopigment. Ultrastructural investigation contributed very little. In the foam cells and in hepatocytes, the vacuoles with limiting membranes were practically translucent with few membrane fragments. In spite of the high concentration of glycolipids, no structures could be observed analogous with the tubules described in Krabbe's cells or in Gaucher's cells. In the brain tissue which was damaged by the formol fixation attention was focused on the dystrophic neurites where dense pleiomorphic granules were found together with proliferation of neurofibrils which sometimes predominated.

The results of histochemical tests are summarised in Table 1 suggesting massive accumulation of neutral glycolipid in the storage elements (hepatocytes, macrophages) in stark contrast to minimal accumulation of sphingomyelin which was not detected expect in macrophages. As with other cases of NPD type C fixed brains (Elleder and Jirásek 1981) the results of histochemistry were minimal.

Chromatographic analysis confirmed the histochemistry in showing neutral glycolipids as the dominant lipid substances (Fig. 2 and Table 3). The spleen exhibited massive glycolipid storage compared with controls, but a slight increase only when compared with a chemically typical case of NPD type C, despite the much lower intensity of the PAS staining of foam cells in the latter. The only explanation is that the degree of foam cell infiltration was low in the patient's spleen (its weight was increased by

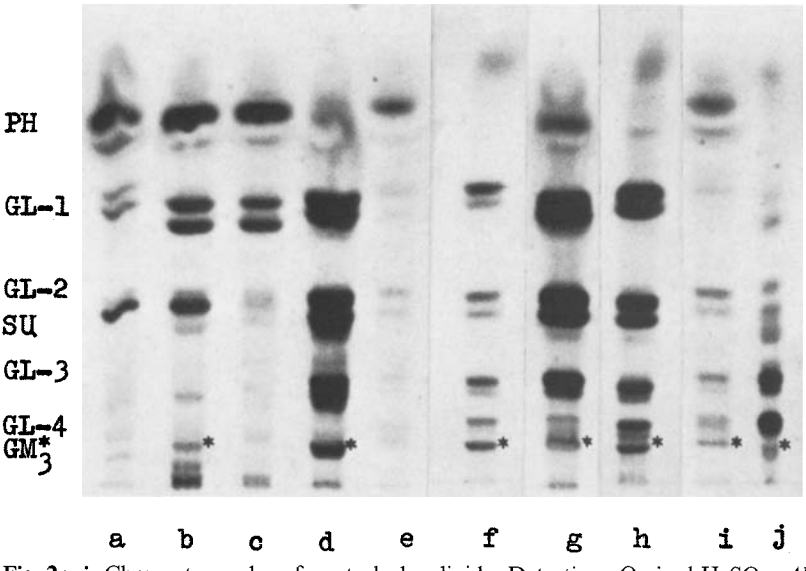

Fig. 2a–j. Chromatography of neutral glycolipids. Detection: Orcinol-H_2SO_4. *Abbreviations: PH* products of alkaline hydrolysis, *C* control, *SU* sulphatides, *GM_3* monosialoganglioside (*asterisk*), *GL-1,2,3,4* glycolipids of the ceramidhexoside series according to the number of hexose moieties (mono-, di-, tri-, tetra). **a–c** Brain cortex (from left: M.Š., NPD type C, control). **d–f** Liver (from left: M.Š., control, NPD type C). **g–i** Spleen (from left: M.Š., NPD type C, control). **j** Glycolipids from the human kidney. Note the increase of the slower fraction of GL-2 in the brain sample of the patient (**a**) and NPD type C (**b**). There is general increase of all the glycolipids in the liver and spleen of the patient even when compared with NPD type C samples. The highest difference is in the liver tissue (**d–f**)

a more 100% whereas in the reference type C spleen the increase amounted to 450% of the weight/age standard). It means that with the roughly equal increase of glycolipids the lipid concentration per patient's foam cell must be three times higher at least, than in the reference case. It accounts for the much more intense reaction of the patient's foam cells as against the classical case of NPD type C. The most prominent increase was in types GL-1, GL-2 and GL-3. The slower fraction in the GL-4 zone, defined as ganglioside GM_3, was increased only moderately. Similar results were described by Dawson (1972). In the liver there was also a massive increase in the above listed glycolipids compared with both the controls and with the reference type C. As it was the only tissue available in sufficient amount quantitation of glycolipids could also be performed (see Table 3). The brain exhibited a slight increase of only the slower fraction of lactosyl ceramide, less expressed than in the fixed specimen of NPD type C. The reduction in GL-1 and in sulphatides was most probably attributable to a low degree of myelination. As for gangliosides there was an mild unspecific increase of GM_2 and GM_3.

Chromatographic tests of glycolipids in the brain, liver and spleen on borate-impregnated plates showed the ceramide monohexoside fraction to

Table 2. Quantity of sphingomyelin (μMol P/gr wet weight)

	Liver	Spleen
Patient	2.53	2.77
Controls ($n=6$)	1.3 ± 0.2	1.6 ± 0.45

Table 3. Composition of major neutral glycolipids in the liver[a]

Glycosphingolipid	nmol/g of wet tissue weight				
	Controls			Patient	NPD type C[b]
	Newborn	1 month	10 years		
Ceramidmonohexoside	53.3	93.3	50.0	3330.0	230.0
Ceramiddihexoside	47.9	47.9	59.9	1978.0	127.0
Ceramidtrihexoside	13.9	8.0	21.9	799.0	45.9
Ceramidtetrahexoside	15.9	8.0	4.0	29.9	12.9
Total amount	132	158	136	6137	417

[a] Analysis was performed in tissues fixed in formaldehyde up to several months
[b] Values in reference case of NPD type C are given for comparison

Table 4. Fibroblast enzyme activities (nmol/min per mg protein)

	Patient's mother	Controls ($n=10$)	
		Mean	Range
β-Galactosidase	9.8	8.9	6.0–11.1
α-Galactosidase	0.53	0.68	0.49–0.89
α-Mannosidase	1.94	1.73	0.94–2.33
α-Fucosidase	0.96	0.95	0.49–2.60
β-Glucuronidase	1.55	2.42	1.03–3.70
β-Hexosaminidase	104	96	62–139
% A form	38%	44%	36–51
Acid esterase	43	27	20–38
Arylsulphatase A	6.7	4.9	3.4–7.6
Galactocerebrosidase[a]	1.52	1.59	0.84–2.56
β-Glucosidase	4.71	4.95	2.21–8.95
Sphingomyelinase[a]	141	84	63–110

[a] Activity units per h

be made up exclusively of glucocerebroside in contrast to controls with their absolute predominance of galactocerebroside.

Phospholipid analysis was restricted, owing to fixation with formaldehyde, to sphingomyelin only and revealed only very slightly increased values (see Table 2). Spots of other phospholipids were either reduced or extinquished and a number of lysoderivatines were present, apparently a consequence of the fixation.

Skin fibroblasts were not available from the patient; however cells from

the mother were assayed for a number of lysosomal enzyme activities. The results are shown on Table 4. All activities fell within, or above, the control range.

Discussion

This particular case reflects both the complexity of diagnosis and our continued pathogenetic embarrassment in the group of NPD type C. Typical cases (i.e. clinically and chemically typical) are easier to diagnose (Elleder and Jirásek 1983) because of the constellation of SM deposition in the liver and spleen, the neurovisceral symptomatology, intactness of sphingomyelinase or decrease of its activity and frequently altered isoenzyme profile restricted to fibroblast cultures (Besley and Moss 1983a and b). However, the degree of sphingomyelinase activity in fibroblast cultures may be the subject of considerable variation among cases and frequently both normal values and normal isoenzyme pattern have been reported (Philippart 1983; Rousson et al. 1983; Vanier et al. 1983b). The same alteration of the sphingomyelinase isoenzyme profile has recently been described in GM_1 gangliosidosies (Vanier et al. 1983b). This, however, invalidates the diagnostic value of the finding and shows clearly it is a remote non-specific manifestation of the altered genetic code.

As for SM, its accumulation in NPD type C may be barely recognizable chemically as a consequence of the decreased quantity of the visceral storage process (Hagberg et al. 1977; Neville et al. 1973) or due to a qualitative difference of storage caused, for instance, by enhancement of glycolipid accumulation. This was shown to be of considerable extent even in typical cases (Philippart 1972; Vanier 1983). Our case serves as an example of the qualitatively different visceral storage process. Since no unfixed tissues were available for an enzyme analysis, diagnosis had to take into account the complex of chemical and morphological findings. Due to the inconstancy of enzymological findings described above this is the major and most valid diagnostic process at present (see also Elleder and Jirásek 1983). The following features were found to be critical: the pattern of the stored lipids corresponding well to the pattern described recently in a series of NPD patients (Vanier 1983) and in the original case of lactosylceramidosis (Dawson 1972), subsequently reclassified as type C of NPD (see Introduction). The stored lipid pattern differed from that in the major glycolipidoses of Gaucher (Kuske and Rosenberg 1972; Nilsson et al. 1982; Suomi and Agronoff 1965) and Fabry (Desnick et al. 1978; Schibanoff, Kamoshita and O'Brien 1969) types. Furthermore, the lack of significant staining of distended neurons, the non-specific pattern of gangliosides in the brain together with the pattern of visceral neutral glycolipid deposition in our case exludes the GM_2 gangliosidosis type 2 (Sandhoff and Harzer 1973). All this, together with a low degree of visceral sphingomyelinosis, foamy character of the storage cells (as distinct from Gaucher's disease) suggests the case belongs to the NPD complex. In favour of type C was the distribution of storage (brain, liver, spleen), the absence of visible storage changes in the adrenal cortex, the restriction of SM storage to macrophages, and minimum lipid involvement

by histochemistry in the fixed brain (Elleder and Jirásek 1981; Elleder and Jirásek 1983). The high degree of neuroaxonal dystrophy frequent in type C brains (Elleder and Jirásek 1981) was also found in the original brain specimen of lactosylceramidosis (Jirásek and Elleder 1983). Also there was no evidence for a partial deficiency of crucial lysosomal enzyme activities in fibroblasts from the patient's mother to suggest she was a carrier of sphingomyelinase (Niemann-Pick types A, B) or beta-glucosidase (Gaucher's disease) deficiencies. All this is in accord with the present day findings in heterozygotes of NPD type C (Besley and Moss 1983). However, one cannot exclude the theoretical possibility of spontaneous mutation in the affected child.

The peculiarities of the chemical pathology of lactosylceramidosis which seem to justify our use of the working term glycolipid (GL) variety of NPD type C are just more evidence of our incomplete knowledge of the true nature of the biochemical defect. No doubt, the excessive accumulation of glycolipids is an enhancement of the normally quite marked tendency of neutral glycolipid to accumulate in NPD type C (Vanier 1983). More attention should be devoted to the specificity of this change throughout the NP complex which does not seem absolute (Philippart 1972; Vanier 1983). We do not know the cause of glycolipid accumulation since the activities of both lactosylceramide beta-galactosidases (Harzer 1978; Tanaka and Suzuki 1975) appear normal (Wenger et al. 1975). Partial deficiencies of beta-glucosidase are, however, reported in fibroblast cultures from type C of NPD patients (Besley and Moss 1983; Rousson et al. 1983) but the significance of this is not clear. Attempts to confirm the assumed deficiency of glucocerebrosidase and sphingomyelinase activators Christomanou 1980) have proved unsuccessful (Philippart 1983; Rousson et al. 1983). Nevertheless it appears that disturbed glycolipid metabolism may become an outstanding and leading feature of the chemical pathology in the group of NPD type C, the pathogenesis of which remains to be elucidated. The data obtained so far point to fact that it is the visceral storage pool which may display enormous variation in this sense as against the brain where no such variation has been described.

To summarize, the glycolipid variant of NPD type C appears to be more frequent than we thought. To a pathologist, massive visceral neutral glycolipidosis contrasting with minimal sphingomyelinosis in macrophages together with storage distribution and cytology inconsistent with Fabry's, Gaucher's or Sandhoff's disease should raise a strong suspicion of the case being a glycolipid variant of NPD type C. May be, for a certain period of time the set of changes of this kind will be more usefull for diagnosis than the hitherto unclear enzymatic alterations.

Acknowledgement. The authors would like to express their thanks to Mr. D.M. Broadhead for perfect technical assistance in the diagnostic assays.

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Accepted November 8, 1983