THE JOURNAL OF INVESTIGATIVE DERMATOLOGY VOL. 43, NO. 3

Printed in U.S.A.

PIGMENT CHANGES INDUCED IN HAIRLESS MICE BY DIMETHYLBENZANTHRACENE*

SIDNEY N. KLAUS, M.D.† AND R. K. WINKELMANN, M.D.

Two distinct pigmentary phenomena may be induced in the skin of hairless mice by the topical application of dimethylbenzanthracene (DMBA). One phenomenon is the development of an unusually intense generalized melanin pigmentation in pigmented hairless mice which begins within a week after the initial application of the carcinogen and remains for a limited time. The second pigmentary phenomenon is the development of discrete pigmented nodules of the skin, which appear 2 to 3 months after the application of the carcinogen and which persist for the life of the animal.

Experimental studies concerning the mode of onset, the gross and microscopic features, the course, and the presumed origin of the DMBA-induced generalized pigmentation will be presented in section I. The second pigmentary phenomenon, the development of discrete pigmentary nodules of the skin, will be discussed in section II.

SECTION I: GENERALIZED PIGMENTATION

Generalized pigmentation as a consequence of application of DMBA to hairless mice was first observed in 1962 during a study of the carcinogenic effects of topical applications of this compound. The pigmentation became apparent within 5 or 6 days after a single application of a 1 per cent solution of DMBA in mineral oil. It developed only in animals which had a large capacity for pigment production, that is, in animals which normally show pigmentation of the feet, ears, tails, and muzzles though the remainder of the skin is white. Further applications of DMBA to the relatively few affected mice in the initial group resulted in gradual increase in intensity of pigment until it reached a plateau of generalized deep black; thereafter it slowly faded if more DMBA was not given. Neither inflammatory changes nor production of tumor was observed during the generalized pigmentation phase. The adrenal glands of several mice were examined and found to be normal, histologically.

Further series of experiments were designed in order to study this unusual pigmentary phenomenon in more detail.

MATERIALS AND METHODS

Thirty hairless mice, ranging in weight from 25 to 30 gm and in age from 10 to 20 weeks, were selected from a colony of Mayo hairless mice. (The mice are hairless offspring of C-57 BL mutants which were crossbred with haired C-57 black mice to introduce pigment capacity into the strain.) The animals selected were designated as D (dark) or L (light) based on the presence or absence of pigmentation of the feet, ears, tails, and muzzles. Not one of the animals was albino. Fifteen dark animals were paired with 15 light ones and treated by schedules in the table. They were kept in separate cages and allowed free access to Purina Laboratory Chow and water.

A 1 per cent solution of DMBA‡ dissolved in light mineral oil (NF) was applied topically to the dorsal surface of each of the animals in the first eight pairs by means of a standard medicine dropper. A single drop of the DMBA solution contains approximately 0.5 mg of DMBA. Pairs A, B, C, and D received four drops every 4 days for four applications; pairs E, F, G, and H received four drops every 5 days for three applications. Pairs I and J received a single drop of 0.1 per cent DMBA solution dissolved in acetone every 4 days for three applications.

Of the remaining five pairs of animals, three pairs K, L, and M, received four drops of mineral oil without DMBA every 4 days for four applications and two pairs, N and O, received one drop of acetone solution every 4 days for three applications (Table I). Changes in skin texture and skin color were recorded daily.

One to 2 mm "pinch" biopsy specimens were obtained from the backs of the animals prior to the application of the solutions and on the second, fourth, sixth, eighth, and tenth days after the initial application of the drug thereafter. The biopsy specimens were frozen, cut at 6 micra (μ) on a cryostat and examined after staining with hematoxylin and eosin, Becker's silver nitrate, dihydroxyphenylalanine (DOPA) oxidase, and tyrosin-

This investigation was supported in part by Training Grant 2A-5299 from the National Institutes of Health, Public Health Service.
Received for publication July 1, 1964.
* From the Section of Dermatology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota.
† Present address: Yale University School of Medicine, New Haven, Connecticut.

‡ 7,12-dimethylbenz (α) anthracene. Prepared by the Eastman Kodak Company.

ase. Appropriate biopsy specimens were sectioned horizontally and stained with dopa-oxidase.

RESULTS

Gross changes.—The first grossly apparent pigmentation in this series was evident in the dark animals of the first four pairs (A, B, C, and D) approximately 4 days after the initial administration of the carcinogen. The pigment was first apparent as a gray-to-brown darkening of the sagittal skin folds along the posterior part of the neck. By the sixth day, the dark animals of the first four pairs showed evidence of pigment over the entire dorsal surface and beginning pigmentation of the ventral surface as well. The ridges of the skin folds were relatively darker than the troughs. In the dark animals of the first four pairs, the pigment reached a maximal degree 10 to 12 days after the first application, remained at a plateau of generalized black until a week after the last application (approximately day 23), and then gradually faded over the subsequent 3 weeks (Fig. 1). The dark animals of the second four pairs became pigmented somewhat less rapidly and less intensely, reached a plateau approximately 4 to 5 days after the last application (day 19), remained unchanged for a week, then gradually faded. The light animals of the first eight pairs failed to show any evidence of pigmentation.

Neither the dark nor the light animals receiving DMBA in acetone showed pigmentation. However, inflammatory changes were seen in these animals within 1 or 2 days after the first application of the acetone solutions. Neither control group became pigmented. In animal pairs N and O, which were treated with acetone only, inflammatory changes developed that were similar to those seen in pairs I and J.

Approximately 2 months after the beginning of the experiment, the mice showing evidence of pigmentation had faded to the color of the control animals except for the gradual appearance of the discrete, deeply pigmented nodules referred to previously.

Histologic changes.—No generalized melanocytic activity was evident in the skin of any of

TABLE I

Hairless mice pairs treated with DMBA

Pairs	DMBA, drops		Frequency	
	In mineral oil	In acetone	Days	Times daily
A	4		4	4
B	4		4	4
C	4		4	4
D	4		4	4
E	4		5	3
F	4		5	3
G	4		5	3
H	4		5	3
I		1	4	3
J		1	4	3
Controls				
K	4		4	4
L	4		4	4
M	4		4	4
N		1	4	3
O		1	4	3

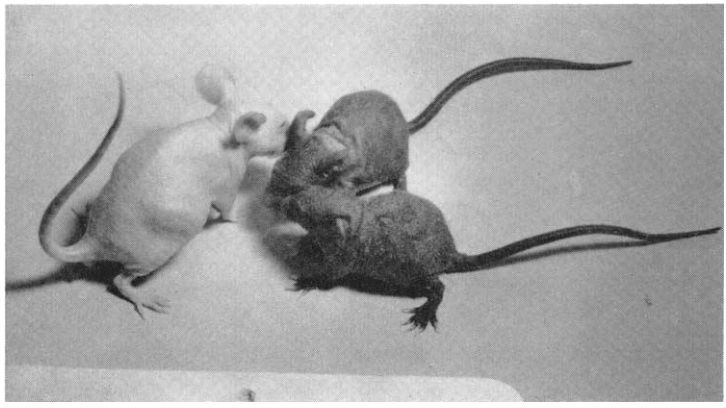


FIG. 1. Generalized pigmentation induced in hairless mice by the topical application of a 1 per cent solution of dimethylbenzanthracene in mineral oil (dark animals, pairs B and D; control animal on the left).

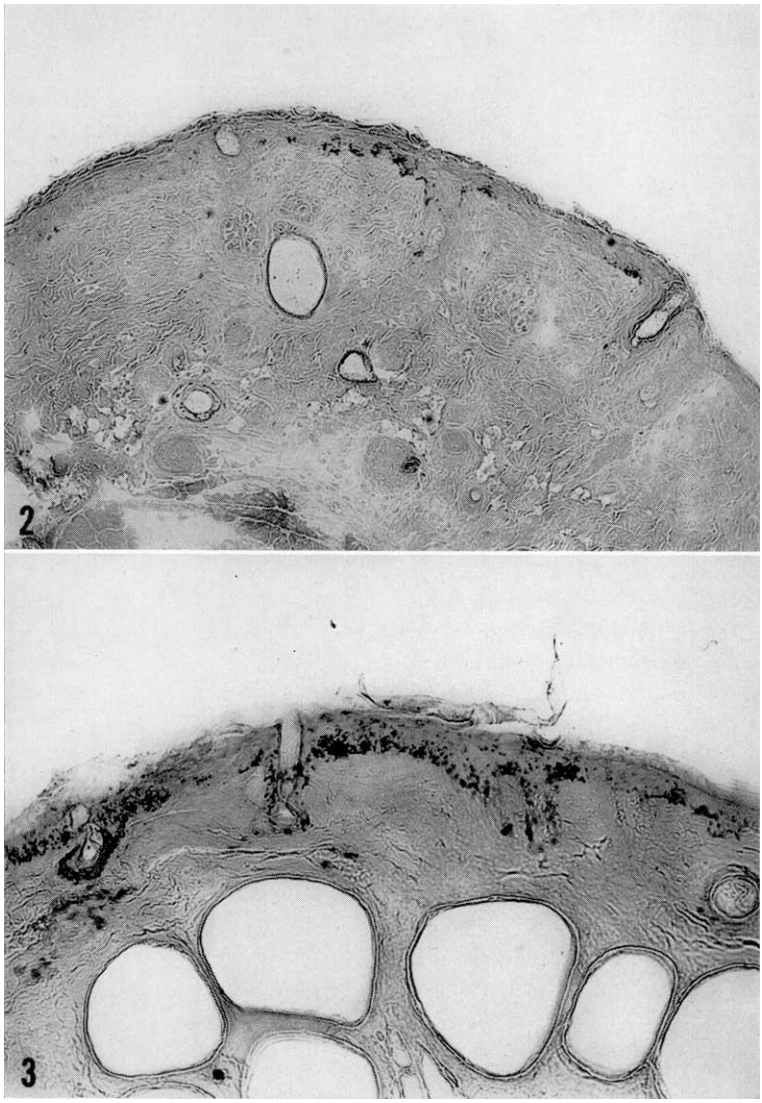


FIG. 2. Pigment production in epidermal melanocytes of hairless mouse 4 days after topical application of 1 per cent dimethylbenzanthracene in mineral oil (DOPA stain; × 100).

FIG. 3. Considerable activity of epidermal melanocytes and minimal activity of dermal melanocytes in hairless mouse skin 8 days after the first application of a 1 per cent solution of dimethylbenzanthracene in mineral oil (DOPA stain; × 100).

the animals in the initial biopsy specimen when a silver or enzyme technic was used. By day two, formation of pigment was not evident on sections stained with hematoxylin and eosin. Rare DOPA-positive dendritic cells were present in the basal layer of the epidermis of the dark animals of pairs B and D. By day four, a few scattered pigment granules were seen in the basal layers of the epidermis in some of the sections stained with hematoxylin and eosin, and DOPA oxidase and tyrosinase stained sections showed moderate degrees of activity in the melanocytes of all the dark animals of the first eight pairs (Fig. 2). The epidermal pigment increased during the next 6 days. In the biopsy specimens taken on day eight, the first evidence of DOPA-positive pigmentation in dermal melanocytes was evident in the dark animals of pairs A, B, and D (Fig. 3). By the tenth day, dermal melanocytic activity was evi-

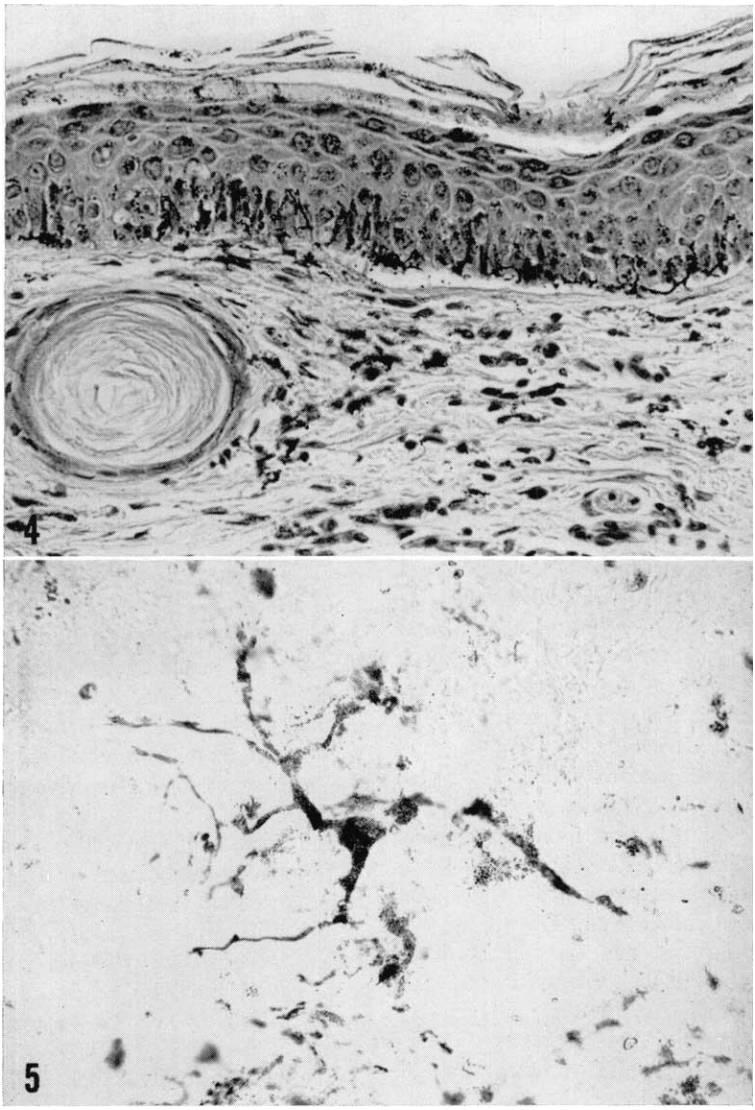


FIG. 4. Large deposition of pigment in both the dermis and epidermis of hairless mouse 16 days after the first application of a 1 per cent solution of dimethylbenzanthracene in mineral oil (Becker's silver stain; × 300).

FIG. 5. Prominent pigment-filled dendritic processes of an epidermal melanocyte, hairless mouse skin, 16 days after the initial application of a 1 per cent solution of dimethylbenzanthracene in mineral oil (horizontal section; DOPA stain; × 425).

dent in varying degrees in all of the dark animals of the first eight pairs. Biopsy study of the specimen taken from the dark animal of pair B during the plateau of intense pigmentation and stained for melanin showed large amounts of pigment throughout all layers of the epidermis and lesser amounts scattered throughout the dermis (Fig. 4). In sections cut horizontally and stained by the DOPA technic, elongation of dendritic processes of epidermal melanocytes was evident which reached a maximal degree by day 16 (Fig. 5).

A few scattered DOPA-positive and tyrosinase-positive epidermal cells were seen by day eight in the lightly pigmented animals of the first eight pairs. By day 15 a few scattered pigment granules also were evident on sections stained with hematoxylin and eosin.

Although an occasional DOPA-positive or tyrosinase-positive epidermal cell was evident in the dark animals receiving DMBA in acetone, the microscopic pigmentary effects were overshadowed by inflammatory infiltrate and moderate acanthosis which appeared within 2 days after the first application. The inflammatory infiltrate was present in all the animals receiving applications of acetone. No pigmentary changes were seen in biopsies from any of the control animals.

COMMENT

When the diffuse nature of the pigmentation after the topical application of DMBA in 1962 was first noted, it was thought to represent a systemic effect of the drug, being absorbed either from the site of the application or by ingestion. However, preliminary studies in which equivalent amounts of the carcinogen were injected parenterally and delivered into the stomach by means of a plastic tube failed to produce any pigmentary effects. This result suggested that the site of primary action was the skin, a conclusion that was confirmed when moderate pigment darkening limited to specific skin sites which were treated with a 1 per cent solution of DMBA in collodion was produced. The degree of pigmentation found subsequent to the application of DMBA in collodion was much less intense than that which followed the use of DMBA in mineral oil. The diffuse appearance of the pigmentation in animals treated with the carcinogen dissolved in mineral oil probably depends on the ease and rapidity with which a thin film of oil spreads over the entire cutaneous surface of the hairless mouse and the ready absorption of the active ingredient percutaneously from the mineral oil vehicle.

The stepwise stimulation of the two systems of melanocytes which are present in these animals (the epidermal and dermal melanocyte systems), as seen in the serial biopsy specimens, also substantiates a topical rather than a systemic effect of the carcinogen.

The intensity of the pigment formation in these animals and the rapidity with which it occurs are unique. In previous studies, diffuse pigmentation in hairless mice has been produced by the combination of topically applied gas-oil and ultraviolet light exposure (1) and through the administration of antimalarial drugs by mouth (2). In neither case, however, was the intensity or rapidity of the pigmentation comparable to that of the present study. The site of pigment formation was also distinctive in each of the three cases. In the present experiment, both dermal and epidermal melanocytes were stimulated; in the gas-oil experiments only dermal melanocytes were involved; and in the antimalarial experiments, predominantly epidermal melanocytes were affected.

Szabo (3), in a recent study of the affects of carcinogen on the melanoctyes of haired mice, demonstrated a similar rapid accumulation of pigmentation 3 to 4 days after a single application of DMBA in acetone. In his preparations, the DOPA-positive cells were located primarily in the outer root sheaths of the hair follicles, and he concluded that the formation of pigment was the result of melanocytes escaping from the hair matrix into the surrounding dermis. In hairless mice, of course, the entire hair matrix, including the pigment component, is absent. Szabo did observe that the DMBA applied to the hairless surface of the tails of the haired animals resulted in an increase in the number of DOPA-positive epidermal melanocytes.

The absence of significant pigment formation in the animals stimulated by DMBA in acetone indicates either that the carcinogen is not well absorbed from this vehicle, or that the associated inflammatory reaction inhibits the pigment activity or, by producing an exfoliative epidermal change, causes its loss from the surface.

The mchanism by which DMBA stimulates melanocytes in the hairless mouse is unknown.

SECTION II: PIGMENTED NODULES

Pigmented tumors which arise in animals after the topical application of certain carcinogenic agents have earned a special place in experimental oncology. As a group, these tumors may be induced by a single application of a carcinogen; they develop without the intervention of a promoting factor, and they act clinically and appear histologically as benign tumors.

This section concerns the formation and histologic appearance of pigmented skin tumors in hairless mice induced by the topical application of dimethylbenzanthracene.

MATERIALS AND METHODS

Ten hairless mice making up the dark half of each of the pairs A through J described previously (Table I) comprise the experimental population studied in this section.

The animals were observed for 18 months, and biopsy specimens of the pigmented tumors were obtained at appropriate intervals.

RESULTS

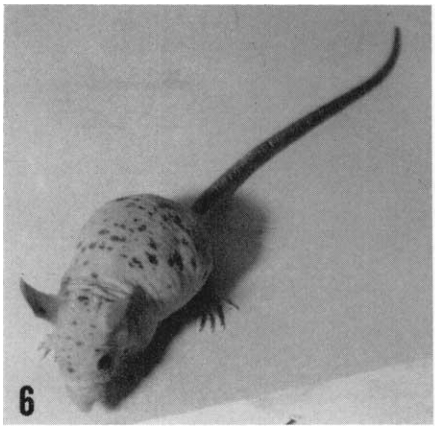
As the striking generalized pigmentation, which developed within a week in some of the dark animals after the topical application of DMBA, began to fade, discrete, slightly raised tumors measuring 1 to 2 mm developed over the backs of seven of the 10 animals. The three animals in which the tumors did not appear included the two receiving DMBA dissolved in acetone. The tumors began to appear 8 to 10 weeks after the initial application of the carcinogen. During the period of observation, squamous and keratotic tumors also developed. No morphologic or functional relationship was apparent between the pigmented and epidermal lesions. The number of tumors per animal varied from 10 to more than 30. They ranged in size from 1 to 3 mm. The majority of the tumors were macular, although a few were slightly raised. They were located primarily over the dorsal surface of the animals (Fig. 6).

After the tumors appeared, they did not change in size. There was no evidence of metastatic dissemination of any of the pigmented lesions. Many of the animals in the study have died subsequently; most showed some involvement by squamous cell carcinoma (Fig. 7).

Histologic Examination.—Biopsy study of representative lesions at varying intervals showed that the earliest lesions were made up of pigmented cells grouped in the mid dermis about the epidermal cystic structures, which had replaced the hair follicles in hairless mice. Later, masses of pigment cells became more compact, pressing the normal dermis both upward and downward. No capsules were seen about the tumors (Fig. 8). The cells of the well-developed lesions consisted of heavily pigmented, rounded or polygonal-shaped cells. After bleaching the pigment with hydrogen peroxide, the cells showed a lightly eosinophilic, granular cytoplasm with centrally placed nuclei and discrete cell borders but without evidence of malignancy (Fig. 9).

COMMENT

The cutaneous pigmented tumors of the hamster induced by DMBA have been studied extensively in recent years. The tumors were originally described by Della Porta and associates (4) and arose characteristically after a single application of the carcinogen. Ghadially and Barker (5) reviewed the histogenesis of these tumors and concluded that they arose from pigment cells situated about the pilosebaceous follicles. More recently Nakai and Rappaport (6) suggested that the tumors induced in Syrian hamsters were of neurogenic origin and, by means of the electron microscope, they demonstrated an abundance of myelinated and nonmyelinated nerves in the early melanotic lesions. They were able to demonstrate nonspecific cho-



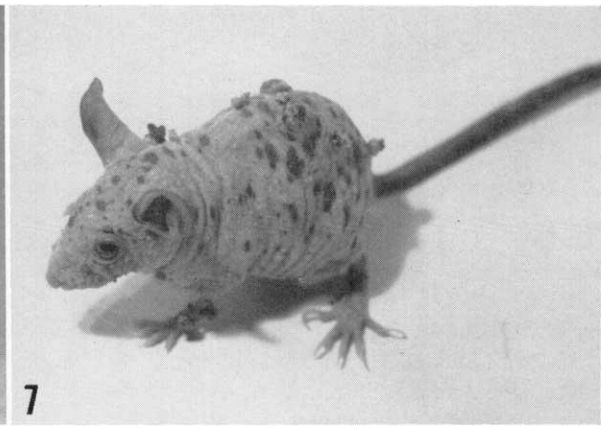


FIG. 6. Hairless mouse 4 months after the initial application of dimethylbenzanthracene in mineral oil. Multiple pigmented tumors appear over the back.

FIG. 7. Hairless mouse 8 months after the application of dimethylbenzanthracene in mineral oil. Squamous cell tumors have developed over the dorsal region.

FIG. 8. Section of pigmented tumor in the skin of the hairless mouse showed massive collection of pigment-containing cells without evidence of encapsulation of the tumor (hematoxylin and eosin; × 25).

FIG. 9. Polyhedral cells with centrally placed nuclei are clearly evident in the pigmented tumor after bleaching with hydrogen peroxide (hematoxylin and eosin; × 85).

linesterase activity in lightly pigmented lesions which pointed to a neurogenic origin.

Similar tumors have been noted in mice but they have been less extensively studied. In 1924, Lipschütz (7) noted the presence of pigmented tumors subsequent to application of a tar solution to the skin of pigmented haired mice. He thought that the tumors arose from dermal melanoblasts. A drawing of a histologic section in Lipschütz' paper correlates closely with the appearance of the tumors which we observed in our study. Burgoyne and co-workers (8) used trimethylbenzanthracene to induce multiple blue nevus-like lesions in the skin of haired mice. They suggested that the cells of origin were macrophages rather than melanocytes.

Szabo (3) also reported that the pigmented tumors, which developed in haired mice after application of DMBA in acetone and of croton oil, may arise from melanocytes of the hair

matrix that migrated into the surrounding dermis.

More recently Epstein and Epstein (9) produced pigmented lesions in hairless mice after the topical application of dimethylbenzanthracene in acetone both with and without subsequent ultraviolet radiation. In two lesions which were produced in that series, an apparent malignant change took place and the tumors became clinically invasive. They reported that no generalized metastasis was seen but that the cells did seed regional lymph nodes. These workers suggested that possibly the cells were melanotic rather than macrophagic.

In the present study the pigmented tumors arose in those animals which showed a capacity for pigment production and especially in those which had undergone diffuse transient cutaneous pigmentation. The pigmented tumors could be distinguished from melanomas. The cells were uniform and there was no cytologic or clinical indication of malignancy.

The production of tumors with the capacity for metastasis by coincident use of ultraviolet light as reported by Epstein and Epstein (9) is significant. It remains to be proved whether the cells of origin of the malignant tumors are similar to those of the benign tumors. Since no follicular melanocytic system exists in hairless mice, one can discount the hair-matrix pigment cell genesis as suggested by Szabo in experiments with haired mice.

A dermal pigment cell, or at least a dermal cell with pigment-forming capacity, remains as the most likely cell of origin for these tumors. Unfortunately, the intense amount of pigment present in the lesion obviated the use of tyrosinase, dopa-oxidase, or cholinesterase staining technics for examination of the functional capacities of these cells.

A human tumor of neuroectodermal origin, the dermal nevus, has recently been shown to contain latent pigment-producing capacity (10). One can speculate that a parallel may exist between the cell of origin of this experimental tumor and the nevus cell.

SUMMARY

Two pigmentary phenomena have been induced in hairless mice by the topical application of dimethylbenzanthracene (DMBA). One is the development of a generalized pigmentation which appears within a week after the initial application and then gradually fades. The other is the production of discrete pigmented nodules which arise later and persist.

Both are probably related to percutaneous absorption of the carcinogen.

REFERENCES

1. Rocha, Glyne and Winkelmann, R. K.: Induced dermal melanocytosis in hairless mice. Arch. Derm. (Chicago), *86:* 229, 1962.
2. Merwin, C. F.: The effects of chloroquine and atabrine on the incidence of ultraviolet light induced squamous cell epitheliomata in skin of the hairless mouse. Thesis, Graduate School, University of Minnesota, 1963.
3. Szabo, G.: The effect of carcinogens on melanocytes. Ann. N. Y. Acad. Sci., *100:* 269, 1963.
4. Della Porta, G., Rappaport, H., Saffiotti, U. and Shubik, P.: Induction of melanotic lesions during skin carcinogenesis in hamsters. Arch. Path. (Chicago), *61:* 305, 1956.
5. Ghadially, F. N. and Barker, J. F.: The histogenesis of experimentally induced melanotic tumours in the Syrian hamster (*cricetus auratus*). J. Path. Bact., *79:* 263, 1960.
6. Nakai, Takashi and Rappaport, Henry: A study of the histogenesis of experimental melanotic tumors resembling cellular blue nevi: The evidence in support of their neurogenic origin. Amer. J. Path., *43:* 175, 1963.
7. Lipschütz, B.: Untersuchungen über die experimentelle Pigmenterzeugung durch Teerpinselung von Mäusen. (Beitrag zur Kenntnis des experimentellen Teercarcinoms der Maus). Arch. f. Dermat. u. Syph., *147:* 161, 1924.
8. Burgoyne, F. H., Heston, W. E., Hartwell, J. L. and Stewart, H. L.: Cutaneous melanin production in mice following application of carcinogen 5,9,10-trimethyl-1,2-benzanthracene. J. Nat. Cancer Inst., *10:* 665, 1949.
9. Epstein, J. H. and Epstein, W. L.: A study of tumor types produced by ultraviolet light in hairless and hairy mice. J. Invest. Derm., *41:* 463, 1963.
10. Klaus, S. N. and Winkelmann, R. K.: Pigment activity in dermal nevi induced by ultraviolet light: An enzyme histochemical study. J. Invest. Derm., *44:* 276, 1965.