Am. J. Hum. Genet. 58:118–125, 1996

Genetic Heterogeneity in Niemann-Pick C Disease: A Study Using Somatic Cell Hybridization and Linkage Analysis

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Summary

The primary molecular defect underlying Niemann-Pick C disease (NPC) is still unknown. A wide spectrum of clinical and biochemical phenotypes has previously been documented. Indication of genetic heterogeneity has recently been provided for one patient. In the present study, somatic cell hybridization experiments were carried out on skin fibroblast cultures from 32 unrelated NPC patients covering the range of known clinical and biochemical phenotypes. The criterion for complementation was the restoration of a normal intracellular fluorescent pattern in polykaryons stained with filipin to document cholesterol distribution. Crosses between the various cell lines revealed a major complementation group comprising 27 unrelated patients and a second minor group comprising 5 patients. Linkage analysis in one multiplex family belonging to the minor complementation group showed that the mutated gene does not map to the 18q11-12 region assigned to the major gene. Patients in the first group spanned the whole spectrum of clinical and cellular phenotypes. No consistent clinical or biochemical phenotype was associated with the second complementation group. Three of the five group 2 patients, however, presented with a new rare phenotype associated with severe pulmonary involvement leading to death within the first year of life. No biochemical abnormality specific of either group could be demonstrated with regard to tissue lipid storage pattern, intralysosomal cholesterol storage, and regulation of cholesterol homeostasis. Mutations affecting at least two different genes have thus been shown to underlie NPC. The two gene products may function together or sequentially in a common metabolic pathway affecting intracellular cholesterol transport.

Received September 5, 1995; accepted for publication November 1, 1995.

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0002-9297/96/5801-0014$02.00

Introduction

Niemann-Pick C disease (NPC) is an autosomal recessive lysosomal storage disease now well distinguished from the primary sphingomyelinase disorders (Niemann Pick disease types A and B). The disease is best characterized by a unique abnormality of intracellular translocation of low-density lipoprotein (LDL)–derived cholesterol (Pentchev et al. 1994, 1995). Variations in disease expression cover a wide spectrum with respect to the age at onset and type of symptoms, but also to severity of the block in intracellular LDL-cholesterol transport (Vanier et al. 1988, 1991*a*, 1991*b*), raising the question of genetic heterogeneity. Somatic cell hybridization experiments were carried out to provide further insight into the number of gene products potentially involved in NPC. Our early preliminary study (Vanier et al. 1991*a*) identified only one complementation group for 11 unrelated patients. The gene implicated in a majority of NPC families was mapped to chromosome 18 (Carstea et al. 1993). Subsequent parallel linkage and complementation analyses, however, gave indication of genetic heterogeneity (Carstea et al. 1994; Vanier et al. 1994). While this work was in progress Steinberg et al. (1994) independently documented existence of a second complementation group represented by one mutant cell line. The results described in the present study establish genetic heterogeneity within NPC. They show that the cell lines studied so far can be divided into one major and one minor complementation group and that the second complementation gene does not map to the 18q 11-12 region assigned to the major gene.

Material and Methods

Subjects and Biological Material

Diploid fibroblast strains from 32 unrelated patients with NPC retrieved from the Fondation Gillet-Merieux laboratory's cell bank were used in the study. The patients covered a broad biochemical and clinical phenotypic spectrum. The individual level of LDL-induced cholesteryl ester formation observed after a 4.5-h LDL pulse (Vanier et al. 1991*b*) and the schematic clinical phenotype of each patient are reported in table 1. Patients have been sorted by increasing level of LDL-in-

Table 1

Biochemical and Clinical Phenotypes of NPC Patients

Patient Identification	LDL-Induced Cholesteryl Ester Formation after 4.5 h[a] and Biochemical Phenotype	Clinical Presentation	Age at Death or at Last Follow-Up (yr)
	Classical		
Case 1	10	Neonatal cholestatic, rapidly fatal form	.3[b]
Case 2	10	Infantile neurological onset	1.66[b]
Case 3	10	Infantile neurological onset	5.33[b]
Case 4	10	Fetal ascites (Manocochie et al. 1989) (case 1)	7
Case 5	10	Infantile neurological onset	5.75[b]
Case 6	10	Infantile neurological onset	2.5
Case 7	10	Infantile neurological onset	6[b]
Case 8	10	HSM at 17 mo—follow up not available	1.42
Case 9	10	Clinical history unknown	?
Case 10	20	Infantile neurological onset	6.25[b]
Case 11	30	Clinical course unknown	11[b]
Case 12	40	Infantile neurological onset	1.08[b]
Case 13	45	HSM at 4 yr—later follow up not available	4
Case 14	50	Infantile neurological onset	4.3[b]
Case 15	50	Late infantile neurological onset	6.5
Case 16	50	Severe pulmonary involvement (Pin et al. 1990)	0.5[b]
Case 17	60	Late infantile neurological onset	4
Case 18	70	Juvenile neurological onset	9
Case 19	75	Severe pulmonary involvement (Steinberg et al. 1994) (NPC-2)	0.5[b]
Case 20	120	Infantile neurological onset	2
Case 21	120	Juvenile neurological onset	20
Case 22	130	Juvenile neurological onset (Pampols et al. 1986) (case 1)	18[b]
Case 23	140	Late infantile neurological onset	9.8[b]
Case 24	165	Fetal ascites (Manocochie et al. 1989) (case 2)	.06[b]
Case 25	170	Juvenile neurological onset	8
	Intermediate		
Case 26	260	Juvenile neurological onset	17[b]
Case 27	280	Juvenile neurological onset	20
Case 28	320	Severe pulmonary involvement (Schofer et al. 1995)	.6[b]
Case 29	330	Juvenile neurological onset	28
Case 30	410	Juvenile neurological onset	9
	Variant		
Case 31	780	Late juvenile neurological onset	16
Case 32	970	Juvenile neurological onset	15

[a] Determination of cholesteryl ester formation and biochemical phenotype assessment as described by Vanier et al. (1991*b*); HSM-hepatosplenomegaly.
[b] Age at death.

duced cholesteryl ester formation. Strains 1–25, 26–30, and 31–32 correspond to the "classical," "intermediate," and "variant" biochemical phenotypes (Vanier et al. 1991*b*), respectively. Clinical phenotyping was done according to a classification scheme described elsewhere (Vanier et al. 1988, 1991*a*; Pentchev et al. 1995). Control cells were obtained from healthy volunteers and children with other lysosomal storage diseases not affecting intracellular transport of LDL-cholesterol.

Material

Polyethylene glycol (PEG) 1000 and dimethylsulfoxide (DMSO) were purchased from Merck and Ficoll 400 from Pharmacia Biotech. Filipin complex and buffered

formalin 10% were purchased from Sigma Chemical Co, and Fluoprep from Biomérieux. LabTek® chambers were from Nunc. Culture media, FCS, and trypsin were from Biomérieux, ICN Pharmaceuticals, or Gibco BRL. Bovine and human lipoprotein-deficient serum (LPDS) and LDLs were prepared in the laboratory from FCS or from fresh human plasma as described by Goldstein et al. (1983). LDLs were used within 1 mo of preparation.

Cell Fusion and Enrichment of Polykaryons

Cultures were grown as monolayers in a humidified incubator (5% CO_2) at 37°C, in minimal essential medium (MEM) supplemented with 10% FCS or LPDS and antibiotics according to standard conditions (Vanier et al. 1985). For each fusion, the two parental cell lines were trypsinized, carefully mixed at a 1:1 ratio (0.7 to 1.10^6 cells of each), seeded in a 25-cm^2 flask, and cocultivated in 10% FCS-MEM medium for 8–16 h. Fusion was carried out by the addition of 41% PEG and 8.75% DMSO in MEM followed by 25% PEG and further dilution steps according to the procedure of Brul et al. (1988). To demonstrate the effects of fusion most effectively, we separated multikaryons from monokaryons. Between 18 and 24 h after fusion, polykaryons were enriched by discontinuous Ficoll gradient sedimentation (layers of 5 ml each of 5.0%, 7.5%, 10%, 12.5%, and 15% w/v Ficoll 400 in MEM supplemented with 10% FCS), essentially as described by Nelson and Carey (1985). After 3 h of sedimentation at $1 \times g$, mono- and polykaryons (7.5% and 12.5% layers, respectively) were collected and suitable aliquots were plated in two-well Labtek chambers.

Assay for Complementation: Cytochemical Visualization of Unesterified Cholesterol Accumulation by Filipin Staining

The fused cells were cultured for 48 h in 10% LPDS-supplemented medium and for an additional 24 h in fresh medium supplemented with 10% LPDS and pure LDL (50 μg/ml). The cell monolayers were fixed, stained with filipin as described by Vanier et al. (1991*b*), mounted with Fluoprep, and examined by fluorescence microscopy using an Ortholux Leitz epifluorescence microscope equipped with a B2 combined filter. The pattern of fluorescent perinuclear vesicles was evaluated either as "negative," as seen in normal or essentially normal cells, or as "positive," corresponding to high fluorescence level as seen in NPC (fig. 1). Only cells with three or more nuclei were evaluated, to increase the probability of studying heteropolykaryons, and results were expressed as percentage of "negative" polykaryons. Restoration of a normal filipin pattern was used as the criterion of complementation.

Genetic Linkage Analysis

Chromosome 18 microsatellites.—The NPC interval of chromosome 18 was defined by loci D18S44 and D18S66 as reported by Carstea et al. (1994). Pericentromeric microsatellites that either map within or flank the NPC interval were used to assess their genetic linkage to the clinical and biochemical disease phenotype presented by the patients. Microsatellite markers D18S53, D18S56, D18S57, D18S66, D18S71, and D18S480 were previously described and mapped by Weissenbach et al. (1992). Markers D18S40 and D18S44 were independently described and mapped by Straub et al. (1993). A comprehensive genetic map was assembled and is accessible through the Cooperative Human Linkage Center via the World Wide Web at http://www.chlc.org/.

DNA preparation and PCR analysis.—DNA was prepared from patient 27, her parents, and her affected sister. Genomic DNA was isolated by standard methods (Sambrook et al. 1989, pp. 273–77). PCR and subsequent polyacrylamide gel electrophoresis of the PCR products were performed as described by Polymeropoulos et al. (1992). Autoradiography was performed at −70°C for periods of 2 h to overnight.

Other Analytical Procedures

Regulation of de novo cholesterol synthesis from [^{3}H]-acetate was studied as described by Rodriguez-Lafrasse et al. (1990). Methods for quantitative lipid studies in liver and spleen were as in the study by Vanier et al. (1983).

Results

Evaluation of the Complementation Assay System

Control crosses were performed to validate the complementation strategy and to define the experimental threshold of positive (C+) and negative (C−) complementation. Crosses between NPC cells and control cells (C+) resulted in 74 ± 8% (n = 8) filipin-negative polykaryons (fluorescent pattern as in fig. 1*D*). In crosses of NPC cells with themselves (C−), an unexpectedly high proportion of essentially filipin-negative polykaryons, ranging between 4% and 25% (12 ± 10; n = 14), was observed (fig. 2). Under our experimental conditions, polykaryons were first cleared of lysosomal cholesterol by maintenance in LPDS-supplemented medium and then secondarily challenged with LDL. One likely explanation for this level of filipin-negative cells is that some polykaryons have a reduced metabolic vitality resulting in diminished LDL uptake. The broad variation observed between repeated experiments using the same cell strains would support this hypothesis. Still, as seen in figure 2, there was a very clear-cut difference between the C+ and C− areas.

Complementation Studies in NPC Cells

Figure 1 displays representative examples of fluorescence patterns observed in the parent cells (panel *A*) and

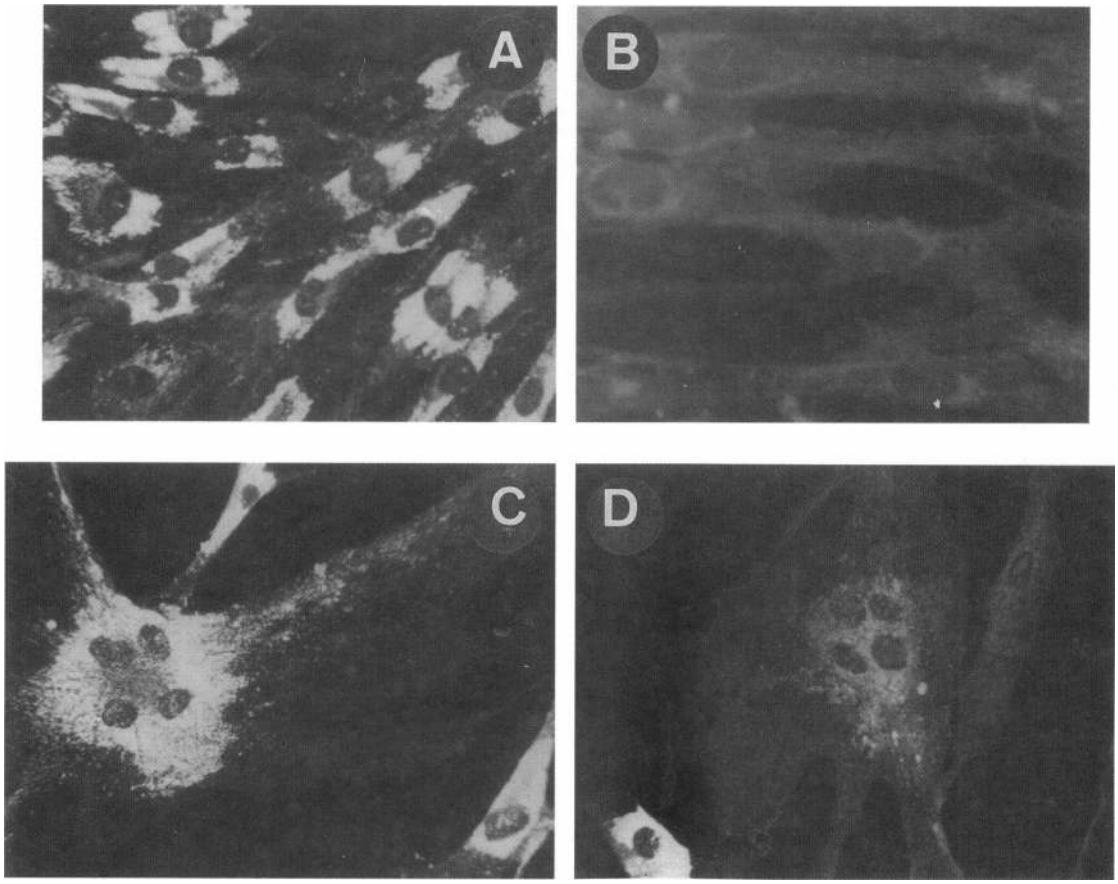

Figure 1 Fluorescence cytochemical analysis of cells challenged with LDL and stained with the cholesterol specific marker, filipin. *A*, Unfused fibroblasts from a "classical" NPC cell line. *B*, Unfused normal fibroblasts. *C*, "Filipin-positive" polykaryon, indicating an absence of complementation after crossing two NPC cell lines. *D*, "Filipin-negative" polykaryon, indicating complementation after crossing two NPC cell lines. A similar pattern is obtained by crossing a NPC line with a normal line.

in test crosses (panels *C* and *D*). Seven different strains assigned to complementation group 1 were successively used during the study. For the sake of clarity, the final results are presented graphically (fig. 2) as the mean result of crosses between each individual NPC strain 1–32 and a NPC strain previously assigned to group 1, expressed in percentage of filipin-negative polykaryons.

In all cell lines except those corresponding to cases 2, 16, 19, 27, and 28, most polykaryons (72%–96%) were strongly filipin positive, indicating an absence of complementation. The finding expands the previous observations of a predominating complementation group reported by us (Vanier et al. 1991*a*) as well as by Steinberg et al. (1994). Two cell lines obtained from patients reported by Manocochie et al. (1989) were included in the series of Steinberg et al. (1994), as well as in ours (cases 4 and 24), establishing that the major groups in both studies were identical. It is interesting that cell lines exhibiting a classical, intermediate, or variant biochemical phenotype coexisted in the same complementation group. The clinical picture within group 1 patients was also very variable. Besides patients with the classical juvenile onset, this group included many patients with an infantile neurological onset and one patient with the rapidly fatal, neonatal cholestatic form.

As shown on figure 2, a clear-cut complementation was found in crosses between cell lines belonging to group 1 and cells derived from individuals 2, 16, 19, 27, and 28. Our next step was to study hybrids between the latter cells. Crosses between cells from case 27 and cases 2, 16, 19, and 28 showed in all instances an absence of complementation (18%, 10%, 22%, and 7% of filipin-negative polykaryons, respectively), indicating that all five individuals belong to the same complementation group. Cell line 19 corresponds to that described as NPC-2 in the study by Steinberg et al. (1994) (A. H. Fensom, personal communication). Therefore, current studies have so far identified two complementation groups in NPC. The occurrence of the second group can be estimated to slightly >10% of the NPC families.

Clinical and Biochemical Characterization of the Second Complementation Group

Clinical presentation.—Three of the cases—19, 16, and 28—had a very unusual clinical presentation, characterized by an early severe pulmonary involvement that

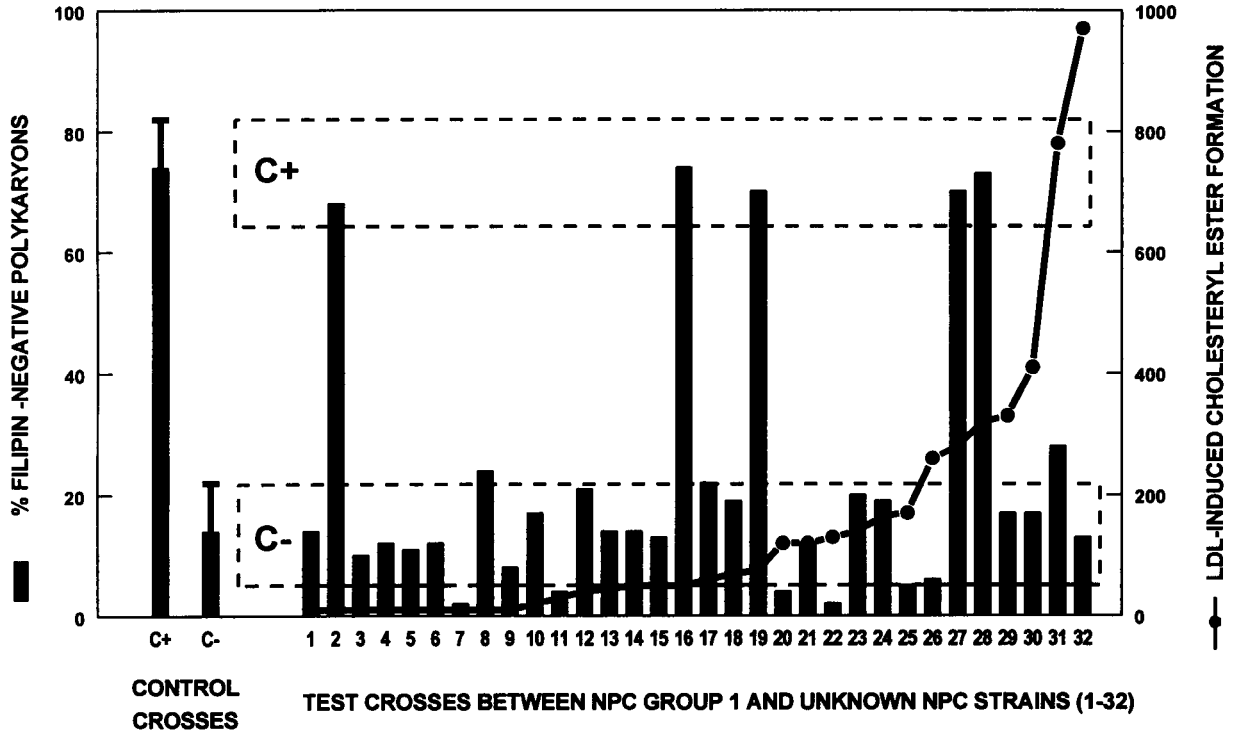

Figure 2 Complementation studies between NPC cell lines. The results are expressed as the percentage of filipin-negative polykaryons, shown as solid bars. Control crosses: C+ (complementation) corresponds to crosses between a NPC strain and a control strain; C− (no complementation) corresponds to crosses of NPC cells with themselves. Seven different strains assigned to complementation group 1 were successively used. The dashed areas correspond to the level expected for noncomplementing (C−) and complementing (C+) crosses. Numbers 1–32 refer to cases described in table 1. The solid line with filled circles indicates the gradient of LDL-induced cholesterol ester formation, expressed as pmol/4.5h/mg cell protein (Vanier et al. 1991*b*)

leads to death at ~6 mo of age, before the onset of neurological symptoms. However, case 2 had a typical neurovisceral disease with infantile neurological onset and rapid course, while case 27 and her affected sister both had a typical juvenile form of the disease. There was no common ethnic origin: case 19 was Italian, case 16 French, case 28 German, and cases 2 and 27 were from North Africa.

Lipid storage pattern.—Liver and spleen tissues could be studied in case 16 and in a fetal sib of case 19. Values were compared to age-matched controls and complementation group 1 NPC patients (table 2). The storage pattern involved free cholesterol and sphingomyelin, but also bis(monoacylglycero) phosphate and glucosylceramide (data not shown), as typically described in the disease.

Intracellular LDL-induced cholesterol homeostasis.—The pattern of intravesicular cholesterol storage visualized after filipin staining and depicted in figure 1*A*, was qualitatively similar in complementation group 1 and group 2 fibroblasts. The intensity of storage (data not shown) appeared to correlate with the level of impairment of LDL-induced cholesteryl ester formation, which was variably affected among different strains belonging to either complementation group 1 or complementation group 2, as shown in figure 2. Down-regulation of de novo cholesterol synthesis from acetate, another homeostatic reaction, was also affected in cells from both complementation groups, again to a varying degree within the same group, as illustrated by cases 19 and 27 (fig. 3).

Linkage Studies in Complementation Group 2

To assess the link between the NPC disease phenotype and the genomic region of chromosome 18q11-12, highly polymorphic microsatellite markers were used. In this study, the resulting genotypes of the family members of patients 10, 13, and 30 indicated identical allele inheritance between affected siblings for the given loci above D18S66 (fig. 4). This finding is consistent with recent mapping evidence that places the NPC gene defect on 18q11-12 between loci D18S44 and D18S66 (Carstea et al. 1994). In contrast, we have found (fig. 4) that patient 27 and his affected sibling do not inherit identical alleles at the broad region encompassing the NPC interval of D18S44–D18S66. This effectively excludes all genes of this region of chromosome 18 as being associated with their NPC phenotype. This evidence conclu-

Table 2

Lipid Studies in Tissues of Complementation Group 2 Patients

Tissue	Cholesterol	Phospholipids	Sphingomyelin
		(μmol/g wet weight)	
Liver:			
Case 16 (group 2)	17.9	41.5	3.9
Case 22 (group 1)	15.2	35.0	3.5
NPC[a] (n = 15)	25 ± 6	43 ± 10	6.2 ± 2.5
Controls[a] (n = 6)	12 ± 3	23 ± 2	1.9 ± 0.3
Fetal sib of case 19 (group 2)	15.5	19.2	4.0
Fetal sib of case 12 (group 1)	17.6	22.3	4.0
Fetal Controls[b] (n = 4)	8.0 ± 1.6	18.1 ± 4.8	1.7 ± 0.5
Spleen:			
Case 16 (group 2)	34.9	41.2	15.6
NPC[a] (n = 8)	49 ± 13	48 ± 6	18.4 ± 5.9
Controls[a] (n = 6)	11 ± 1	15 ± 2	3.0 ± 0.6

[a] From Vanier (1983).
[b] From Vanier et al. (1985); NPC = children with Niemann-Pick C disease, complementation group unknown.

sively establishes the existence of a second NPC gene locus.

Discussion

The wide range of phenotypic variation observed in NPC has suggested that more than one gene could underlie the disease. To get further insight on the number of gene products potentially involved, complementation analysis in cultured skin fibroblasts appeared to be the method of choice, since cell lines from all patients shared a biochemical abnormality closely linked to the primary defect (i.e., abnormal LDL-cholesterol processing) (Pentchev et al. 1995). Either the measure of LDL-induced cholesteryl ester formation or the vesicular storage of unesterified cholesterol evidenced by filipin staining could in theory be used as a complementation test. The latter appeared best suited for our purpose, since it is more prone to give clear-cut results in the study of patients with a "variant" biochemical phenotype (i.e.,

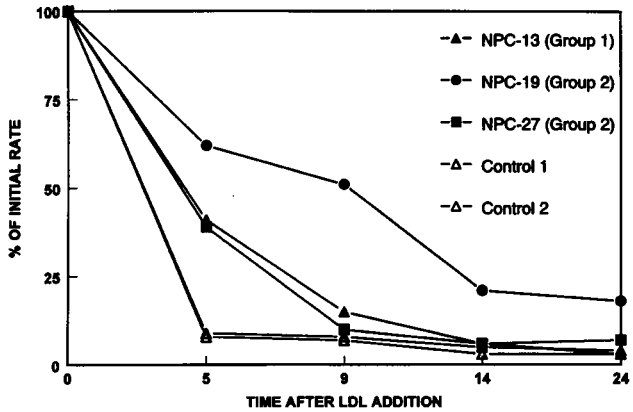

Figure 3 Time course (h) of LDL-mediated suppression of de novo cholesterol synthesis in normal and NPC cell lines. For each cell type, results have been normalized and expressed as percent of the response obtained for cells maintained in LPDS-supplemented medium.

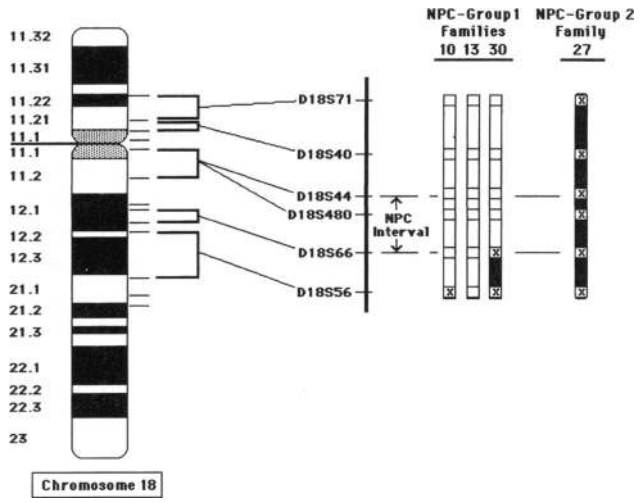

Figure 4 Use of polymorphic microsatellites to assess the inheritance of chromosome 18 alleles in affected siblings of four NPC families. The NPC interval, as shown elsewhere (Carstea et al. 1993, 1994), is defined by the region between D18S44 and D18S66. The columns (containing boxes) depict the region of chromosome 18 that was assessed for each given family. Empty boxes indicate that the NPC-affected siblings inherited identical alleles at that locus. Boxes containing an X indicate nonidentical inheritance among the affected siblings at that locus. Patient 27 and his affected sibling showed nonidentical allele inheritance both within and around the NPC interval. This event effectively excludes the possibility that this chromosome region carries the gene associated with their NPC phenotype.

showing only minor alterations of cholesterol esterification) (Argoff et al. 1991; Vanier et al. 1991*b*). Another advantage of the cytochemical test was the possibility to evaluate multinucleated cells even in the presence of a significant number of unfused parent mononucleated cells. This strategy was validated in our preliminary study (Vanier et al. 1991*a*), and an essentially similar approach was used later by Steinberg et al. (1994).

Our early study (Vanier et al. 1991*a*) identified only one complementation group on 11 cell lines from unrelated patients spanning the classical, intermediate, and variant biochemical phenotype. The subsequent work of Steinberg et al. (1994) on 10 additional families with a classical phenotype confirmed the finding that a majority of patients belonged to the same complementation group and disclosed existence of a second group represented by a single case. We have now extended the latter observation to a total of five families, including their patient NPC-2 (our case 19), and attempted to more closely define complementation group 2.

NPC patients belonging to complementation group 1 covered the whole range of clinical phenotypic expression in the present study as well as in that of Steinberg et al. (1994). Preliminary reports further indicate that Nova Scotian "type D" patients belong to this main group (Sidhu et al. 1993; Greer et al. 1995). We have found that a wide clinical spectrum also applies to group 2. Nevertheless, the observation that all three presently studied cases with a rare severe pulmonary form of the disease belonged to the latter group is intriguing and worth a more detailed investigation.

In complementation group 2 patients, biochemical abnormalities did not differ from those found in group 1. The type of the lipid storage pattern in liver tissue and its developmental profile were similar. In cultured fibroblasts, examination of the intracellular cholesterol storage did not disclose any specific pattern. Disruption of intracellular LDL-mediated cholesterol homeostatic responses was shown to involve regulation of de novo cholesterol synthesis as well as that of cholesteryl ester formation.

At variance with Steinberg et al. (1994), who selected cell lines with uniform severe abnormalities, our initial goal in complementation studies was to better understand the varying severity in impairment of the early homeostatic responses (Bowler et al. 1990; Argoff et al. 1991; Vanier et al. 1991*b*), which we described as classical, intermediate, and variant phenotypes (Vanier et al. 1991*b*). A significant finding of the present work is the observation that phenotypic variation may occur similarly in both complementation groups. In mutant Chinese hamster ovary cells defective in the intracellular transport of LDL-derived cholesterol (Cadigan et al. 1990; Dahl et al. 1992), two complementation classes have also been disclosed (Dahl et al. 1994). One showed a phenotype very similar to classical NPC, while the single class 2 mutant was characterized by expression of a variant NPC phenotype. This finding is not necessarily incompatible with the situation in human NPC, and the authors have indeed discussed that the allelic variation of the class 2 mutant may mask the true phenotype of this class.

Impaired intracellular LDL-cholesterol processing is a constant abnormality in NPC cells, but the exact nature of the primary lesion is unknown. Current complementation studies have given good indication that a deficiency of two different gene products may cause the disease. Some caution in interpretation of complementation studies is needed, however, especially when the biological criterion is not the direct consequence of the gene defect, as proved by the recent example of ataxia telangiectasia (Savitsky et al. 1995). Our results are considerably strengthened by the linkage study in a family representative of complementation group 2, showing recombinations throughout the genetic interval D18S44–D18S66 and thus ruling out the region of chromosome 18 (Carstea et al. 1993, 1994) containing the gene involved in complementation group 1 patients.

In conclusion, the present data have established that mutations affecting at least two genes may underlie NPC. The similar biochemical phenotypes observed in the two known complementation groups suggest that the products may function together or sequentially in a common metabolic pathway affecting intracellular cholesterol transport. Further work is in progress to find other group 2 multiplex families and to define whether additional complementation groups exist within NPC.

Acknowledgments

We wish to thank Dr. A. H. Fensom and Dr. S. Steinberg, London, for informing us of the identity between their strain NPC-2 and our strain 19. We are also greatly indebted to all colleagues who provided us with biological material and clinical data from their patients. The expert technical assistance of Ms. M. C. Juge and Ms. G. Bailloud is gratefully acknowledged. The study was supported by INSERM, the National Niemann-Pick Disease Foundation, and Vaincre les Maladies Lysosomales. S.D. was the recipient of a fellowship from Vaincre les Maladies Lysosomales.

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