CONTROL OF PIGMENT PRODUCTION IN MOUSE MELANOMA CELLS IN VITRO

Evocation and Maintenance

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ABSTRACT

A clonally derived amelanotic melanoma cell line repeatedly has been forced to produce pigment by the inhibitor of DNA synthesis, I-β-D-arabinofuranosylcytosine (ara-C) at sublethal levels. One ara-C–derived melanotic line has been cloned, and has continued to produce pigment for 2 years on normal medium. The inhibitor is most effective when administered to synchronized cells in four pulses on successive days at 1.8×10^{-5} M during the S phase of the cell cycle. Colcemid at a sublethal concentration, and growth on medium solidified with agar also evoked pigment production in this line, but a large number of other inhibitors of biosynthetic processes did not, under the conditions tested. The melanotic lines are active producers of tyrosinase (DOPA oxidase), whereas the amelanotic line produces an inhibitor of tyrosinase activity. Both enzyme and inhibitor are labile at 4° C and −20° C, and decay of the inhibitor in homogenates of amelanotic cells reveals a low level of residual DOPA oxidase activity. The mean population doubling time of a cloned melanotic line is 23 hr, and that of a cloned amelanotic line 16.5 hr. A similar decrease in rate of growth is found in other melanotic lines and is believed to be a significant factor in maintaining this differentiated function. Rapid growth may be related to the production of an inhibitor by the amelanotic cells.

INTRODUCTION

Study of the mechanisms of differentiation is facilitated in a cell culture system permitting precise control of environmental conditions. Many problems have been encountered in maintaining specific differentiated function in vitro, since most cells in long term culture revert to a dedifferentiated state maintaining themselves, but not producing their specialized macromolecules.

The investigation reported here describes a model system with such a "dedifferentiated" cell line, an amelanotic melanoma growing in vitro. This line was repeatedly forced into producing melanin by the use of an inhibitor of DNA synthesis, a mitotic poison, and growth on medium solidified with agar. The growth rate of the melanin-producing cells is lower than that of the amelanotic cells. The activities of DOPA oxidase and an inhibitor of the enzyme were compared in melanotic and amelanotic cells in an effort to relate the observed phenomena to biochemical parameters.

MATERIALS AND METHODS

Cell Culture

The cell line used, originally developed by Hu (1), was derived from a mouse melanoma (B16). It was completely amelanotic, giving negative results

when tested for melanin (2, 3) or tyrosinase activity (4). Cells were maintained in monolayer culture in Eagle's minimal medium (MEM) (5) with 10% fetal calf serum, 10^2 units/ml penicillin, 10^2 μg/ml streptomycin (all from Grand Island Biological Company, Grand Island, N.Y.), 2.5 μg/ml fungizone (E. R. Squibb & Sons, New York) and 50 μg/ml chlortetracycline HCL (Lederle Laboratories, Pearl River, N. Y.). They were grown in plastic flasks or petri dishes at 37° C in a humidified atmosphere of 5% CO_2 in air. When melanotic lines were developed, subcultures were prepared by scraping selected pigmented areas. Amelanotic lines were subcultured by trypsinization (0.25% trypsin, Grand Island Biological Company). Chromosome preparations were made and studied as previously described (6).

Cloning

Cells were cloned by Puck's technic (7). Preliminary experiments were done on the uncloned amelanotic line. This line was then cloned. All clones were completely unpigmented and one such clone, B78, was used in all subsequent experiments, both on agar and with inhibitors, and in the enzyme studies. Three of the pigmented lines that were derived were also cloned, and all enzyme studies were done on cloned line N87.

Growth of Cells on Agar

Medium (5 ml) containing 1% purified agar (Difco Laboratories, Inc., Detroit) was allowed to harden in 60 mm petri dishes. Cells were trypsinized, centrifuged (800 rpm), and the supernatant was aspirated. Thick cell suspensions were dotted on the agar surface and incubated.

Treatment with Inhibitors

The inhibitors tested are listed in Table I. All were tested over a wide range of concentrations in unsynchronized populations to determine their lethal and sublethal concentrations. The effect of a narrow range of sublethal concentrations of each inhibitor on growth and pigment production was determined. Cells were treated one day after plating at a cell density of 2 to 4 × 10^4/ml in 5 ml of medium. Cells in at least three circumscribed areas in each replicate flask were counted daily and the entire flask was scanned microscopically for signs of pigment in individual cells.

The only inhibitors tested in parasynchronous (partially synchronized) populations were 1-β-D-arabinofuranosylcytosine (ara-C) and Colcemid (Ciba Pharmaceutical Company, Summit, N.J.). Ara-C was added at 2 μg/ml and at 5 μg/ml for 4 successive days. One group of cells received ara-C for 2 hr each day during the S period, on Day 0 starting 6.5 hr after initiation of the experiment, and at the same time on Days 1, 2, and 3. In another group, ara-C was added for 16.5 hr on Day 0 beginning 6.5 hr after initiation of the experiment, and for 2 hr on Days 1, 2, and 3. The 16.5 hr included portions of two S periods. Colcemid (0.06 μg/ml) was used both continuously for 24, 48, and 72 hr and for similarly pulsed treatment.

Parasynchronization (Partial Synchronization)

Two methods were used: collection of mitotic cells without any inhibitor (8, 9) and after 3 to 3.5 hr of treatment with 0.06 μg/ml of Colcemid (10). With either method cells were first brought into active division in 32 oz prescription bottles by trypsinizing every second or third day, and splitting each culture into two or three parts. When mitotic cells were collected without Colcemid, the bottles were shaken and the cells were rinsed off the surface of the actively dividing monolayers with MEM, collected, and rinsed in a minimal amount of medium so that the final cell

TABLE I

Inhibitors Tested

Inhibitor	Type of synthesis inhibited	Concentrations tested
Actinomycin D	RNA	0.01 μg/ml—1 μg/ml
Puromycin	protein	0.1 μg/ml—1 μg/ml
Cycloheximide	protein	10^{-8} M—10^{-4} M
p-Fluorophenylalanine	protein	0.2 μg/ml—40 μg/ml
5-Fluorodeoxyuridine (FUdR)	DNA	10^{-8} M—10^{-5} M
5-Bromodeoxyuridine (BUdR)	DNA	10^{-8} M—5 × 10^{-4} M
5-Iododeoxyuridine (IUdR)	DNA	4 × 10^{-9} M—4 × 10^{-4} M
Amethopterin	DNA	4 × 10^{-7} M—4 × 10^{-6} M
Edeine	DNA	0.01 μg/ml—10 μg/ml
Mitomycin C	DNA	0.01 μg/ml—1 μg/ml
1-β-D-Arabinofuranosylcytosine (ara-C)	DNA	1 μg/ml—10 μg/ml
Colcemid	mitotic poison	0.06 μg/ml

density would be 2×10^4/ml. The cell suspension was concentrated when necessary by centrifugation at 800 rpm for 5 min and resuspended. Falcon plastic flasks (30 ml) were inoculated with 5 ml of this cell suspension. When Colcemid was used, it was added as described, after which the mitotic cells were collected, rinsed, centrifuged, and resuspended. Synchronization was partial. In different experiments, mitotic cells comprised 75 to 90% of the cell population initially plated in the flasks (Fig. 2). All experimental flasks were set up in triplicate or duplicate with appropriate controls.

In the cell synchrony experiments, the percentage of mitotic cells was scored at intervals ranging from 30 to 90 min by staining aliquots with aceto-orcein. At zero time, aliquots were seeded into replicate plastic flasks marked with nine numbered squares. Cells in each square were counted at 1.5 hr intervals with the inverted microscope, and the changes in their number were thus scored.

Radioautography

Cells grown on coverslips were labeled with tritiated thymidine (0.5 μc/ml, 6.7 c/mmole, New England Nuclear Corp. Boston, Mass.) for 20 min at specified times (Table II and Fig. 2). They were then rinsed with phosphate-buffered saline (PBS), fixed in acetic acid–methanol (1:3), dried, mounted face-up on slides, and stained with aceto-orcein. The slides were dipped in Eastman Kodak emulsion Type NTB2 (Eastman Kodak Co., Rochester, N.Y.), developed after 9 to 10 days at 4° C in the dark, and the percentage of nuclei taking up the labeled thymidine was determined. After fixation, some cover slips were extracted with ice-cold 5% trichloroacetic acid for 15 min, rinsed with distilled water, dried, and dipped in emulsion.

Preparation of Homogenates and Enzyme Assays

Monolayers were washed with PBS, trypsinized, and centrifuged at 800 rpm for 5 min. All subsequent operations, until the enzyme assay, were carried out at 0 to 4° C. The pellet was washed 3 times with cold PBS, and then homogenized with a hand homogenizer with Teflon pestle for 5 min in sodium phosphate buffer (0.1 M, pH 6.8) with final ratio of pellet to buffer 1:20 (v/v). To obtain a soluble fraction the homogenate was centrifuged at 10,000 *g* at 0° C for 10 min to remove melanin granules, nuclei, mitochondria, etc. (11). The supernatant solution was decanted and respun, and the pellet was again extracted with buffer and centrifuged at 10,000 *g* for 10 min. The supernatants were pooled and the sediments were discarded. This resulted in a protein concentration of about 2 mg/ml. The soluble fraction obtained under these defined conditions contained maximal enzyme activity.

DOPA oxidase activity in the combined supernatant solutions was determined with L-DOPA (3,4 dihydroxyphenyl-L-alanine) (Nutritional Biochemicals Corporation, Cleveland, Ohio) as substrate. A Klett-Summerson photoelectric colorimeter, Klett microtubes and No. 42 blue filter were used (4). The standard reaction mixture consisted of between 0.05 and 0.2 ml of the cell extract (enzyme), 0.5 ml L-DOPA (2 mg/ml in 0.1 M sodium phosphate buffer (pH 6.8)) and buffer to a final volume of 3 ml. All mixing was done at 0 to 4° C and incubation was done at 37° C. Appropriate autoxidation controls for DOPA were run in triplicate and enzyme tests in duplicate. The soluble fraction of amelanotic cells, obtained as described, was tested for DOPA oxidase activity alone and in combination with 0.1 ml of active enzyme (from melanotic cells) in the proportion of 1:1. The preliminary experiments which were done, and in which the amount of amelanotic extract varied, indicated that the inhibitory effect was proportional to the amount of amelanotic extract. Protein concentration was determined by the method of Lowry et al. (12) with crystalline bovine albumin (Armour Pharmaceutical Company, Inc., Chicago, Ill.) as a standard.

RESULTS

Determination of Amelanotic Nature of Cells

In its original form, the cell line had no visible pigment (Fig. 1 *a*), did not stain for melanin by the ferrous ion (13) or Nile blue sulfate (14) techniques, and fresh homogenates did not contain any detectable tyrosinase activity when DOPA was used as substrate in enzymatic assay (4). When 2 to 4 $\times 10^5$ cells were injected into ten C57BL/6 mice, all the mice developed partially or fully melanotic tumors. This proved that these cells retained the capacity to produce pigment, although this capacity was not being expressed in vitro.

Growth on Agar

Cells grown on medium solidified with agar formed colonies with melanotic sectors. When these melanotic sectors were explanted into liquid medium, a mixture of pigmented and unpigmented cells grew out into a monolayer. The pigmented cells usually were overgrown quickly by unpigmented cells despite rigorous selection by scraping of pigmented cells for subculture. One pigmented line was, however, developed by such selection and then cloned. Fig. 1 *b* shows cells from the

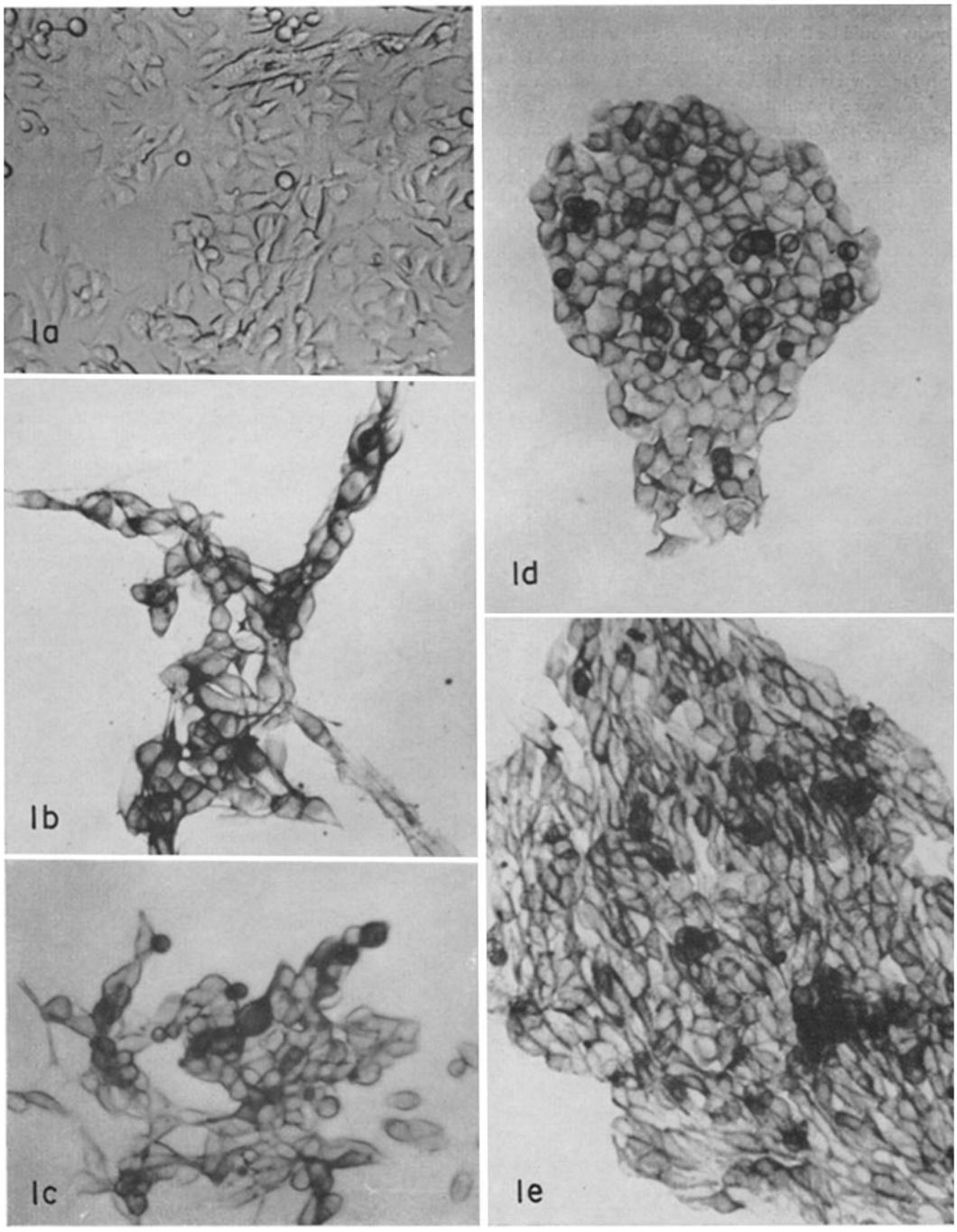

FIGURE 1 Living melanoma cells growing in culture dishes viewed with Wild inverted microscope. Bright field, × 125. Note that each cell line has a characteristic growth pattern and that all lines exhibit lack of contact inhibition. *1 a*, Cloned amelanotic cell line B78; *1 b*, Clone of cell line derived from amelanotic cells which pigmented while growing on agar. Photographed after maintenance on normal medium for over 1.5 yr; *1 c*, Cell line pigmented after a 72 hr treatment with 0.06 μg/ml Colcemid and photographed after 7 wk of continuous culture on normal medium; *1 d*, Clone N87 of cell line pigmented during 10 wk of maintenance on sublethal concentrations (0.5 μg/ml-2 μg/ml) of ara-C. Photographed after more than 2 yr on normal medium; *1 e*, Cells pigmented after 4 days of pulsed ara-C treatment during S period of a parasynchronously growing cell population. (See Table III, experiment 2). Photographed after 6 wk of continuous culture on normal medium.

pigmented clone after maintenance for 1.5 years in vitro. In order to determine what factors might permit evocation of pigment in unpigmented cells grown on agar, a number of experiments were performed. To test whether aggregation was the inducing factor, unpigmented cells were grown: (a) in extremely dense culture (15), (b) as aggregates in roller tubes (16), (c) with conditioned medium (17), and (d) on irradiated feeder layers. No pigment appeared under any of these conditions. Nor did pigment appear when the growth rate was decreased when cells were grown at 25, 30 and 32°C, or when serum was removed from the medium (18–20).

Use of Metabolic Inhibitors

The inhibitors (Table I) were tested at sublethal levels to prevent rapid mitosis in order to examine the hypothesis that rapid division time in vitro and the synthesis of products needed for specific differentiated function are mutually exclusive. Of the inhibitors only two, ara-C and Colcemid, evoked pigment. Colcemid at 0.06 μg/ml for 48 and 72 hr evoked some pigment. Usually, unpigmented cells outgrew the pigmented ones despite repeated selective scraping of pigmented areas. With rigorous selection, two continuously growing pigmented cell lines were derived from cells treated with Colcemid for 72 hr (Fig. 1 *c*) and frozen in liquid nitrogen.

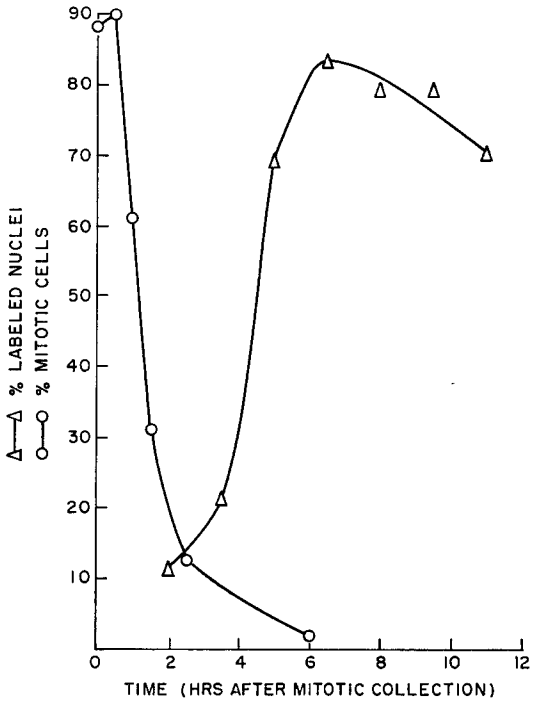

FIGURE 2 Curves showing mitotic index (○——○) and percentage of cells in the S phase of the cell cycle (△——△) in cells synchronized by harvest and reincubation of untreated mitotic cells. Scoring is described under Materials and Methods.

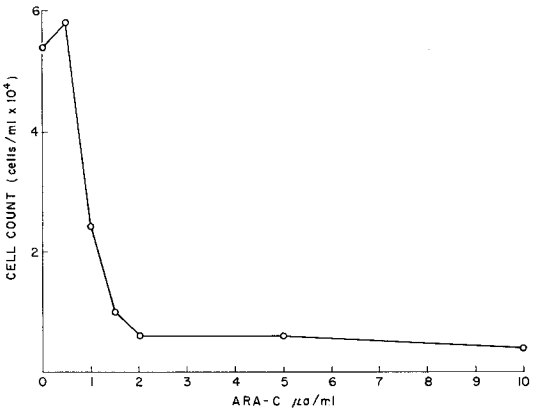

FIGURE 3 Survival of amelanotic cells after 48 hr in the presence of different concentrations of ara-C. Ara-C at 1 μg/ml inhibits the rate of growth by 50% in 48 hr. Cells were grown from an inoculum of 2×10^4 cells/ml in 5 ml of medium in Falcon flasks.

Ara-C is a specific inhibitor of DNA synthesis, but does not affect RNA or protein synthesis for at least 24 hr after completely and immediately shutting off DNA synthesis when used continuously in various cell lines at a concentration of 3.6×10^{-5} M (10 μg/ml) (21–24). Ara-C inhibits cell reproduction in many different kinds of organisms and in mammalian cells in culture. In these amelanotic cells which grow logarithmically, 1 μg/ml inhibits cell multiplication by 50% in 48 hr (Fig. 3), and 5 μg/ml effectively inhibits DNA synthesis (Table II). After 24 hr of treatment at 2 μg/ml to 10 μg/ml the cells show the marked increase in cell size which is characteristic of cells inhibited in DNA synthesis that continue RNA and protein syntheses (23, 24).

Ara-C was used at many concentrations and different lengths of exposure and a variety of combinations proved successful in evoking pigment production. The first experiments were performed on the uncloned line. After cells in 54 flasks had pigmented, subsequent experiments were performed on cloned line B78. In early experiments, all done with unsynchronized cells, 1 μg/ml and

2 μg/ml were used continuously, then 2 μg/ml and 5 μg/ml were used for several consecutive days, and then the same concentrations in short pulses over a few days. Of the 116 flasks in which pigment developed in several such experiments, 35 of them had cells which were maintained with pigment for at least 6 weeks. From this group, 14 melanotic lines were developed and maintained for 3–5 months in normal medium. Three of these lines were frozen in liquid nitrogen. One was cloned, designated N87, and is still being maintained in a highly pigmented state in normal medium more than 2 years after it was derived by ara-C treatment of unpigmented cells (Fig. 1 *d*). When repeated trials with this procedure gave consistently successful results, experiments were designed to determine whether the cells were more vulnerable during the S stage of the cell cycle.

TABLE II

Radioautography of Ara-C-Treated Synchronized Cells

	Hours after mitotic collection		Percentage of cells in S phase			
	Before ara-C treatment*	At end of ara-C treatment period‡	Before ara-C treatment*	At end of ara-C treatment period‡		
				Heavily labeled§	25–50 grains/cell	5–25 grains/cell
Exp. 1	6.5	8.5	83	0	0	8
	29.5	31.5	66	0	0	28
	53	55.0	67	0	0	40
	77	79	69	0	0	34
Exp. 2	6.5	22.5	83	0	0	30
	29.5	31.5	72	0	17	36
	53	55	38	0	13	9
	77	79	56	0	27	22

* ^{3}H-TdR was added to untreated cells during the first 20 min of period during which other cells were treated with ara-C, at 5 μg/ml.

‡ ^{3}H-TdR was added for 20 min to treated cells after exposure to ara-C.

§ Heavily labeled cells contained over 100 grains/cell.

Cell Synchrony

The number of cells in each flask that became pigmented was far greater when parasynchronized cells were treated with ara-C (2 and 5 μg/ml) for 4 successive days at timed intervals than when unsynchronized cells were similarly treated. The percentage of flasks containing pigmented cells remained essentially unchanged (Table III). In early synchronization experiments, mitotic cells were accumulated by pretreatment with Colcemid before collection. When it was found that 0.06 μg/ml Colcemid used alone could evoke some pigment, later synchronizations omitted Colcemid. Thus the effect of treatment with ara-C alone during S in evoking pigment production was verified. In the unsynchronized amelanotic cells in logarithmic growth the mitotic index was 4.6%; in parasynchronized cells the mean mitotic index was 82%.

Radioautography with thymidine-H^3 was used to determine the S period of untreated cells (Fig. 2) and of cells synchronized by mitotic collection without Colcemid, and treated with ara-C. Table II shows the results of such autoradiography with cells treated with ara-C (5 μg/ml) for 2 hr on 4 consecutive days (experiment 1), and for 16.5 hr the first day, thus proceeding through parts of two S periods, and 2 hr on 3 subsequent days (experiment 2). It should be noted that although

TABLE III

*Ara-C-Induced Pigmentation in Amelanotic Cells**

	Asynchronous cells			Synchronous cells short pulse	
	Long treatment	2–3 days treatment	Short pulse	Colcemid induced	Mitotic collection
Number of successful trials	54	18	44	75	12
Percentage successful trials	64	100	96	97	100
Day of first appearance of pigment	12–45 (mode 23)	6–7	6–9	6	3–6

* All experiments, except for those in the first column, were performed on a cloned amelanotic cell line. The long-term treatments were performed on the uncloned amelanotic line.

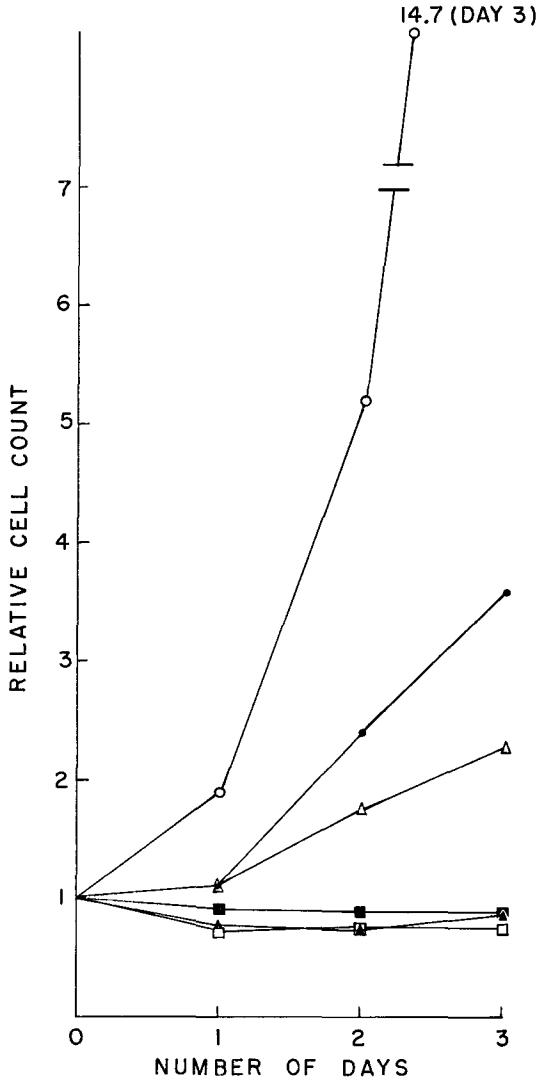

FIGURE 4 Growth curves of cell populations of B78 amelanotic line synchronized by mitotic collection after 3 hr of treatment with 0.06 μg/ml Colcemid. ○——○, Control 1, no Colcemid used; ●——●, Control 2, with Colcemid; △——△, ara-C 5 μg/ml Days 0–3, for 2 hr daily; ▲——▲, ara-C 5 μg/ml Day 0 for 16.5 hr, Days 1, 2, 3 2 hr daily; □——□, ara-C 2 μg/ml Day 0 16.5 hr, Days 1, 2, 3 2 hr daily; ■——■, Colcemid 72 hr continuous.

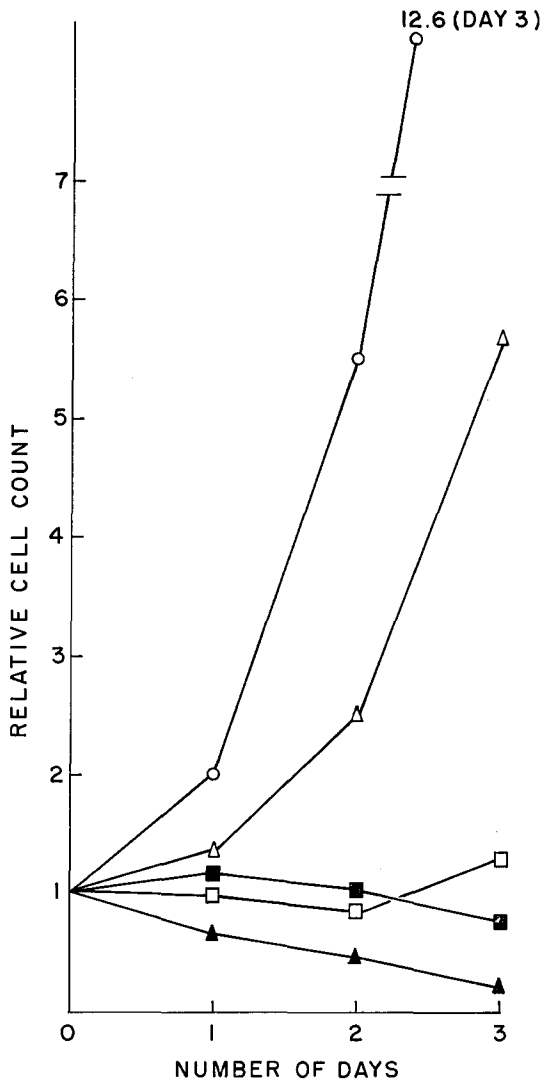

FIGURE 5 Growth curves of cell populations of B78 amelanotic line synchronized by mitotic collection of untreated cells. Symbols are the same as those used in Fig. 4.

these treatments temporarily reduced DNA synthesis almost to 0, there was minimal uptake of thymidine-H^3 in a fraction of the cells when put on normal medium for 20 min after exposure to ara-C. Within 24 hr after each treatment, a high percentage of the cells had recovered sufficiently to carry on normal thymidine uptake into DNA. Fig. 4 shows the growth curves of treated and untreated cells synchronized with Colcemid, and Fig. 5 shows those of cells synchronized by mitotic collection alone. From these figures, it can be inferred that in most cases enough DNA synthesis was going on to maintain the cell population at approximately its original level for several days, new cells replacing those killed by the ara-C. This was verified by microscopic observation. Six highly

pigmented continuous lines were derived from parasynchronized cloned cell populations and frozen in liquid nitrogen (Fig. 1 *e*).

Population Doubling Time and Plating Efficiency

The amelanotic cells have a mean population doubling time during the log growth phase of 16.5 hr with a range of 16–18 hr. The mean doubling time of the pigmented clone (derived by ara-C treatment) that has been maintained for over 2 years is 23 hr with a range of 20–28 hr. All the melanotic lines grow more slowly than the amelanotic line, taking much longer to reach maximum growth. Exact doubling time data are not available for them. The plating efficiency of the cloned amelanotic line is 79%, that of the pigmented ara-C derived clone is 61%, and that of a pigmented clone derived from the agar grown line is 65%.

Chromosome Studies

The chromosome number was the same in the amelanotic cell line, the melanotic line derived from cells pigmented during growth on agar, and in melanotic cells which grew from explants of pigmented tumors which had developed in mice injected with amelanotic cells. The mode was 60, the range 52–65.

Melanotic cell lines derived after ara-C treatment of the amelanotic cells had an increased chromosome number. This was true of such cells whether they had been subjected to long term treatment or had been given four pulses on successive days. The pigmented tumors that developed after injection of these cells also had high chromosome numbers. Amelanotic cells derived from ara-C pigmented lines also showed evidence of chromosome breakage and rearrangement.

Ara-C, like many other inhibitors, induces chromosome breakage (25). The breaks and rearrangements which occurred in the melanoma cells under the influence of ara-C did not appear to bear any direct relationship to the presence or absence of melanin, since both pigmented and unpigmented cell lines were derived by the different methods described with the chromosome number of the original amelanotic line and with the increased chromosome number of the ara-C-treated cells.

Isolation of Unpigmented Clone from Pigmented Clone

Attempts to clone unpigmented lines directly from the ara-C pigmented clone N87 proved unsuccessful since all clones had some pigment.

A largely unpigmented line was isolated after a combination of selective scrapings and trypsinization of N87 to select for rapidly growing unpigmented cells. Four months after initiating this procedure, one clone, AN87, was isolated which appeared to be unpigmented when observed with the inverted microscope. By the eighth subculture after cloning, occasional pigment cells were observed, although most of the cells remained unpigmented.

Experiments were undertaken to repigment these cells by using ara-C inhibition. Both unsynchronized cells and cells synchronized by mitotic collection with Colcemid were used. In all cases pigment developed between Day 4 and Day 6. Pigmented foci developed more readily than in

TABLE IV

DOPA Oxidase Activity of Cloned Pigmented Cell Line (N87)

	Condition of homogenate	Specific activity*	Percentage of original activity
Exp. 1	Fresh	625	100
	−20°C, 3 days	262	42
Exp. 2	Fresh	650	100
	−20°C, 6 days	190	29
	4°C, 6 days	396	61
	4°C, 47 days	430	66
Exp. 3	Fresh	2526	100
	4°C, 1 day	1644	65

* Specific activity is defined as corrected optical density/mg protein/per hr. (optical density is corrected for autoxidation of DOPA).

TABLE V

DOPA Oxidase Activity and Inhibitor Activity in Cloned Unpigmented Cell Line (B78)

	Condition of homogenate	Specific activity*	Percentage inhibition of active enzyme‡
Exp. 1	Fresh	−15	45
	−20°C, 3 days	31	52
	−20°C, 10 days	95	0 (+12%)
	4°C, 56 days	41	0 (+6%)
Exp. 2	Fresh	−67	44
	−20°C, 6 days	−20	33
	4°C, 6 days	13	16
	4°C, 46 days	27	0 (+16%)
Exp. 3	Fresh	−90	30
	4°C, 36 days	80	9
	4°C, 10 months	33	0
Exp. 4	Fresh	−100	27
	4°C, 8 days	0	2

* Specific activity is defined as corrected optical density/mg protein/per hr. (See Table IV).

‡ Tested in a 1:1 mixture with N87 homogenate.

flasks of similarly treated B78 cells. One highly pigmented line was developed, and the rest were discarded.

DOPA Oxidase

DOPA oxidase activity in homogenates of pigmented cells was linear with time for the first 90 min and with enzyme concentration up to 200 μg of protein. The enzyme prepared as described was labile at both 4° C and −20° C. It lost as much as 35% of its activity within 24 hr and 40% within 6 days when stored at 4° C. At −20° C, it lost 70% of its activity within 6 days. The results of three typical experiments are shown in Table IV.

The amelanotic cells contain an inhibitor of DOPA oxidase and of DOPA autoxidation (Table V). Storage of the homogenates at 4° C or −20° C gradually inactivates the inhibitor and reveals a low level of enzyme activity in the extract (Table V). Since the enzyme itself is labile under these conditions, the original level of enzyme activity is unascertainable. It is probably very low, as can be seen by comparing the specific activity of a homogenate of the pigmented line (430 after 47 days at 4° C) with the specific activity of a homogenate of an unpigmented line (27 after 46 days at 4° C) which is only 6% of the former (Experiment 2 in Tables IV and V).

DISCUSSION

The cell line used had originated from a melanotic mouse melanoma (B16) and originally had formed pigment in vitro (1). It had no detectable pigment or DOPA oxidase activity in freshly prepared homogenates when received by the author. Partially or completely melanotic melanomas developed in all C57BL/6 mice into which these cells were injected, thus showing retention of pigment-forming capacity. It proved possible to force pigmentation repeatedly in a high percentage of these cultured amelanotic cells, both cloned and uncloned, by three means: growing on medium solidified with agar, using 0.06 μg/ml Colcemid continuously for 72 hr and using sublethal levels of ara-C.

The literature reports many studies of the loss of pigment in embryonic chick iris and retina cells (3, 17–19, 23, 26). In most of these studies, suppressing growth i.e. by removal of embryo extract or growth as monolayer or pellets in organ culture restored pigment. If the cells were allowed to grow again, they once more lost pigment. This effect is not completely analogous to the effects studied in the melanoma cells, since the repigmented melanoma cells did and do divide in normal medium and remain pigmented under these conditions. Pigmented cells in all stages of mitosis are commonly seen (Fig. 6).

The studies of Cahn et al. (27) on clonal growth of retinal pigment cells are pertinent to this study only in that the melanoma cells also pigment better when growing in a clonal fashion from a small inoculum. Since these melanoma cells do not require chick embryo extract (CEE), no experiments were done with it. It might be noted,

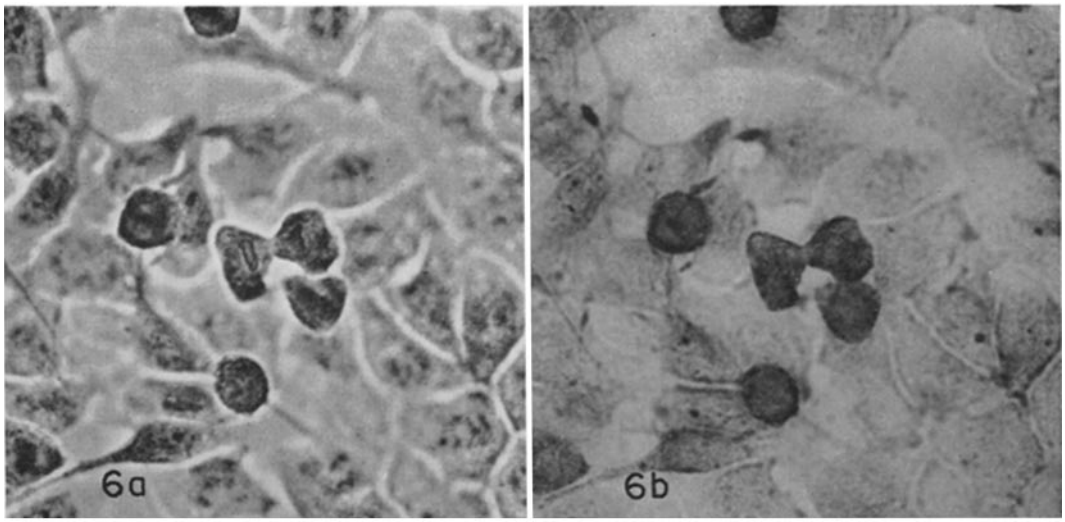

FIGURE 6 Pigmented cells (N87) stained for melanin by ferrous ion method. Note telophase in center of frame. × 551. *6 a*, Phase contrast; *6 b*, Bright field.

though, that Coon and Cahn (28) found that the division time on the "L" fraction of CEE on which pigmentation was good was 28 to 36 hr, whereas that on the "H" fraction on which pigmentation was poor was 16 to 24 hr. This difference in rates is similar to that between the melanotic and amelanotic melanoma lines described in this paper.

A hypothesis based on the data presented should attempt to provide a unifying framework and answer the following three questions: (a) Why is pigmentation not expressed in the amelanotic cells? (b) What induces its reexpression? (c) How is the reexpression maintained?

(a) The answer to the first question may well reside in a relationship between the more rapid growth rate of the amelanotic cells and their production of an inhibitor of enzyme activity. The metabolism of cells in culture growing logarithmically and selected for maximum multiplication is devoted primarily to those biosynthetic processes necessary for cell division. Economy would dictate omission of those molecules involved solely in differentiated function. In these cells, it is possible that the substance which inhibits tyrosinase activity serves also, in some unknown fashion, to prevent continuous production of enzyme. The fact that a low level of enzyme activity is revealed in the amelanotic cells after decay of the enzyme-activity inhibitor, would indicate that the tyrosinase gene is not completely repressed. It is possible, though not proved, that once a small amount of enzyme is produced in rapidly growing cells that are also producing large quantities of inhibitor, a feedback mechanism, possibly an enzyme-inhibitor complex, operates to suppress further enzyme production.

(b) The reexpression of pigment occurred in clonally derived amelanotic cells subjected to three different treatments. The clonal origin of the treated cells would indicate that there was no question of selection from a heterogeneous population either of preexisting pigmented cells or of cells differing in genome. The fact that every cell did not form pigment may reflect differences in microenvironment. All treatments were alike in evoking pigment in cells so inhibited in growth that the population remained virtually constant (Figs. 4 and 5). This effect is sublethal and reversible.

The studies with ara-C, a specific inhibitor of DNA synthesis, afford some insight into a possible mechanism for the evocation of this differentiated function. The fact that parasynchronized cells which were intermittently inhibited for 4 days during part of the S phase of the cell cycle by ara-C at 1.8×10^{-5} M (5 μg/ml) or 7.2×10^{-6} M (2 μg/ml) produced the greatest amount of pigment, indicates that S is the period when the cells are most vulnerable to ara-C's effect. This coincides with the findings of Young and Fischer (29) that one hour's treatment with ara-C at 1×10^{-4} M specifically and irreversibly stops replication in Don C (hamster) cells during the S phase of the

cell cycle. The same concentration and time of treatment during G_1 and G_2 or a lower concentration during S produces an effect on cell multiplication that can be reversed by an appropriate mixture of deoxynucleosides.

Why ara-C was successful in evoking pigment production whereas other inhibitors of DNA synthesis were not is unclear.

All successful treatments drastically inhibited growth. If rate of growth is indeed related to production of enzyme inhibitor, the following hypothesis relating to evocation of pigment can be formulated. Under conditions of virtually stationary growth the production of enzyme inhibitor would decrease or cease. This would permit the tyrosinase gene to continue to function, reevoking its expression.

(c) To maintain this differentiated function in normal medium, rigorous selection of the more slowly-growing pigmenting cells is necessary. Pigmented cells are scraped, and subcultured by using low inocula. Thus, clonal growth may be favoring those cells in which the balance between growth rate, production of tyrosinase and its inhibitor permits maintenance of a highly pigmented cell line.

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