MITOTIC ACTIVITY IN NON-NEOPLASTIC MELANOCYTES

IN VIVO AS DETERMINED BY HISTOCHEMICAL,

AUTORADIOGRAPHIC, AND ELECTRON MICROSCOPE STUDIES

KOWICHI JIMBOW, SANFORD I. ROTH, THOMAS B. FITZPATRICK, and GEORGE SZABO. From the Department of Dermatology and the Department of Pathology, Harvard Medical School and Massachusetts General Hospital, Boston, Massachusetts 02114, and the Department of Oral Biology and Pathophysiology, Harvard School of Dental Medicine, Boston, Massachusetts 02115

Although ultraviolet irradiation and other external stimuli can induce an increase in the number of functioning melanocytes in skin, the mechanism of this multiplication of melanocytes in vivo is still a matter of speculation. Some light microscope studies have indicated mitosis of epidermal "clear" cells after stimulation (1, 11, 26–28). It has not been established, however, whether these dividing clear cells are melanocytes or one of the other types of clear cells (i.e., [a] Langerhans' cells, [b] "indeterminate" cells, or [c] dormant melanocytes) (2, 4, 10, 33). Only rare melanocytes are "lost" from normal epidermis (8); therefore if normal melanocytes with tyrosinase activity can divide mitotically in vivo as do melanoma cells (3, 18, 20), then stimulation could promote additional mitotic activity of mature melanocytes. It would then not be necessary to postulate the presence of undifferentiated tyrosinase-inactive precursors in the normal epidermis (23, 26, 28, 35). Mitosis

would also provide an acceptable mechanism for the formation of new melanocytes to replace the small number of melanocytes lost during hair and feather shedding (14, 19, 31, 32).

This study was undertaken to determine whether mitotic divisions can occur without external stimuli in differentiated melanocytes that contain immature and mature melanosomes and active tyrosinase.

MATERIALS AND METHODS

The neural crest-derived melanocytes of human skin, hair of black mice, and feathers of black chicks, and the non-neural crest-derived melanocytes of the retinal pigment epithelium of the eye were examined by light and electron microscopy. Human skin was obtained from the nonexposed skin of Caucasoid adult volunteers. Hairs in various growth phases (anagen stages I-VI) (25) from C57BL/6J mice (Jackson Laboratory, Bar Harbor, Maine), feathers of Black Minorca embryos, 9-21 days old (gift from the Massachusetts Audubon Society,

Lincoln, Mass.), and eyes of White Leghorn embryos, 3–21 days old (Spafas, Inc., Norwich, Conn.), were examined.

The specimens were fixed in Karnovsky's cacodylate-buffered glutaraldehyde-paraformaldehyde mixture (17). Half of the specimen was postfixed in 2% osmium tetroxide in the same buffer solution; the other half was washed with 0.1 M phosphate buffer, pH 7.4, incubated with 0.1% L-3,4-dihydroxyphenylalanine (DOPA) in 0.1 M phosphate buffer, pH 7.4, for 4–5 h at 37°C (15), before postfixation with osmium tetroxide, for the demonstration of tyrosinase (DOPA-oxidase) in tissues. Controls for the DOPA reaction were tissues whose tyrosinase was inactivated by boiling for 3 min (15). All tissues were finally stained en bloc with 1.5% uranyl acetate in 0.1 M Veronal buffer (pH 5.0) for 20 min, dehydrated in graded ethanol solutions, embedded in epoxy resin, and sectioned on the LKB microtome (LKB Instruments, Rockville, Md.). Sections were stained with lead citrate (30) and examined in a Siemens Elmiskop I electron microscope.

To identify population density of the epidermal melanocytes in the S (synthetic) phase of the cell cycle, 5-wk old black mice (C57BL) were injected intravenously (tail vein) with 4 μCi/g weight of [methyl-^{3}H]thymidine (New England Nuclear, Boston, Mass., sp act 20 Ci/mmol) in normal saline. 24 h before injection, the tail skin was epilated by wax plucking (13). 6 h after thymidine injection, the tail skin was biopsied and fixed with 4% formaldehyde in 0.1 M phosphate buffer, (pH 7.0) for 1 h. The biopsy specimens were incubated with 0.1% DOPA in 0.1 M phosphate buffer (pH 7.4), for 3–4 h at 37°C postfixed with 10% Formalin in 0.1 M phosphate buffer (pH 7.0), dehydrated in alcohol solutions and embedded in paraffin. Sections were processed for autoradiography (21) using a 1:1 dilution of Kodak NTB-2 emulsion (Eastman Kodak Co., Rochester, N.Y.), and counterstained with hematoxylin solution. Only nuclei with 10 or more grains were counted as labeled. 5 sections from each of six mice (30 sections total) were examined. To identify the population of melanocytes in the whole skin, some of the tissues were treated with 2 N NaBr for 2 h at 37°C, and the split preparation of epidermis thus obtained was incubated with 0.1% DOPA in 0.1 M phosphate buffer (pH 7.4) at 37°C for 3–4 h, fixed with 10% formalin, dehydrated, and mounted on slides.

RESULTS

Epidermis

In the nonstimulated, unexposed human skin, two dividing melanocytes were found after extensive sectioning (280 sections) of multiple blocks from 18 Caucasoid subjects (5–6 sections from each block, and 3 blocks from each subject, were examined). One mitotic figure was found in a cell which, in contrast to the surrounding keratinocytes, contained a large "clear" perikaryon and a number of pigment granules (Fig. 1 a). Serial thin sectioning showed that this dividing cell was devoid of tonofilaments but did contain 100-Å filaments, ribosomes, and melanosomes (Fig. 2). There was little granular endoplasmic reticulum. Golgi apparatuses were simple and inconspicuous. The presence of nonaggregated and singly distributed melanosomes in various stages of melanization indicated that this dividing cell was the melanocyte. The second melanocyte was in late telophase with beginning nuclear organization and peripheral membrane construction, but no well-developed nuclear membrane. Keratinocytes in mitosis were occasionally found in the same materials (Fig. 3). The dividing melanocytes contained no Langerhans granules.

Autoradiographic study of the mouse tail skin demonstrated that 9 of 1,322 melanocytes were in the S phase of the cell cycle (0.75 ± 0.28%) (Fig. 1 b and c). In the same specimens, however, 15.0% ± 5.5% of the keratinocytes showed an incorporation of thymidine. Split-DOPA preparation revealed that the population density of the melanocytes in the skin is 722 ± 7.6 per mm^2 of epidermis.

Hair

Mitotic divisions in melanocytes were found in the normal growing (anagen) hair of mice (Fig. 1 d). Electron microscope examination of the melanocytes during mitosis demonstrated a large number of immature and mature melanosomes (Fig. 4). Histochemical studies indicated that the tyrosinase (DOPA-oxidase) was present not only in the melanosomes and smooth endoplasmic reticulum but also in small amounts in the entire perikaryon. Granular endoplasmic reticulum was sparse, and Golgi apparatuses were much less complex than they were in the nondividing melanocytes. The mitoses in melanocytes were more frequent in the hair follicles during anagen stages III and IV, when the hair bulb becomes tyrosinase positive and begins to extend downward into the deep dermis (19). Mitosis in the melanocytes was rarely found in the hair follicles during anagen stages I and II, when the hair bulb is still tyrosinase negative. Langerhans' cells were not noted in their hair bulb. Tyrosinase activity was never found in other mitotic cells, i.e., keratinocytes or fibroblasts.
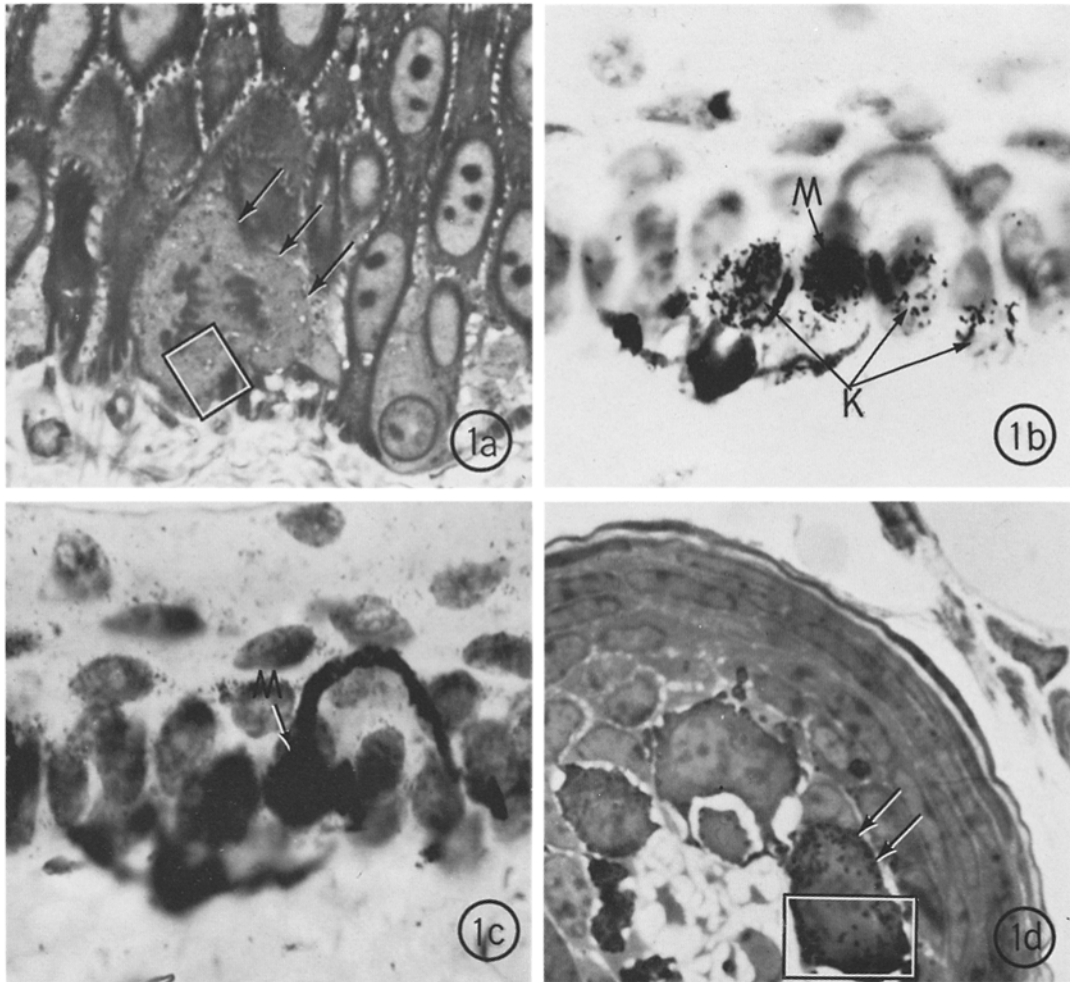
FIGURE 1 (a) A light micrograph of the normal buttock epidermis of a Caucasoid showing a mitotic figure in a clear cell (arrows). Toluidine blue and azure II. × 1,600. (b and c) Autoradiograph of C57BL/6J mouse skin showing uptake of [³H]thymidine in the melanocyte (M). Adjacent keratinocytes (K) also show uptake. (b) Focused on grains in emulsion. × 1260. (c) Focused on melanocyte body. × 1260. (d) A light micrograph of a normal mouse hair bulb in an anagen stage III showing a mitotic figure in a DOPA-positive cell (arrows). DOPA reaction. × 1,300.

Feather

Dividing melanocytes in the growing feathers of chicks were most prominent at days 9–11 of embryonic growth, when the feathers become tyrosinase-positive and start to extend downward into the epidermis as well as upward from the epidermal surface (14, 29). There were more melanosomes and rough endoplasmic reticulum in the dividing melanocytes in feathers than in those of skin and hair (Fig. 5). Langerhans' cells were not seen in the feathers.

Eye

In the retina of the chick eye, melanocytes in various stages of mitosis were most frequently seen between the 4th and 7th embryonic days (Fig. 6 a and b). At this time, the melanocytes had begun the synthesis of melanosomes and tyrosinase, and had formed junctional complexes with the neighboring cells. Fewer sacs of granular endoplasmic reticulum and less well-formed Golgi apparatuses were seen in these dividing melanocytes than in the later stages of embryonic growth, i.e., at day 10,
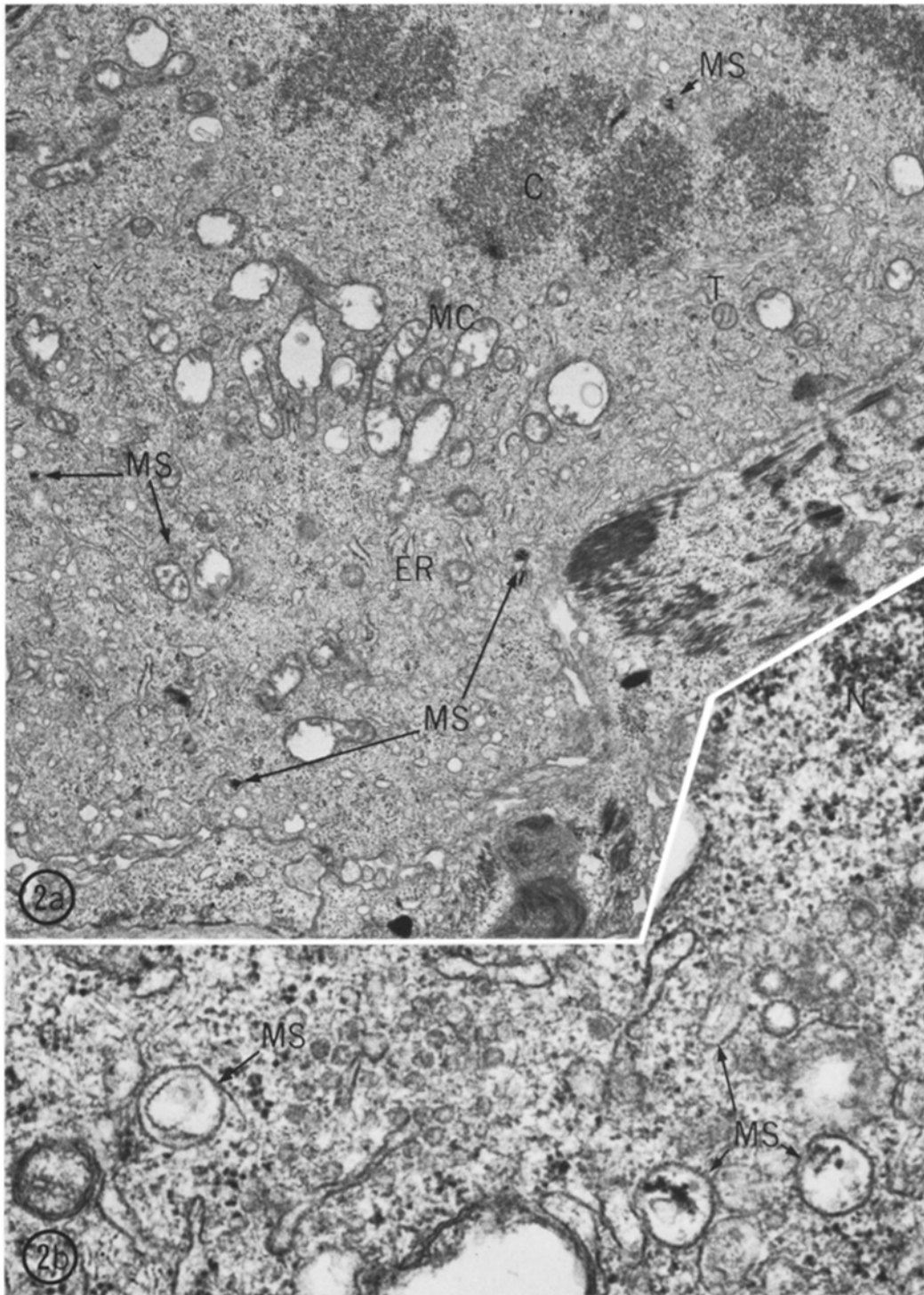
FIGURE 2   (a) An electron micrograph of the melanocyte in mitosis seen in Fig. 1 a. The nuclear chromatin (C) is separated into chromosomes. No nuclear membrane is seen. The microtubules (T) of the spindle and microfilaments are present. Individual melanosomes (MS) are scattered throughout the cytoplasm. Granular endoplasmic reticulum (ER) and mitochondria (MC) are seen. Lead citrate. × 27,000. (b) A high-power electron micrograph of the dividing cell shown in Fig. 2 a demonstrates the individual immature melanosomes (MS). Lead citrate. × 128,000.
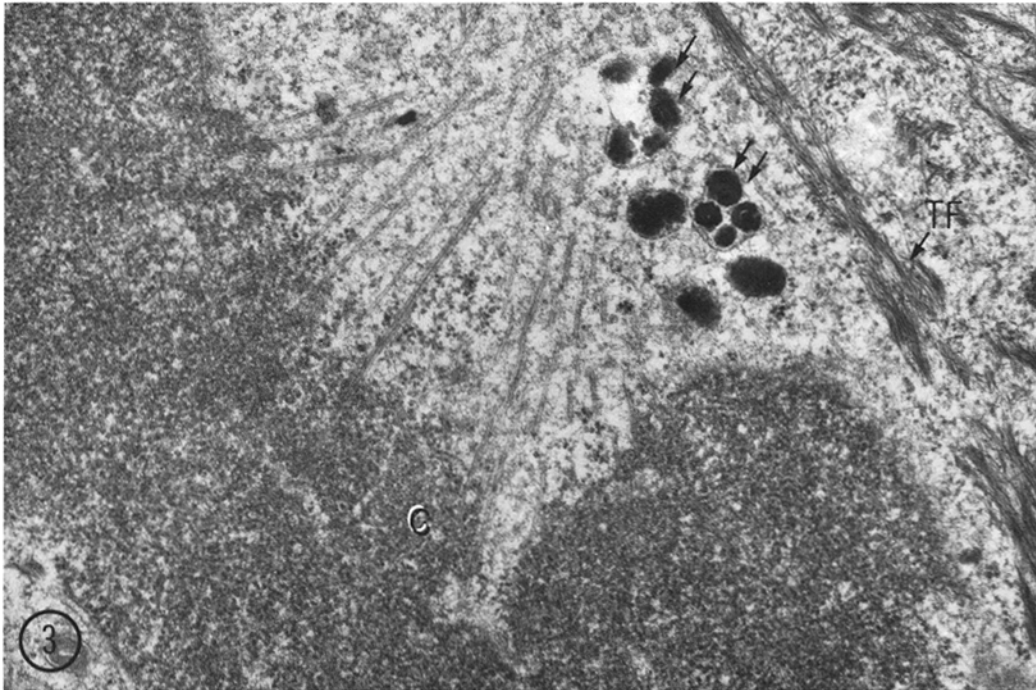
FIGURE 3 An electron micrograph of a dividing keratinocyte illustrating the aggregation of the fully melanized melanosomes (i.e., melanosome complex) within membrane-limited vacuoles (arrows), tonofilaments, (TF) and the chromosomes (C). × 18,750.

when the synthesis of tyrosinase and melanosomes is maximally visible (24). Golgi apparatuses were even smaller in size and less complex during metaphase than during the other stages of mitosis. No Langerhans' cells were seen.

DISCUSSION

This study confirms the light microscope finding of Coulombre et al. (5) that the increase in the number of non-neural crest melanocytes in the embryonic retinal pigment epithelium occurs by mitotic division of the melanocytes after the development of such differentiated features as melanosomes, tyrosinase, and junctional complexes. The specific differentiated features are not lost during mitosis.

Further, we have shown that the presence of such differentiating features as melanosomes and tyrosinase in melanocytes derived from the neural crest (29, 36) does not preclude the mitotic division of melanocytes in vivo under physiological conditions without external stimulation. It is not clear from the present study whether the non-neoplastic epidermal melanocytes synthesize melanosomes and tyrosinase during mitosis, although dividing

melanoma cells in vitro synthesize active tyrosinase during G_2 phase after administration of melanocyte-stimulating hormone (34). The Golgi apparatus in dividing melanocytes was generally less developed during mitosis than during the interphase, indicating that "packaging" of tyrosinase may not be as active, although melanosomes and tyrosinase were still present.

In the normal epidermis, there is little need for melanocyte replication, since, although there are 35 keratinocytes to each melanocyte in the human germinal stratum (9), melanocytes of normal epidermis are almost never shed while one keratinocyte is generally shed for each one generated (6, 8). In the hair and feather follicles, the exact population of melanocytes has never been determined, although it has been presumed that some melanocytes are lost during feather and hair growth (19, 29, 31). Also, there are no data on the number or percentage of melanocytes lost during each hair and feather cycle. However, some melanocytes are apparently retained in the follicles, since Silver et al. (32) found by light microscopy clear cells with aggregated pigment granules in the telogen hair, and Jimbow et al. (14) found by electron micros-
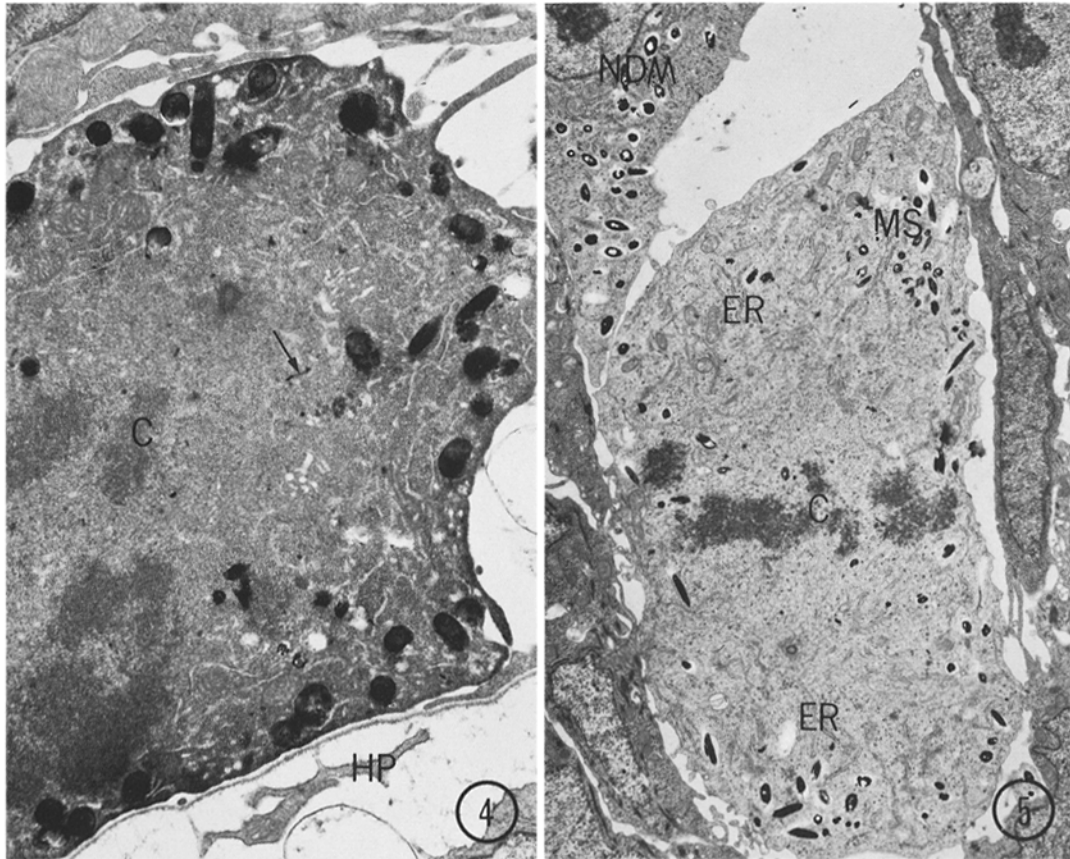
FIGURE 4 An electron micrograph of the dividing cell seen in Fig. 1 d. Most of the melanosomes are extremely electron dense because of the DOPA reaction in which DOPA melanin has been synthesized in the presence of active tyrosinase (DOPA oxidase). Arrow indicates DOPA-positive smooth endoplasmic reticulum. Chromosomes (C). Hair papilla (HP). DOPA reaction. × 32,000.

FIGURE 5 Electron micrograph of a dividing melanocyte in a feather of a Black Minorca, at day 11 of embryonic growth. An adjacent nondividing melanocyte (NDM) is also seen. Melanosomes (MS). Granular endoplasmic reticulum (ER). Chromosomes (C). × 14,000.

copy a few remaining melanocytes with mature melanosomes in resting feathers. The demonstration that mature melanocytes of hair and feather are capable of mitosis suggests that replenishment of melanocytes during the growth phase in these tissues could be the result of division of mature melanocytes. It is thus not necessary to postulate that the "indeterminate" cells of Zelickson and Mottaz (35) or the "α-dendritic" cells of Mishima et al. (22) are melanocyte precursor cells not yet producing melanin, and the "melanoblasts" of Silver et al. (32) are likely to be just mature melanocytes in mitotic division.

Our studies also indicated a much lower thymi-

dine index (0.75%) for epidermal melanocytes than for epidermal keratinocytes (15%) under conditions (epilation) known to produce mild stimulation of keratinocyte mitotic indices (13). The frequency of mitosis in completely unstimulated melanocytes is not known, although it may be as low as 0.1% if epilation or plucking increases the mitotic index of melanocytes by an amount (5–10 times) similar to that produced in the keratinocytes (7, 12, 16). Since unstimulated melanocytes are capable of mitotic division and mildly stimulated ones are capable of DNA synthesis, one may postulate that a need for additional melanocytes could be met by stimulation of the DNA synthesis,

thus the mitotic capability of these mature cells. Therefore, ultraviolet light or other external stimuli that increase the number of melanocytes in skin (23, 26, 28) may do so by directly stimulating DNA synthesis and mitosis in differentiated melanocytes, as these stimuli do in the keratinocytes. Replacement of epidermal melanocytes that are destroyed and extruded with the keratinocytes
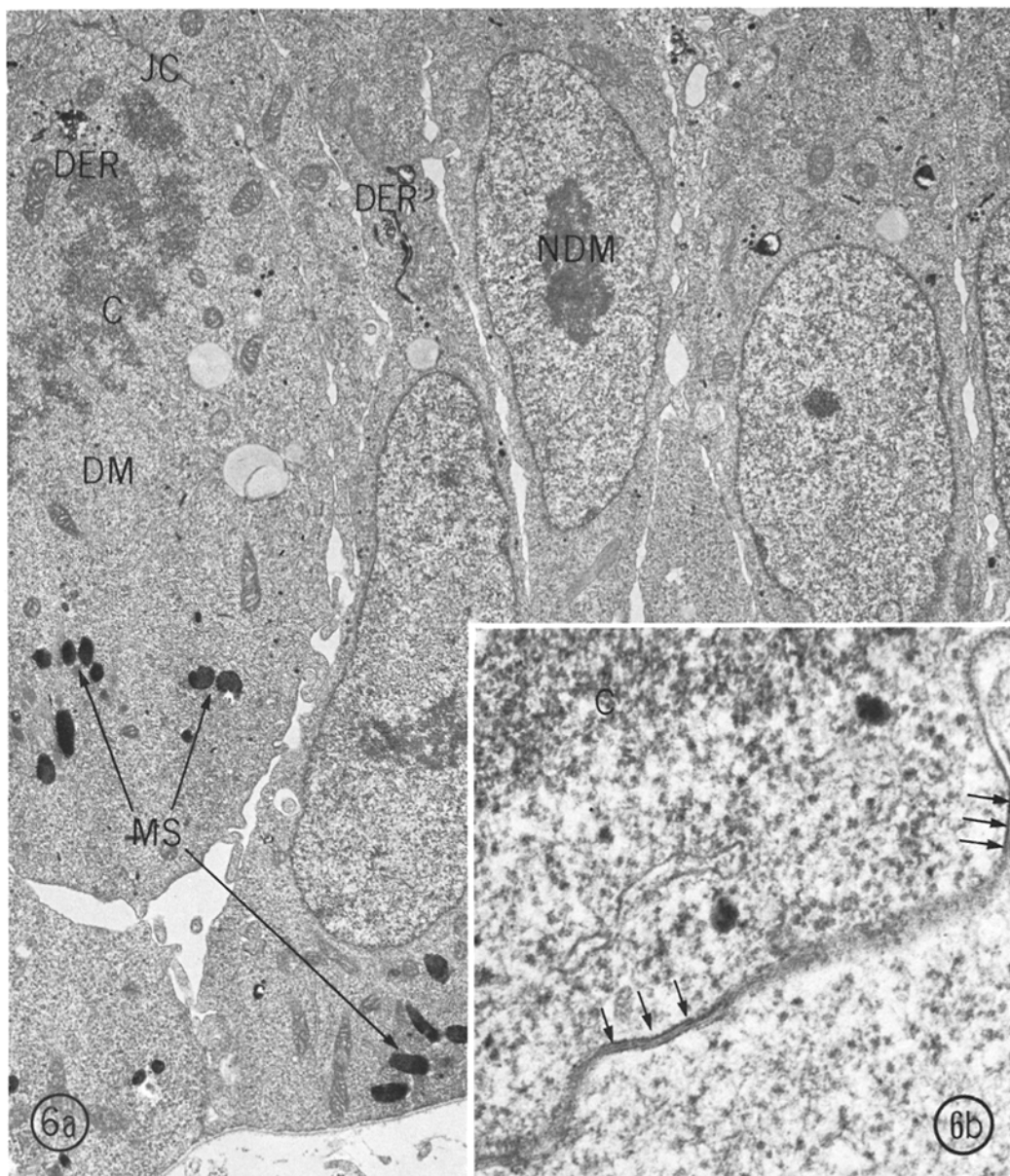

FIGURE 6 (a) An electron micrograph of a dividing (DM) and nondividing (NDM) melanocyte in the retinal pigment epithelium of a White Leghorn. The melanosomes (MS) and other organelles of the two cells are similar. Junctional complexes (JC) are present between the two cells. Chromosomes (C). DER = DOPA-positive endoplasmic reticulum. Lead citrate. × 9,200. (b) A high-power electron micrograph of a dividing melanocyte in the retinal pigment epithelium showing nexus junctions (arrows) with the adjacent nondividing melanocytes. Lead citrate. × 72,000.

during chemically induced depigmentation (13) might also be the result of activation of mitosis in the remaining differentiated melanocytes.

SUMMARY

Mitotic figures were demonstrated in the differentiated melanocytes of normal epidermal and nonepidermal tissues without the presence of external stimuli. These dividing melanocytes were present in adult human and mouse skin, mouse hair, chick feathers, and embryonic chick retinal pigment epithelium. In normal adult human epidermis, dividing melanocytes, though rare, were found in the nonstimulated areas. L-3,4-dihydroxyphenylalanine reaction on the melanocytes during mitosis demonstrated activity of the melanin-forming enzyme, tyrosinase, and ultrastructural studies demonstrated the characteristic melanosomes in variour stages of maturation. Other ultrastructural characteristics of the melanocytes during mitosis, except for the Golgi apparatus, which was smaller and less complex, were similar to those seen in well-differentiated nondividing melanocytes. Autoradiographic studies of thymidine incorporation into mouse skin indicated that 0.7% of epidermal melanocytes, when slightly stimulated, are in the S phase. Thus, in vivo differentiation of non-neoplastic melanocytes (to produce tyrosinase and melanosomes) does not preclude their replication by mitotic division.

REFERENCES

1. BECKER, S. W., T. B. FITZPATRICK, and H. MONTGOMERY. 1952. Human melanogenesis: cytology and histology of pigment cells (melanodendrocytes). *Arch. Dermatol.* **56**:511.
2. BREATHNACH, A. S., M. S. BIRBECK, and J. D. EVERALL. 1963. Observations bearing on the relationship between Langerhans cells and melanocytes. *Ann. N. Y. Acad. Sci.* **100**:223.
3. CHANG, J. P., T. SAITO, M. M. ROMSDAHL, and W. O. RUSSELL. 1972. Electron microscopy of human melanoma tissues and cells during mitosis. *In* Pigmentation, Its Genesis and Biologic Control. V. Riley, editor. Appleton-Century-Crofts, Inc., New York. 51.
4. CLARK, W. H., M. C. WATSON, and B. E. M. WATSON. 1961. Two kinds of "clear" cells in the human epidermis. *Am. J. Pathol.* **39**:333.
5. COULOMBRE, A. J., S. M. STEINBERG, and J. W. COULOMBRE. 1963. The role of intraocular pressure in the development of the chick eye. *Invest. Ophthalmol.* **2**:83.
6. DOWNING, S. W., and S. I. ROTH, 1974. The derivation of the cells of the epidermal strata of the boa constrictor. *J. Invest. Dermatol.* **62**:450.
7. FISHER, W. B. 1968. Determination of the normal rate and duration of mitosis in human epidermis. *Br. J. Dermatol.* **80**:24.
8. FITZPATRICK, T. B., and A. S.BREATHNACH. 1963. Das epidermale Melanin-Einheit-System. *Dermatol. Wochenschr.* **147**:481.
9. FRENK, E., and J. P. SCHELLHORN. 1969. Zur Morphologie der epidermalen Melanineinheit. *Dermatologica (Basel).* **139**:271.
10. GIACOMETTI, L., and R. CAPUTO. 1969. Skin ultrastructure in Hand-Schüller-Christian disease. *Arch. Dermatol.* **100**:342.
11. GIACOMETTI, L., W. MONTAGNA, M. BELL, and F. HU. 1972. Epidermal melanocyte proliferation in skin wound healing and after ultraviolet irradiation in rhesus monkeys. *In* Pigmentation, Its Genesis and Biologic Control. V. Riley, editor. Appleton-Century-Crofts Inc., New York. 451.
12. HEGAZY, M. A. H., and J. F. FOWLER. 1973. Cell population kinetics and desquamation skin reaction in plucked and unplucked mouse skin. *Cell Tissue Kinet.* **6**:587.
13. JIMBOW, K., H. OBATA, M. A. PATHAK, and T. B. FITZPATRICK. 1974. Mechanism of depigmentation by hydroquinone. *J. Invest. Dermatol.* **62**:436.
14. JIMBOW, K., G. SZABO, and T. B. FITZPATRICK. 1974. Ultrastructural investigation of autophagocytosis of melanosomes and programmed death of melanocytes in White Leghorn feathers: a study of morphogenetic events leading to hypomelanosis. *Dev. Biol.* **36**:8.
15. JIMBOW, K., M. TAKAHASHI, S. SATO, and A. KUKITA. 1971. Ultrastructural and cytochemical studies of melanogenesis in melanocytes of normal human hair matrix. *J. Electron Microsc.* **20**:87.
16. KAHN, G., G. D. WEINSTEIN, and P. FROST. 1968. Kinetics of human epidermal cell proliferation: diurnal variation. *J. Invest. Dermatol.* **50**:459.
17. KARNOVSKY, M. J. 1965. Formaldehyde-glutaraldehyde fixation of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**(2, Pt. 2):137 *a*. (Abstr.).
18. KITANO, Y., and F. HU. 1970. DNA, RNA and protein synthesis of pigment cells in culture. *J. Invest. Dermatol.* **54**:463.
19. KUKITA, A. 1958. Demonstration of tyrosinase in

melanocytes using C-14 labeled tyrosine. *Jpn. J. Dermatol.* **68**:915.

20. MASSON, P. 1951. My concept of cellular naevi. *Cancer.* **4**:9.

21. MESSIER, B., and C. B. LEBLOND. 1969. Cell proliferation and migration as revealed by radioautography after injection of thymidine-³H into male rats and mice. *Am. J. Anat.* **106**:247.

22. MISHIMA, Y., H. KAWASAKI, and H. PINKUS. 1972. Dendritic cell dynamics in progressive depigmentation: distinctive cytokinetics of α-dendritic cells revealed by electron microscopy. *Arch. Dermatol. Forsch.* **243**:67.

23. MISHIMA, Y., and S. WIDLAN. 1967. Enzymatically active and inactive melanocyte populations and ultraviolet irradiation: combined DOPA-premelanin reaction and electron microscopy. *J. Invest. Dermatol.* **49**:273.

24. MIYAMOTO, M., and T. B. FITZPATRICK. 1957. On the nature of the pigment in retinal pigment epithelium. *Science (Wash. D. C.).* **126**:449.

25. MONTAGNA, W. 1956. Structure and Function of Skin. Academic Press Inc., New York. 234.

26. PATHAK, M. A., S. T. SINESI, and G. SZABO. 1965. The effect of a single dose of ultraviolet radiation on epidermal melanocytes. *J. Invest. Dermatol.* **45**:520.

27. POTTEN, C. S., L. P. MERKOW, and H. PARDO. 1971. Ultrastructural changes in mouse hair follicle pigment cells after plucking, X-rays, and actinomycin D treatments. *Lab. Invest.* **21**:607.

28. QUEVEDO, W. C., JR., G. SZABO, J. VICKS, and S. J. SINESI. 1965. Melanocyte population in UV-irradiated human skin. *J. Invest. Dermatol.* **45**:295.

29. RAWLES, M. E. 1948. Origin of melanophores and their role in development of color patterns in vertebrates. *Physiol. Rev.* **20**:283.

30. REYNOLDS, E. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.

31. SILVER, A. E. 1958. Origin and identity of clear cells found in hair bulbs of albino mice. *Anat. Rec.* **130**:135.

32. SILVER, A. F., H. B. CHASE, and C. F. POTTEN. 1969. Melanocyte precursor cells in the hair follicle germ during the dormant stage (telogen). *Experientia (Basel).* **25**:299.

33. SNELL, R. B. 1965. An electron microscopic study of the dendritic cells in the basal layer of guinea pig epidermis. *Z. Zellforsch. Mikrosk. Anat.* **66**:457.

34. WONG, G., J. PAWELEK, M. SANSONE, and J. MOROWITZ. 1974. Response of mouse melanoma cells to melanocyte stimulating hormone. *Nature (Lond.).* **248**:351.

35. ZELICKSON, A. S., and J. H. MOTTAZ. 1968. Epidermal dendritic cells. *Arch. Dermatol.* **38**:612.

36. ZIMMERMAN, A. A., and T. CORNBLEET. 1948. The development of epidermal pigmentation in the Negro fetus. *J. Invest. Dermatol.* **11**:383.