Complementation studies in Niemann-Pick disease type C indicate the existence of a second group

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Abstract

Niemann-Pick disease type C is a clinically heterogeneous storage disorder with an unknown primary metabolic defect. We have undertaken somatic cell hybridisation experiments using skin fibroblast strains from 12 patients representing a wide clinical spectrum. Preliminary experiments using filipin staining of free cholesterol as a marker for complementation indicated the existence of one major group (group α) and one minor group (group β) represented by one mutant strain. Subsequent experiments in which sphingomyelinase activity was measured as a marker for complementation using five mutant strains showing activity consistently <40% control levels confirmed the existence of the second group.

(J Med Genet 1994;31:317-320)

The basic defect in the autosomal recessive storage disorder Niemann-Pick disease type C (NPC) is unknown, but appears to be related to a perturbation in the processing of exogenously derived cholesterol.¹ Affected patients can be diagnosed biochemically using cultured skin fibroblasts by showing a low esterification rate of exogenously derived cholesterol with [³H]-oleic acid or by testing for lysosomal accumulation of free cholesterol with the fluorescent antibiotic filipin.² Some patients have a partial deficiency of acid sphingomyelinase (EC 3.1.4.12) in their cultured fibroblasts arising as an effect which is probably secondary to the basic defect.³ NPC contrasts with Niemann-Pick disease types A and B (NPA and NPB) in that acid sphingomyelinase is markedly deficient in tissues of the latter patients as a primary effect caused by mutations in the acid sphingomyelinase gene. This gene has been cloned⁴ and mutations leading to NPA and NPB have been identified.⁵ Recent work has suggested that the gene implicated in some NPC patients is on chromosome 18.⁶ However, since NPC is phenotypically very heterogeneous⁷ more than one gene may be involved in the aetiology of the disease. To investigate this possibility we have conducted somatic cell hybridisation experiments using skin fibroblasts from 12 patients covering a wide clinical spectrum of NPC. The initial strategy entailed the filipin staining of unesterified cholesterol as a marker for complementation; subsequent confirmatory experiments were performed by

measuring sphingomyelinase as a marker in hybrids derived from parental strains exhibiting a consistently low level of activity (<40% of controls). A similar analysis using filipin as a marker for complementation by Vanier *et al*⁸ identified only one complementation group from 11 unrelated patients spanning the classical, intermediate, and variant biochemical phenotypes.

Materials and methods

SPECIAL MATERIALS

The following materials were obtained from the listed sources: ICN Flow: Eagle's minimum essential media with Earle's salts (EMEM), fetal bovine serum (FBS); Sigma: lipoprotein deficient serum (LPDS), pure low density lipoprotein (LDL), filipin III, Ficoll 400, Hank's balanced salt solution (HBSS), dimethylsulphoxide (DMSO); NEN: [N-methyl-¹⁴C]-sphingomyelin; Merck: polyethyleneglycol (PEG) 4000; NBS Biologicals: 4-methylumbelliferyl- β -D-galactopyranoside; BDH: Giemsa stain.

SOURCE AND MAINTENANCE OF CULTURED CELLS

Skin fibroblast cultures were initiated from 12 patients (10 pedigrees including two sib pairs) with NPC, one each of NPA and I-cell disease patients, and several healthy controls. Strains were maintained up to the twentieth passage in 10% FBS-EMEM supplemented with glutamine (580 μ g/ml), penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a humid 5% CO₂ incubator.

NIEMANN-PICK DISEASE TYPE C PATIENTS

Individual NPC strains are identified by an Arabic numeral after the "NPC" abbreviation; sib pairs are distinguished by a small case letter after this numeral (for example, NPC-5a and NPC-5b). The NPC patients covered a broad phenotypic spectrum encompassing neurological, visceral, or neurovisceral pathology. The main clinical features of the patients are summarised in table 1. The 12 NPC strains were confirmed to be of a classical biochemical phenotype in the laboratory of Dr M T Vanier (Lyon, France) by showing a massive accumulation of free cholesterol in cells stained with filipin and a markedly reduced rate of cholesterol esterification.

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Received 6 September 1993
Revised version accepted for publication 10 November 1993

Table 1 The origin and clinical details of the NPC patients studied are shown

Case identification	Origin	Sex	Age at presentation	Presenting symptoms	Neurological involvement	Visceral involvement	Age at last follow up	Reference
NPC-1	UAE	M	Neonatal	HSM at 2 days	+	+	8 y	9 (case 2)
NPC-2	Italy	F	Neonatal	Persistent jaundice, HSM	—	+	*6 mth	
NPC-3	UK	F	1 y	DD, ataxia	+	+	*9 y	
NPC-4	UK	F	13 y	Progressive neurological deterioration at 13 years (mild neonatal jaundice)	+	+	17 y	
NPC-5a	UK	F	Prenatal	Neonatal ascites, HSM	—	+	*3 wk	10 (case 2)
NPC-5b	UK	F	Fetal sib	NA	NA	NA	Terminated at 17 wk	
NPC-6	UK	F	Prenatal	Neonatal ascites, HSM	—	+	7 y	10 (case 1)
NPC-7a	UK	M	?	?	+	?	17 y	
NPC-7b	UK	M	?	?	+	?	19 y	
NPC-8	UK	F	Neonatal	Jaundice, HSM	+	+	*4 y	
NPC-9	UK	F	Neonatal	Liver disease	+	+	11 y	
NPC-10	UK	F	1.5 y	Lost sitting, DD	+	+	2.5 y	

NA = not applicable, + = definitely present, — = definitely not present, ? = uncertain or unknown, HSM = hepatosplenomegaly, DD = developmental delay, * = died at...

SOMATIC CELL HYBRIDISATION

The protocol entailed a three step approach essentially as described by Roscher *et al.*¹¹: co-cultivation of two mutant strains in an even ratio on day 1; treatment of the co-cultivated cells with 42% PEG on day 2 to induce cellular fusion; and separation of mono- and multikaryotic cells on a discontinuous Ficoll gradient (0–15%) on day 3. Mono- and multikaryotic fractions (fractions I and II, respectively) were subsequently cultured and analysed as described below.

ANALYSIS OF HYBRID CELLS

Filipin staining of unesterified cholesterol

Mutant strains were co-cultivated using approximately 5×10^5 cells per strain; cell separation was on a 30 ml Ficoll gradient. Fractions I and II were cultured for 96 hours in 5% LPDS-EMEM on coverslips and subsequently challenged with 50 µg pure LDL per ml 5% LPDS-EMEM for 24 hours. Cells were fixed in 10% phosphate buffered formalin (pH 7.2) and treated with 0.01% filipin in phosphate buffered saline: DMSO (4:1, v/v) essentially as described by Vanier *et al.*² Cells were visualised by fluorescence microscopy using a Zeiss Axioplan microscope and filter set 02. The pattern of fluorescence for each cell examined was graded as one of four types: A = no enhanced fluorescence (as observed in control fibroblasts); B = intense punctate perinuclear fluorescence (NPC); C = intense contiguous fluorescence (I-cell disease); and D = equivocal. In order to increase the likelihood that the cells studied were heterokaryons we scored only multinuclear cells with clearly discernible boundaries and three or more nuclei.

Sphingomyelinase activity in vitro

Crosses prepared for enzyme analysis were co-cultivated using approximately $25\text{--}30 \times 10^5$ cells per strain; cells were separated on a 48 ml Ficoll gradient. Postgradient fractions were cultivated for 2.5 days in 10% FBS-EMEM, harvested, and stored at -70°C until analysed. Parental strains were cultured for 2.5 days in 10% FBS-EMEM and analysed simultaneously during the enzyme analysis of respect-

ive crosses. An aliquot from each fraction was concurrently cultured on a coverslip and stained with 10% Giemsa at the same time as cells for enzyme analysis were harvested. The fusion efficiency [(number of nuclei in multinuclear cells ÷ total number of nuclei) × 100] was determined for multinuclear fractions and was at least 60% in reported crosses; the mononuclear fractions contained >95% monokaryotic cells. Sphingomyelinase activity was determined using [N-methyl- ^{14}C]-sphingomyelin as previously described¹² and protein was determined by the method of Lowry *et al.*¹³ β-galactosidase was assayed as a reference enzyme using a 4-methylumbelliferyl substrate.¹⁴

Results and discussion

FILIPIN STAINING OF FREE CHOLESTEROL

The figure (A-C) displays examples of the characteristic staining patterns observed in unfused mononuclear cells in control, NPC, and I-cell disease fibroblasts. I-cell disease fibroblasts were found to exhibit a staining pattern distinct from NPC as reported and discussed previously.²

Control crosses were established to assess the suitability of the strategy for measuring complementation. Control crosses, shown at the top of table 2, were made between an I-cell disease and an NPC mutant strain for expected positive complementation and between NPC sibs from two sib pairs for expected negative complementation. The co-cultivated mononuclear cells in both instances retained their characteristic staining pattern. In the NPC × I-cell cross, 57% of the multinuclear cells showed a normal staining pattern (figure D); none of the multinuclear cells from the sib crosses corrected.

Crosses between the different sib pairs (NPC-5 × NPC-7) indicated an absence of complementation between them and established one major group (group α) which could be used to cross against the other mutant strains. The test crosses displayed in table 2 show that seven further NPC strains failed to complement with group α (example, figure E); however, strain NPC-2 exhibited positive complementation with any group α strain with which it was hybridised (example, figure F). These hybridisation experiments indicate the

Cells stained with filipin after lipoprotein deprivation and LDL challenge. Mononuclear cells from parental strains are shown in frames A to C: A=healthy control; B=NPC; C=I-cell disease. Cells from the multinuclear fractions of mutant crosses are displayed in frames D to F: D, I-cell $\times$ NPC-7b, shows a normalised multinuclear cell; E, NPC-1 $\times$ NPC-7b, displays several intensely stained multinuclear cells; F, NPC-5b $\times$ NPC-2 shows a normalised multinuclear cell at the top of the field. Images were exposed on HP5 film.

existence of a second complementation group (group β) represented by the single strain NPC-2.

IN VITRO SPHINGOMYELINASE ACTIVITY

To confirm the above results we selected the measurement of sphingomyelinase as a marker for complementation using NPC-2 and four other strains (NPC-1,3,4, and 8) which consistently exhibited sphingomyelinase activity $<40\%$ of control levels.

Control crosses using parental self-crosses for negative complementation and NPC versus NPA for positive complementation confirmed the efficacy of our protocol and results pre-

viously reported by Besley *et al.*¹⁵ Absolute levels of sphingomyelinase compared to theoretical activities (determined from the mean activity measured in the individual parental strains) did not rise significantly in fraction II of the parental self-crosses but did increase two to three times in fraction II in crosses of NPA $\times$ NPC (table 3).

In test crosses between NPC-2 and group α NPC strains, the absolute sphingomyelinase activity increased beyond the control range (200–276 *v* 178–215 nmol/hour/mg protein). In crosses between group α strains, the fraction II enzyme levels remained $<60\%$ of control activity, although some of these activities were higher than the theoretical levels. In addition, the β -galactosidase activity in some multinuclear fractions, especially those crosses including NPC-2, yielded a raised absolute activity compared to the theoretical level.

In order to account for possible non-specific changes in lysosomal enzyme activity induced by the experimental method and co-cultivation conditions, we chose to express these results as an index abbreviated MMI (multinuclear:mononuclear index). This index (see legend to table 3 for equation) accounts for these potential alterations in enzyme activity by expressing the fraction II: fraction I sphingomyelinase activity relative to the reference lysosomal enzyme β -galactosidase. The MMI for parental NPC self-crosses was 0.4–1.1 and for NPA $\times$ NPC crosses 2.3–2.5. The NPC group α crosses yielded MMIs between 0.8 and 1.1. The crosses between group α and group β NPC strains yielded MMIs of 1.5–2.2. These results show that sphingomyelinase activity has increased relative to non-specific alterations in lysosomal enzyme activity in both NPA $\times$ NPC crosses and NPC- α $\times$ NPC- β crosses.

Conclusion

Strain NPC-2 is from an Italian patient with an early onset of disease. Liver disease was apparent at birth, but neuronal involvement was not confirmed. Severe lung involvement

Table 2 Complementation analysis of hybrid cells by the staining of free cholesterol with filipin. Percentage of cells exhibiting a normal staining pattern (that is, grade A, as described in the methods section) was determined for multinuclear cells and co-cultivated mononuclear cells for each cross. Control crosses for negative and positive complementation are distinguished from subsequent test crosses between mutant strains

Cross		Mononuclear cells		Multinuclear cells		Interpretation for complementation
Strain 1	Strain 2	No of cells	% normal	No of cells	% normal	
Control crosses						
I-cell	NPC-3	68	0	125	57	Positive
NPC-5a	NPC-5b	118	0	73	0	Negative
NPC-7a	NPC-7b	323	1	82	0	Negative
Test crosses						
NPC-5b	NPC-2	246	7	180	48	Positive
NPC-6	NPC-2	452	1	121	27	Positive
NPC-7a	NPC-2	769	1	121	50	Positive
NPC-8	NPC-2	376	2	39	77	Positive
NPC-5a	NPC-7a	415	0	120	0	Negative
NPC-5a	NPC-3	103	1	75	0	Negative
NPC-5b	NPC-4	313	0	117	0	Negative
NPC-7a	NPC-6	120	2	107	0	Negative
NPC-7b	NPC-1	120	1	85	0	Negative
NPC-1	NPC-8	470	0	120	0	Negative
NPC-1	NPC-9	119	0	255	0	Negative
NPC-1	NPC-10	115	0	284	0	Negative

Table 3 Complementation analysis of hybrid cells by measurement of sphingomyelinase activity

		Mononuclear fraction				Multinuclear fraction				
Cross		Sphingomyelinase (nmol/h/mg protein)		β -galactosidase (nmol/h/mg protein)		SM ^{ase} ÷ β -Gal	Sphingomyelinase (nmol/h/mg protein)		β -galactosidase (nmol/h/mg protein)	
Strain 1	Strain 2	A	T	A	T				SM ^{ase} ÷ β -Gal	MMI
NPC-2	NPC-2	53	48	361	438	0.15	62		999	0.06
NPC-8	NPC-8	35	62	217	432	0.16	60		359	0.17
NPA	NPC-1	26	20	604	506	0.04	64		623	0.10
NPA	NPC-8	38	32	518	532	0.07	74		451	0.16
NPC-1	NPC-4	58	58	373	535	0.16	64		383	0.17
NPC-1	NPC-8	63	70	253	530	0.25	110		533	0.21
NPC-3	NPC-8	37	61	267	486	0.14	83		578	0.14
NPC-2	NPC-1	55	33	277	349	0.20	237		590	0.40
NPC-2	NPC-3	45	65	225	496	0.20	200		696	0.29
NPC-2	NPC-8	73	66	434	442	0.17	276		751	0.37
Cell strain		Sphingomyelinase (nmol/h/mg protein)		β -galactosidase (nmol/h/mg protein)		Sphingomyelinase ÷ β -galactosidase				
Control 1		215		488		0.44				
Control 2		178		425		0.42				

Sphingomyelinase and β -galactosidase activities were determined for mono- and multinuclear fractions from mutant crosses as described in the methods section. Ratios expressing sphingomyelinase activity relative to β -galactosidase activity were calculated for each fraction and the final index for interpretation, the MMI (multinuclear:mononuclear index), was determined from the equation:

$$MMI = \frac{(\text{fraction II sphingomyelinase} \div \text{fraction II } \beta\text{-galactosidase})}{(\text{fraction I sphingomyelinase} \div \text{fraction I } \beta\text{-galactosidase})}$$

Theoretical activities of co-cultivated mononuclear cells for each cross are shown for each enzyme based on simultaneous analysis of parental strains. (A = absolute activity, T = theoretical activity, SM^{ase} = sphingomyelinase, β -Gal = β -galactosidase).

was responsible for the patient's death at a few months of age and is an unusual feature of NPC. Biochemically the patient was classified as a classical NPC patient and was indistinguishable from other diagnosed NPC patients. NPA and NPB had been excluded unequivocally in this patient based on sphingomyelinase activity, which was only partially deficient in skin fibroblasts (26% of the controls in NPC-2 compared to <2% of the controls in NPA or NPB) and normal in leucocytes.

Although the primary biochemical defect in NPC remains unknown, these results indicate the existence of two complementation groups within NPC. This work, in combination with the earlier study reported by Vanier *et al.*⁸ suggests the existence of one major group and at least one minor group.

The work of Carstea *et al.*⁶ suggested that the gene implicated in NPC (based on linkage studies in 12 families) is located on chromosome 18. The large number of affected patients included in this study would suggest that this unidentified gene corresponds to patients in the major complementation group (group α). The existence of a second, rare complementation group (group β) indicates that there is a second gene in which mutations can lead to NPC; its chromosomal location is undetermined.

We would like to thank Dr A P Kent for his advice on cell staining and fluorescent microscopy and for the use of his Zeiss microscope; Professor M Adinolfi for the use of his Zeiss microscope; C M Slade and A R Gran for assistance in tissue culture; Drs P F Benson, G Miele-Vergani, O Gibby, N F Lawton, J Collins, and Professor A P Mowat for biopsies from their patients; Dr M Vanier for initial biochemical confirmation of the NPC patients; and Adrienne Knight for help in manuscript preparation. This work has been supported by Wellcome

Trust grant numbers 034087 and 037623. The Paediatric Research Unit is supported by the Generation Trust.

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