Cholesterol accumulates to massive levels in cells from Niemann-Pick type C (NP-C) patients and in cells treated with class 2 amphiphiles that mimic NP-C disease. This behavior has been attributed to the failure of cholesterol released from ingested low density lipoproteins to exit the lysosomes. However, we now show that the rate of movement of cholesterol from lysosomes to plasma membranes in NP-C cells is at least as great as normal, as was also found previously for amphiphile-treated cells. Furthermore, the lysosomes in these cells filled with plasma membrane cholesterol in the absence of lipoproteins. In addition, we showed that the size of the endoplasmic reticulum cholesterol pool and the set point of the homeostatic sensor of cell cholesterol were approximately normal in NP-C cells. The plasma membrane cholesterol pools in both NP-C and amphiphile-treated cells were also normal. Furthermore, the buildup of cholesterol in NP-C lysosomes was not a physiological response to cholesterol overload. Rather, it appeared that the accumulation in NP-C lysosomes results from an imbalance in the brisk flow of cholesterol among membrane compartments. In related experiments, we found that NP-C cells did not respond to class 2 amphiphiles (e.g. trifluoperazine, imipramine, and U18666A); these agents may therefore act directly on the NPC1 protein or on its pathway. Finally, we showed that the lysosomal cholesterol pool in NP-C cells was substantially and preferentially reduced by incubating cells with the oxysterols, 25-hydroxycholesterol and 7-ketocholesterol; these findings suggest a new pharmacological approach to the treatment of NP-C disease.

Niemann-Pick type C (NP-C) disease is a rare, fatal, and presently untreatable homozygous recessive disorder. Characteristically, NP-C cells accumulate massive amounts of cholesterol in abundant cytoplasmic vacuoles; these bear lysosomal markers, are packed with membrane whorls, and are of low buoyant density (1, 2). These NP-C vacuoles resemble lysosomes (3), late endosomes (4), and/or the lamellar bodies derived therefrom (2, 5); for simplicity, we will call them lysosomes. The trans-Golgi cisternae in NP-C cells are also enriched in cholesterol (1, 6). NPC1, the affected gene in this disease, encodes a polytopic integral membrane glycoprotein that is concentrated in small vesicles bearing lysosomal markers but not much cholesterol (7–11). It has been speculated that these vesicles facilitate the transport of endocytic cholesterol to the plasma membrane; however, the mechanisms mediating intracellular cholesterol transport are not well understood (12).

From the start, the experimental hallmark of NP-C cells has been a delay of several hours in the exit of ingested LDL cholesterol from the lysosomes (13–16). However, this lag is seen only in cells that have been incubated for several days in the absence of LDL. Because of this unphysiological treatment, the significance of the lag phenomenon has been questioned (17). Indeed, the efflux of lysosomal cholesterol in NP-C cells under typical culture conditions has not been clearly determined. The observation that the entire cholesterol pool in cultured fibroblasts circulates between lysosomes and plasma membranes suggests that it may not be trapped (5). Similarly, cholesterol exits the lysosomes promptly both in vitro (18) and in human patients (17) and is completely cleared when cultured NP-C cells are starved of LDL for several days (3).

Many of the key features of the NP-C phenotype are mimicked by fibroblasts treated with a variety of drugs and experimental agents called class 2 amphiphiles; these compounds are typically steroids or hydrophobic amines (4, 19–24). It is therefore possible that class 2 amphiphiles act upon the NPC1 protein or along its pathway (25).

Given these unsettled issues, we re-examined the movement of cholesterol in NP-C and amphiphile-treated cells in order to address the following questions. (a) Is there a block in lysosomal cholesterol transport in NP-C cells? (b) Is the extensive accumulation of cholesterol a misguided homeostatic response of NP-C cells to the underestimation of their cholesterol load? (c) In particular, is the putative sensor of cell cholesterol perturbed in NP-C cells? (d) In that case, does the altered sensor cause an expansion of the plasma membrane cholesterol pool, leading to spillover of cholesterol to the lysosomes? (e) Do class 2 amphiphiles act on the NPC1 protein pathway? (f) Can the pool of excess lysosomal cholesterol be reduced pharmacologically?

### EXPERIMENTAL PROCEDURES

**Materials**—[oleoyl-1-14C]Coenzyme A was purchased from NEN Life Science Products; [1α,2α-3H]cholesterol oleate and [1α,2α-3H]cholesterol were from Amersham Pharmacia Biotech. 4-Methylumbelliferyl β-D-galactopyranosidase was from Molecular Probes. Hydroxypropyl-β-cyclodextrin (HPCD) from Research Plus, Inc. (Bayonne, NJ), was complexed with cholesterol as described (26). LPDS was prepared from FBS (27).

**Cell Culture and Treatments**—Normal human foreskin fibroblasts were obtained as described (28). Human NP-C fibroblast cell lines 93.59 and 93.41 were generously provided by Peter Pentchev (National Insti-
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tutes of Health); the two lines lacked detectable NPC1 protein and behaved similarly. All fibroblasts were cultured in Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. 25-RA, a mutant Chinese hamster ovary cell line generously provided by T.-Y. Chang (Dartmouth University), was grown in Ham’s F-12 medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, and 10% FBS. For cholesterol depletion experiments, 10% FBS was replaced by 5% LPDS. Controls for incubations with drugs contained the solvent at <1% of volume. The cholesterol content of cells (plasma membranes) was reduced by HPCD extraction and augmented by incubation with HPCD cholesterol complexes (29).

Gradient Fractionation (30)—Homogenates of cell suspensions were mixed with 30% Percoll in 0.25 M sucrose, 5 mM NaPi (pH 7.5), 1 mM EDTA and layered on a cushion of 56% sucrose in 5 mM NaPi (pH 7.5). The tubes were centrifuged for 30 min at 35,000 rpm at 4 °C in a Beckman SW 50.1 rotor and the gradients fractionated from the bottom.

Assays—Error estimates are given as standard deviations. Determinations of cholesterol and protein mass (28, 31), the activity of acid lysosomal β-galactosidase (30), and in vivo cholesterol esterification (29) were as described. ER cholesterol was taken as the total cholesterol esterified when whole cell homogenates were reacted to completion with 30 μM [14C]cholesterol-CoA for 2 h at 37 °C (32). For this determination, a [3H]cholesterol oleate standard was added to correct for subsequent losses. Cholesterol esters were isolated by thin layer chromatography, and their radioactivity was determined. The mass of cholesterol esterified was calculated from the [14C]radioactivity using the known specific activity of the [14C]cholesterol-CoA added (32).

Kinetics of Efflux of Lysosomal [3H]Cholesterol (Fig. 2)—Because aqueous solutions of HPCD efficiently extract plasma membrane but not intracellular cholesterol (9), we used this agent to determine the time course of transfer of lysosomal cholesterol to the plasma membrane, as follows. Cells in 75-cm² flasks were incubated at 37 °C for 10% of volume. The cholesterol content of cells (plasma membranes) was reduced by HPCD extraction and augmented by incubation with HPCD cholesterol complexes (29).

Kinetics of Efflux of Lysosomal [3H]Cholesterol (Fig. 2)—Because aqueous solutions of HPCD efficiently extract plasma membrane but not intracellular cholesterol (9), we used this agent to determine the time course of transfer of lysosomal cholesterol to the plasma membrane, as follows. Cells in 75-cm² flasks were incubated at 37 °C for 10% of volume. The cholesterol content of cells (plasma membranes) was reduced by HPCD extraction and augmented by incubation with HPCD cholesterol complexes (29).

Because HPCD samples only the plasma membrane, the specific activity of the cholesterol in the medium equals that of the plasma membrane at all times. That is, 

$$\eta(t) = H(t)/M(t) + \eta(t) = H(t)/M(t)$$  

By definition:

$$\eta(t) = H(t)/M(t) + \eta(t)$$

Since the plasma membrane is the sole source of HPCD-cholesterol in the medium.

$$M_0(t) = M_0(0) - M(t)$$  

That is, 

$$M_0(t) = M_0(0) - M(t)$$

From Equation 5, we get 

$$M_0(t) = H(t)/\eta(t)$$

The fraction of cell cholesterol initially residing in the plasma membrane of the cells, R, is given by 

$$R = M_0(0)/M(t) + M_0(0)$$

The numerator in Equation 9 can be substituted from Equation 7, and the denominator can be replaced by the expression $M(t) + M(t) + R(t)$. Thus Equation 9 can be written:

$$R = [M(t) + M(t)]/[M(t) + M(t) + M(t)]$$

Substituting from Equation 8 into the numerator and Equation 6 into the denominator:

$$R = ([H(t)/\eta(t)] + M(t))/[H(t)/\eta(t)] + M(t)$$

Assay of the cell pellet provided both $H(t)$ and $\eta(t)$. $M(t)$ and $\eta(t)$ were determined by assaying the medium.

RESULTS

Quantitation of the Distribution of Cholesterol in NP-C Cells—We determined the distribution of cholesterol between plasma membrane and intracellular compartments by a new method (see “Experimental Procedures”). As illustrated in Table I, 53% of the cholesterol in the normal human fibroblasts was found in the plasma membrane. This value is in excellent agreement with those determined by three other methods (34, 35). The present method has the advantage of simplicity and avoids the problem of cross-contamination of organelles en-
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TABLE I

| Fibroblasts | Total cell cholesterol | Plasma membrane cholesterol | Intracellular cholesterol | Fraction in plasma membrane | Fraction intracellular | Fraction intracellular (published) |
|-------------|------------------------|----------------------------|--------------------------|-----------------------------|-----------------------|-----------------------------------|
| A           | n | µg cholesterol/mg cell protein (S.D.) | %  | % | % | n | Ref.           |
| Control     | 5 | 35 (2) | 29 (1) | 6 (1) | 83 | 17 | 10 | 34 |
| NP-C 93.59  | 6 | 88 (11) | 34 (7) | 54 (8) | 39 | 61 | 65 | 9 | 5 |
| NP-C 93.41  | 14 | 119 (20) | 37 (4) | 82 (17) | 31 | 69 | 66 | 3 | 5 |
| B           | Control + U18666A | 4 | 37 (4) | 31 (4) | 6 (0.5) | 84 | 16 |
| C           | Control + U18666A | 3 | 36 (2) | 30 (5) | 6 (1) | 83 | 17 |

Part A, normal and NP-C fibroblasts were analyzed as described under "Experimental Procedures." Part B, same as in part A, except normal fibroblasts were preincubated in growth medium + 10% FBS + 2.5 µM U18666A for 18–22 h. Part C, same as in part B, except that the preincubation with U18666A was for 26–72 h to allow the formation of buoyant lysosomes. The right-most column gives values calculated from published data obtained by sucrose density gradient fractionation. Values are means ± S.D. in n separate experiments.

The cholesterol in the plasma membrane of NP-C fibroblasts was about 20% greater than normal (Table I, part A). In contrast, the intracellular cholesterol pools in the NP-C cells were increased roughly 10-fold. These results are consistent with those obtained by density gradient fractionation, where about two-thirds of NP-C cell cholesterol was in buoyant vacuoles containing acid hydrolases, and about one-third was in the plasma membrane fraction (Table I, part A, last column).

It is well known that class 2 amphiphiles induce the steady accumulation of lysosomal cholesterol in normal fibroblasts (22–24, 29). We now show that plasma membrane cholesterol is not altered by U18666A, a prototypic class 2 agent (Table I, parts B and C). Once again, the distribution of cellular cholesterol obtained by the new method agreed well with data from density gradient fractionation (Table I, part C, last column).

Transport of Intracellular Cholesterol to the Plasma Membrane in NP-C Cells—As one approach to determining the rate of exit of cholesterol from the lysosomes of NP-C cells, we followed its redistribution following the acute depletion of surface cholesterol by HPCD extraction. Most of the cholesterol extracted came from within the cell (Fig. 1). Furthermore, the average absolute rate of loss of intracellular NP-C cholesterol over the 5-h incubation, 5 µg of cholesterol/mg of protein/h, is comparable to the rate of movement of lysosomal cholesterol to the plasma membrane determined for normal human fibroblasts (5). The present value presumably underestimates the true rate of transport of lysosomal cholesterol to the plasma membrane, since (a) it is somewhat limited by the submaximal rate of extraction by the HPCD, and (b) it disregards plasma membrane cholesterol movement back to the lysosomes.

Note that the loss of cholesterol from the NP-C plasma membrane pool in Fig. 1 slowed greatly after about an hour while exit from the intracellular pool continued. To test the implication that the plasma membranes were replenished from the lysosomes, we removed a portion of the plasma membrane pool by briefly exposing the cells to HPCD. The redistribution of cholesterol during a chase in medium lacking cholesterol was then followed (Table II). The initial depletion reduced plasma membrane but not intracellular cholesterol. This result corroborates the selectivity of the HPCD extraction and the ability of the method to distinguish cell surface from intracellular cholesterol. During the 3-h chase, plasma membrane cholesterol was restored at the expense of the intracellular pool. The average rate of transfer from the intracellular compartment to plasma membranes, −6 µg of cholesterol/mg of protein/h, was comparable to the value obtained in Fig. 1. Again, the true initial rate might be faster than this estimate given that transfer may have slowed during the 3-h incubation, and cholesterol from plasma membranes could have partially replenished the lysosomes (5).

We also determined the export of cholesterol from the lysosomes of NP-C cells by isotope flux (Fig. 2). Cells were incubated with exogenous HPCD-[3H]cholesterol, either briefly to label the plasma membrane preferentially or for a prolonged period to allow equilibration of the probe throughout the cell. The kinetics of transfer of the cellular probe to an "infinite sink" of unlabeled extracellular cholesterol-HPCD was then followed. The behavior of the briefly labeled NP-C cells (○) resembled that of normal fibroblasts (●); these time courses are close to first-order and presumably reflect the flux of plasma membrane cholesterol to HPCD. The rate constants determined by best fit for the normal (1.9 h⁻¹) and NP-C cells (1.8 h⁻¹) were indistinguishable. These kinetics (half-time <30 min) are considerably faster than seen with other extracellular acceptors (36).

In the case of NP-C cells pre-labeled to equilibrium, the time course was more complex (○ and ▭ in Fig. 2), presumably because of the large influence of the slow transport of lysosomal cholesterol to the plasma membrane. To obtain the kinetic constant for this process, we fit the data to a three-compartment model. We specified the relative sizes of the intracellular and cell surface cholesterol compartments using the values given in Table I, and we applied the rate constant for transfer out of the plasma membrane estimated above. The rate constant for lysosome to plasma membrane transport thus ob-
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Replicate flasks were preincubated for 30 min at 37 °C in growth medium containing 5% LPDS ± 2.5% HPCD and then chased in growth medium containing 5% LPDS for 0 or 3 h. The distribution of cell cholesterol was then determined as described under “Experimental Procedures.” The values shown are averages of duplicate determinations that agreed to within 5%; two other experiments gave comparable results.

| Preincubation | Chase Total cell cholesterol | Plasma membrane cholesterol | Intracellular cholesterol |
|---------------|-----------------------------|----------------------------|--------------------------|
| LPDS alone    | 0 h 107 39 68               | 39 68                       |                          |
| LPDS + HPCD   | 3 h 99 29                   | 29 70                       |                          |
| LPDS + HPCD   | 3 h 92 38                   | 38 53                       |                          |

Since the intracellular cholesterol pool in the NP-C cells contained was 0.14 h⁻¹, corresponding to a half-time of exit of 5 h. Since the intracellular cholesterol pool in the NP-C cells contained ~82 μg of cholesterol/mg of protein (Table I), we calculate that approximately 0.14 × 82 = 11.5 μg of cholesterol/mg of protein moved from lysosomes to plasma membrane/h. This rate is about double that estimated above for NP-C cells and that reported previously for normal fibroblasts (5). A slightly enhanced rate of efflux of cholesterol from the lysosomes of cells lacking functional NPC1 protein has also been reported recently (18). These data all suggest that the transport of cholesterol to the plasma membrane from its principal intracellular locus in NP-C cells is at least as great as in normal fibroblasts.

**Is Ingested LDL Needed to Provide Cholesterol to NP-C Lysosomes?**—An overnight incubation of NP-C cells with HPCD diminished the intracellular pool somewhat more than the plasma membrane pool (Table III, experiments 1 and 2; lines 1 and 2). A subsequent pulse of cholesterol delivered by HPCD in the absence of LDL went to both the intracellular and the plasma membrane pool (Table III, experiments 1 and 2, line 3). Sucrose density gradient centrifugation verified that the intracellular pool of cholesterol was associated with buoyant lysosomes (data not shown but see Ref. 5). These results demonstrate that the cholesterol that accumulates in NP-C lysosomes can be derived from sources other than LDL.

**Does the Accumulation of Lysosomal Cholesterol in Cells Treated with Class 2 Amphiphiles Depend on Ingested LDL?**—Normal fibroblasts were treated in four ways prior to the determination of the shift of acid β-galactosidase to a low buoyant density, an indicator of lysosomal cholesterol accumulation (5). As shown in Fig. 3, panel A, cells starved of LDL for 40 h had only dense lysosomes, i.e., the normal pattern (●). A 40-h incubation with the amphiphile, U18666A, induced significant lysosomal buoyancy, even in the absence of LDL (○). A brief pulse of exogenous HPCD-cholesterol prior to the 40-h incubation with U18666A (▲) fostered almost as much lysosomal buoyancy in the absence of LDL as did incubation in its presence (△). It appears that class 2 agents induce the accumulation of lysosomal lipids when cells are provided with LDL, as well as in the absence of LDL, both following a pulse of exogenous cholesterol to the plasma membrane and even when exogenous cholesterol is omitted altogether.

**Is the Accumulation of Cholesterol in NP-C Cells the Result of False Signals to the Regulatory ER Pool?**—Normally, cellular cholesterol levels are maintained by a feedback system in which the ER cholesterol pool varies in response to the level of plasma membrane cholesterol relative to a threshold set point, thereby regulating the homeostatic elements in the ER (29). Since the cholesterol in NP-C cells is typically several times greater than normal (Table I), we examined the possibility that this excess results from false signals of a cholesterol deficit due to an inappropriate reduction of the ER pool size. We found, however, that the ER pool in the NP-C cells (0.49 ± 0.2 nmol of cholesterol/mg of cell protein (n = 10)) is the same as that in

![Figure 2](Image 317x390 to 544x517)

**FIG. 2.** Flux of NP-C intracellular [3H]cholesterol to the plasma membrane. The transfer of [3H]cholesterol from labeled fibroblasts to extracellular HPCD was determined as described under “Experimental Procedures” (●, normal fibroblasts surface labeled for 1 h. In the other three experiments, line 93.41 NP-C cells were incubated with exogenous [3H]cholesterol for 40 min (□), 70 h (○), or 113 h (◇). A best fit curve is shown for the NP-C cells labeled for 113 h using the values given in the text.

![Figure 3](Image 105x428 to 242x566)

**FIG. 3.** Buoyancy of lysosomes from fibroblasts starved of LDL. Fibroblasts were incubated for 40 h at 37 °C in growth medium ± 5 μM U18666A and either 5% LPDS (to starve them of LDL) or 10% FBS (to provide LDL). Cell homogenates were fractionated on Percoll gradients and assayed for the distribution of acid β-galactosidase. Fractions 2 and 9 are the normal positions of dense lysosomes and lipid-filled lysosomes, respectively (5). Panel A, □, LDL starvation minus U18666A; ●, LDL starvation plus U18666A. Panel B, Δ, LDL feeding plus U18666A. A, pre-enrichment by incubation of cells for 20 min at 37 °C in growth medium containing HPCD-cholesterol complexes (final concentration, 5 mg of HPCD bearing 0.3 mg of cholesterol/ml), followed by incubation for 40 h at 37 °C in growth medium lacking LDL and containing U18666A.
Panel A

in both panels, values for both ER and cell cholesterol (in \( \mu g \) of cholesterol/mg of protein) were expressed relative to unmodified cells. The 1.0/1.0 points (\( \bullet \)) mark the superposition of the untreated control values for all experiments. The averages of the values at this point were as follows: panel A, 40 \( \pm \) 5 \( \mu g \) of cholesterol/mg of protein and 0.47 \( \pm \) 0.2 nmol of ER cholesterol/mg of protein; panel B, 106.4 \( \pm \) 3.5 \( \mu g \) of cell cholesterol/mg of protein and 0.48 \( \pm \) 0.2 nmol of ER cholesterol/mg of protein.

normal fibroblasts (0.50 \( \pm \) 0.2 nmol of cholesterol/mg of cell protein (n = 7)).

Is the Sensor for Plasma Membrane Cholesterol Defective in NP-C Cells?—Fibroblast cholesterol is maintained at a set point near which small changes in total cholesterol elicit large changes in the ER pool (29). The relationship between the major plasma membrane pool and the minute ER pool is J-shaped in normal fibroblasts; the vertex of this curve is centered on an apparent threshold residing at the physiological cholesterol level (\( \bullet \)) in Fig. 4, panel A). We now show that this relationship has the same form in NP-C cells as in the control (Fig. 4, panel B). Once again, the vertex of the J curve coincided with the ambient cholesterol level (\( \bullet \)). Furthermore, the magnitude of the variation of ER cholesterol in response to changes in cell cholesterol was about normal in the NP-C cells.

We also tested the dependence of the rate of esterification of cholesterol on total cell cholesterol, in vitro esterification is thought to depend strongly on the size of the substrate pool in the ER (29). We found that the rate of esterification of plasma membrane \([3H]\)cholesterol in intact NP-C cells varied with plasma membrane cholesterol (Fig. 5) with the same threshold behavior as that seen in the in vitro runoff assay (Fig. 4). Once again, the vertex of the J curve (\( \bullet \)) coincided with the ambient cholesterol level in NP-C cells (Fig. 5, panel B), just as in control fibroblasts (Fig. 5, panel A). The magnitude of the NP-C response also was normal.

Is Excess Cell Cholesterol Normally Dumped to Endocytic Spaces?—Unesterified cholesterol might be transferred to lysosomes as a physiologic response that defends the plasma membrane against harmful over-accumulation (3). To determine whether human fibroblasts normally send excess cholesterol to lysosomes, cell cholesterol was enriched with a pulse of exogenous HPCD-cholesterol (29). The cells then were rinsed and pulse-labeled with \([3H]\)cholesterol. Medium containing 5% LPDS was added to the flasks which were incubated for 2.5 h to allow esterification of the plasma membrane \([3H]\)cholesterol. Finally the cells were dissociated, washed, and extracted for the determination of cholesterol esterification as described (29). The 1.0/1.01.0 points (\( \bullet \)) mark the superposition of the untreated control values for all experiments. The mean control values for the cell cholesterol in normal and NP-C fibroblasts were 34.2 \( \pm \) 0.4 \( \mu g \) of cholesterol/mg of protein (n = 4) and 90 \( \pm \) 5 \( \mu g \) of cholesterol/mg of protein (n = 3), respectively.

Fig. 4. Dependence of ER cholesterol on total cell cholesterol. Normal (panel A) or NP-C fibroblasts (panel B) were either depleted of cholesterol by incubation for 20–60 min with 2% HPCD in medium containing 5% LPDS or loaded with cholesterol by incubation for 20–30 min in medium containing 10% serum + plus HPCD-cholesterol (29). The cells then were rinsed and pulse-labeled with \([3H]\)cholesterol. Medium containing 5% LPDS was added to the flasks which were incubated for 2.5 h to allow esterification of the plasma membrane \([3H]\)cholesterol. Finally the cells were dissociated, washed, and extracted for the determination of cholesterol esterification as described (29). The 1.0/1.01.0 points (\( \bullet \)) mark the superposition of the untreated control values for all experiments. The mean control values for the cell cholesterol in normal and NP-C fibroblasts were 34.2 \( \pm \) 0.4 \( \mu g \) of cholesterol/mg of protein (n = 4) and 90 \( \pm \) 5 \( \mu g \) of cholesterol/mg of protein (n = 3), respectively.

Fig. 5. Dependence of \([3H]\)cholesterol esterification in vivo on plasma membrane cholesterol. Normal (panel A) or NP-C fibroblasts (panel B) were either depleted of cholesterol by incubation for 20–60 min with 2% HPCD in medium containing 5% LPDS or loaded with cholesterol by incubation for 20–30 min in medium containing 10% serum + plus HPCD-cholesterol (29). The cells then were rinsed and pulse-labeled with \([3H]\)cholesterol. Medium containing 5% LPDS was added to the flasks which were incubated for 2.5 h to allow esterification of the plasma membrane \([3H]\)cholesterol. Finally the cells were dissociated, washed, and extracted for the determination of cholesterol esterification as described (29). The 1.0/1.01.0 points (\( \bullet \)) mark the superposition of the untreated control values for all experiments. The mean control values for the cell cholesterol in normal and NP-C fibroblasts were 34.2 \( \pm \) 0.4 \( \mu g \) of cholesterol/mg of protein (n = 4) and 90 \( \pm \) 5 \( \mu g \) of cholesterol/mg of protein (n = 3), respectively.

Fig. 6. Buoyancy of lysosomes from normal fibroblasts loaded with exogenous cholesterol. Duplicate flasks of human fibroblasts were loaded with cholesterol by incubation for 20 min at 37 °C in medium containing 10% serum + plus HPCD-cholesterol. The cells were rinsed and overlaid with medium containing 5% LPDS lacking (panel A) or containing 5 \( \mu g \) U18666A (panel B). The flasks were incubated for 48 h at 37 °C; then the cells were homogenized; the homogenates were fractionated on Percoll gradients, and \( \beta \)-galactosidase activity was determined as described under “Experimental Procedures.” The cells without and with the U18666A treatment contained 58 and 61 \( \mu g \) of cholesterol/mg of protein, respectively.

Fig. 7. Dependence of \([3H]\)cholesterol esterification on intracellular cholesterol. The treatment of normal fibroblasts with U18666A or imipramine caused cell cholesterol to rise steadily (Fig. 8, panel A) and ER cholesterol to decline acutely (Fig. 8, panel C) (29). In contrast, NP-C cells showed no consistent response to U18666A (Fig. 8, panels B and D). The ER cholesterol in NP-C cells was similarly not reduced (and perhaps was marginally increased).
of protein (normal fibroblasts treated with 2.5 μM U18666A. Replicate flasks of 25-RA cells were incubated at 37 °C in medium containing 10% serum or 2.5 μM Sandoz 58035 (panel B), or 5 μM U18666A (panel C). The cells were homogenized; homogenates were fractionated on Percoll gradients, and acid β-galactosidase activity was determined as described (29). The cells in panels A–C contained 33.3, 66.7, and 61.1 μg of cholesterol/mg of protein, respectively.

by a battery of other class 2 compounds that lowered ER cholesterol in normal fibroblasts (Table IV). These data support the hypothesis that this very diverse set of agents might act upon cholesterol homeostasis at the same site (21, 38).

Response of NP-C Cell Cholesterol to Oxysterols—Certain oxysterols (particularly, 25-hydroxycholesterol and 7-ketocholesterol) signal cholesterol excess, rapidly down-regulating its accumulation through several homeostatic ER mechanisms (29, 39). Since these agents counter the accumulation of lysosomal cholesterol in normal fibroblasts treated with class 2 amphiphiles (5), we examined their effect on both ER and total cell cholesterol in NP-C cells.

Fig. 9, panel A, shows that 25-hydroxycholesterol caused a rapid rise in NP-C ER cholesterol to ~2.5 times its ambient level, just as in normal fibroblasts (29). The elevated ER cholesterol then remained constant over at least the subsequent 15 h. 25-Hydroxycholesterol also caused a steady decline in cell cholesterol (Fig. 9, panel B), the result expected when ER cholesterol is elevated (29). Cell cholesterol continued to fall for many hours after the ER pool reached its new (increased) steady state so that, after 3 days of incubation, NP-C cell cholesterol had decreased to about half of its original value. The observed loss was chiefly from the intracellular (lysosomal) pool and not the plasma membrane (Table V). 7-Ketocholesterol and 25-hydroxycholesterol were the most potent of the oxysterols tested in this way; 7-α-hydroxycholesterol and 7-β-hydroxycholesterol had no such effect.

DISCUSSION

We provide two lines of evidence that a functional NPC1 protein is not required for the robust exit of cholesterol from NP-C lysosomes under standard culture conditions. First, the cholesterol in the NP-C lysosomes rapidly replenished depleted plasma membrane pools (Fig. 1 and Table II). Second, the rate of transport of cholesterol from lysosomes to plasma membranes in NP-C cells (Figs. 1 and 2) was as least as great as that estimated for control fibroblasts (5, 18, 36, 40). That cholesterol is readily exported from NP-C lysosomes was also suggested by

In several experiments, pairs of flasks of NP-C fibroblasts were incubated for 2.5–3.0 h in growth medium ± class 2 agents. ER cholesterol was then determined by an in vitro run-off assay (32). Values (in nanomoles of [14C]cholesterol ester formed per mg of cell protein) were the means of duplicates that agreed within 5%; each was normalized to its untreated control, and two such experiments were averaged. (The average deviation among pairs was 6%). Values for the normal fibroblasts are from Ref. 29; the average ER cholesterol in these untreated normal cells was 0.50 ± 0.2 (n = 7) nmol/mg cell protein. The average value for ER cholesterol in the untreated NP-C cells (below) was 0.49 ± 0.2 (n = 10) nmol/mg cell protein.

| Agent          | Concentration | Relative ER cholesterol |
|----------------|---------------|-------------------------|
|                | μM            | Normal cells | NPC cells |
| None           | 1.0           | 1.0         | 1.0       |
| Monensin       | 2             | 0.58        | 1.2       |
| U18666A        | 2.5           | 0.36        | 1.1       |
| TriFluoperazine| 20            | 0.41        | 1.1       |
| Imipramine     | 80            | 0.58        | 1.1       |
| Chloroquine    | 80            | 0.39        | 1.0       |

FIG. 7. Buoyancy of lysosomes from 25-RA cells treated with Sandoz 58035 or U18666A. Replicate flasks of 25-RA cells were incubated for 20 h in growth medium and either <1% ethanol (panel A), 1 μg/ml Sandoz 58035 (panel B), or 5 μM U18666A (panel C). The cells were then determined as described (30). Panel A, cell cholesterol in normal fibroblasts treated with 2.5 μM U18666A (●) or 80 μM imipramine (○). Panel B, cell cholesterol in NP-C fibroblasts treated with 2.5 μM U18666A. Panel C, ER cholesterol in normal fibroblasts treated as in panel A. Panel D, ER cholesterol in NP-C fibroblasts treated with 2.5 μM U18666A. Values are plotted relative to their untreated controls (A); for untreated normal fibroblasts, these were 40 ± 5 μg of cell cholesterol/mg of protein (n = 10) and for untreated NP-C fibroblasts, these were 102 ± 21 μg of cell cholesterol/mg of protein (n = 9) and 0.61 ± 0.04 nmol of ER cholesterol/mg of protein (n = 6). The data in panels A and C are from Ref. 29.

FIG. 8. Response of cholesterol in NP-C cells to amphiphiles. Replicate flasks of NP-C cells were incubated in medium containing 10% serum ± amphiphiles for the times indicated. ER and cell cholesterol were then determined as described (30). Panel A, cell cholesterol in normal fibroblasts treated with 2.5 μM U18666A (●) or 80 μM imipramine (○). Panel B, cell cholesterol in NP-C fibroblasts treated with 2.5 μM U18666A. Panel C, ER cholesterol in normal fibroblasts treated as in panel A. Panel D, ER cholesterol in NP-C fibroblasts treated with 2.5 μM U18666A. Values are plotted relative to their untreated controls (A); for untreated normal fibroblasts, these were 40 ± 5 μg of cell cholesterol/mg of protein (n = 10) and 0.47 ± 0.2 nmol of ER cholesterol/mg of protein (n = 10), and for untreated NP-C fibroblasts, these were 102 ± 21 μg of cell cholesterol/mg of protein (n = 9) and 0.61 ± 0.04 nmol of ER cholesterol/mg of protein (n = 6). The data in panels A and C are from Ref. 29.

In experiments, pairs of flasks of NP-C fibroblasts were incubated for 2.5–3.0 h in growth medium ± class 2 agents. ER cholesterol was then determined by an in vitro run-off assay (32). Values (in nanomoles of [14C]cholesterol ester formed per mg of cell protein) were the means of duplicates that agreed within 5%; each was normalized to its untreated control, and two such experiments were averaged. (The average deviation among pairs was 6%). Values for the normal fibroblasts are from Ref. 29; the average ER cholesterol in these untreated normal cells was 0.50 ± 0.2 (n = 7) nmol/mg cell protein. The average value for ER cholesterol in the untreated NP-C cells (below) was 0.49 ± 0.2 (n = 10) nmol/mg cell protein.

FIG. 9. Response of cholesterol in NP-C cells to treatment with 25-hydroxycholesterol. Panel A, ER cholesterol. In four experiments (represented by the various symbols), flasks of NP-C cells were incubated at 37 °C in medium containing 10% serum ± 10–15 μg of 25-hydroxycholesterol/ml. At the times indicated, ER and cell cholesterol/mg of cell protein were then determined and the values plotted relative to untreated controls (29). The data were fit to a first-order expression with a half-time of 40 min and a plateau value of 2.6. The mean value for ER cholesterol in the untreated cells was 0.5 ± 0.1 nmol of cholesterol/mg of protein. Panel B, cell cholesterol. In 17 experiments, flasks of NP-C cells were incubated at 37 °C in medium containing 10% serum ± 10–15 μg of 25-hydroxycholesterol/ml for the stated times. Cell cholesterol/mg of cell protein was determined, and the values were plotted relative to untreated controls (○). The mean cholesterol content of the untreated cells at zero time was 101 ± 16 μg of cholesterol/mg of protein (n = 17).
Cholesterol Movement in NP-C and Amphiphile-treated Cells

TABLE V
Effect of oxysterols on cholesterol distribution in NP-C cells

| Exp. Incubation with 7-KC | mg cell cholesterol | Plasma membrane cholesterol | Intracellular cholesterol |
|--------------------------|---------------------|-----------------------------|--------------------------|
| h                        | µg cholesterol/mg protein |                             |                          |
| 1                        | 0                   | 107                         | 33                       | 74                       |
| 41                       | 70                  | 28                          | 43                       |
| 2                        | 0                   | 131                         | 35                       | 96                       |
| 46                       | 90                  | 33                          | 57                       |
| 3                        | 0                   | 116                         | 33                       | 82                       |
| 70                       | 67                  | 38                          | 29                       |
| 4                        | 0                   | 136                         | 42                       | 94                       |
| 70                       | 114                 | 45                          | 69                       |

Earlier findings (3, 5, 17).

Our data also demonstrate that the accumulation of excess cholesterol in NP-C lysosomes (Table III) and in the lysosomes of fibroblasts treated with class 2 amphiphiles (Fig. 3) does not require the ingestion of LDL, as long surmised (23, 24). Rather, plasma membrane cholesterol (to which lysosomal cholesterol, of course, contributes) seems to be a major source (5). It could be argued that our in vitro results do not reflect the natural pathophysiology of the disease. However, the accumulation in NP-C lysosomes of other lipids characteristic of the plasma membrane (e.g., sphingomyelin and glycosphingolipids (2)) also implicates the plasma membrane as a source of the lysosomal lipid. Similarly, a variety of sphingolipid storage diseases lead indirectly to the accumulation of cholesterol and other plasma membrane lipids in lysosomes (41). Furthermore, it has been shown recently that the build up of excess cholesterol in various organs of NP-C mice is the same whether they have or lack LDL receptors (42). All of these data suggest that a major fraction of the cholesterol in NP-C lysosomes derives from the plasma membrane both in vivo and in vitro.

Our findings could signify that the excess cholesterol in NP-C cells is a high throughput pool in competent lysosomes. Since the exit of cholesterol from this compartment is robust, perhaps lysosomal accumulation reflects an abnormally high influx. Consider that about 1 eq of plasma membrane is internalized per h by endocytosis in fibroblasts (43). A portion of the plasma membrane bilayer cholesterol endocytosed thereby cycles through the lysosomes at ~0.05 plasma membrane equivalents/h (5). The observed rate of lysosomal cholesterol accretion in NP-C cells (5, 42) as well as in cells treated with amphiphiles (Fig. 8, panel A), 1–2% of cell cholesterol/h, is only a fraction of the endocytic throughput. It is therefore possible that defective NPC1 protein undermines the pre-lysosomal recycling of internalized plasma membrane cholesterol, perhaps from late endosomes (4). If so, large amounts of the endosomal cholesterol might pass to lysosomes by default, overwhelming their export capacity and promoting lysosomal cholesterol build up and the formation of lamellar bodies (29). Ingested LDL would intensify this process.

This hypothesis is in accord with the suggestion that the NPC1 protein resides in shuttle vesicles that return endocytic cholesterol to the cell surface (9). The putative “sterol-sensing domain” of NPC1 might thus play an informational or regulatory role rather than serve a transport function (7, 8). In that case, the NPC-C phenotype could represent a dynamic imbalance in cholesterol allocation arising from defective sensing or recycling of endocytic plasma membrane sterol.

The original concept of a lysosomal transport block derives mainly from the prolonged lag observed between the ingestion of LDL and the homeostatic responses it engenders in cultured NP-C cells (15, 24). However, this lag is only seen after several days of LDL deprivation in vitro. (Reduced movement of ingested LDL [3H]cholesterol to the plasma membrane in vivo has been reported (17); however, this effect could result from isotope dilution of the endocytic probe in cholesterol-packed lysosomes rather than a transport defect.) The delay in cellular responses to LDL refeeding observed in vitro might not be due to a loss of intracellular cholesterol transport capacity in NP-C lysosomes but rather could reflect the time course of cell recovery from LDL deprivation. This recovery period might entail the expression or activation of a protein. Alternatively, the export of cholesterol from NP-C lysosomes might depend on their level of cholesterol, hence, the requirement for prolonged LDL refeeding (44).

Given this uncertainty, it seemed important to test some alternative hypotheses concerning the NP-C phenotype. For example, a sensor defect could cause NP-C cells to underepress their cholesterol and mistakenly build up an excess. Indeed, NP-C cells synthesize cholesterol and ingest LDL at elevated rates both in cultured cells (15, 45) and in vivo (42, 46). On the other hand, our data suggest that the threshold setting of the putative cholesterol sensor is normal (Figs. 4 and 5). Current concepts of cholesterol homeostasis suggest that an inappropriately low level of cholesterol in the ER could signal the regulatory elements therein to drive excess cholesterol accumulation (47, 48). However, we found the size of the ER cholesterol pool in NP-C fibroblasts to be normal. Furthermore, a mistaken perception by the cell of a need for more cholesterol should bring about an increase in the plasma membrane pool, but this was not observed (Table I). It could be countered that the NP-C plasma membrane does receive excess cholesterol but that a physiological mechanism rapidly removes it to lysosomes. However, no such lysosomal loading was detected in normal cells when we imposed high levels of plasma membrane or intracellular cholesterol (Figs. 6 and 7).

The near-normal levels of plasma membrane cholesterol in NP-C and amphiphile-treated cells (Table I) verify the premise that cholesterol homeostasis is not pegged to the cell total (which is greatly elevated in these cells) but rather to a specific subfraction, perhaps that in the plasma membrane or a representative thereof (29, 45). The increased biosynthesis of cholesterol and ingestion of LDL observed in cultured NP-C cells (15) and in vivo (42, 46) appear to just compensate for cholesterol deposition in intracellular compartments, so that the plasma membrane pool is maintained close to normal.

The approximately normal level of plasma membrane cholesterol found in NP-C and drug-treated cells (Table I) was unexpected because these cells are characteristically resistant to amphotericin B (12, 49, 50). The lytic action of this antibiotic depends acutely upon membrane cholesterol (51), so that cellular resistance has been interpreted to reflect a reduction in plasma membrane cholesterol. An alternative mechanism consistent with all of these data is that the large intracellular cholesterol pool in NP-C cells protects the plasma membrane by taking up most of the amphotericin B. The various NPC1-deficient cell lines identified by their amphotericin B resistance (12) might then have been selected on the basis of their large intracellular pools rather than reduced plasma membrane cholesterol.

Various lines of evidence suggest that class 2 amphiphiles mimic NP-C disease by acting on the NPC1 protein itself or somewhere along its pathway. First is the fact that normal fibroblasts treated with these agents show a pattern of cholesterol accumulation similar to that in NP-C cells (4, 22, 23). Second, in both systems, cell cholesterol circulates briskly between the intracellular and plasma membrane compartments (see “Results”
and Ref. 5). Third, the plasma membrane cholesterol content is essentially normal in both NP-C and amphiphile-treated cells (Table I). Fourth, the threshold behavior (J curve) that relates ER cholesterol to plasma membrane cholesterol is normal in NP-C cells (Figs. 4 and 5) and in fibroblasts treated with amphiphiles (29). Fifth, NPC1 expression is increased in cells treated with class 2 amphiphiles (10). Sixth, both NP-C cells and normal cells treated with class 2 amphiphiles accumulate the same types of membrane lipids as follows: biosynthetic sterol precursors (52), GM2 gangliosides (53), and bismonoacylphosphatidate (lysobisphosphatidic acid) (2, 4). Seventh, amphiphiles alter the intracellular distribution of the NPC1 protein (9). Eighth, we now show that the ability of class 2 amphiphiles to reduce the size of the regulatory ER cholesterol pool and to induce cholesterol accretion is abrogated in cells lacking the NPC1 protein (Fig. 8 and Table IV) as would be expected if the action of these agents required this protein or pathway.

Finally, the action of the oxysterols, 25-hydroxycholesterol and 7-ketocholesterol, on NP-C cells appeared to be normal in this study and previously (16). That is, these rapidly acting oxysterols increased the level of ER cholesterol, thereby promoting cholesterol esterification and down-regulating cholesterol accretion through the various ER control elements (Fig. 9). These findings provide further support for the hypothesis that the central mechanism of cholesterol homeostasis are not perturbed in NP-C disease. Of practical importance was the finding that the oxysterols reduced the pool of lysosomal cholesterol in preference to that in the plasma membrane in NP-C cells (Table V). Perhaps lysosomal cholesterol replenished the plasma membrane cholesterol pool as it became depleted (as suggested by Table II). The causal chain might be oxysterols → elevation of ER cholesterol → down-regulation of cholesterol accretion → reduction of plasma membrane cholesterol → compensatory shift of lysosomal cholesterol stores to the plasma membrane → differential reduction of lysosomal cholesterol. It is therefore conceivable that oxysterols or agents with a similar action could lower the intracellular cholesterol burden in the cells of patients with NP-C disease while maintaining a normal level of cholesterol in their plasma membranes.

After the submission of this report, a related study was published that also concluded that cholesterol moves freely from the lysosomes to the plasma membrane in NP-C cells and that a transport defect could exist in an endocytic compartment (18). On the other hand, the conclusion of those authors that cholesterol accumulates in late endosomes, as suggested earlier (4), is not consistent with evidence that the excess intracellular cholesterol in NP-C cells is associated with the bulk of the lysosomal hydrolases and LAMP 2 antigen (5, 9). Furthermore, our results do not support the inference of a defect in the delivery of cholesterol to the ER of NP-C cells (18).

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