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Cultured Skin Fibroblasts Derived from Patients with Mucolipidosis 4 Are Auto-Fluorescent

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ABSTRACT

Mucolipidosis 4 (ML4) is an autosomal recessive disorder with both lipid and mucopolysaccharide storage. The disease is characterized by severe visual impairment and psychomotor retardation. In our effort to find a phenotypic marker for ML4 fibroblasts, living cells were stained with fluorescent compounds. The staining pattern in cells was complicated by auto-fluorescence. A careful study revealed that auto-fluorescence by itself was a sufficient marker for viable ML4 fibroblasts. ML4 cells in cultures obtained from four unrelated patients contain auto-fluorescent material. Auto-fluorescence was noted over a wide range of excitation wavelengths from ~365 to ~546 nm. The most intense fluorescence was observed in the lower wavelength range. Cultured fibroblasts from normal individuals or obligate ML4 heterozygotes did not fluoresce under adequately controlled culture conditions. High passage number or inadequate feeding caused a small proportion of fibroblasts obtained from normal individuals to auto-fluoresce. The auto-fluorescent material co-localized with phase-dense inclusion bodies, shown to be lysosomes by staining with LAMP-ab. These findings imply that fluorescence may relate to the specific compound(s) stored in the lysosomes. In a comparative study, neuronal ceroid lipofuscinosis fibroblasts were also fluorescent. Fibroblasts from other diseases such as Gaucher disease and glycogenosis type 2 did not show any fluorescence. These findings are currently used in our functional cloning strategy for determining the gene involved in ML4. ***(Pediatr Res* 37: 687–692, 1995)**

Abbreviations

ML4, mucolipidosis 4
NCL, neuronal ceroid lipofuscinosis

ML 4 is a metabolic disorder transmitted in an autosomal recessive manner (1). Clinically, the patients suffer visual impairment and psychomotor retardation starting within the first year of life (2, 3). The diagnosis of the disease has been based on clinical and ultramicroscopic parameters (4, 5). Numerous storage bodies of varying size and shape are found throughout many of the tissues as evident by electron microscopic observations (6). The gene involved in the disease has not been identified, and the primary storage material is also unknown. A number of studies have described subtle biochemical differences between ML4 and normal fibroblasts, but these were not sufficiently unique to easily identify the mutant cells in culture. Indications of abnormal lipid metabolism in ML4 cells have been reported (7, 8). We undertook an investigation to further characterize the abnormal biochemistry associated with ML4 findings by labeling living fibroblasts with hydrophobic fluorescent compounds. We found that this technique was unnecessary because ML4 fibroblasts show marked auto-fluorescence under normal culture conditions.

METHODS

Cell cultures. Human skin fibroblasts were grown in Earl's modified Eagle's medium (Biofluids, Rockville, MD), in 10% fetal bovine serum (Hyclone, Logan, Utah), with penicillin and streptomycin. Normal cultures were obtained from the Human Genetic Mutant Cell Repository (no. 5659) (Institute of Medical Research, Camden, NJ) and from normal individuals, less than one year to 28 y of age, under approved National Institutes of Health guidelines. They were viewed between passages two and 14. Fibroblast cultures obtained from four ML4 patients ages 3–11 y (91.70, 91.85, 92.23, and 92.73) were viewed between passages two and 11. Obligate heterozygote fibroblast cultures from parents of patients 91.71 and 92.24 (passages two to five) were also obtained. Neuronal ceroid lipofuscinosis cultures were obtained from affected children with the three types of the disease; 9404 representing the late infantile form was from the Human Genetic Mutant Cell Repository, no. 2334 infantile, and no. 2207 juvenile forms were kindly provided by

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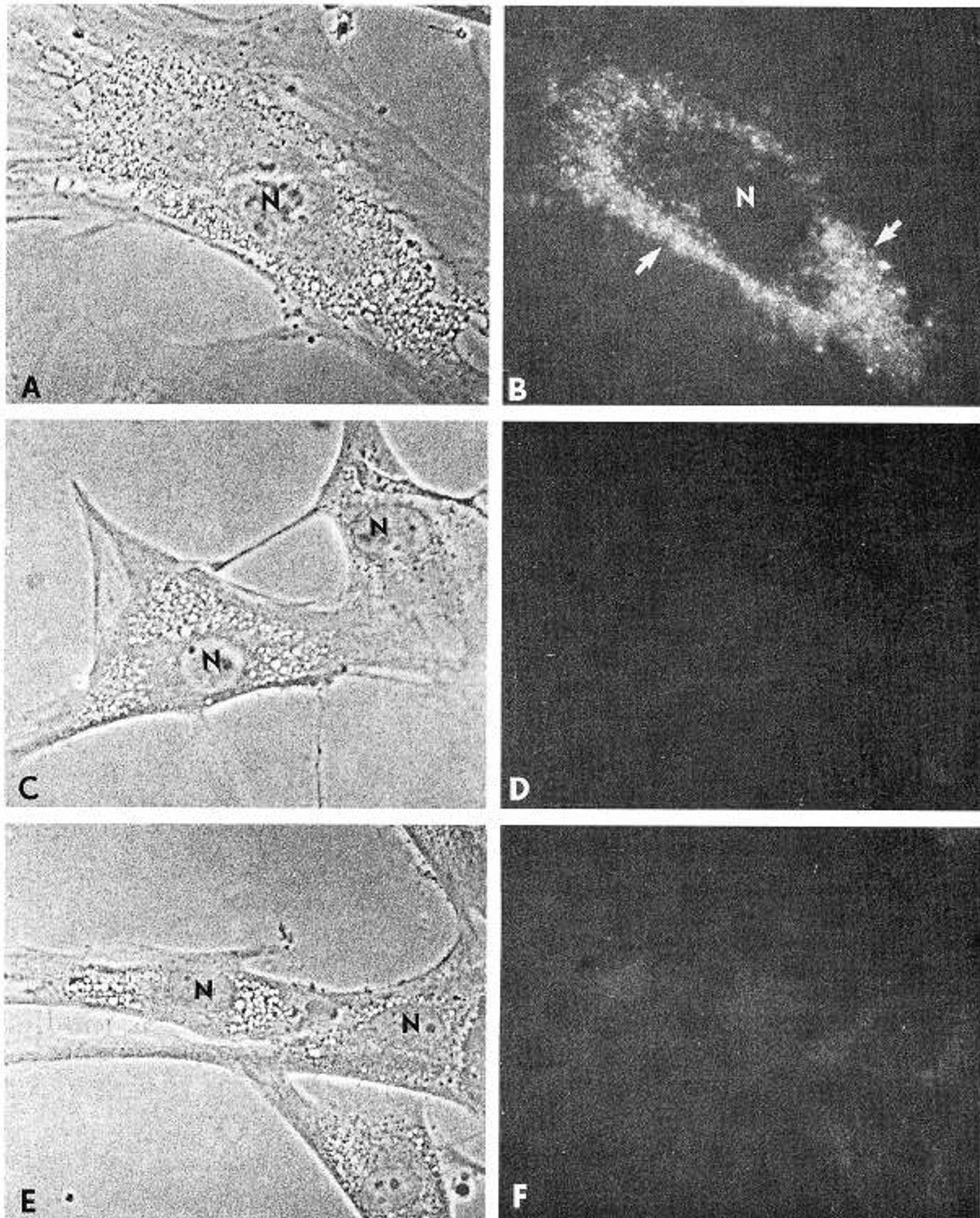

Figure 1. Phase and fluorescence micrographs of fibroblasts from ML4 patient (*A* and *B*), a heterozygous carrier for ML4 (*C* and *D*) and normal control (*E* and *F*). Fluorescence was obtained with a 450–490-nm excitation filter and 515-nm absorption filter. Auto-fluorescent granules (arrows) are present in fibroblasts from the ML4 patient (*B*) but not in fibroblasts from heterozygous (*D*) or normal (*F*) donors. *N*, nucleus; magnification, ×460.

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Fluorescence microscopy. Fibroblasts from confluent growth cultures were seeded in two-chamber glass slides (Lab-Tek, Nunc, Naperville, IL) at 20,000–40,000 cells per chamber. Cells were observed after 24–48 h on the slides. Glass coverslips were placed on the slides using the culture medium as mounting medium, and they were immediately examined and photographed. Fibroblasts were examined with a Leitz Labolux microscope equipped with phase and epifluorescence optics using two different filter combinations, a BP 350–410 excitation filter, RKP 455 dichroic beam splitter, LP470

absorption filter (UV+ violet), a BP 450–490 excitation filter, RKP 510 dichroic beam splitter, LP 515 absorption filter (blue), and a mercury vapor lamp. Photographs were made on TMAX film (Eastman Kodak, Rochester, NY) using a 400 ASA exposure setting and developed for an 800 ASA setting. Auto-fluorescence in ML4 cells was photographed first, and the same exposure time was used to photograph control and NCL fibroblasts. To identify lysosomes, rat antibody against human lysosomal protein was used (9), and secondary antibody (Jackson Laboratories, Avondale, PA) labeled with rhodamine was visualized with excitation filter BP 530–560, KRP 580 beam splitter, and absorption filter LP 580 (green). Rat MAb to human lysosomal protein was a gift from Dr. T. August, Johns Hopkins University, Baltimore, MD. Confocal fluorescence microscopy was performed on a Nikon Optiphot microscope equipped with a Bio-Rad MRC-600 confocal imaging system, krypton argon laser, 488 DF10 excitation filter DR 560LP dichroic reflector, and 522 DF35 emission filter (Bio-Rad Microscience, Hercules, CA). Cultures showing the same phenotype were observed at least twice at different times under the same conditions.

RESULTS

Mutant ML4 fibroblasts (Fig. 1*A*) have intracellular auto-fluorescence (Fig. 1*B*), whereas heterozygous (Fig. 1, *C* and *D*) and normal cells (Fig. 1, *E* and *F*) are rarely fluorescent. Appearance of fluorescent cells in normal cultures is probably an indication of a stressful environment, *i.e.* seeding cells in clusters and keeping them for prolonged periods without medium changes. In contrast, 20–100% of ML4 cultured fibroblasts were always fluorescent, under various culture conditions, including overnight incubation in serum-free medium, or when the medium was supplemented with lipoprotein-deficient serum (not shown). The fluorescence in ML4 fibroblasts (Fig. 2*C*) is easier to photograph at the shorter excitation wavelength 350 nm (Fig. 2*A*), 15-s exposure, than at the longer wavelength of 530 nm (Fig. 2*B*) requiring 60 s of exposure. Sporadically, mutant cells were fluorescent even at longer excitation wavelength using a rhodamine filter set (not shown).

Confocal fluorescence microscopy was used to examine the auto-fluorescent properties of living normal and mutant ML4 fibroblasts (Fig. 3). Normal fibroblasts (Fig. 3*A*) showed a faint diffuse auto-fluorescence (Fig. 3*B*), whereas mutant ML4 fibroblasts (Fig. 3*C*) contained intensely fluorescent spherical granules of varied sizes (Fig. 3*D*). These intracellular fluorescent granules (note *arrow* in Fig. 3*D*) co-localize with phase-dense granules (*arrow* in Fig. 3*C*). Mutant ML4 fibroblasts immunostained with antibodies against lysosomal membrane protein contained fluorescent rings (*L* in Fig. 3*F*) which co-localized with the surface of the phase-dense granules (Fig. 3*E* at *arrows*). Thus, phase-dense auto-fluorescent granules in ML4 fibroblasts (Fig. 3, *C* and *D*) are lysosomal organelles (Fig. 3, *E* and *F*).

We examined the specificity of ML4 auto-fluorescence by studying several other lysosomal storage diseases. Three types of neuronal ceroid lipofuscinosis, infantile, late infantile, and juvenile, show intense auto-fluorescence (Fig. 4). Auto-fluorescence in storage bodies is a well known characteristic of NCL that has been previously reported in living NCL cells in culture only in the juvenile form (10). Fibroblasts obtained from mucolipidosis 2 (I cell disease), Gaucher's disease, cysteinosis, or glycogen storage disease type 2 patients, did not have any auto-fluorescence (data not shown). Our results sug-

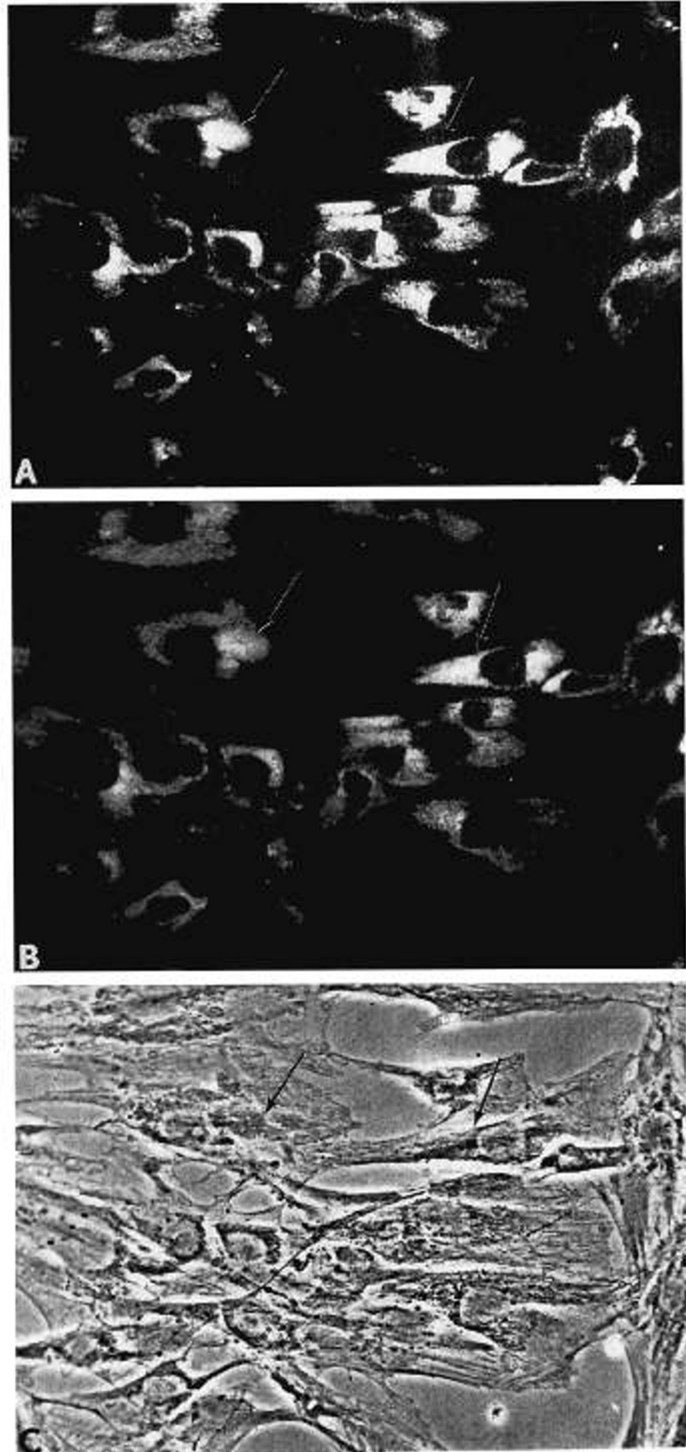

Figure 2. Phase and fluorescence micrographs of fibroblasts from an ML4 patient. Fibroblasts shown in the phase micrograph (*C*) contain phase-dense granules (*arrows*) which exhibit intense auto-fluorescence with 350–410-nm excitation filter and 470-nm absorption filter (*A*, *arrows*) and less auto-fluorescence with 450–490-nm excitation filter and 515-nm absorption filter (*B*, *arrows*). Magnification, ×185.

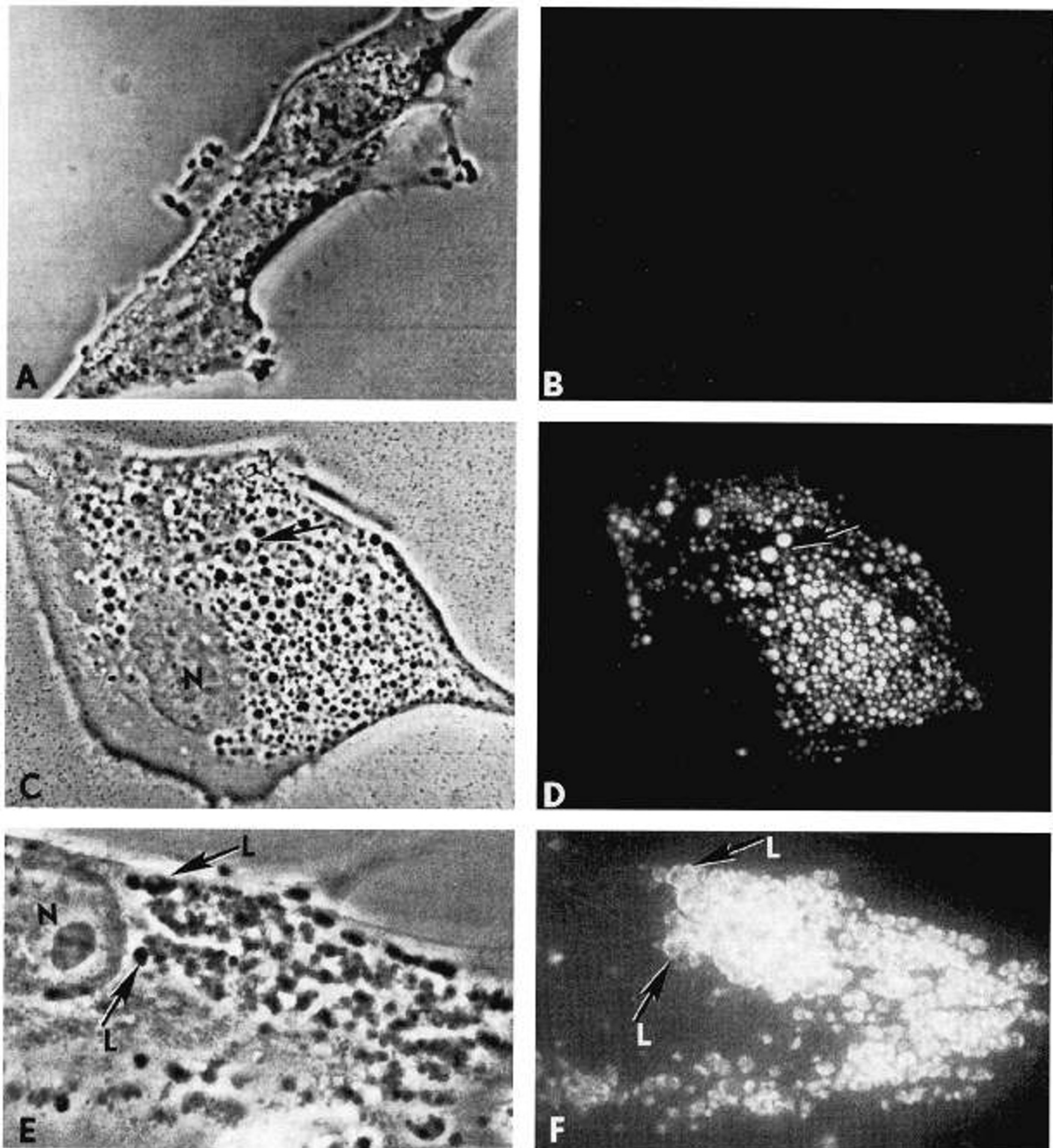

Figure 3. Phase and fluorescence micrographs of fibroblasts from normal (*A* and *B*), and ML4 (*C–F*) patients. Phase (*A*) and fluorescence images (*B*) of the same normal fibroblast, taken with the confocal microscope, show no auto-fluorescent inclusions. Phase (*C*) and fluorescence (*D*) images of an ML4 fibroblast, taken with the confocal microscope, show that cytoplasmic inclusions which are spherical and electron dense are also auto-fluorescent (*arrows*). Phase-dense inclusions (*L*) present in ML4 fibroblasts (*E*) are identified as lysosomes because they immunofluoresce when stained with antibodies to lysosomal membrane protein (*F*). Note the ring-shaped membrane staining of lysosomes (*L*) can be seen at *arrows*. *N*, nucleus; magnification: confocal images, *A–D*, ×925; *E* and *F*, ×1175.

gest that the presence of large storage bodies which are prevalent in I cells as well as in ML4 fibroblasts does not cause auto-fluorescence *per se*. Moreover, several different kinds of storage components such as amino acids and glycoproteins that accumulate in other diseases do not seem to be directly linked to the auto-fluorescence.

DISCUSSION

In this report we describe a simple and unambiguous method to positively identify ML4 fibroblasts in culture. Although the autofluorescence that we observed in living cultured ML4 cells was more intense with UV+ violet excitation, we have chosen to use blue excitation as this is the more commonly available wavelength routinely used to observe fluorescein fluorescence. One important caution is that cells should be well fed and of a relatively low passage to prevent false positives. This technique provides promise for relatively simple laboratory diagnostic for ML4 that could complement and, in some instances, potentially replaces the need for the currently used and more labor-intensive electron microscopic assay (4). The fluores-

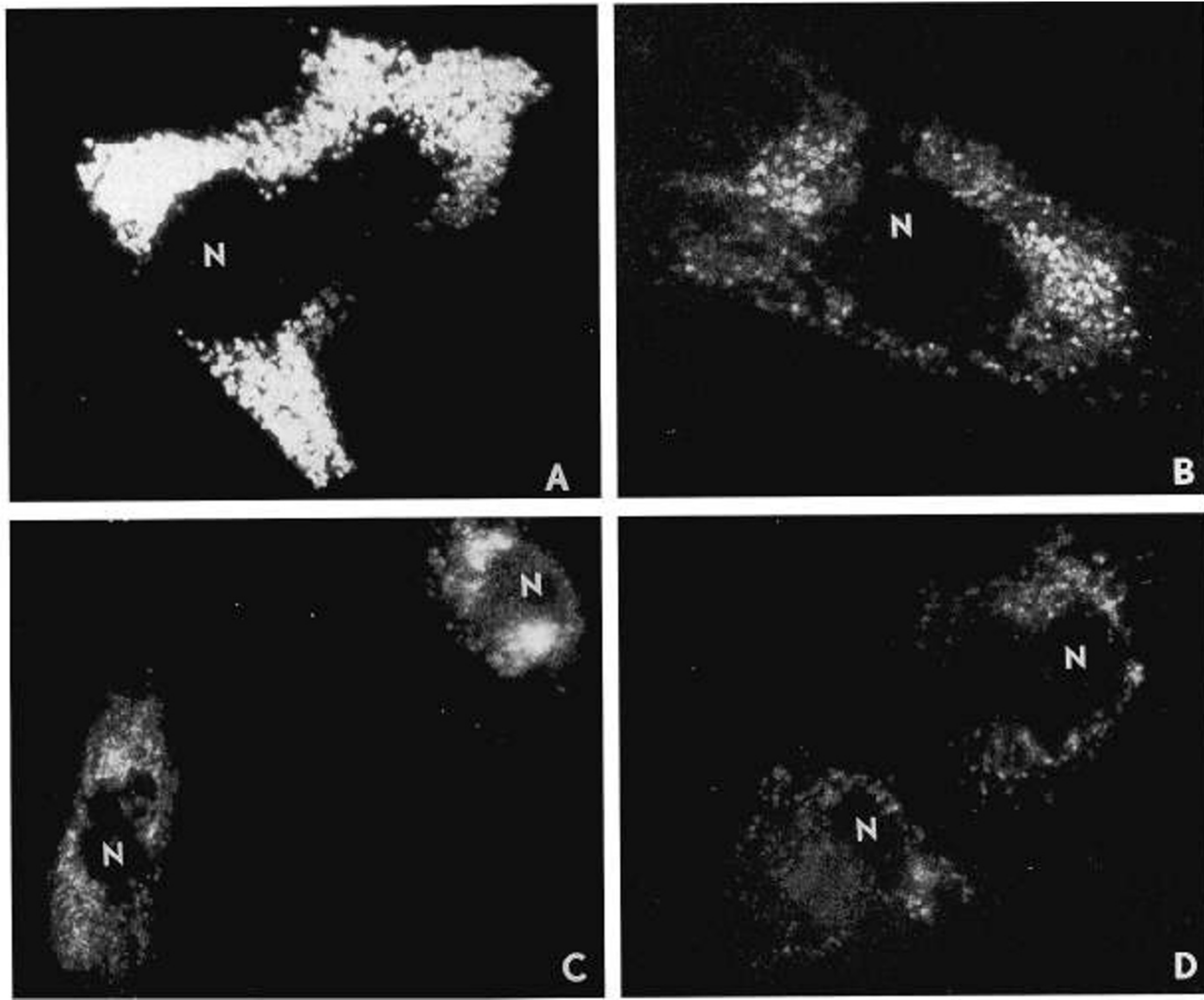

Figure 4. Auto-fluorescence in fibroblasts from an ML4 patient (*A*) and NCL patients (*B*, late infantile; *C*, juvenile; *D*, infantile). Note the large accumulation of intensely fluorescent paranuclear (*N*) lysosomes in the ML4 cell and the relatively less auto-fluorescent granules in NCL fibroblasts (*B–D*). Fluorescence was obtained with a 450–490-nm excitation filter and 515-nm absorption filter. Magnification: *A*, ×750; *B*, ×1000; *C*,×700; *D*, ×880.

cence method may also prove to be useful for prenatal diagnosis of ML4 with cultured amniotic cells. This possible clinical application requires additional validation studies and confirmation by other investigators before routine clinical implementation.

Auto-fluorescence of storage bodies in affected tissue is well documented in NCL (11, 12). This phenomenon was also mentioned with regard to storage bodies in the brain of an ML4 patient (6). The substances causing the fluorescence in the storage bodies are unknown. Efforts to identify the fluorophore in purified storage bodies of ovine NCL tissue were unsuccessful (12, 13). The results obtained by Palmer *et al.* (12, 13) lead to the speculation that the proteins accumulating in NCL storage bodies are stacked in a way that causes the fluorescence. However, it is not known whether proteins accumulate in ML4 cells.

The only additional evidence we could find for auto-fluorescent storage material in cultured cells was published by Dyken (10) in brain cells and fibroblasts obtained from a patient with juvenile NCL. Finding auto-fluorescence in fibroblasts is interesting in light of the assumption that storage material is expected to be more abundant in aged lysosomes of tissue specimens than in cultured cells. This may indicate that a rather limited storage is sufficient to produce detectable fluorescence, either because the fluorophore is one of the primary storage materials, or because of its strong fluorescence properties.

Another immediate use for the fluorescence method is screening for correction by candidate cDNA clones. This is of particular value in the study of NCL where positional cloning has led to a limited number of possible genes. Functional cloning using these candidate genes and screening for correction using the simple method described here will provide a facile way to find the gene responsible for the disease. We propose to use this strategy as part of our study to pursue the defective gene in ML4.

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