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THE INFLUENCE OF CERTAIN HORMONES AND CHEMICALS ON MAMMALIAN PIGMENT CELLS*

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The role of hormones and chemicals on melanogenesis in mammalian pigmentation has been speculated on the basis of *in vivo* observations but so far has not been shown at the cellular level. The use of a mammalian pigment cell strain grown in cultures appears to be an ideal approach for such studies.

MATERIALS AND METHODS

Cell strains both pigmented (HFH-14 and HFH-18) and non-pigmented (HFH-18NP) derived from B16 transplantable mouse melanomas and established in our laboratory were employed in this study (1). The hormones and chemicals which were used are listed in Table I.

The experiments were designed as follows:

A. Effects of agents which are known to influence melanin pigmentation.

I. Short Term Effects:—Agents incorporated in the culture medium at the initiation of the culture.

The melanoma cells were grown on coverglasses in the Yerganian culture tubes (2). Controls and test cultures were fixed and stained at the end of 5–7 days of incubation and examined microscopically for extent of growth, relative numbers of pigmented and non-pigmented cells, and other cellular changes.

II. Long Term Effects:—Agents incorporated in the initial cultures and in subsequent subcultures.

Cells were grown in 2 ounce prescription bottles. 300,000 cells were inoculated into each culture bottle. In 7–9 days, compact sheets of cells were formed on the glass bottom at which time the cultures were ready for subcultures.

For subcultures, 0.01% trypsin in Hank's balanced salt solution was used to disperse the cells. When a cell suspension was obtained, a cell count was done to determine the extent of growth. 300,000 cells were then inoculated to another 2 ounce bottle to start the subculture.

For this study, at least 3 subcultures or more were done for each test agent. For each subculture cytological examinations of stained as well as unstained slides were made.

III. Immediate Direct Effects:—Cinemicrographic study.

Cells were grown on coverglasses in Yerganian culture tubes or in petri dishes.

At the end of 5–7 days of incubation, aggregates of pigmented cells were present overlying the non-pigmented cells in sheet formation on the glass surface. At this time, the coverglass with the cells in monolayers was transferred and mounted in a perfusion chamber devised specially to accommodate the rectangular coverglasses we used. Movie sequences of these cells prior to treatment were made as controls, followed by sequences immediately following treatment, and overnight after treatment. In some, the medium containing the test agent was removed and replaced by normal growth medium; sequences were made following this change of medium to see whether the cells could recover from the effect of the treatment.

All cultures were fixed at the end of movie taking, mounted on glass slides, and examined for evidence of cellular damage. The results were analyzed and compared between the treated and untreated cultures.

B. Effect of colchicine on pigment cell formation.

The pigmented strains were used in this study. The cells were grown on coverglasses in Yerganian culture tubes for 3–5 days until reasonably good aggregates of cells in monolayers were seen. At this time, growth medium containing 0.3 μg/ml of colchicine (Colcemid, Ciba) was introduced to the test culture. Also, the medium was changed in the controls, but without the incorporation of colchicine. The treatment schedule is outlined in Table II.

At the end of the experiments, all cultures with appropriate controls were fixed and stained for cytological examinations.

RESULTS

The results are summarized in Table II, III, and IV.

In general, it appeared that the pigmented cells were more susceptible to the effect of chemicals than the non-pigmented cells in the same culture. Following trypsinization, most of the pigment-containing cells disappeared; only a few were seen in the fresh subculture in the first few days.

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TABLE I

Agents Used in This Study

ACTH (Lerner)
α-MSH (Lerner)
Copper sulfate (Mallinckrodt, Lot #4844)
DOPA (Dow, Lot #1)
L-Tyrosine (Matheson, Coleman & Bell, Lot #344033)
Ascorbic acid (Parke-Davis, Stock 3-193-10)
Hydroquinone (White Laboratories, WL-3030)
Melatonin (Upjohn)
Colchicine (Colcemid, Ciba)

TABLE II

The Effect of Colchicine on Pigment Cell Formation

Colcemid 0.3 μg/ml	Results as compared with untreated controls
24 hours	Decrease in numbers of cells. Increase percentage of large epithelial-like cells.
48 hours	Large epithelial-like cells. Increase in the number and size of pigmented cells.
72 hours	Similar to above.
Normal medium after colcemid treatment for 48 hours	
24 hours	Large numbers of large cells, pigmented and nonpigmented.
48 hours	Similar to above. Size of cells slightly reduced.
72 hours	Further reduction in cell size, but still larger than untreated controls. More pigmented cells than controls.

ACTH, MSH, and copper sulfate appeared to have some stimulating effect on the pigment cells. However, the difference was not of sufficient magnitude to be considered significant.

Tyrosine, at a concentration of 0.08 mg/ml, even though it did not seem to affect the cells immediately, did have some delayed effect on the cells as shown by the failure of those treated cells to survive a second subculture. Also, at a concentration of 0.06 mg/ml, growth was somewhat inhibited. However, the cells did survive several subcultures. In each subculture there was no increase in the number of pigmented cells in the treated cultures; in fact, often the number of the pigmented cells was actually less than in the untreated controls.

Dopa at a concentration of 100 μg/ml had no effect on the cells. The cells grew well in the dopa-containing medium and survived several subcultures without evidence of apparent damage.

Melatonin and ascorbic acid at high concentrations were definitely detrimental to the viability of these cells. On initial treatment, at lower concentrations, they inhibited growth to some extent and prevented subsequent successful subcultures. Neither showed evidence of "lightening" effect on the pigmented cells.

On one occasion melatonin at 100 μg/ml stopped the granule movement in the pigmented cells but had no effect on the nonpigmented cells. At 200 μg/ml, it caused slowing of granule movement in both the pigmented and nonpigmented cells. This effect was reversible; the granules resumed their normal movement when fresh normal medium without melatonin was introduced to the chamber. A concentration of 300 μg/ml produced complete irreversible damage to these cells.

Ascorbic acid at concentrations of 0.3 and 0.4 mg/ml had no effect on the cells which were established as monolayers on glass in our time-lapse movie study.

Hydroquinone inhibited growth of cells *in vitro*, particularly the pigmented cells, at a concentration of 0.625 μg/ml. When compared with control cultures, the treated cultures grew much slower, less extensively, and with only a few pigment-containing cells in contrast to the controls which had many aggregates of pigmented cells. When these treated cells were ready for subculture, the subcultures were also slow in growing and with a definite loss of pigmented cells. At concentrations of 1.25 and 2.5 μg/ml, hydroquinone inhibited growth completely when it was incorporated in the culture at the beginning of cultivation.

When the culture was well established in normal medium such as that used in the movie study, hydroquinone had no effect on the granule movement of the cells at 1 μg/ml, but it induced cessation of granule movement at 2.5 μg/ml. This effect was found to be reversible at certain occasions. In addition to its effect on the granule movement, hydroquinone also induced vacuolization of the cytoplasm and clumping of melanin granules. At 20–40 μg/ml, hydroquinone abruptly stopped granule move-

TABLE III

The Effect of Certain Hormones and Chemicals on Pigment Cells in Vitro

Agent	Cell Line	One Single Subculture	Repeated Subcultures
ACTH			
0.2 μg/ml	Pigmented	Like control	3–4 subcultures. Like control. ? more mitoses. Slightly larger pigmented cells.
	Nonpigmented	Like control	5 subcultures. Like control
α-MSH			
0.2 μg/ml	Pigmented	Like control	2–4 subcultures. Slightly more, larger, and darker pigmented cells.
	Nonpigmented	Like control	4 subcultures. Control—0 pigmented cell. Treated 2–5 pigmented cells
Copper sulfate			
1 μg/ml	Pigmented	Like control	2–4 subcultures. Slightly more and larger pigmented cells
DOPA			
100 μg/ml	Pigmented	Like control	2–4 subcultures. Like control
Tyrosine			
0.08 mg/ml	Pigmented	Less growth than control	Poor growth. Did not survive second subculture.
0.06 mg/ml	Pigmented	Slightly less growth than control	2 subcultures. Slightly less growth. No increase of pigmented cells
Ascorbic Acid			
0.025 mg/ml	Pigmented	Complete degeneration	
0.0125 mg/ml	Pigmented	Growth slightly less. More large epithelial-like cells with vacuolated cytoplasm	Growth less than control. Did not survive third subculture
Hydroquinone			
2.5 μg/ml	Pigmented	Complete degeneration	
1.25 μg/ml	Pigmented	Almost complete degeneration	
0.625 μg/ml	Pigmented	Growth slightly less than control. Almost no pigmented cells in contrast to controls which have many aggregates of pigmented cells	1 subculture*. Growth similar to controls, but definitely less pigmented cells
Melatonin			
10 μg/ml	Pigmented	Like control	4 subcultures like control
25 μg/ml		Much less growth than control	Did not survive subculture
100 μg/ml		Almost complete degeneration	

TABLE IV

Time-lapse Cinephase Study on the Direct Effect of Certain Hormones and Chemicals on the Pigment Cells

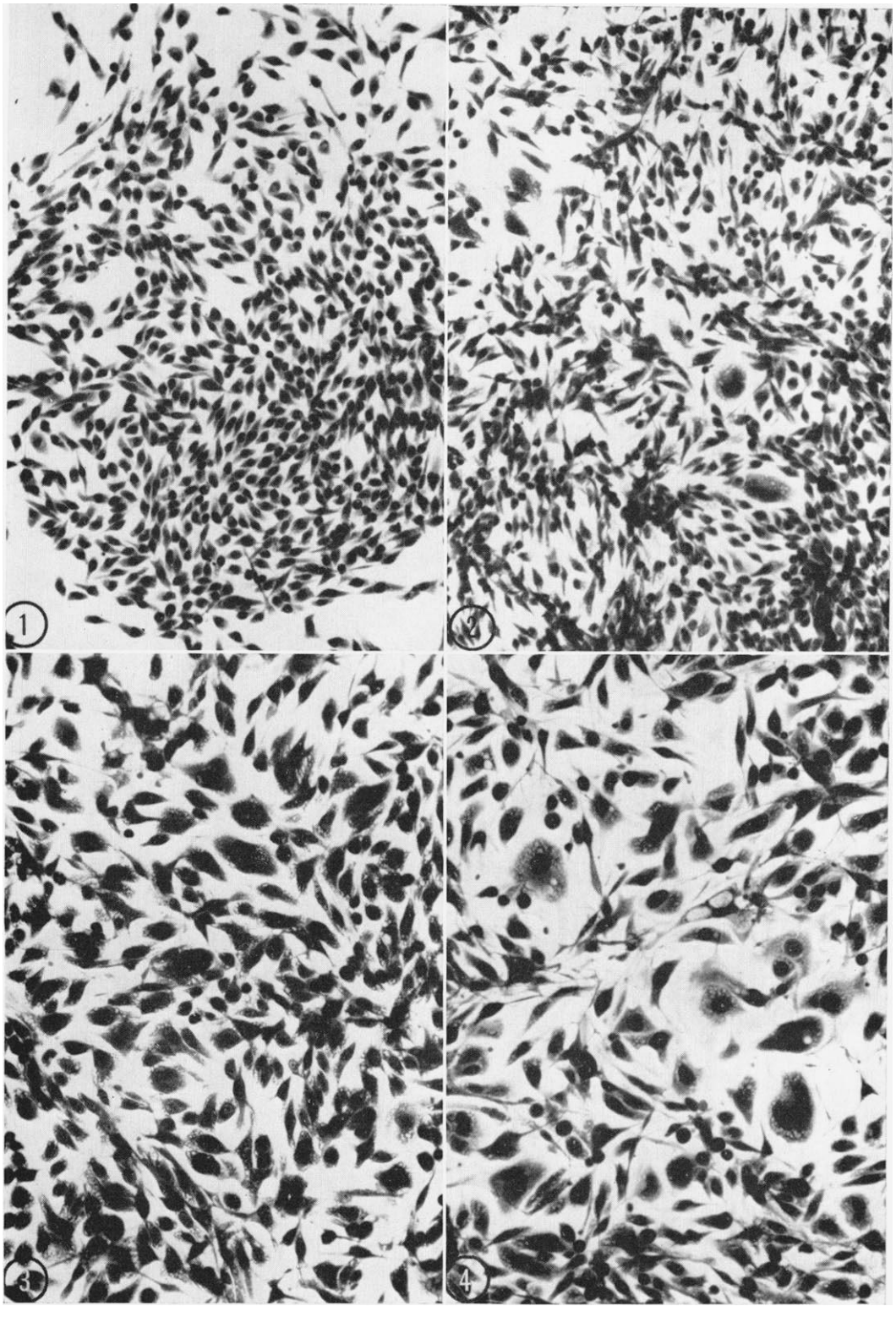
Agent/Concentration	On Melanized Cells	On Non-Melanized Cells	Remarks
α-MSH 3 μg/ml	No effect. Movement of pigment granules unchanged overnight	No effect	
4 μg/ml	No apparent change except processes slightly longer and darker	No effect	
Tyrosine 0.08 mg/ml	No effect	No effect	
Ascorbic Acid			
0.3 mg/ml	No effect	No effect	
0.4 mg/ml	No effect	No effect	
Hydroquinone			
1 μg/ml	No effect	No effect	
2.5 μg/ml	Granule movement slowly stopped	Granule movement slowly stopped	
20-40 μg/ml	Granule movement abruptly stopped	Granule movement abruptly stopped	Cell outline preserved. Not reversible
Melatonin			
100 μg/ml	Granule movement stopped on one occasion. No effect on others	No effect	
200 μg/ml	Slowing of granule movement	Slowing of granule movement	Reversible by introduction of normal medium
300 μg/ml	Degeneration	Degeneration	

ment in all cells in the same manner as a fixative affects cells.

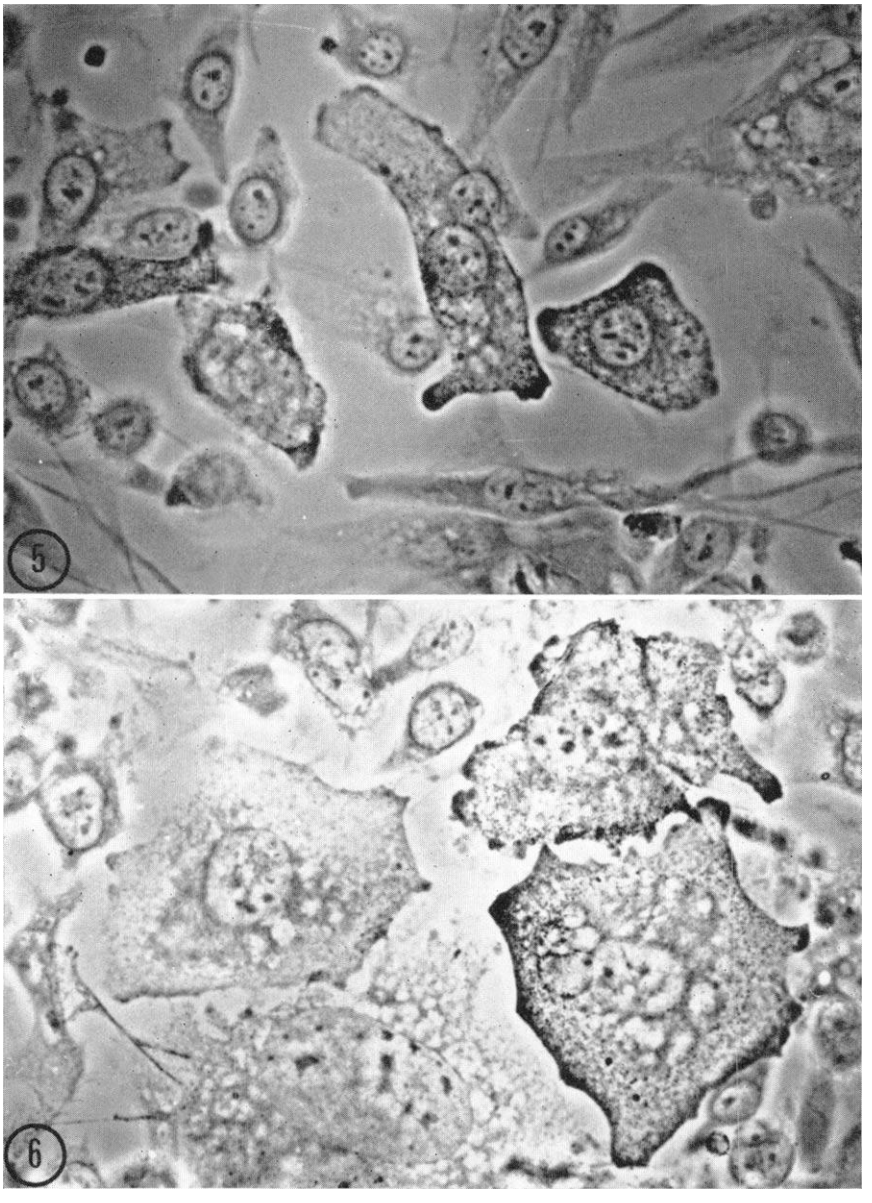
With colchicine treatment, the cells increased greatly in size and contained intracytoplasmic vacuoles (Figs. 1–4). These changes were partially reversible when normal growth medium was introduced into the culture system and when colchicine-containing medium was removed from the culture system. Figures 5 and 6 illustrate the difference in cell sizes in the untreated controls and colchicine treated cells. Normally when the incubation time was prolonged, these large epithelial cells were found in the untreated controls. However, they were not seen until the cultures were kept for several weeks. When treated with colchicine, these large cells appeared within 48 hours following treatment. Also, the number of pigmented cells in the treated cultures was always much more than that in the untreated controls.

DISCUSSION

The results obtained from those experiments can be considered, at best, suggestive but not conclusive. Due to the selective destructive effect of trypsin on the melanized cells, the cell count obtained after trypsinization during each subculture represents essentially the non-pigmented population of the growth. Similarly, microscopic examinations of specimens after each subculture again may not represent the true picture due to the elimination of most of the pigment-containing cells. The lack of a definite increase in the numbers of the pigmented cells following incorporation of the known melanocyte-stimulating agents such as ACTH, MSH, copper, etc. in the culture system may in part be the result of partial destruction of the pigmented cells in these experiments. It is necessary to devise a more reliable quantitative method in order to measure the



FIGS. 1–4. Cell strain HFH-18. May-Gruenwald-Giemsa stain
FIGS. 1–2. Untreated controls
FIGS. 3–4. Colcemid treatment for 48 hours, normal medium 72 hours. Note the large cells with vacuolated cytoplasm, in contrast to the relatively uniform small cells in the untreated controls.



FIGS. 5–6. Cell strain HFH-18. Phase contrast microscopy
FIG. 5. Untreated control
FIG. 6. Colcemid treatment for 48 hours. Note the large pigmented and nonpigmented epithelial-like cells.

difference more accurately; at present such studies are in progress in our laboratory.

With the use of cinemicrographic recordings, the direct effect of certain agents on the cells may be observed. These observations are reproducible and can give an accurate account of the direct effect of that particular agent on these cells shown on the recordings under the specified experimental conditions. The disadvantage is that only a limited number of the cells may be examined with this technic. Its special advantage is well illustrated in the case of hydroquinone, which at a certain concentration, immediately kills the cells like a fixative

does without changing the cell outline and internal structures. Without these recordings, one is apt to be misled to the erroneous conclusion that the cells were unaffected by such treatment. With the recordings, the cessation of granule movement in the cells following such treatment is a good indication of cellular damage. Other signs of cell injury, such as cytoplasmic vacuolization, bubbling, retraction of cellular sheets or rounding up of individual cells may also be shown. Whether these changes are reversible or not, may be determined by recordings following the removal of the agents with replacement of fresh growth medium.

Under the conditions of our experiments, it is concluded that the responses of mouse pigment cells to some of the hormones, *i.e.*, MSH and melatonin, differ from the known effects of those agents on the fish or amphibian melanophores (3, 4). In the time-lapse recordings, it was clearly shown that there was no rapid dispersion of melanin granules in these cells with MSH treatment, nor aggregation of granules with melatonin treatment. ACTH and MSH have been shown to induce pigment cell formation in goldfish tailfin cultures (5); the incorporation of these agents in the culture medium failed to show a definite increase in the number of the pigmented cells, even though with prolonged treatment, ACTH and α-MSH appeared at times to have some melanin stimulating effect on cells which were potentially capable of pigment formation.

Hydroquinone in appropriate concentrations, was found to have selective injurious effect on the melanotic cells. Similar effect on goldfish pigment cells has been reported by Chavin (6).

The enhancement of pigment cell formation in cultures following colchicine treatment is interesting. It appears that inhibition of mitotic activity of the cells favors melanin pigment formation, which presumably is a feature of pigment cell differentiation. This is not surprising, since it is known that often proliferation and differentiation occur at the expense of each other. Other mitotic inhibitors will be tried to determine whether the effect on these cells is the same as that observed with colchicine.

Tyrosine treatment did not appear to injure these cells drastically, but it did have some delayed effect on the cells. At a concentration of 0.08 mg/ml, there was less growth as compared with untreated controls, and the treated cells failed to survive a second subculture. At a concentration 0.06 mg/ml, growth was inhibited to some extent, but the cells did survive several subcultures. However, in each subculture, the number of pigmented cells was less than the untreated cultures. The effect of tyrosine on the pigment cell strains was rather unexpected. In contrast to the reported pigment-enhancing effect of this amino acid in the organ culture of eye tissues from mice with pink-eyed dilution genes (7), its incorporation in the culture medium was found to decrease the number of pigmented cells in our cultures. At present, we can offer no satisfactory explanation for this.

SUMMARY

The influence of certain chemicals and hormones on B16 mouse melanoma cell strains were studied. The immediate direct effect of these agents on these cells were compared by the use of cinemicrographic recordings; the short term and long term effects were studied by treating the cells either for one single subculture or for repeated subcultures.

Under the conditions of the experiments, it was concluded that the responses of mouse pigment cells to some of the hormones, *i.e.*, MSH and melatonin, differed from the known effects of those agents on the fish or amphibian melanophores. Hydroquinone in appropriate concentrations selectively inhibited the melanotic cells; colchicine treatment appeared to stimulate pigment cell formation.

The significance and possible explanation of these findings are discussed.

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