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Effects of Centrophenoxine on Lipofuscin Formation in Neuroblastoma Cells in Culture[1]

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C1300 mouse neuroblastoma cells in culture were used to study cytoplasmic lipofuscin pigment accumulation and the ability of centrophenoxine to alter its formation. The pigment was detected by its autofluorescence, by histochemical staining for acid phosphatase, and by positive staining with the periodic acid Schiff procedure. The percentage of cells with pigment increased from 25% at 5 days of culture to 60% at 25 days of culture. Pigment formation was enhanced by treatment for 9 days with either 1.1×10^{-5}M papaverine or 5.6×10^{-5}M prostaglandin E_1. Pigment formation in papaverine-treated cells was markedly reduced by treatment for 9 days with either 1.0 or 3.4×10^{-4}M centrophenoxine. It is concluded that neuroblastoma cells in culture provide an *in vitro* model with which to study lipofuscin pigment formation and its manipulation by pharmacological agents.

PROGRESSIVE deposition of lipofuscin pigment in the cytoplasm of neurons, as well as in other nondividing cells, has been shown to be a consistent cytological finding correlated with aging in several animal species, including man (Nandy, 1968, 1971; Pearse, 1964). Lipofuscin pigment is identifiable by its characteristic autofluorescence and histochemical properties (Nandy, 1968) and by its appearance at the electron microscopic level (Hasan & Glees, 1972; Hasan, Glees, & El-Ghazzawi, 1974; Riga & Riga, 1974). Furthermore, it gradually accumulates in cells with age (Nandy, 1971). These same approaches have been used to investigate lipofuscin formation *in vitro*. Spoerri and Glees (1974) have shown at the electron microscopic level that lipofuscin pigment accumulates over a 4-week period in rat dorsal root ganglion cells in culture. While lipofuscin pigment has also been demonstrated in cultured human glial cells, as shown by acid phosphatase-positive staining and by electron microscopy, it accumulated most markedly when cell division was reduced at confluent densities (Brunk, Ericsson, Ponter, & Westermark, 1973). Cultured WI-38 human fibroblasts also accumulate lipofuscin, especially in late passage, nondividing cells (Deamer & Gonzales, 1974).

Lipofuscin pigment also accumulates in mouse C1300 neuroblastoma cells in culture (Nandy & Schneider, 1976), developing gradually over a 3-week period, and its accumulation was enhanced upon treatment of the cells with papaverine, an inhibitor of the enzyme phosphodiesterase.

Another characteristic of lipofuscin pigment is the ability of dimethylaminoethyl-p-chlorophenoxyacetate (centrophenoxine) to reduce its formation *in vivo* (Chemnitius, Machnik, Low, Arnrich, & Urban, 1970; Hasan, Glees, & Spoerri, 1974; Meier & Glees, 1971; Nandy, 1968; Nandy & Bourne, 1966; Spoerri & Glees, 1974). Centrophenoxine is an ester of dimethylaminoethanol and p-chlorophenoxyacetic acid. The former is a biological precursor of acetylcholine and the latter is chemically related to members of the plant growth hormones known as auxins.

Improvement of certain clinical conditions such as psychosomatic asthenia of presenescence, disturbances of memory, intellectual concentration, and states of confusion in old age has been reported by several investigators (Destrem, 1961; Thuillier, Rumpf, & Thuillier, 1959) after treatment for several weeks at a dose of 80-100 mg/kg of body weight. The drug has also been shown to improve learning and

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memory in 12-mo.-old mice, compared to the corresponding controls, after 3 mo. of treatment with 80 mg/kg body weight (Nandy & Lal, 1977).

The mechanism by which centrophenoxine acts is not known, due in part to the difficulties of working with the *in vivo* model systems available for investigating the long-term actions of agents which may influence aging. Drug metabolism and distribution, assessment of drug concentration at the sites of action, and the prolonged time required for age-related cellular changes *in vivo* contribute to these difficulties. The use of cell culture systems for studying effects of chemicals on cellular aging could circumvent some of these difficulties. In this study, we found that centrophenoxine reduces lipofuscin pigment formation in mouse neuroblastoma cells in culture.

METHODS

The uncloned T59 line of mouse C1300 neuroblastoma cells was used in this study. Cells were grown in monolayer in Falcon plastic flasks or dishes in Ham's chemically defined synthetic medium (GIBCO Ham's F-12) containing $NaHCO_3$ (1.176 gm/L, 10% gamma-globulin-free newborn calf serum (GIBCO Laboratories), penicillin (100 U/ml) and streptomycin (100 μg/ml) and were maintained at 36 C in humidified air containing 5% CO_2. Cells used for maintaining this cell line in continuous culture were routinely subcultured every 4 or 5 days in order to maintain the cells in logarithmic growth; their doubling time varied between 19 and 26 hours.

Cells were harvested for transfer or for analysis by exposure for 6 min. at 36 C to 2 ml. of .25% Viokase (GIBCO) in phosphate-buffered saline (PBS; 8.0 gm NaCl, .2 gm KCl, 2.8 gm $Na_2HPO_4 \cdot 12\ H_2O$, .2 gm KH_2PO_4 in distilled H_2O to give 1 L). Cell counts were obtained with a Model D2 Coulter automatic blood cell counter. The number of cells contained in the medium before treatment with Viokase were counted separately from those harvested from the flask surface; the total number of cells in each flask represents the cells in the medium plus those harvested. The percentage of cells in the medium was used as the value for cell sloughing. Cell viability was measured by the trypan blue exclusion method (Phillips, 1973) using .04% trypan blue in PBS for 2 min. at 36 C. Protein content was measured by the Lowry procedure (Lowry, Rosebrough, Farr, & Randall, 1951). Cells harvested with Viokase were washed two times with PBS and incubated overnight in 5% cold trichloroacetic acid and the resulting precipitate was dissolved in 3% sodium hydroxide for analysis.

Cells for histochemical studies were grown on sterile Corning no. 2 (18 mm sq.) glass coverslips contained in LabTek 100 × 15 mm polystyrene petri dishes. The coverslips were incubated in serum-free F12 medium for 1 hour prior to fixation for histochemistry.

Lipofuscin pigment in cells was demonstrated using previously published methods (Nandy, 1968, 1971). The histochemical stains included the periodic acid Schiff stain after diastase digestion and the azo-dye method for acid phosphatase. Fluorescence analysis was carried out by exposure of cells on coverslips to ultraviolet light at a wavelength of 3650 Å. and an emission range of 2800-3800 Å. as described for brain tissue (Nandy, 1971). The following counts were made on cells grown on coverslips which had been stained for pigment or prepared for fluorescence study: (1) total number of cells in ten fields for each coverslip; (2) cells containing pigment which stained positively for acid phosphatase or with periodic acid-Schiff; (3) cells containing characteristic autofluorescent pigment. An ocular grid on a light microscope was used to facilitate cell counting.

RESULTS

Neuroblastoma cells harvested from cultures in log-phase growth and replated at a lower density in fresh medium grow with an average generation time of approximately 24 hours. Saturation density (around 4×10^5 cells/cm^2) is generally reached in 6 to 7 days after initial plating at 3×10^3 cells/cm^2. Thereafter, this high density can be maintained for as long as 30 days, and perhaps longer, when the medium is changed every day. T59 neuroblastoma cells maintained at confluent density were subjected to fluorescence and histochemical analyses in order to assess formation of lipofuscin pigment. Histochemical and fluorescence analyses showed a marked increase in pigment upon maintaining the cells in culture. The pigment was detected with each of the histologic analytical procedures employed as shown in the micrographs in Fig. 1 and 2. Acid phosphatase-positive material was detectable in cells after 3 days in culture (Fig. 1A) but was present to a greater extent after 10 days (Fig. 1B). Histo-

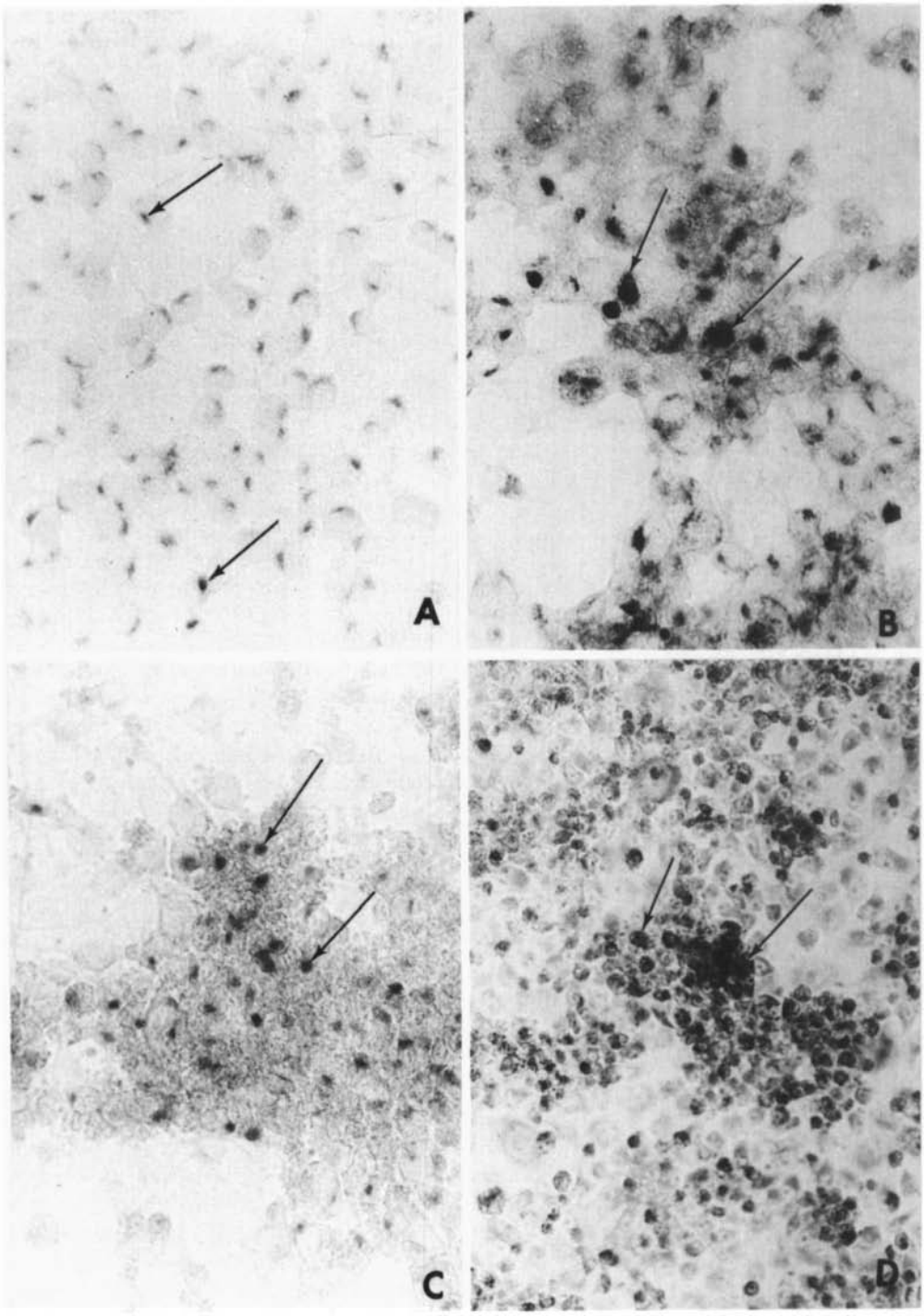

Fig. 1. Note the acid phosphatase-positive lipofuscin pigment (arrows) in small amounts in 3 day-old untreated cultures (1A) and the increased amount of pigmentation (arrows) in day 10 untreated (1B) neuroblastoma cells. 1C shows the marked increase in acid phosphatase-positive pigment (arrows) in cells treated with 1.1×10^{-5}M papaverine for 10 days. Papaverine treatment began 48 hr. after the cells were plated. ×600

Fig. 1D-2A. Note the periodic acid-Schiff-positive pigment (arrows) in day 3 untreated cells (1D) and the markedly increased acid-Schiff-staining pigmentation (arrows) in day 10 untreated neuroblastoma cells (2A). ×370

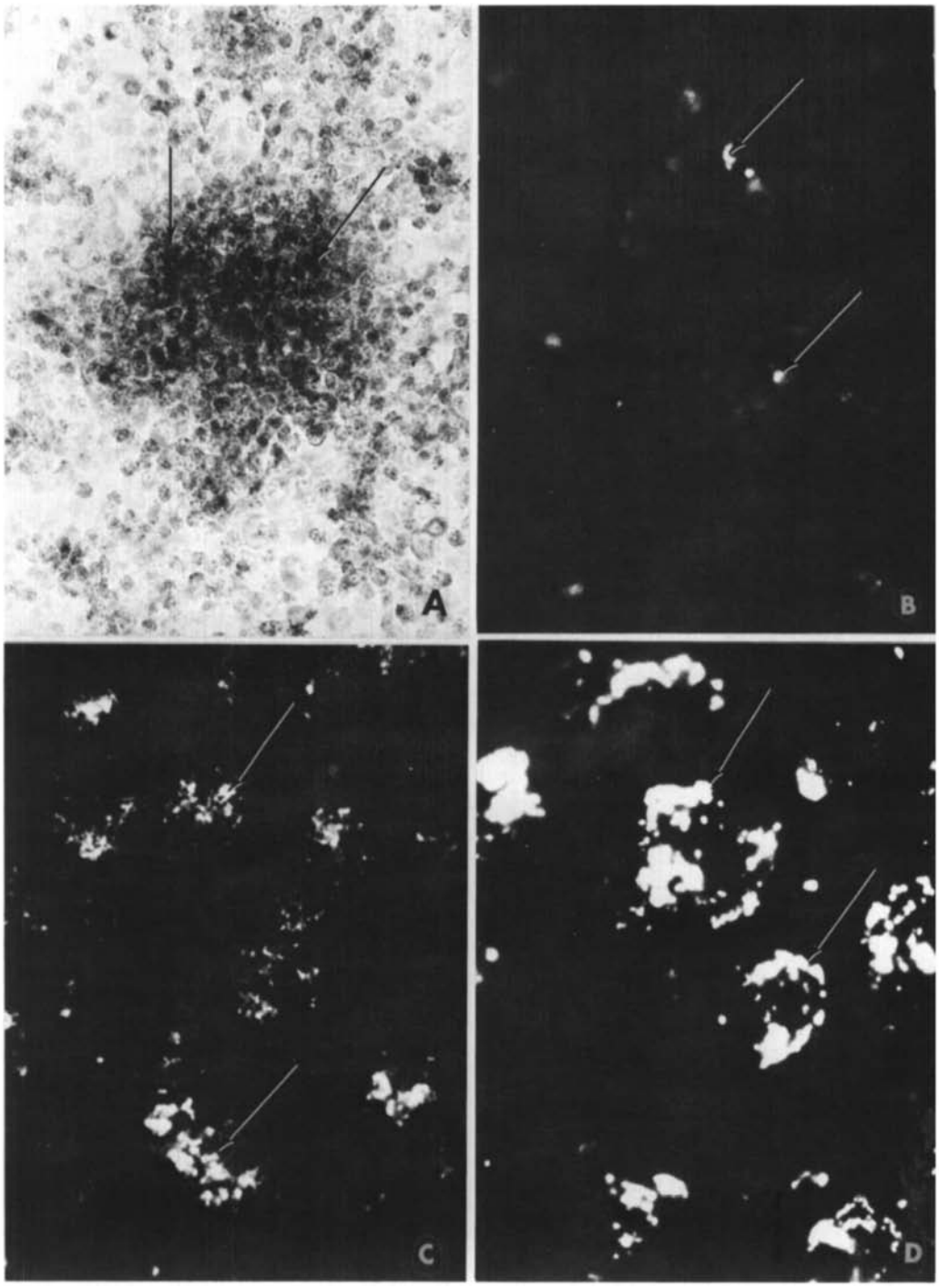

Fig. 2B-2D. Note the small amount of autofluorescent lipofuscin in day 3 cultures (2B) and the increase in pigment formation in day 10 untreated cells (2C). Pigmentation was markedly increased in cells treated with 1.1×10^{-5}M papaverine for 10 days. Papaverine treatment was begun 2 days after the cells were plated (2D). ×600

chemical staining with the periodic acid-Schiff reagent also showed the presence of pigment-like material, detectable after 3 days in culture (Fig. 1D), and to a greater extent after 10 days (Fig. 2A). An important criterion for establishing the presence of lipofuscin is a characteristic yellow autofluorescence upon exposure to ultraviolet light of the appropriate wavelength. There was a small amount of autofluorescent pigment in 3-day old cultures (Fig. 2B), but this was markedly enhanced after 10 days in culture (2C).

Quantitative data showing an increase in the percentage of cells having detectable pigment over a 25-day period are shown in Fig. 3. The cells having lipofuscin material ranged between 19-28% after 5 days in culture and increased to between 53-55% by day 25. Light microscopic examination of the confluent aging cultures, which were stained with the vital dye trypan blue, showed a gradual development of dense clumps of cells. These dense clumps sloughed from the flask surface and left sparsely populated areas. Surrounding cells migrated into these areas and presumably underwent division, since they were no longer under contract inhibition. In an attempt to prevent cell division in these cultures, and at the same time to prevent clump formation, the cultures were treated with 1.1×10^{-5}M papaverine, a phosphodiesterase inhibitor which inhibits growth of neuroblastoma cells in culture. Fig. 3 also shows that pigment formation was greater in papaverine-treated cultures than in corresponding untreated cultures, in some cases with pigment in sixty to seventy percent of the cells. Acid Phosphatase-positive and fluorescent material are shown in the micrographs in Figs. 1C and 2D, respectively. Prostaglandin E_1 (5.6×10^{-5}M), an agent which stimulates adenylate cyclase activity in neuroblastoma cells and reduces cell division, also promoted pigment formation (Fig. 3). The percentage of cells having pigment reached a maximum or near maximum value by 10 days in culture. Each of the microscopic procedures demonstrated the presence of pigment in untreated and treated cultures. Cell viability ranged between 83% on day 5 and 45% by day 25.

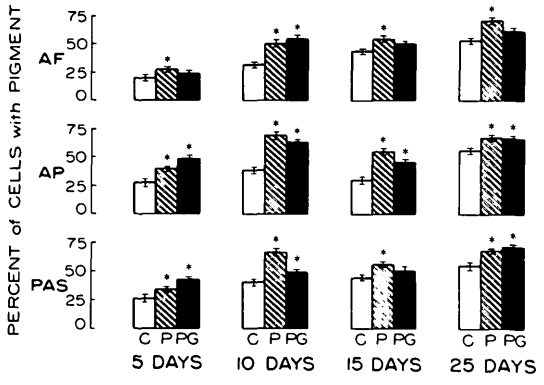

Fig. 3. Effects of 1.1×10^{-5}M papaverine (P) and 5.6×10^{-5} prostaglandin E_1 (PG) on pigment formation in T59 neuroblastoma cells grown on glass coverslips. Exposure to drugs was started 1 day after plating. AF means autofluorescence, AP means acid phosphatase stain and PAS means periodic acid-Schiff stain. The height of each bar represents the mean of between 40 and 80 counts for 2 to 4 experiments; the bracketed lines represent standard errors and the asterisk indicate significant difference from control (C) at the $p < .05$ level. Details of the histochemical procedures are described in the methods section.

The ability of 10^{-4}M and 3.4×10^{-4}M centrophenoxine to inhibit pigment formation was tested in cultures which were treated with papaverine for 18 days; centrophenoxine was present during the final 9 days in some cases. The combined data from two separate experiments presented in Table 1 show that 3.4×10^{-4}M centrophenoxine reduced cell pigment content as measured by each of the three histologic methods. Centrophenoxine at 10^{-4}M caused a statistically significant reduction of equal degree when pigment was measured by acid phosphatase or by PAS staining, but not as detected by autofluorescence. The micrographs in Fig. 4 show pigment content in papaverine-treated cells maintained in the absence or presence of 3.4×10^{-4}M centrophenoxine for the final 9 days and illustrate the decrease in pigment caused by centrophenoxine. A reduced amount of pigment was evi-

Table 1. Effect of Centrophenoxine on the Percentage of T59 Neuroblastoma Cells, Growing on Glass Coverslips, Which Contain Pigment[a].

Treatment	Fluorescence	APase	PAS
Regular media	33 ± 2*	59 ± 2*	70 ± 3*
Papaverine	51 ± 4	87 ± 1	88 ± 1
Papaverine + centrophenoxine (10^{-4}M)	45 ± 3	28 ± 2*	27 ± 3*
Papaverine + centrophenoxine (3.4×10^{-4}M)	28 ± 3*	29 ± 2*	34 ± 3*

[a] Papaverine was present at 1.1×10^{-5}M for 9 days starting on day 9 after plating; in the centrophenoxine-treated cultures centrophenoxine was also added on day 9 and continued for an additional 9 days. Media were changed everyday. The numbers are means ± standard errors, and an asterisk indicates a value which is different from papaverine treatment at $p < .001$ level. See methods section for details of procedures.

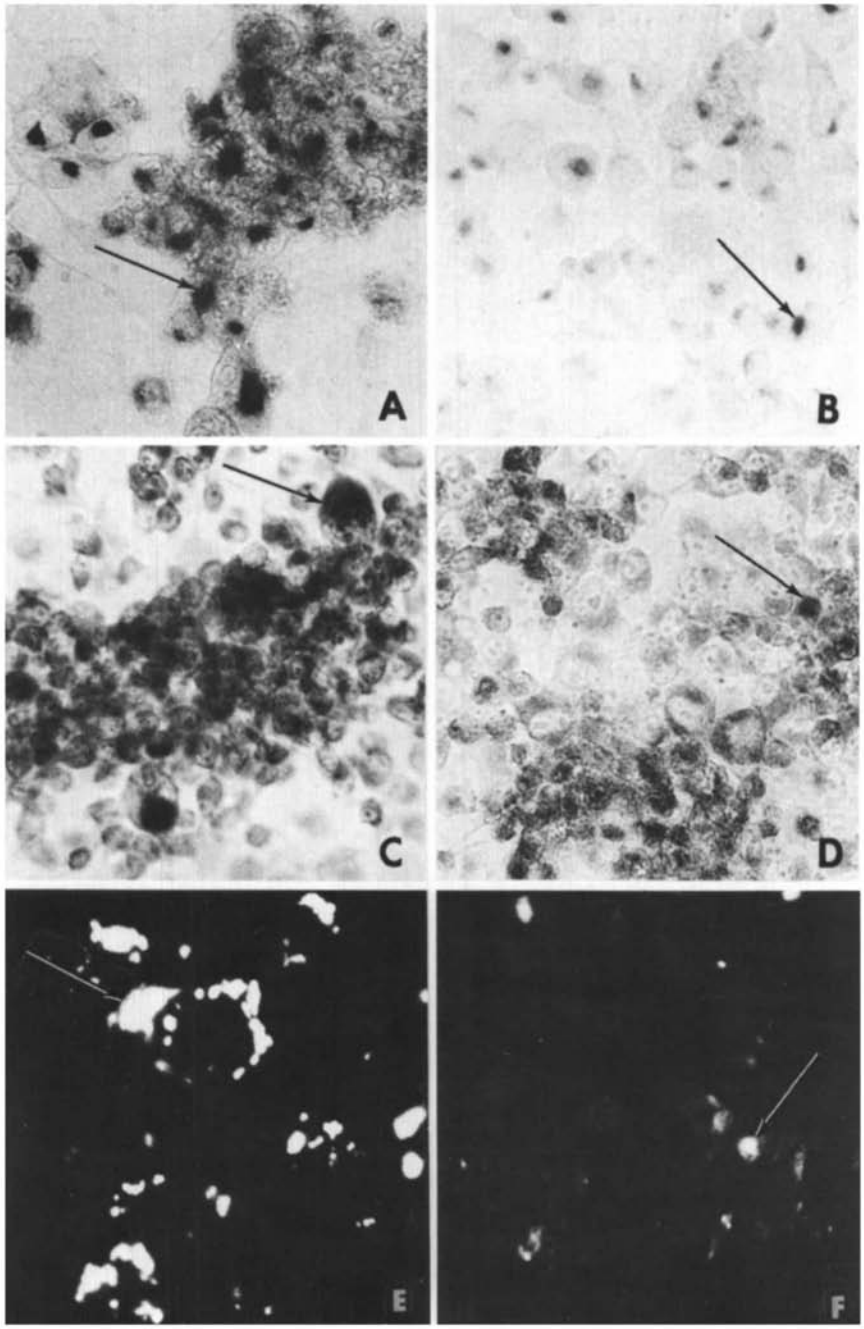

Fig. 4A-B. Strong acid phosphatase-positive lipofuscin pigment is evident in the papaverine-treated cells (4A). There is a marked reduction in the pigment due to treatment with centrophenoxine (3.4×10^{-4}) for 9 days (4B). ×600.

Fig. 4C-D. Note the periodic acid-Schiff positive pigment in day 18 papaverine-treated cells (3C) and the reduction of the number of pigmented cells (3D) upon addition of centrophenoxine to the cultures for 9 days. ×600

Fig. 4E-F. Note the large number of cells containing lipofuscin with typical autofluorescence in papaverine-treated cultures (3E) and the marked reduction in the pigmented cells due to the addition of 3.4×10^{-4}M centrophenoxine. See text for a description of the time course of the experiment depicted in Fig. 4.

dent upon examination by the acid phosphatase method (Fig. 4A,B), with the periodic acid-Schiff reagent (Fig. 4C,D), and by autofluorescence (Fig. 4E,F).

Centrophenoxine at 10^{-4} or 3.4×10^{-4} M did not decrease cell viability, increase cell sloughing, or significantly alter cell growth over the time period of these studies.

Discussion

The results of this study show that mouse neuroblastoma cells in culture accumulate intracellular lipofuscin pigment. The presence of the pigment was demonstrated with standard histochemical and fluorescence procedures (Nandy & Schneider, 1976). These analyses show that the pigment in neuroblastoma cells is similar to that of animal neurons *in vivo*. Pigment formation in the neuroblastoma cells increased progressively with time when the cultures were maintained at confluent density, a condition which results in a reduction of cell division in a large proportion of the cells. Lipofuscin formation was enhanced even further in cultures when cell division was reduced with papaverine or with prostaglandin E_1. Furthermore, a higher degree of pigment formation is reached sooner in the treated cultures than in the untreated cultures.

Inhibition of cell division in cultured neuroblastoma cells can result in a variety of other cellular changes as well. For example, cells develop neurite-like processes, and show increases in several neuronal enzymes, in intracellular cyclic AMP and in cell adenylate cyclase activity (Prasad, 1975). Whether the rate of lipofuscin pigment formation actually increases in nondividing cells or relatively increases because it is not diluted by cell division, is not known. Lipofuscin formation in neuroblastoma cells in culture is consistent with similar pigment formation in cultured fibroblasts (Deamer & Gonzales, 1974) and glial cells (Brunk et al., 1973).

Earlier studies have shown that centrophenoxine reduces the rate of lipofuscin formation in neurons of aging mice, rats and guinea pigs (Nandy, 1968, Nandy & Bourne, 1966). Neither the mechanism of this reduction of lipofuscin nor its possible physiopathologic significance is understood at the current time. This is largely due to the lack of knowledge about the mode of formation of the pigment and about its possible physiological significance.

Some questions may be raised about the applicability of neoplastic cells in the study of the effects of drugs on aging. However, a number of studies have shown that these cells, following differentiation, behave like normal neurons in many ways (Breakefield, 1976; Prasad, 1975) and develop lipofuscin pigment with aging (Nandy & Schneider, 1976). This study indicates that neuroblastoma cells in culture may provide a model to examine the cellular events involved in lipofuscin formation and its modification by pharmacological agents.

Summary

C1300 mouse neuroblastoma cells gradually accumulate lipofuscin-like pigment when they are maintained in culture up to 25 days. Pigment was demonstrated by specific autofluorescence and positive staining for acid phosphatase and with periodic acid-Schiff stain. Pigment formation is enhanced by treatment of the cells with 1.1×10^{-5}M papaverine or 5.6×10^{-5}M prostaglandin E_1. Pigment formation in papaverine-treated cells was reduced by exposure of the cells to 1.0 or 3.4×10^{-4}M centrophenoxine. It was concluded from this study that neuroblastoma cells provided an *in vitro* model to study lipofuscin pigment formation and its alteration by pharmacological agents.

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