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A defect in cholesterol esterification in Niemann–Pick disease (type C) patients

(cholesterol-storage disorder/fluorescence microscopy/non-lipoprotein cholesterol)

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ABSTRACT **The demonstration of a defect of cholesterol esterification in a mutant strain of BALB/c mice with an attendant reduction of sphingomyelinase activity [Pentchev, P. G., Boothe, A. D., Kruth, H. S., Weintroub, H., Stivers, J. & Brady, R. O. (1984) *J. Biol. Chem.* 259, 5784–5791] prompted us to examine the capacity of cultured human Niemann–Pick fibroblasts to esterify exogenously derived cholesterol. Cholesterol was supplied to cell cultures in the form of native or chemically modified, positively charged low density lipoprotein or as non-lipoprotein cholesterol. Cholesterol esterification was not impaired in cell cultures derived from patients with type A or B Niemann–Pick disease. However, esterification of exogenously administered cholesterol was deficient in 20 type C Niemann–Pick cell lines that were available for testing. Fluorescence histochemical staining of unesterified cholesterol in type C cells suggested that these cells were able to internalize and lysosomally process lipoprotein cholesterol. Acyl-CoA:cholesterol acyltransferase activity did not appear deficient in type C cell extracts. The error in cholesterol esterification may provide an opportunity for probing the molecular lesion in this disorder and may afford a useful and reliable means for establishing diagnosis.**

Niemann–Pick disease is an inborn error of metabolism associated with sphingomyelin accumulation and sphingomyelinase deficiency (1). The two classic forms, types A and B, represent primary lysosomal storage disorders of sphingomyelin caused by severe deficiencies of sphingomyelinase activity (2, 3). Other affected individuals have also been classified as Niemann–Pick patients (types C, D, and E) even though no clear and consistent deficiency of sphingomyelinase has been documented for these particular phenotypes (1). The common characteristics among this latter group are chronic neurological deterioration associated with hepatomegaly, foamy macrophage infiltration of tissues, sea-blue histiocyte staining in bone marrow, and a modest accumulation of sphingomyelin in certain tissues (1).

A murine lipid-storage disorder with some of the features of Niemann–Pick disease has been documented (4, 6). Among the abnormal phenotypic manifestations found in mice affected with this autosomal recessive, neurovisceral storage disorder are a partial deficiency of sphingomyelinase and a modest accumulation of sphingomyelin (4). The deficiency of sphingomyelinase does not appear to be a primary feature of the murine disorder, since the levels and properties of this enzyme in tissue extracts of heterozygous mice appeared normal (4). However, both *in vivo* and in tissue cultures, a specific and extensive block in the esterification of exogenously derived cholesterol was apparent in the homozygous murine mutants. Further studies have revealed that the murine esterification error is partially expressed (50% of normal esterification) in fibroblasts derived from heterozygous mice (unpublished observations). It appears that the primary genetic lesion of the murine lipid-storage disorder is closely linked to an error in cholesterol esterification and storage.

Because of the similarities in some of the phenotypic presentations of the human and murine sphingomyelin storage disorders, we examined the ability of cultured fibroblasts derived from Niemann–Pick patients to esterify exogenously derived cholesterol. All 20 type C Niemann–Pick cell cultures examined showed a major deficiency in cholesterol esterification that could not be accounted for by an apparent deficiency in acyl-CoA:cholesterol *O*-acyltransferase (EC 2.3.1.26) activity. Esterification of cholesterol was normal in types A and B Niemann–Pick cell cultures.

MATERIALS AND METHODS

Cells. Niemann–Pick C (NPC) cell lines NPC-1, -3 to -6, -8 to -10, -12, and -15 to -20 were provided from the laboratory of M.T.V. Cell lines NPC-2 and -7 were provided by D.A.W. NPC-11 (GM0110) and NPC-13 (GM3123) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The cell line NPC-14 was generously provided by J. F. O'Brien (Mayo Clinic, Rochester, MN). Control cells (normal and non-Niemann–Pick) and types A and B Niemann–Pick cells originated from the Human Genetic Mutant Cell Repository or from the laboratory of M.T.V. These skin fibroblasts were obtained under procedures approved by the clinical research committees of the respective medical centers.

Lipoprotein Preparations and Lipoprotein-Deficient Serum. Fresh human low density lipoprotein (LDL) (d = 1.019–1.063 g/ml) was prepared by sequential ultracentrifugation from a KBr/plasma solution (7) and was obtained from Meloy Laboratories (Springfield, VA). Positively charged LDL was prepared from fresh LDL by covalent coupling with the tertiary amine *N,N*-dimethyl-1,3-propanediamine as described by Basu *et al.* (8). Lipoprotein-deficient serum ($d <$ 1.20 g/ml) was prepared from fetal bovine serum by ultracentrifugation from KBr/serum and was purchased from Meloy Laboratories.

Radioisotopes. [1,2-^{3}H]Cholesterol (60–90 Ci/mmol; 1 Ci = 37 GBq) and [9,10-^{3}H]oleic acid (2–10 Ci/mmol) were

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Abbreviation: LDL, low density lipoprotein(s).
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obtained from New England Nuclear. [^{3}H]Cholesterol was repurified by reversed-phase thin-layer chromatography prior to use.

Tissue Culture and Lipid Analysis. Cells were maintained in Eagle's minimal essential medium with 10% (vol/vol) normal fetal bovine serum, 1% (wt/vol) L-glutamine, penicillin (100 units/ml), and streptomycin (100 mg/ml). Cells were grown in humidified 95% air/5% CO_2 at 37°C. Cells were harvested by trypsinization for 5 min at 37°C. Washed cell pellets were sonicated with 100 μl of H_2O, and an aliquot of the fibroblast extract was delipidated by addition of 4 ml of chloroform/methanol (2:1) and 1 ml of H_2O. The two-phase lipid-extraction system (9) was centrifuged and the lower, lipid-containing phase removed. Following addition of carrier lipid and evaporation, the lipids were analyzed by thin-layer chromatography on 0.25 mm thick, precoated silica gel 60 plates, (Merck, Darmstadt, F.R.G.). Neutral lipids were separated in a solvent system of hexane/ether/glacial acetic acid (90:10:1), and the locations of labeled cholesterol, cholesteryl ester, and triacylglycerol were outlined with iodine vapor. The lipids were scraped from the plates and radioactivity was measured by liquid scintillation spectroscopy in 10 ml of Aquasol.

RESULTS

Fluorescence Histochemical Staining for Cholesterol in Cultured Human Fibroblasts. The ability of the fluorescent antibiotic filipin to form stable and specific complexes with unesterified cholesterol (10) has been utilized to develop a method to monitor the intracellular accumulation of cholesterol by fluorescence microscopy (11). This technique was employed in the present experiments to examine the intracellular level of unesterified cholesterol when cultured fibroblasts are grown in medium containing 10% fetal bovine serum. The photomicrographs in Fig. 1 show that sparse cultures of type C Niemann–Pick fibroblasts develop a relatively intense fluorescence with filipin that is not observed with either normal or type A or B Niemann–Pick fibroblasts. These findings indicate that in type C Niemann–Pick cells there is an abnormal storage of lipoprotein-derived cholesterol in the unesterified form.

Esterification of Lipoprotein Derived-Cholesterol. As outlined and developed by Goldstein, Brown, and their coworkers, uptake and normal processing of LDL by fibroblasts leads to intracellular esterification of LDL-derived-cholesterol which may be isotopically monitored by measuring the increased synthesis of cholesteryl esters in cell cultures incubated with LDL (12). This LDL stimulation of cholesteryl ester synthesis was utilized to compare esterification of cholesterol in various Niemann–Pick cell lines (Table 1). With types A and B Niemann–Pick cell cultures, LDL enhancement of cholesteryl ester synthesis was within the normal range. However, in 10 of 11 Niemann–Pick C cell lines, the esterification of LDL cholesterol was substantially lower than that of the control cells.

It has been shown that chemical modification of native LDL from a negatively charged complex to that of a polycationic form causes enhanced uptake of the lipoprotein by a mechanism that bypasses the normal LDL receptor (8). As with native LDL, such cationic LDL leads to enhanced intracellular esterification of cholesterol. The esterification response with cationic LDL in types A and B Niemann–Pick cells was somewhat higher than that of the control cells (Table 2). Of the eight Niemann–Pick C cell lines tested with cationic LDL, two responded in the normal range and six showed responses that ranged from 0% to 54% of the control response.

Esterification of the Non-Lipoprotein [^{3}H]Cholesterol. Although intracellular entry of lipoprotein and non-lipoprotein cholesterol has been shown to differ in several major ways (13–15), many of the same cellular responses to exogenously derived sterol are elicited with both forms of cholesterol (13, 14). Esterification of a portion of the exogenously derived cholesterol is one of these commonly shared responses. The esterification of non-lipoprotein [^{3}H]cholesterol by types A and B Niemann–Pick cell cultures was within the range found in control cells (Table 3). However, all 20 Niemann–Pick C cell lines tested showed a marked deficiency in esterification of non-lipoprotein cholesterol (11% of control; range 1–26%).

DISCUSSION

Storage of exogenous cholesterol as esters is one of the homeostatic responses available to cells to maintain relatively constant levels of membrane-associated cholesterol (12). The current study with 31 separate Niemann–Pick cell lines derived from the three major forms of the disorder (types A–C) has revealed that intracellular cholesterol esterification is severely and uniquely compromised in cultured fibroblasts derived from patients with type C Niemann–Pick disease that were examined. All 20 type C Niemann–Pick cell lines studied showed substantial deficiencies in esterification of cholesterol derived from at least one of the three exogenous cholesterol sources provided to the cells. With three exceptions, all type C cell lines examined showed a lesion in esterification irrespective of the source of the exogenous cholesterol. In contrast to the refractive response of type C cells, esterification of exogenously derived cholesterol appeared normal in types A and B Niemann–Pick cells.

The well-defined physiological pathway for exogenous cholesterol processing in cultured fibroblasts involves binding of lipoprotein cholesterol to LDL receptors, endocytosis of the ligand–receptor complex, and subsequent hydrolysis of the esterified LDL cholesterol in lysosomes prior to its reesterification by microsomal acyl-CoA:cholesterol acyltransferase (12). Histochemical studies with filipin showed that type C Niemann–Pick fibroblasts accumulated unesterified cholesterol when cultured with whole serum, suggesting that uptake and initial lysosomal processing of lipoprotein cholesterol are not compromised and do not account for the lack of cholesterol esterification observed in this metabolic disorder. A lesion in cholesterol processing subsequent to internalization of exogenous cholesterol is strongly supported by the fact that all type C Niemann–Pick cells examined showed a deficiency in the esterification of non-lipoprotein [^{3}H]cholesterol (Table 3). This form of cholesterol has been shown to diffuse readily into the cellular cholesterol pools of cultured fibroblasts independent of the LDL receptor (13, 14) or endocytosis in general (15). At the terminus of the

FIG. 1 (*on opposite page*). Fluorescent filipin staining for unesterified cholesterol in normal and types A, B, and C Niemann–Pick fibroblasts grown in medium with 10% fetal bovine serum. Normal (N) and Niemann–Pick A (NP-A), B (NP-B), and C (NP-C) fibroblasts were plated in single glass slide chambers (34,000 cells per chamber) and grown for 2 days in minimal essential medium supplemented with 10% fetal bovine serum. These nonconfluent cultures were rinsed, fixed in 10% phosphate-buffered formalin, and stained with filipin (11). (*Left*) Phase photomicrographs. (*Right*) Respective fluorescence photomicrographs of the same microscopic fields. N, NP-A, and NP-B fibroblasts showed minimal filipin-stained intracellular unesterified cholesterol. In contrast, all NP-C fibroblasts contained numerous filipin-stained, unesterified cholesterol-containing, intracellular inclusions. In some cells, individual inclusions are not resolved photographically because of their high numbers. (×50.)

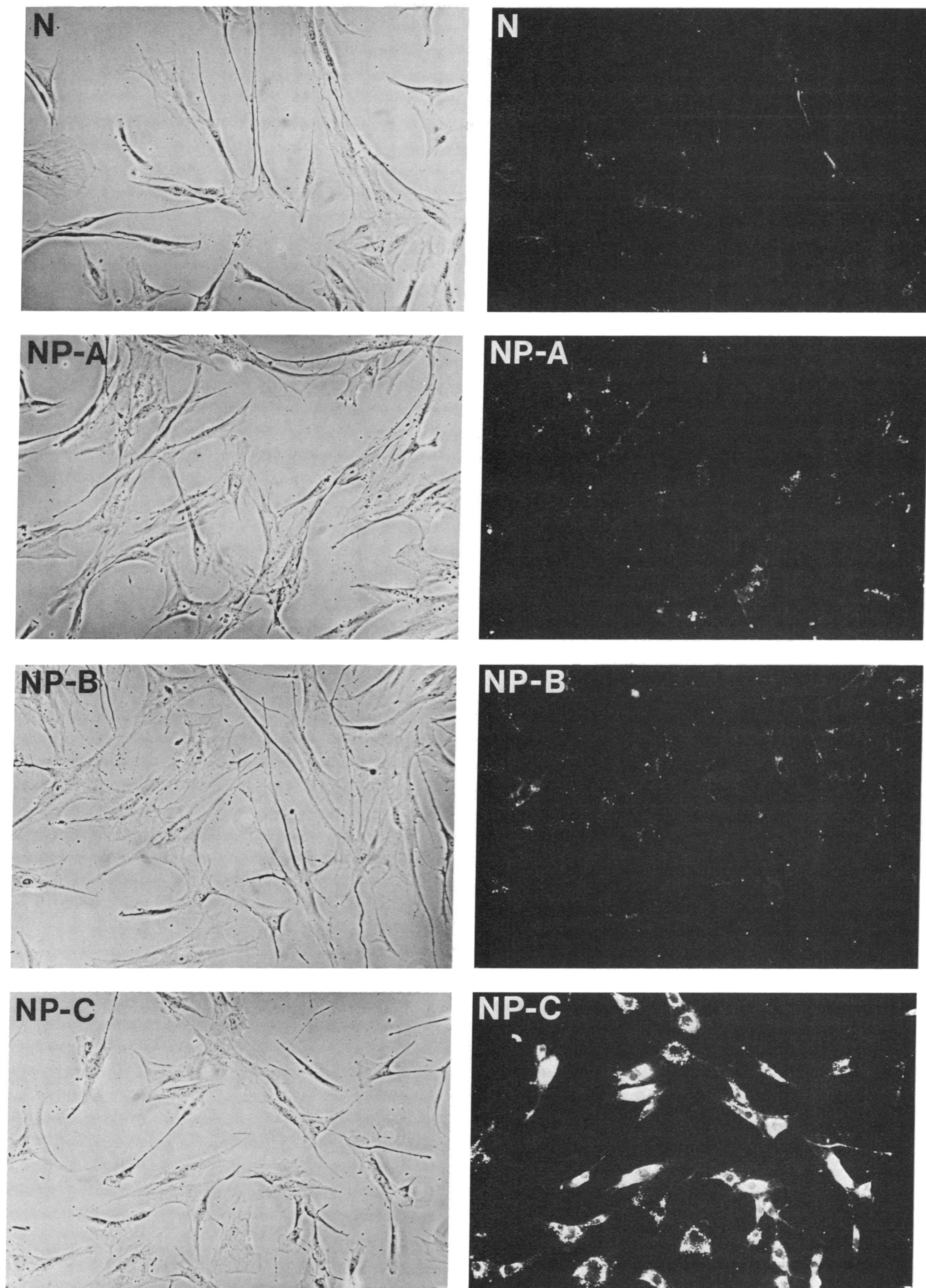

Fig. 1. (*Legend appears at the bottom of the opposite page.*)

Table 1. Esterification of lipoprotein-derived cholesterol in cultured human fibroblasts

Cell line	Cholesteryl [^{3}H]oleate formation, nmol/mg of protein			Esterification, % of control
	+LDL	−LDL	Δ (±LDL)	
Control (n = 5)	6.0 ± 2.0	0.10 ± 0.06	5.9 ± 2.0	
Niemann–Pick A (n = 4)	6.2 ± 3.2	0.18 ± 0.14	6.0 ± 3.4	102 ± 58
Niemann–Pick B (n = 7)	4.0 ± 1.6	0.10 ± 0.05	3.9 ± 1.5	65 ± 26
Niemann–Pick C				
NPC-1	3.0	0.10	2.9	49
NPC-4	6.9	0.18	6.7	114
NPC-16	1.5	0.10	1.4	24
NPC-19	1.4	0.20	1.2	20
NPC-20	1.3	0.13	1.2	20
NPC-13	1.1	0.10	1.0	17
NPC-5	1.0	0.05	0.9	19
NPC-17	0.9	0.10	0.8	14
NPC-15	0.4	0.02	0.4	6
NPC-10	0.3	0.05	0.2	4
NPC-11	1.2	2.0	0	0

Normal and affected fibroblasts (3×10^5) were seeded in 75-cm^2 flasks and incubated for 24 hr in 5 ml of McCoy's medium supplemented with penicillin, streptomycin, L-glutamine, and 10% lipoprotein-deficient fetal bovine serum as described (6). Further intracellular cholesterol depletion and maintenance of a low cell density was achieved by 72 hr of incubation in fresh medium containing no serum. Cell cultures were given fresh serum-free medium containing LDL (100 μg/ml), incubated for 24 hr, and then supplemented with 40 μl of 12.5 mM [^{3}H]oleate [200 dpm/pmol in 12% (wt/vol) bovine serum albumin] for 2 hr. Cell washing, harvesting, and lipid analysis were as described in *Materials and Methods*.

cholesterol-processing pathway, the catalytic potential for cholesterol esterification appears undisturbed, since the levels of acyl-CoA:cholesterol acyltransferase activity in cell-free extracts of type C fibroblasts was determined to be 85 ± 20% (mean ± SEM, n = 7) of control cell extracts (data not shown). Taken together, the findings indicate that the lesion in cholesterol esterification associated with type C Niemann–Pick disease lies subsequent to initial processing of cholesterol and prior to the interaction with the acyltransferase.

The error in esterification associated with type C Niemann–Pick fibroblasts was not uniformly expressed with all forms of exogenous cholesterol tested. For example, Niemann–Pick C cell lines NPC-4 and NPC-8 showed normal esterification of native and cationic LDL, respectively, even though esterification of non-lipoprotein cholesterol was 15% and 7% of normal, respectively. These apparent discrepancies are unexplained at present. Perhaps further studies with such cell lines will show that their mutation affects only the pathway that leads to interaction of non-lipoprotein cholesterol with cholesterol acyltransferase. In any case, the degree of blockage in cholesterol esterification varied significantly among the 20 Niemann–Pick C cell lines studied. Further studies will be required to determine to what extent this variation in response may reflect genotypic heterogeneity. Type C Niemann–Pick disease has in fact been documented to represent a disorder with considerable biochemical and clinical heterogeneity (5, 16).

The murine cholesterol-storage disorder that prompted the examination of cholesterol esterification in the human Niemann–Pick disorders has recently been shown to involve an as yet unrecognized primary molecular alteration that can partially compromise cholesterol esterification in the heterozygous mutant genotype (unpublished observations).

Table 2. Esterification of cationic-lipoprotein-derived cholesterol in cultured fibroblasts

Cell line	Cholesteryl [^{3}H]oleate formation, nmol/mg of protein			LDL-stimulated cholesteryl ester formation, % of control
	+LDL	−LDL	Δ (±LDL)	
Control (n = 3)	2.0 ± 1.1	0.3 ± 0.3	1.7 ± 0.9	
Niemann–Pick A (n = 2)	3.1	0.5	2.6	153
Niemann–Pick B (n = 4)	3.0 ± 1.3	0.3 ± 0.3	2.7 ± 1.1	161 ± 66
Niemann–Pick C				
NPC-1	1.73	0.16	1.6	94
NPC-8	2.31	0.37	2.0	118
NPC-15	1.16	0.25	0.9	53
NPC-20	0.95	0.22	0.7	41
NPC-19	0.54	0.79	0.2	12
NPC-13	0.55	0.30	0.2	12
NPC-10	0.38	0.19	0.2	12
NPC-16	0.23	0.26	0.0	0

Normal and affected fibroblasts (5×10^5) were seeded in 25-cm^2 flasks and incubated in 3 ml of McCoy's medium supplemented with penicillin, streptomycin, L-glutamine, and 10% lipoprotein-deficient fetal bovine serum for 48 hr. Cationic LDL (10 μg/ml) and 40 μl of 12.5 mM [^{3}H]oleate [200 dpm/pmol in 12% (wt/vol) bovine serum albumin] in fresh medium were added to the cells for 24 hr. Cultures then were harvested and lipid analysis was carried out.

Table 3. Esterification of non-lipoprotein-derived [^{3}H]cholesterol in cultured fibroblasts

Cell line	[^{3}H]Cholesterol, nmol/mg of protein		Esterification	
	Free	Ester	% of total	% of control
Control (n = 5)	183 ± 44	32 ± 16	15 ± 5	
Niemann–Pick A (n = 5)	158 ± 56	17 ± 20	10 ± 6	70 ± 30
Niemann–Pick B (n = 8)	247 ± 143	39 ± 35	14 ± 6	93 ± 30
Niemann–Pick C				
NPC-1	386	4	1.0	6
NPC-2	115	4	3.4	22
NPC-3	150	6	3.8	26
NPC-4	379	9	2.3	15
NPC-5	210	5	2.3	15
NPC-6	368	5	1.3	9
NPC-7	114	1.4	1.2	8
NPC-8	351	4	1.1	7
NPC-9	362	3	0.8	5
NPC-10	482	2	0.4	3
NPC-11	128	0.4	0.3	2
NPC-12	464	1.3	0.3	2
NPC-13	102	0.3	0.3	2
NPC-14	381	1.0	0.3	2
NPC-15	629	1.0	0.2	1
NPC-16	279	0.6	0.2	1
NPC-17	339	0.5	0.2	1
NPC-18	244	0.4	0.2	1
NPC-19	243	0.4	0.2	1
NPC-20	279	0.3	0.1	1

Cells were prepared and cultured for 48 hr as described for Table 2. Fresh medium containing [^{3}H]cholesterol (50 μg/ml) was prepared by gradually adding, to medium rapidly mixed on a Vortex, a stock solution of [^{3}H]cholesterol in ethanol (32 mM, 30 dpm/pmol) in the proportion 12 μl of ethanol/3 ml of medium. Cells were incubated with the above medium for 24 hr and analyzed for free and esterified [^{3}H]cholesterol.

This has been taken as a strong evidence that the primary molecular defect of the murine disorder is closely linked to the error in cholesterol esterification. Similar studies with fibroblast cultures derived from obligate type C Niemann–Pick carriers can be employed to determine the proximity and relevance of the blocked esterification to the primary genetic lesion and may provide useful direction to establishing both the primary molecular defect itself and the relationship between the lesion in cholesterol esterification and the phenotypic abnormalities that characterize type C Niemann–Pick disease.

Although further surveys and studies are needed, it appears as if fluorometric histochemical or biochemical monitoring of exogenous cholesterol esterification will prove to be useful in establishing and/or confirming at-risk cases of type C Niemann–Pick disease. The potential application in prenatal diagnosis should be investigated.

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