THE REVERSIBLE INHIBITION OF MYOBLAST PROLIFERATION BY γ-HEXACHLOROCYCLOHEXANE[1]

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ABSTRACT

The effects of treating non-fusing myoblast variants, fu-1 and M_3A, with two levels (1×10^{-4} M and 2×10^{-4} M) of γ-hexachlorocyclohexane, an inhibitor of phosphatidylinositol synthesis, on myoblast proliferation were evaluated by measuring myoblast proliferation (counting cells) and visual inspection via phase microscopy. In the presence of γ-hexachlorocyclohexane, these cells were arrested, presumably in G_1. The inability of these cells to replicate did not appear to be due to a toxic effect of γ-hexachlorocyclohexane, because these cells were capable of resuming proliferation once they were transferred to media lacking γ-hexachlorocyclohexane. Cells were grown in media containing myo-[2-^{3}H] inositol and the radioactive content of water-soluble metabolites, the end product of phosphatidylinositides hydrolysis, was quantitated. Cells that were grown in the presence of γ-hexachlorocyclohexane, in addition to the loss of proliferative ability, also contained significantly less water-soluble metabolites. Therefore, it appears that there is a direct relationship between phosphatidylinositol metabolism and cell proliferation in the cell lines studied. (Key Words: Cell Culture, Cell Growth, Phosphatidylinositols.)

Introduction

Since the initial isolation of phosphatidylinositol from the brain (Folch, 1949), a great deal has been learned about these unique and minor constituents of the cellular phospholipids (for review, see Berridge, 1984). It is generally accepted that there are three species of phosphatidylinositols (phosphatidylinositol, phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate), and that these species are interconvertible through phosphorylation (Berridge, 1984; Berridge et al., 1984). It is also well-documented that the hydrolysis of these phospholipids leads to the mobilization of intracellular Ca^{++} (Rebecci et al., 1983; Berridge et al., 1984; Biden et al., 1984; Burgess et al., 1984a,b; Irvine et al., 1984; Joseph et al., 1984a,b; Kolesnick and Gershengorn, 1984; Aub and Putney, 1985). Among the different species of inositol phosphates generated through hydrolysis of phosphatidylinositols (Berridge, 1984), inositol 1,4,5-triphosphate seems to serve as the secondary messenger for the mobilization of Ca^{++} from the intracellular stores, particularly those associated with the endoplasmic reticulum (Streb et al., 1983; Burgess et al., 1984a,b; Dawson and Irvine, 1984; Joseph et al., 1984a,b; Prentki et al., 1984).

The function(s) of the secondary messenger, i.e., the mobilized Ca^{++}, generated through this pathway remains unclear. Michell (1979) proposed that a change in cytosol Ca^{++} concentration can exert an appreciable effect on the progress of cell proliferation, and that proliferation was initiated by a cytosol Ca^{++} concentration in excess of a certain critical level (Kishimoto et al., 1980). Other studies have shown that rate of phosphatidylinositol hydrolysis is directly proportional to rate of chicken ventricular cell proliferation (Ishima et al., 1982), and that turnover of phosphatidylinositol in rapidly dividing lens epithelial cells ($t_{1/2}$ approximately 5 h) was much greater than

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in non-proliferating and differentiating fiber cells ($t_{1/2}$ approximately 63 h; Zelenka, 1980). Moreover, Ristow et al. (1973) observed enhanced incorporation of ^{32}p and ^{3}H-inositol into phosphatidylinositol when G_1-arrested embryonic rat fibroblasts were stimulated to grow by addition of serum. It was also observed that up to 15 to 20% of the total radioactivity of embryonic rat fibroblasts prelabelled with ^{3}H-inositol was eliminated from the phospholipid during 20 to 40 min after the addition of serum (Ristow et al., 1975).

Because other studies suggested that enhanced phosphatidylinositol turnover is related to cell proliferation, we investigated the relationship between cell growth and phosphatidylinositol hydrolysis utilizing γ-hexachlorocyclohexane, a drug with the same configuration as myo-inositol and known to inhibit the synthesis of phosphatidylinositol (Hokin and Brown, 1969; Fisher and Mueller, 1971; Ristow et al., 1973; Hoffmann et al., 1980; Vu et al., 1983; Zelenka and Vu, 1984). Hoffman et al. (1980) reported that γ-hexachlorocyclohexane inhibits phosphatidylinositol synthesis as well as DNA synthesis and mitosis. Our experiments using non-fusing myoblast variants indicate these cells lose the ability to divide when phosphatidylinositol synthesis is prevented by γ-hexachlorocyclohexane. Moreover, we have demonstrated that these cells will regain their ability to proliferate if they are cultured in the absence of γ-hexachlorocyclohexane, even after extended exposure to γ-hexachlorocyclohexane.

Materials and Methods

Cell Culture Conditions. Fu-1, a non-fusing variant of L_8 myoblasts was obtained from Dr. Kaufman (University of Illinois) and M_3A, a non-fusing variant of L_6 myoblasts was obtained from Dr. Schubert (Salk Institute). Growth characteristics of these cells were described by Kaufman et al. (1980) and Shubert and Lacorbienic (1982), respectively. Cell lines were cloned and maintained by serial passage as described by Kaufman et al. (1980) and Schubert and Lacorbienic (1982). Cells were grown in Dulbecco's modified Eagle's Medium[6] supplemented with 10% fetal calf serum (DMEM + 10% FBS) at 37 C in a 10% CO_2 high-humidity atmosphere.

Cell Growth. Approximately 5×10^4 cells were plated in 35-mm^2 tissue culture dishes[7] in 2 ml of DMEM + 10% FBS and allowed to attach. After attachment, the media was removed and new media corresponding to the appropriate treatment added. Control media consisted of DMEM + 10% FBS and .035% dimethylsulfoxide (DMSO). To study the effect of blocking phosphatidylinositol synthesis on the cell growth, both cell lines were grown in DMEM + 10% FBS containing 1×10^{-4} M and 2×10^{-4} M γ-hexachlorocyclohexane[8]. γ-hexachlorocyclohexane was dissolved in DMSO and appropriate volumes were added to the media to give the desired final concentrations of γ-hexacholorcyclohexane, i.e., 1×10^{-4} M and 2×10^{-4} M. This media was then sterilized by filtration (.2 μM, Millipore) and added to the dishes (both control and treatments contained .035% DMSO). Twenty-four hours after original cell plating, media was removed and new media corresponding to appropriate treatment was added to the dishes. At 0, 24, 48, 72 and 96 h after the original plating, cells were trypsinized (.05% trypsin in Ca^{++}, Mg^{++}-free Earle's Salt Solution) and counted in a ZBI Coulter Counter[9].

Labelling and Extraction of Water-Soluble Metabolites. At the same time cells were plated to measure growth rate, parallel sets of 35-mm^2 plates were prepared by the same procedure for labelling and extraction of water-soluble metabolites according to the procedure of Berridge et al., 1982. Four hours after plating, media was removed and fresh media, corresponding to the appropriate treatment (control, 1×10^{-4} M and 2×10^{-4} M γ-hexachlorocyclohexane), containing 1 μCi/plate of myo-[2-^{3}H] inositol (specific activity 14 Ci/mmol, Amersham) was added. Twenty-four hours after plating, the media was removed and fresh media corresponding to appropriate treatment and containing myo-[2-^{3}H] inositol (1 μCi/plate) was added to the dishes. After 72 h of pre-labelling with myo-[2-^{3}H] inositol, the labelled media was removed, cells were rinsed twice and incubated for 1 h with 2 ml of phosphate-buffered saline (PBS), pH 7.4 (Berridge et al., 1984). After an additional rinse with 2 ml of PBS, the reaction was stopped by addition of 1 ml of ice-cold 15% trichloroacetic

[6] Gibco, Grand Island, NY.
[7] Costar, Cambridge, MA.
[8] Sigma Chemical Co., St. Louis, MO.
[9] Coulter Elect. Inc., Hialeah, FL.

acid (TCA). The dishes were then left on ice for 30 min. Cells were then harvested with rubber policemen and centrifuged at 2,000 ×g for 5 min. This procedure was repeated two additional times with the addition of 1 ml of H_2O each time for maximum extraction of the water-soluble metabolites. The supernatants from the TCA extractions and H_2O washes were combined and TCA was removed by extraction with diethylether (four extractions). The final extract was neutralized and the ^3H content was determined.

Results and Discussion

Results presented in figures 1 and 2 document the effect of γ-hexachlorocyclohexane on the proliferative capacities of the non-fusing myoblast variants, fu-1 and M_3A. Growth curves for control cells are similar to those of Kaufman et al. (1980) for fu-1 and Schubert and Lacorbienic (1982) for M_3A cells lines. These results (figures 1 and 2) clearly indicate that the presence of γ-hexachlorocyclohexane in the media negatively affected the ability of these cells to divide. For example, for M_3A cells, cell population in the media lacking γ-hexachlorocyclohexane increased 7.6 times after 96 h of incubation, but only 2.4 and 2.1 times in the presence of 1×10^{-4} M and 2×10^{-4} M γ-hexachlorocyclohexane, respectively. The same analysis for fu-1 cells were 7.5, 2.6 and 2.1 times for control, 1×10^{-4} M and 2×10^{-4} M γ-hexachlorocyclohexane, respectively.

Hoffmann et al. (1980) reported that 3×10^{-4} M concentration of γ-hexachlorocyclohexane was near the toxic level for rat embryo fibroblasts and led to significant cell death in some of their experiments. In order to ensure that the effects of γ-hexachlorocyclohexane observed in our experiments (figures 1 and 2) was not due to cell death, we monitored the appearance of the treated cells via phase microscopy (figures 3 and 4). Although cell proliferation was substantially attentuated, the cells remained viable and did not become refractile and release from the substrate with γ-hexachlorocyclohexane treatment. Moreover, when the γ-hexachlorocyclohexane media was removed after 96 h and was replaced by fresh media (DMEM + 10% FBS alone), we were able to reverse the inhibitory effect of γ-hexachlorocyclohexane (figure 5) and show a dramatic increase in M_3A proliferation after 48 h in normal media. The fact that these cells regained normal proliferative rates suggest that the γ-hexachlorocyclohexane arrested the cells in G_1, stage of cell cycle. Phase micrographs of cells after 96 h of incubation in the presence of γ-hexachlorocyclohexane (figure 6a) and 48 h in normal media (figure 6b) show the ability of these cells to regain their normal proliferative properties after the removal of γ-hexachlorocyclohexane. In addition to resuming normal

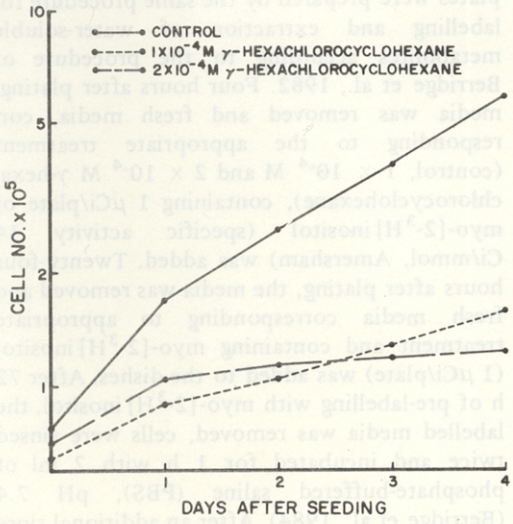

Figure 1. Growth curve of fu-1 cells in the presence and absence of γ-hexachlorocyclohexane.

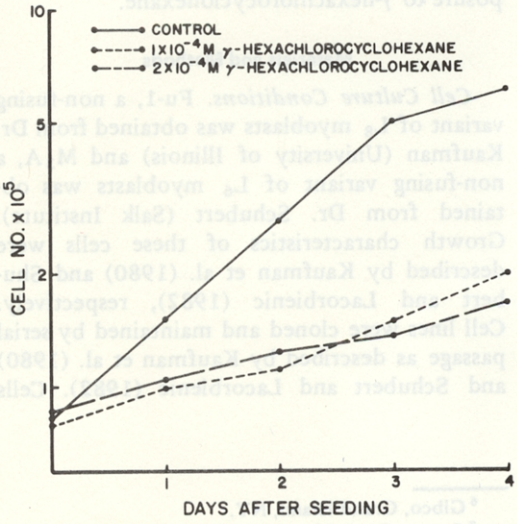

Figure 2. Growth curve of M_3A cells in the presence and absence of γ-hexachlorocyclohexane.

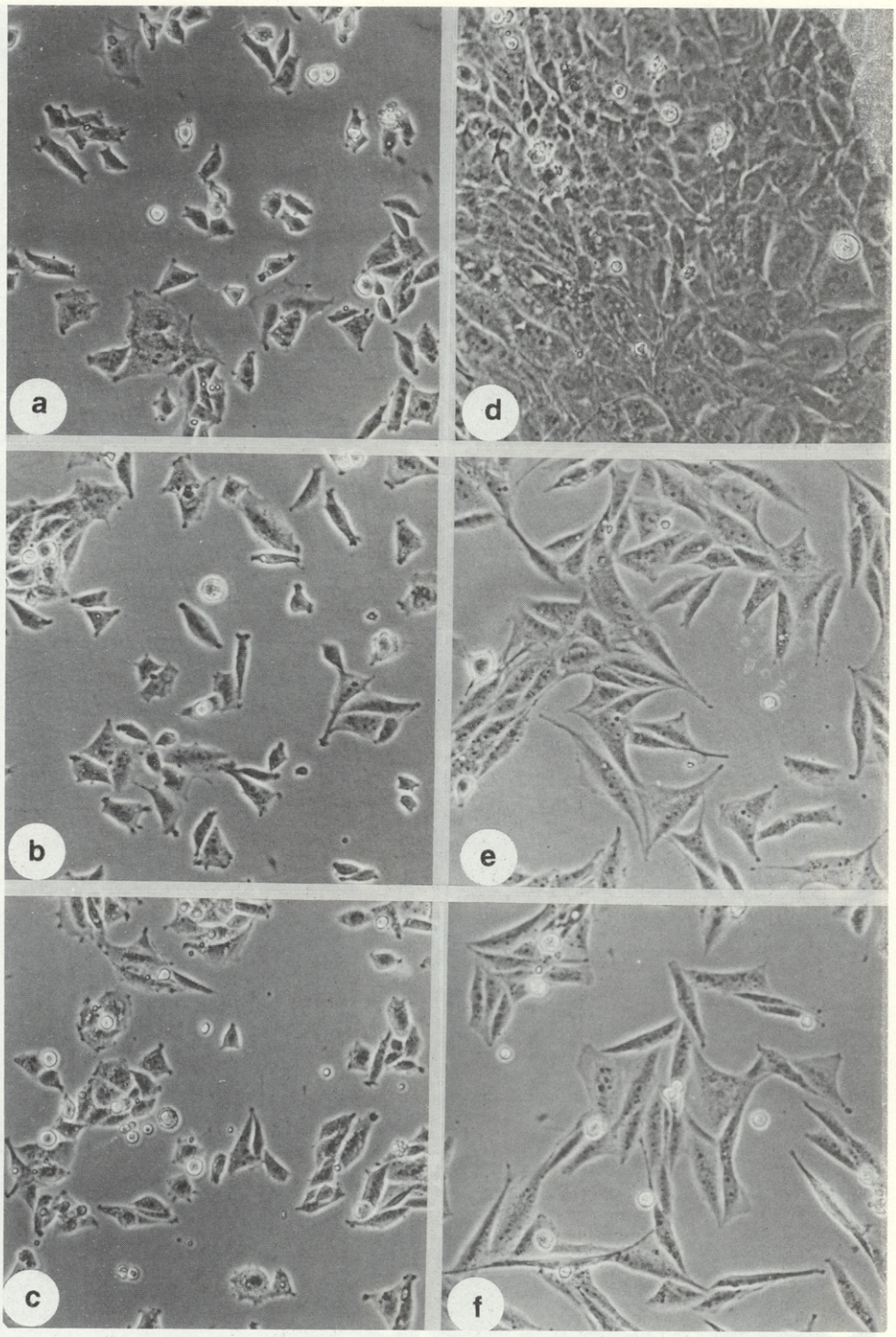

Figure 3. Phase contrast micrograph of fu-1 cells grown in the presence and absence of γ-hexachlorocyclohexane (× 120). a, b and c represent control, 1×10^{-4} M and 2×10^{-4} M γ-hexachlorocyclohexane, respectively at 0 h. d, e and f represent control, 1×10^{-4} M and 2×10^{-4} M γ-hexachlorocyclohexane, respectively at 96 h.

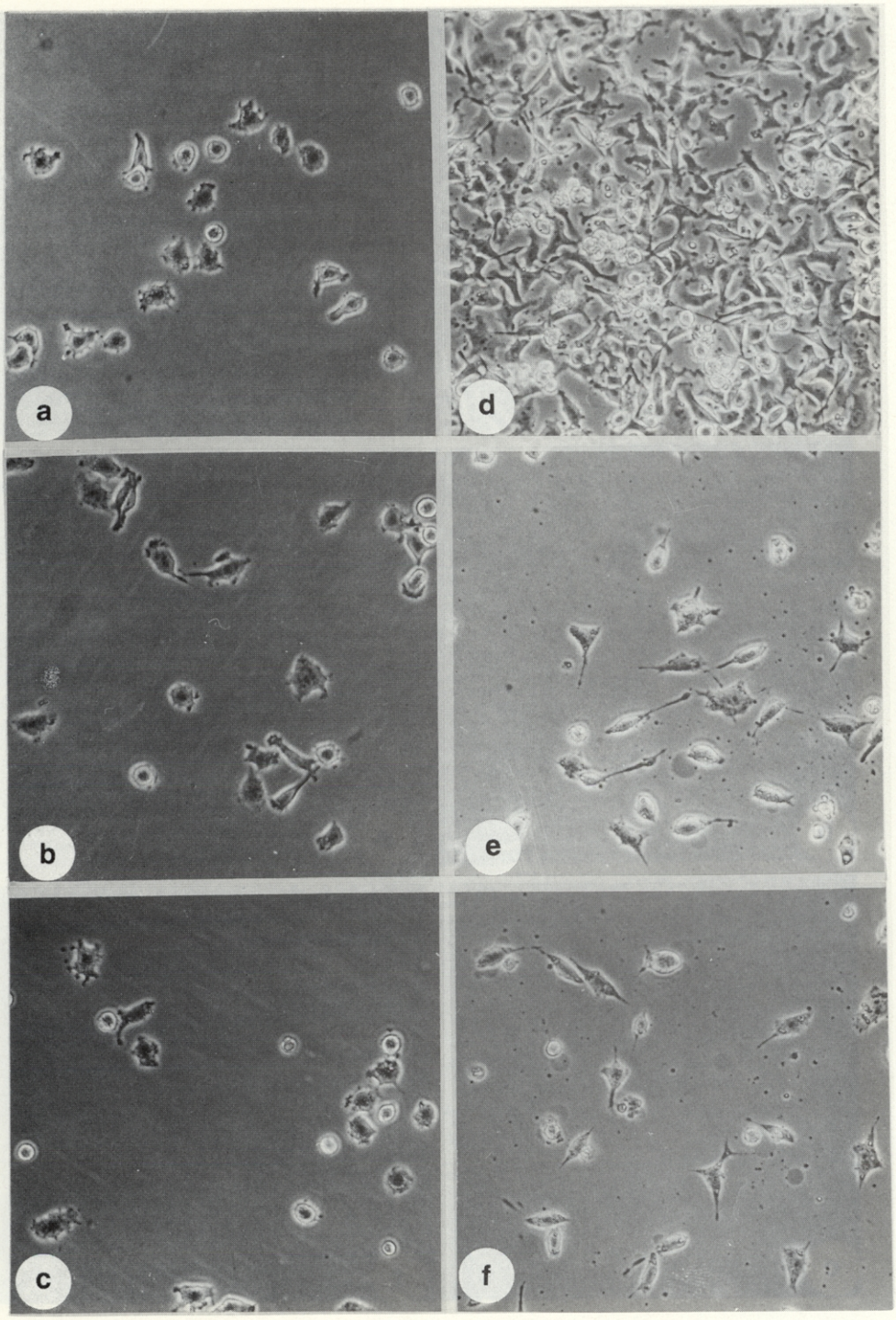

Figure 4. Phase contrast micrograph of M_3A cells grown in the presence and absence of γ-hexachlorocyclohexane (× 120). a, b and c represent control, 1×10^{-4} and 2×10^{-4} M γ-hexachlorocyclohexane, respectively at 0 h. d, e and f represent control, 1×10^{-4} M and 2×10^{-4} M γ-hexachlorocyclohexane, respectively at 96 h.

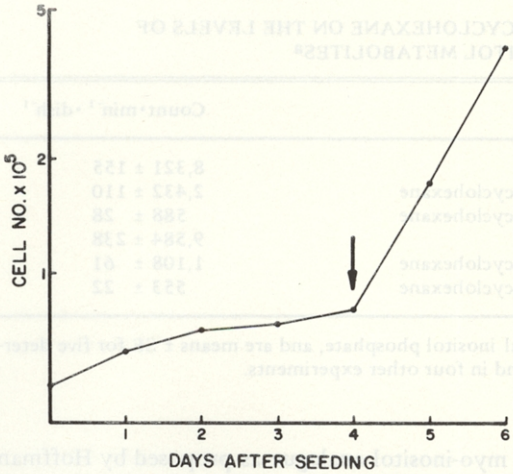

Figure 5. Growth curve of M_3A cell grown in the presence of 2×10^{-4} M γ-hexachlorocyclohexane, then in its absence. Arrow indicates start of growth in the absence of inhibitor.

proliferative activities, removal of γ-hexachlorocyclohexane resulted in generation of water-soluble inositol metabolites at a rate similar to that in control cells (table 1). The results of this experiment provide evidence that the effect of γ-hexachlorocyclohexane could not be due to a toxic effect.

Although the hexachlorocyclohexane-treated cells remained viable even after 96 h of treatment, treated cells, both fu-1 and M_3A cells, displayed substantial morphological differences when compared with the control cells. Fu-1 cells appear to be more elongated, flattened and somewhat larger (figures 3e and f). This latter impression may result from the fact that these cells are more flattened. Treated M_3A cells also exhibit an altered morphology (figures 4e and f), again demonstrating some elongation and extensive pseudopod formation. M_3A cells treated for 96 h with 2×10^{-4} M hexachlorocyclohexane (figure 6a) appear almost arborized. However, these cells, when refed with normal media ffor 48 h, certainly have regained their proliferative ability and morphologically are indistinguishable from control M_3A cells seen in figure 4d. The results presented in figures 3 and 4 constitute the first evidence documenting morphological changes associated with γ-hexachlorocyclohexane treatment.

Causes of the morphological changes observed in treated cells were not investigated in this study. However, other researchers have noted that diacylglycerol, an end-product of phosphatidylinositol hydrolysis, may effect membrane enzymes and can induce membrane fusion (Das and Rand, 1984). Thus, it is possible that by inhibiting phosphatidylinositol metabolism, thereby reducing the amount of

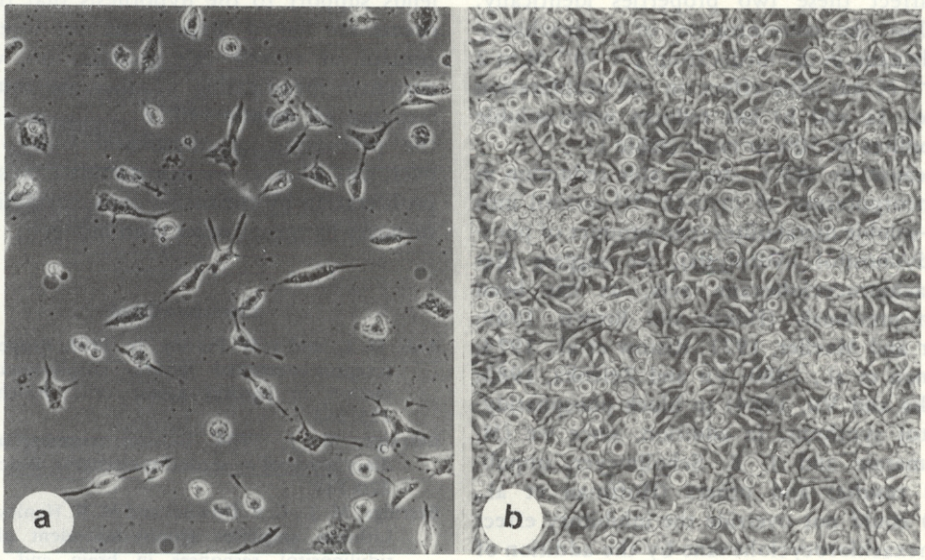

Figure 6. Phase micrograph (X 120) of M_3A cells grown in presence of 2×10^{-4} M γ-hexachlorocyclohexane (96 h; a) and then in its absence (48 h; b).

TABLE 1. EFFECT OF γ-HEXACHLOROCYCLOHEXANE ON THE LEVELS OF WATER-SOLUBLE INOSITOL METABOLITES[a]

Cell line	Treatment	Count·min^{-1}·dish^{-1}
Fu-1	Control	8,321 ± 155
Fu-1	1×10^{-4} M γ-hexachlorocyclohexane	2,432 ± 110
Fu-1	2×10^{-4} M γ-hexachlorocyclohexane	588 ± 28
M_3A	Control	9,584 ± 238
M_3A	1×10^{-4} M γ-hexachlorocyclohexane	1,108 ± 61
M_3A	2×10^{-4} M γ-hexachlorocyclohexane	553 ± 22

[a]The results are expressed as radioactivity in the total inositol phosphate, and are means ± SE for five determinations in a single experiment. Similar results were found in four other experiments.

diacylglycerol available, we have in some way altered the plasma membranes.

Assay of the effects of γ-hexachlorocyclohexane on phosphatidylinositol metabolism showed that, at 1×10^{-4} M γ-hexachlorocyclohexane, the ^{3}H content (i.e., water-soluble metabolites) in fu-1 and M_3A cells was 29.2 and 7.0%, respectively, of that of control cells (table 1). At 2×10^{-4} M γ-hexachlorocyclohexane, the ^{3}H content were 11.6 and 5.8 % of control for fu-1 and M_3A cells, respectively. The results in table 1 also indicate that M_3A cells in DMEM + 10% FBS generate water-soluble metabolites more rapidly that fu-1 cells (9,584 vs 8,321 cpm). Comparing these results on phosphatidylinositol metabolism with the results on cell proliferation (figures 1a and b) indicates that γ-hexachlorocyclohexane does not affect these two properties identically. Doubling the concentration of γ-hexachlorocyclohexane in the media from 1×10^{-4} M to 2×10^{-4} M has little effect on cell proliferation (figures 1a and b) but reduces water-soluble metabolites by 50% or more (table 1).

Because we saw a considerable reduction in the incorporation of myo-[2-^{3}H] inositol into water-soluble metabolites (end-product of phosphatidylinositol hydrolysis), we suggest that γ-hexachlorocyclohexane acts, as has been shown previously (Hokin and Brown, 1969; Fisher and Mueller, 1971; Ristow et al., 1973; Hoffman et al., 1980; Vu and Zelenka, 1983; Zelenka and Vu, 1984) to block phosphatidylinositol synthesis. In addition, since we (M. Koohmaraie and J. E. Schollmeyer, unpublished results) and others (Hoffman et al., 1980) could not override the inhibitory effect of γ-hexachlorocyclohexane by the addition of excess inositol, we conclude that γ-hexachlorocyclohexane could not be functioning as a myo-inositol analogue as proposed by Hoffman et al. (1980). The ability of these cells to continue to proliferate for the first few hours in the presence of γ-hexachlorocyclohexane suggests that there are sufficient stores of inositol lipids to support that proliferation.

These results, when considered collectively with similar results with other cell types (Zelenka, 1980; Ishima et al., 1982), lend substantial support to the hypothesis that cell proliferation and phosphatidylinositol turnover are linked. The precise manner in which these two events are interrelated is not yet understood. However, the fact that various cell types, when presented with mitogenic stimuli, display elevated phosphatidylinositol hydrolysis (Ristowet al., 1973; Hoffman et al., 1974; Habenicht et al., 1981; Sawyer and Cohen, 1981; Berridge et al., 1984) lends support to the hypothesis that the two events are linked. Because of our interest in control of muscle cell proliferation, we are currently investigating whether the mitogenic response of muscle cells depends, in part, on the promotion of phosphatidylinositol turnover, and whether we can stimulate muscle cell proliferation by enhancing phosphatidylinositol turnover by means other than mitogens.

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