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NORMAL MURINE MELANOCYTES IN CULTURE

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SUMMARY

A major obstacle to applying the techniques of molecular biology to the genetics and cell biology of pigmentation has been our inability to grow normal murine melanocytes in culture. We report here the establishment and characterization of continuously proliferating cultures of cutaneous pigment cells from seven strains of mice. Melanocytes were grown from the dermis of newborn mice in medium containing 12-0-tetradecanoyl-13-phorbol-acetate; a substance, such as melanotropin, that raises intracellular levels of cyclic AMP; and an extract made from human placenta.

Key words: murine melanocytes; cell proliferation; melanotropin.

INTRODUCTION

Techniques for growing human melanocytes were developed over the last few years (2,3,6–8,12), but it has not been possible until now to culture non-malignant murine melanocytes in large numbers. Research has been limited to melanocytes derived from melanomas. There have been three obstacles to growing non-malignant murine melanocytes in culture: uncertainty regarding an optimal source for melanocytes; what mitogens were required; and how to prevent contamination by fibroblasts.

We describe here a technique that overcomes these problems and yields pure cultures of continuously proliferating murine melanocytes. The source of cells is dermis from newborn mice. Three mitogens are used in concert to produce optimal proliferation: first and foremost, 12-0-tetradecanoyl-phorbol-13-acetate (TPA); second, a substance that raises intracellular levels of adenosine 3′,5′-cyclic monophosphate (cAMP) such as melanotropin (melanocyte stimulating hormone, MSH), dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP) or isobutylmethyl xanthine (IBMX); and third, a source for a natural growth factor, provided in form of an extract made from human placenta. Fibroblasts are destroyed by intermittent treatment of early cultures with the antibiotic geneticin (G418 sulfate) (6).

MATERIALS AND METHODS

Cell Cultures. One- to three-day-old mice were killed with pentobarbital and rinsed immediately with 70% ethanol in water and with buffered saline (PBS) in quick succession. Skin was collected from the dorsal area of each mouse. Piebald zones of the vitiligo mice were not included. The skins were incubated individually in 3 ml of 0.25% trypsin in Eagle's minimal essential medium without calcium or magnesium (MEMS, GIBCO Laboratories, Grand Island, NY) at 37° C for 2–4 hrs, or at 4° C overnight. The epidermis was then removed and discarded. The dermis was placed into TIP medium. *TIP* is Ham's F-10 medium (American Biorganics, Inc., Tonawanda, NY) with the following additions: 48 nM *T*PA (Consolidated Midland Corporation, Brewster, NY); 0.1 mM isobutylmethyl xanthine, or *I*BMX (Sigma Co., St. Louis, MO); 50 μg protein/ml human *P*lacental extract (7); 1 mM L-glutamine; 200 units/ml penicillin; 100 μg/ml streptomycin (GIBCO Laboratories, Grand Island, NY); 10% newborn calf serum (JR Scientific, Inc., Woodland, CA).

Details of procedure differed slightly for the dissociation of the dermis from dark and light strains. Tissues from highly pigmented strains were teased apart with jeweler's forceps in 1 ml TIP medium, whereas tissues from lightly pigmented or amelanotic strains were shaken vigorously on a Vortex mixer in 2 ml TIP medium. Dissociated dermis from each mouse was transferred into a 25 cm^2 flask (Falcon, Oxnard, CA) into a final volume of 2 ml to allow for optimal attachment of the tissue. The tissues were then triturated gently and incubated at 37° C in humidified air with 5% CO_2. After 2 days, tissue debris was removed by suction and the medium was changed and supplemented with 100 μg/ml geneticin (G418 sulfate, GIBCO Laboratories, Grand Island, NY) (6). The treatment with geneticin lasted 2-3 days and was repeated intermittently as long as fibroblasts persisted.

Once pure colonies of melanocytes appeared, the medium was modified by two substitutions. Fetal calf serum (20%, Whittacker MA Bioproducts, Inc., Walkersville, MD) was substituted for the newborn calf- and Nu-serum. A medium containing 15% fetal calf serum, 2.5% newborn calf serum, and 2.5% Nu-serum can also be used. N^6-2′-dibutyryl adenosine 3′:5′-cyclic monophosphate (dbc*A*MP, 0.1 mM,

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Sigma, St. Louis, MO) or beta-*M*SH (11) (0.2 μM) were substituted for the IBMX. The modified medium was called *TAP* or *TMP*, respectively. This medium was changed 2x/week. If fibroblasts continued to contaminate the melanocyte cultures, the concentration of geneticin during the intermittent treatments was raised to 200 or 300 μg/ml.

When the cultures became confluent, they were detached by incubation with the above 0.25% trypsin solution, supplemented with 3 mM ethylene diaminetetraacetic acid disodium salt (EDTA), at 37° C for 3-5 min, and the cells were collected and replated at one third their previous density. After two or three such splits, cells were passed at a ratio of 1:4 to 1:8 every 4-10 days. The presence of mycoplasma was tested with Mycotrim-TC (NEN Research Products, Boston, MA), following the manufacturer's instructions.

Chromosome spreads for karyotyping were prepared as described (4).

Melanocyte Proliferation. To measure proliferative activity, incorporation of ^{3}H-thymidine and changes in cell number were used as indices. Melanocytes were plated in 4 cm^2 cluster wells (Costar, Cambridge, MA) in TAP or TMP medium and on the following day switched to fresh medium or experimental medium. The latter media were renewed every 2-3 days over a period of 6-7 days. During the last 3 hr, the cells were incubated with MEMS supplemented with 3% dialysed calf serum and 5 μCi/ml ^{3}H-thymidine (90 Ci/mmole, Amersham, Arlington Heights, IL). The cells were then detached from the culture dishes with the trypsin-EDTA solution and trapped onto #30 glass filters in the minifold apparatus of Schleicher and Schuell. The filters were washed three times with distilled water, dried, placed in scintillation fluid, and radioactivity was determined in a scintillation counter.

To determine the kinetics of cell proliferation, melanocytes were grown in experimental and control media and at 2-3 day intervals were detached from the culture dishes, suspended in isotonic buffer and counted with a Coulter counter.

Results

All successful melanocyte cultures originated from dermis and, therefore, the epidermis is now being discarded routinely. As can be seen in Fig. 1A, during the first few days fibroblasts dominate the cultures, and melanocytes are distributed sparsely among them. Colonies of melanocytes become visible within 1-3 weeks after treatment with geneticin and finally yield pure cultures of proliferating pigment cells as seen in Fig. 1B. Cultures established in this manner were derived from the following murine strains: C57BL/6J (B6), B10.BR, B10.D2, C3H/HeJ, the two c-albino locus pigmentation mutants Himalayan C57BL/6J-c^h and Chinchilla 129/J (1) and the vitiligo mouse C57BL/6J-Ler *vit/vit* (9). All were obtained from the Jackson Laboratory, Bar Harbor, Maine.

In order to establish the relative importance of each growth-promoting additive, the components were added to the culture medium individually and in different combinations. As illustrated in Table 1 and Fig. 2, TPA was obligatory for obtaining a mitogenic response. The requirement for substances that increase intracellular levels of cAMP varied with the strain and/or the age of the cultures. Melanocytes derived from Chinchilla and Himalayan mice required the presence of cAMP stimulators throughout the 7 months of cultivation. The

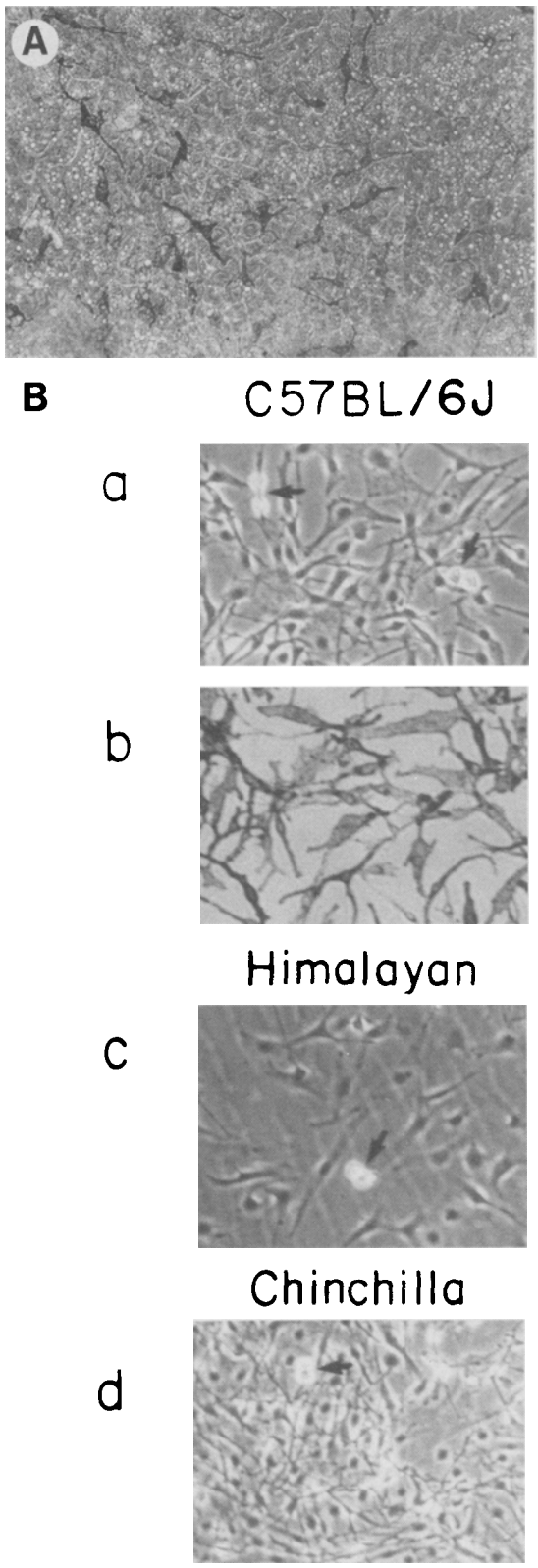

FIG. 1. Murine melanocytes in culture. A. Phase contrast photomicrograph of a typical mixture of murine fibroblasts and pigmented dermal melanocytes after two days in culture. Magnified 60×. B. Murine melanocytes after being cultured in TAP medium for 2-3 months. a, c, d: phase contrast; and b: bright-field photomicrograph to show color due to melanin. a, b; C57BL/6J; c: Himalayan; d: Chinchilla. Black arrows point to mitotic figures. Magnified 150×.

TABLE 1

GROWTH RESPONSES OF MURINE MELANOCYTES[a]

Additions	^{3}H-thymidine incorporation (cpm/well)		
	Strain of origin C57BL/6J		Chinchilla
	Time in culture (months)		
	3	7	3
TMP	9,550	6,150	17,000
TAP	6,430	7,720	24,000
TIP	4,850	4,850	10,300
TP	6,670	9,510	5,780
TM	1,230	5,340	10,150
TA	1,180	6,880	14,340
TI	1,500	4,150	6,250
P	1,360	110	150
T	580	8,050	4,030
M or A or I or MP or none	90-830	140-180	60-350

[a]Incorporation of ^{3}H-thymidine into melanocyte cultures incubated in experimental media for 7 days. The number of melanocytes one day after plating into the 4 cm^2 cluster wells was 32,000 and 10,000 cells/well for the experiments done 3 and 7 months in culture, respectively. Numbers are averages of triplicate wells; standard deviation did not exceed 12%. T: 48 nM TPA (12-0-tetradecanoyl-phorbol-13-acetate); M: 0.2 μM β-MSH (melanocyte stimulating hormone); P: 50 μg protein/ml human placental extract; A: 0.1 mM dbcAMP (dibutyryl cyclic 3′,5′-adenosine monophosphate); I: 0.1 mM IBMX (isobutylmethyl xanthine).

most effective cAMP stimulators were MSH and dbcAMP. IBMX was less effective, at the concentration presented in Table 1 as well as at higher (0.2 mM) or lower (0.05 and 0.01 mM) concentrations (data not shown). Human placental extract (7) augmented the synergism between TPA and each of the cAMP stimulators. Basic fibroblast growth factor (bFGF), known to substitute for the TPA requirement in cultures of normal human melanocytes (8), was an ineffective substitute for TPA in the murine system (data not shown). However, bFGF substituted for placental extract in cultures of B10.BR and BG melanocytes (data not shown), suggesting that the mitogenic activity in this extract is due to the presence of bFGF. There was no mitogenic stimulation by any one of the substances when added without TPA, even though there had been a report that dbcAMP had a stimulatory effect on murine melanocytes cultured from 14-day-old embryos (10). Melanocytes from the B10.BR strain, which proliferated more rapidly than any of the others did not need the cAMP stimulator. Melanocytes from the C57BL/6J strain at first required all three factors, i.e., TPA, a cAMP stimulator and placental extract for optimal proliferation. However, when tested again seven months later, they grew as well in TPA alone as in TPA plus other additions (Table 1). None of the strains were inhibited by MSH, which is known to inhibit Cloudman melanoma cells under some conditions (5). At optimal conditions for growth, the most rapidly proliferating strains were B10.BR, Himalayan, and Chinchilla with population doubling times of 1.5, 1.7, and 2 days respectively (Fig. 2). B10.BR and C57BL/6J mice are congeneic, with H-2 being the only divergent locus. The differences we found in the proliferation rates and initial growth requirements

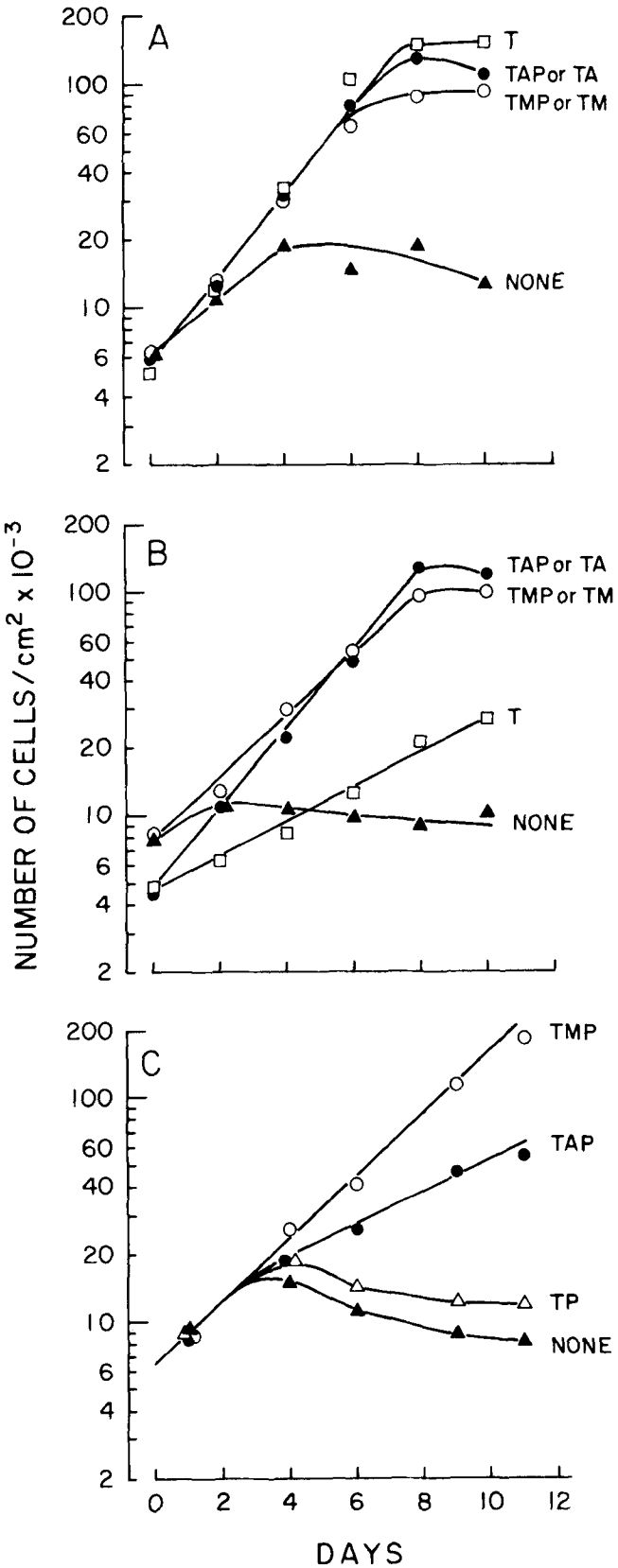

FIG. 2. Kinetics of growth in culture of melanocytes from strains B10.BR (A), Himalayan (B), and Chinchilla (C). T: 48 nM TPA; M: 0.2 μM MSH; P: 50 μg protein/ml human placental extract; A: 0.1 mM dbcAMP. None: medium containing serum but none of the above additions.

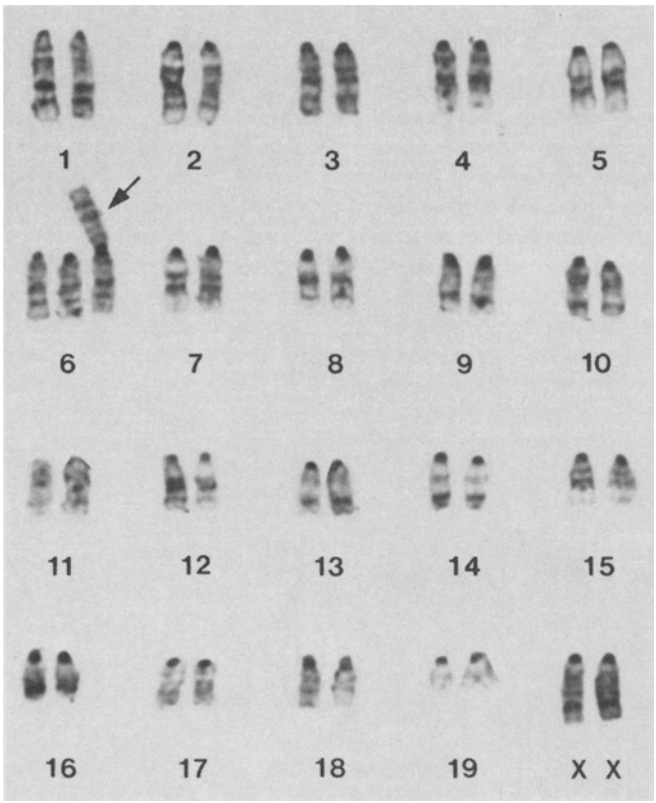

FIG. 3. Karyotype of B10.BR melanocytes, with an additional isochromosome (*arrow*).

of the respective melanoyctes cannot be explained on that basis. Analysis of mitotic chromosomes from melanocytes of the Himalayan and Chinchilla strains after 3-6 months in culture showed normal karyotypes. B10.BR melanocytes were found to have an abnormal karyotype, with 41 chromosomes (Fig. 3). The extra chromosome was metacentric and was identified as an isochromosome 6. It is thus possible that gene dosage contributed by the extra chromosomes 6 may have affected the growth of B10.BR melanocytes. In cultures of common origin, only cells of one sex (male or female) were observed.

There was no contamination with mycoplasma at the end of 7 months in culture.

DISCUSSION

Our studies show that normal murine melanocytes in culture require mitogens similar to those required by human melanocytes (2,3,7,8). In general, the obligatory ingredients for growth are TPA, a natural growth factor, or a combination of TPA and a substance that increases intracellular levels of cAMP. bFGF could not substitute for TPA. With regard to details, each strain has its own requirements. Optimal growth of melanocytes from the two albino mutants requires the simultaneous presence of all three agents, with MSH being the most effective stimulator of cAMP in all the murine cultures. The highly melanized strains B10.BR and C57BL/6J were either independent of the cAMP stimulators or became so with time. Development of independence from cAMP stimulators in late cultures of C57BL/6J melanocytes might be due to cellular changes that occurred during the period of proliferation in culture. Because these cells were kept continually in medium supplemented with MSH or dbcAMP and placental extract, it is unlikely that we selected for a clone that had become independent of these factors. It is also possible that differences in growth requirements with time were due to a change in the serum supplementation. Different batches of serum are known to contain different levels of growth factors. Testing the growth responses of early and late cultures of melanocytes from the C57BL/6J strain, using the same batch of serum both times, may explain the discrepant growth responses.

The cultures of murine melanocytes from a variety of mice with genetically defined pigmentation defects will enable us to apply the powerful techniques of molecular biology to bear on the mechanisms of normal and abnormal pigmentation, vitiligo, piebaldism and the generation of melanomas.

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