Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 38-45

TYPE C NIEMANN-PICK DISEASE: DOCUMENTATION OF ABNORMAL LDL PROCESSING IN LYMPHOCYTES

Charles E. Argoff[1*], Christine R. Kaneski[1], E. Joan Blanchette-Mackie[2], Marcella Comly[1], Nancy K. Dwyer[2], Anthony Brown[1], Roscoe O. Brady[1], and Peter G. Pentchev[1]

[1]Developmental and Metabolic Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

[2]Endocrinology Section, Laboratory of Cellular and Developmental Biology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Received July 2, 1990

Type C Niemann-Pick disease (NPC) is an autosomal recessive neurovisceral storage disorder in which defective intracellular cholesterol processing has been demonstrated in fibroblasts from NPC patients and obligate heterozygotes. In the present paper, the ability to esterify LDL-cholesterol was examined in cultured lymphocytes from 8 NPC patients, 8 obligate heterozygotes and 8 controls. Cholesteryl ester synthesis was 8% (±5%) and 45% (±16%) of controls in homozygous and heterozygous cell lines, respectively. Histochemical and electron microscopic examinations confirmed that this biochemical lesion was associated with abnormal intracellular accumulation of unesterified cholesterol in mutant lymphocytes. These results demonstrate that measurement of cholesterol esterification in cultured lymphocytes offers a quick and reliable means of confirming the diagnosis of NPC and that these cells may be useful for probing the primary molecular lesion of NPC.

Type C Niemann-Pick disease (NPC) is an autosomal recessive neurovisceral storage disorder (1). Despite heterogeneous clinical presentations, cultured fibroblasts from NPC patients are all deficient in their intracellular processing of cholesterol (2). As described by Brown and Goldstein, the cellular pathway for LDL cholesterol processing involves initial binding of LDL cholesterol to LDL-receptors on the cell surface, endocytosis of the receptor-ligand complex and subsequent lysosomal processing of the internalized LDL (3). In normal fibroblasts, these events induce cellular cholesterol homeostatic responses including down-regulation of the LDL receptor, down-regulation of HMG CoA reductase activity and up-regulation of acyl-CoA:cholesterol acyl transferase activity. These responses act in

* To whom all correspondence should be addressed.

0006-291X/90 $1.50

concert to prevent excessive intracellular accumulation of LDL derived unesterified cholesterol (3).

Studies by Pentchev et al. have shown that following normal endocytic uptake and lysosomal hydrolysis of LDL, NPC fibroblasts exhibit uniform delays in the induction of cellular cholesterol homeostatic responses resulting in intracellular accumulation of excess unesterified cholesterol (4,5). These earlier biochemical findings have led to effective diagnostic detection of NPC homozygotes and identification of heterozygotes and have aided in an improved understanding of the pathogenic basis of this metabolic disorder. The present study reports differences in the processing of LDL-cholesterol in normal and mutant lymphocytes. The successful documentation of a lesion in cholesterol processing in lymphocytes will now allow biochemical diagnosis to be carried out in one week instead of the eight weeks currently required for fibroblasts. The method described will also provide the opportunity to probe the molecular lesion of NPC in cells other than fibroblasts.

MATERIALS AND METHODS

Patients. Venous blood samples were obtained from 8 normal controls, 8 NPC patients and 8 obligate heterozygotes. The phenotype of these NPC patients and heterozygotes had previously been confirmed clinically and biochemically (6).

Preparation of Lymphocytes. Lymphocytes were isolated from 30 ml of heparinized blood by centrifugation on a layer of sodium daitrizoate - Ficoll (Organon Teknika, Durham, NC) (7). Cell pellets were subsequently washed twice by centrifugation and suspended in 14 ml of RPMI-1640 medium (Biofluids, Rockville, MD) supplemented with 5% (vol/vol) lipoprotein deficient serum (LPDS, Bionetics Research Institute, Rockville, MD), 2% (vol/vol) 200mM L-glutamine (Biofluids, Rockville, MD) and 1% Phytohemagglutinin (Life Technologies, Grand Island, NY). Lymphocyte cultures were established by equally dividing each cell suspension into 2 T75 flasks and placing these in a humidified incubator (5% CO_2) at 37°C. After 5 days, each set of duplicate T75 flasks was pooled and a cell pellet obtained by centrifugation (10 minutes, 470 x *g*). Each cell pellet was then washed twice by centrifugation and resuspended in RPMI-1640 supplemented with 5% (vol/vol) LPDS, 2% (vol/vol) 200mM L-glutamine and 10% (vol/vol) human interleukin-2 (Advanced Biotechnologies, Columbia, MD). Each culture was incubated for 24 additional hours prior to cholesterol esterification assay or histochemical staining. On the sixth day, cell viability was assessed by Trypan blue exclusion and was always greater then 90%. Cell viability was further assessed by measuring the incorporation of [^{14}C]leucine into protein over 4 hours. All cultures demonstrated equal viability by showing comparable levels of protein synthesis (data not shown).

Cholesterol esterification assay. An aliquot of cholesterol depleted cells (3.0 x 10^6 cells) was taken from each culture and equally distributed among six 16 mm wells (5 x 10^5 cells/ml). Measurement of cholesteryl ester synthesis during the next 24 hours was carried out in triplicate by incubating cell cultures with or without LDL (50μg/ml, Bionetics Research Institute, Rockville, MD) in the presence of 100μm [^{3}H]oleate ((9,10-[^{3}H])oleate, New England Nuclear, Boston, MA), added from a stock solution (200 DPM/pmol) in 14% BSA.

Lipid analysis. The contents of each well were placed into individual 15 ml conical tubes and cell pellets obtained by centrifugation (10 minutes, 400 x *g*). After the pellets were washed twice with phosphate-buffered saline, they were suspended in 250µl of 2-propanol and briefly sonicated with a microprobe. The 2-propanol cell extract was incubated at 37°C for 30 minutes and then centrifuged (10 minutes, 470 x *g*). The supernatants were used for lipid measurements and the remaining pellets were dissolved in 250 µl of 0.5M NaOH for protein quantitation (8). For measurement of cholesteryl[^{3}H]oleate synthesis, an aliquot of the supernatant was transferred to a 12x75 glass tube and triolein and cholesteryl oleate (10µg each) were added as carriers. After the samples were dried by a vortex-evaporator, they were dissolved in 50µl of chloroform/methanol (2:1 vol/vol) and applied to silica gel 60 plates (precoated, 0.25mm thick, E. Merck, Darmstadt, F.R.G.). Lipids were separated using a solvent mixture of hexane/ether/glacial acetic acid (90:10:1 vol/vol) and the positions of cholesteryl oleate and triolein were established with iodine vapor. These positions as well as the origin were scraped from the silica plates and their respective amount of radioactivity determined by scintillation spectroscopy.

Filipin Staining. Filipin (Sigma, St. Louis, MO) was dissolved in DMSO at a concentration of 2.5%. This stock was diluted to 0.05mg/ml in 3% paraformaldehyde in PBS at ph 7.4 immediately before use. Lymphocytes were prepared as for cholesteryl ester measurements with the exception that no [^{3}H]oleate was added. Following the 24 hour incubation with or without LDL, cell pellets were obtained by centrifugation and as much as the supernatant as possible was removed. The cells were resuspended in 200µl of the filipin-paraformaldehyde solution. The cell suspensions were kept on wet ice for a minimum of 30 minutes and up to 1 hour before examination with fluorescence microscopy. Samples compared were exposed to the filipin/paraformaldehyde solution for the same amount of time (Figure 2). Cells were observed on glass slides, under coverslips, with a Zeiss microscope, using a 100 oil objective. Filipin fluorescence was visualized with filters of excitation wavelength 350-410, and absorbance wavelength 470, as well as neutral density filter of 50% absorbance. Color transparencies were taken on Kodak Ektachrome® at ASA 800 at the same exposure and black and white prints were made from the transparencies of the filipin fluorochrome under identical exposure conditions.

Electron microscopy. Cells were fixed in suspension in Karnovsky fixative in NaCacodylate buffer at pH 7.4, washed in buffer and exposed to filipin solution at a concentration of 0.25mg/ml NaCacodylate at pH 7.4 for one hour (9). The cells were pelleted in a Beckman microfuge in 400 µl tubes. Subsequent preparatory steps were done on cell pellets. The pellets were post-fixed in 0.2% osmium tetroxide in NaCacodylate buffer at pH 7.4 for 2 hours at 4°C, dehydrated in acetones and embedded in resin. Sections of cell pellets were examined in a Phillips 400 Electron Microscope.

RESULTS

Esterification of exogenously derived cholesterol. The level of cholesteryl[^{3}H]oleate formation upon addition of LDL to control cell lines was 6.4 ± 1.7 nmoles/mg protein/24 hours. LDL induced cholesteryl[^{3}H]oleate synthesis was 8% (±5%) and 45% (±16%) of controls in homozygote and heterozygote cell lines respectively (Figure 1). Synthesis of triglycerides and polar lipids from the added [^{3}H]oleate was comparable in all cell cultures (data not shown).

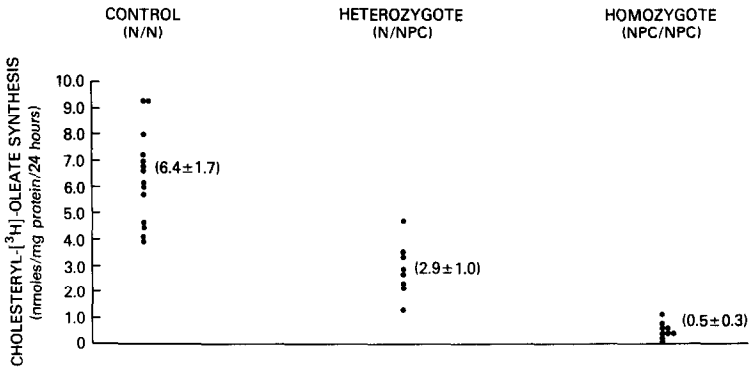

Figure 1. **Results of LDL-stimulated esterification of cholesterol in cultured control, heterozygous and NPC lymphocytes.** Cells prepared as described in methods. Data represents combined results of four separate assays. Values in parentheses are means ± SD.

Fluorescent histochemical staining. The ability of the fluorescent antibiotic filipin to form a stable and specific complex with unesterified cholesterol was utilized to demonstrate that the abnormalities of LDL processing observed in NPC lymphocytes were associated with abnormal intracellular accumulation of unesterified cholesterol (Figure 2) (10). Almost all NPC lymphocytes which were incubated with LDL for 24 hours and subsequently stained with filipin exhibited abnormal accumulation of unesterified cholesterol clearly visible by fluorescent microscopy and located in intracellular organelles with sizes and shapes of lysosomes. In 50% of the heterozygote cell lines similarly studied, a few lymphocytes exhibited intracellular fluorescence. In contrast, none of the control cell lines treated with filipin and LDL showed abnormal intracellular fluorescence.

Electron microscopic analysis of filipin treated lymphocytes. With electron microscopy, filipin-cholesterol complexes were seen in multilamellar vesicles within NPC lymphocytes in locations coincident with the intracellular fluorescence previously described (Figure 3A). At higher magnifications, these vesicles showed the characteristic corrugated membrane caused by the filipin-cholesterol complex (Figure 3B) (10). Fewer similar vesicles were identified in the one heterozygote cell line studied. There were no abnormal intracellular filipin-cholesterol complexes visualized by electron microscopy in control cell lines.

DISCUSSION

Cholesteryl [^{3}H]oleate synthesis in lymphocytes following 24 hour incubation of these cells with LDL and [^{3}H]oleate was severely restricted in all NPC cell lines and was partially restricted for obligate heterozygotes,

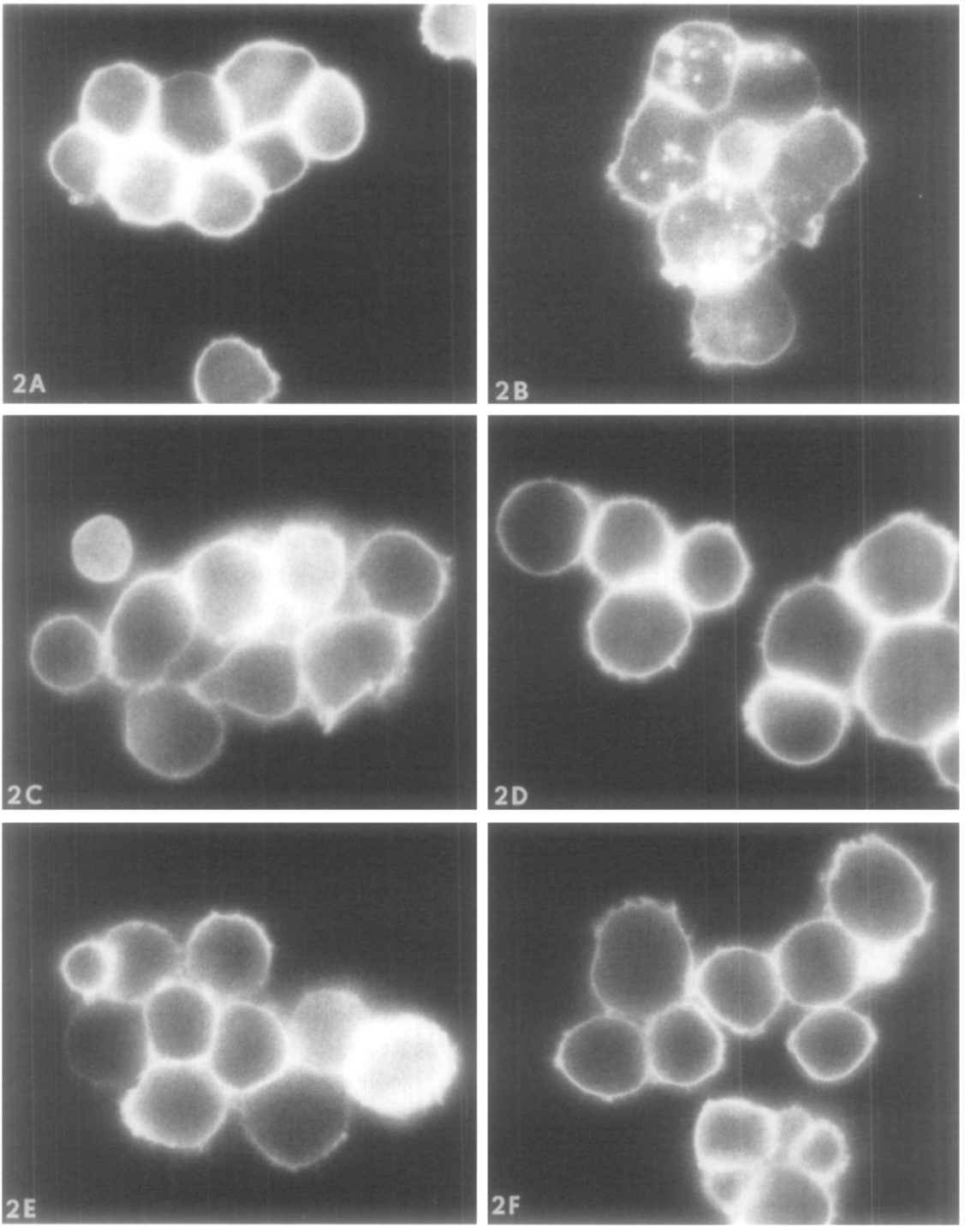

Figure 2. **Lymphocytes exposed to filipin for 60 minutes.** NPC lymphocytes incubated in the absence of (A) or the presence of LDL (B). NPC lymphocytes incubated without LDL show only plasma membrane filipin fluorescence while those incubated with LDL also show filipin fluorescent intracellular granules. Heterozygote lymphocytes incubated in the absence of (C) or the presence of LDL (D) and normal lymphocytes incubated in the absence of (E) or the presence of LDL (F). (Compare with figure 2B.) x 664.

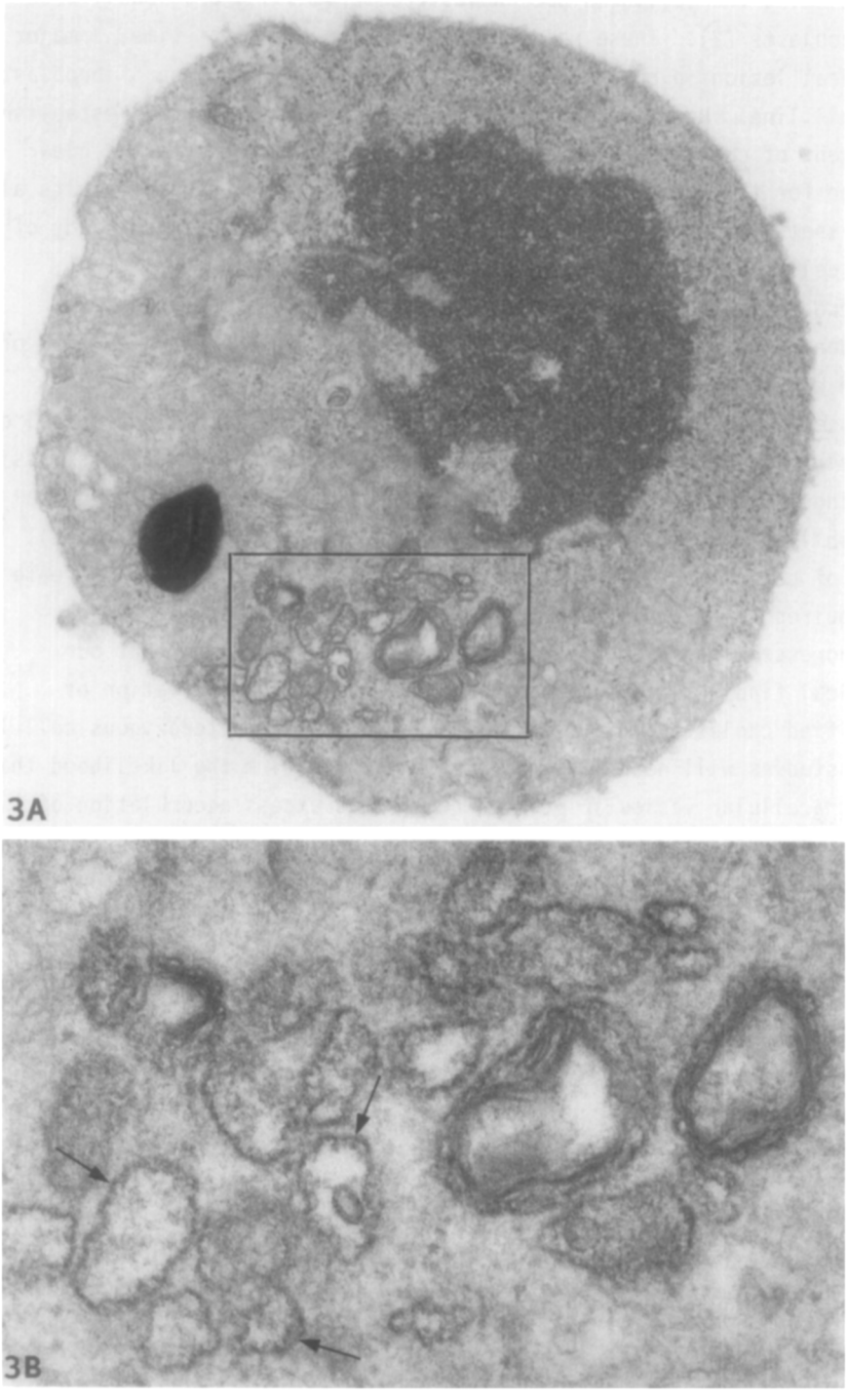

Figure 3A. **NPC fibroblast cultured with LDL.** Cells were prepared as described in Methods. Intracellular granules (outlined in rectangle) show filipin reaction. x 184,500.
Figure 3B. **Area in rectangle in Figure 3A at higher magnification.** The corrugated membranes (arrows) of the intracellular granules result from insertion of filipin into the membranes (filipin/cholesterol complexes). x 410,000.

thus mimicking the lesion in LDL-cholesterol processing previously observed in NPC fibroblasts (2). These results document for the first time, a major biochemical lesion in cholesterol processing for NPC in a non "fibroblast-like" cell line. Because fibroblast cultures take 4-6 weeks to establish, measurement of cholesterol esterification in lymphocytes offers a clear advantage for the more rapid (1 week) diagnosis of NPC. These results also suggest that lymphocytes may be a useful tool for the continued study of the primary cellular lesion of this metabolic disorder.

Since cationized LDL is known to be taken up by a LDL-receptor independent endocytic pathway, we have also begun to study the ability of this modified LDL to induce cholesterol esterification in freshly isolated lymphocytes which have not had to be depleted of cholesterol by several days of *in vitro* incubation (11). Our preliminary results suggest it is possible to distinguish between the cholesterol esterification response of normal and NPC fresh lymphocytes when cationized LDL is employed (data not shown). Thus, the use of cationized LDL with freshly isolated lymphocytes can decrease the time required for diagnosis from 6-8 weeks for fibroblasts to 2 days.

Fluorescent and electron microscopic studies confirmed that our biochemical findings are associated with an abnormal accumulation of unesterified cholesterol in all homozygous and 50% of heterozygous cell lines. Further studies will need to be carried out to confirm the likelihood that this intracellular vesicular storage represents excess accumulation of cholesterol in lysosomes as previously documented in fibroblasts (12).

Current data from different laboratories suggest that impaired intracellular trafficking of cholesterol plays a major role in the cellular pathology of NPC. Since one of the principle functions of lymphocytes is to package and secrete substances in a timely fashion, these cells may be especially important to further study of the specific cholesterol trafficking error associated with NPC.

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