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Neural Differentiation in the OTT-6050 Mouse Teratoma: Enzymatic and Immunofluorescence Characterization of a Tumor Fraction Showing Melanogenesis in Neuroepithelial Cells

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Summary. A pigmented tumor fraction, designated IB-9, obtained following cellular dissociation and elutriation procedures applied to the solid transplants of the OTT-6050 mouse teratoma cell line, was characterized enzymatically and by immunofluorescence for the presence of tyrosinase and tyrosine hydroxylase (TH). Enzymatic assays of the pigmented tumors were compared with those obtained on non-pigmented teratoma-derived tumors, on pigmented tumors obtained from the mouse melanoma B16 line as a control for tyrosinase activity, and on whole brains of adult 129/J mice as a control for TH activity.

All the teratoma-derived tumors, including the IB-9 fraction, showed a predominance of TH over tyrosinase activity. The levels of TH activity appeared independent of the presence or the extent of melanin pigment. All pigmented teratoma-derived tumors showed low levels of tyrosinase activity.

On the basis of the enzymatic assays, the IB-9 tumors were divided into two groups: group I, which showed low enzyme activity, almost certainly entirely tyrosinase; and group II, in which the enzyme activity appeared largely due to TH, with presumably a very low background of tyrosinase activity. Immunofluorescence demonstrated the localization of TH activity to non-pigmented cells of the IB-9 fraction, whereas the pigmented cells showed absence of TH activity.

These findings, taken in conjunction with the presence by electron microscopy of premelanosomes and melanosomes, indicate that pigment formation associated with melanosomal differentiation in the neural cells of IB-9 with the histologic patterns of primitive CNS neuroepithelium results from tyrosinase activity only and is therefore unrelated to the metabolic pathways

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involved in catecholamine synthesis and degradation. It is suggested that, at this stage of differentiation and in this system, the expression of catecholamine synthesis via tyrosine hydroxylase in neuroepithelial cells, and of melanin pigment via tyrosinase, are probably mutually exclusive.

Key words: Melanin – Teratoma – Tyrosinase – Tyrosine hydroxylase – Immunofluorescence

Introduction

A companion report (VandenBerg et al. 1981) has described the production, following cellular dissociation and elutriation procedures applied to the solid transplants of the OTT-6050 mouse teratoma cell line, of a tumor fraction, labelled IB-9, which on subsequent subcapsular renal implantation in syngeneic hosts consistently showed the presence of melanin. The pigment described in that tumor fraction proved on electron microscopy to be melanosomal, a finding which is of interest in view of the fact that the neoplastic cells containing melanin showed the histologic pattern of primitive central nervous system (CNS) neuroepithelium.

This paper describes the enzymatic and immunofluorescence characterization of the IB-9 tumor fraction. The characterization was performed in an attempt to elucidate the probable biochemical pathways by which melanosomal differentiation might occur in primitive neoplastic neuroepithelial cells, and specifically the putative roles of two enzymes generally presumed to be involved in cellular melanogenesis: tyrosinase, which does not participate in catecholamine synthesis and is widely regarded as the primary enzyme in the synthesis of melanosomal melanin (Edelstein 1971; Swan 1974), and tyrosine hydroxylase (TH), which is a rate-limiting enzyme in catecholamine synthesis. For this purpose, determinations of the activities of the two enzymes were performed on fragments of the IB-9 tumor fraction, and the localization of TH activity was studied by immunofluorescence in the cells of the tumor, specifically in those cells in which melanin pigment was or was not demonstrable. The results suggest that the presence of melanosomal melanin in the primitive neuroepithelial component of the IB-9 fraction is apparently independent of the levels of TH activity in the tumor, and therefore presumably unrelated to catecholamine synthesis or degradation.

Materials

Mouse Teratomas. A pigmented OTT-6050 teratoma-derived tumor, designated IB-9, was produced by cell dissociation and elutriation and serially transplanted beneath the renal capsule as described elsewhere (VandenBerg et al. 1981).

The non-pigmented OTT-6050 teratoma-derived tumors included both an elutriation-produced tumor similar to IB-9 in the spectrum of neuroepithelial differentiation except that it lacked pigment, and embryoid body-produced solid tumors (VandenBerg et al. 1975). Both types of non-pigmented tumor were transplanted subcutaneously into the flanks of adult syngeneic females.

Murine Melanoma. As a positive control for tyrosinase-containing tumor cells, the P/51/502 HE line of B16 mouse melanoma was generously supplied by Dr. F. Hu and, after growing sufficient

numbers of cells in vitro (White et al. 1979), was transplanted into C57 BL/6 mice as previously described (White and Hu 1977).

Murine Adult Brains. As a positive control for TH-containing tissue, brains from adult 129/J females, 2–5 months old, removed in toto without the dura mater, were used.

Methods

Enzymatic Assays. Tyrosine hydroxylase (TH) activity was assayed by the modified method of Waymire et al. (1971). Tissues were homogenized in 5 volumes of 0.05 M Tris-acetate pH 6.0 buffer, containing 0.2% Triton X-100. The homogenates were centrifuged at 10,000 g for 10 min, and 25 µl of the supernatant was used for the assay. The assay mixture contained the following in 75 µl: potassium phosphate buffer, pH 6.0, 0.2 M; catalase, 7,000 units/ml; saturating sheep liver dihydropteridine reductase; nicotinamide-adenine dinucleotide-reduced, 1 mM; L-(1-^{14}C) tyrosine (58 mCi/mM), 13 µM; 6-methyl 5,6,7,8 tetrahydropterine, 2 mM. Following a 25 min incubation at 37° C, a mixture of pyridoxal phosphate (140 µm), a saturating amount of hog kidney decarboxylase, and 3-iodotyrosine (3 mM) was added, and the incubation was continued for another 30 min. $^{14}CO_2$ was trapped and counted according to the procedure of Ichiyama et al. (1970).

In order to differentiate between tyrosine hydroxylase (E.C. 1.14.16.2) and tyrosinase (E.C. 1.14.18.1) activities, $^{14}CO_2$ production was measured under the following conditions: 1) Addition of 3-iodotyrosine, an inhibitor of TH (Waymire et al. 1971); 2) Addition of thiourea or diethyldithiocarbamate, inhibitors of tyrosinase, and the latter reversible by supplemental Cu(II) (Pomerantz 1963); 3) Change in reaction pH to the optimum for TH, pH 6.0, and to the optimum for tyrosinase, pH 7.0 (Nagatsu et al. 1964) and 4) Addition of DL-DOPA, which inhibits TH and activates tyrosinase. Omisscion of 6-methyl 5,6,7,8 tetrahydropterine, a cofactor for TH, eliminated any enzymatic activity in all tissues, as detected by $^{14}CO_2$ production.

Preparation of Tyrosine Hydroxylase-Antiserum. Tyrosine hydroxylase was purified from rat pheochromocytoma according to the procedure of Edelman et al. (1981) with the exception that isoelectric focusing in polyacrylamide gels took the place of the gradient centrifugation. Polyacrylamide gel slices containing the tyrosine hydroxylase activity were homogenized with an equal volume of Freund's complete adjuvant and injected (50 µg per injection) into multiple subcutaneous sites on the back of New Zealand white rabbits. After 3 booster injections (2 weeks apart) the animals were bled by cardiac puncture and the cells removed by centrifugation. The antiserum cross-reacted with rat and bovine striatal tyrosine hydroxylase and, in addition, to extracts from the mouse neuroblastoma clone NIE-115 (provided by Dr. M. Nirenberg, NIH). Double immunodiffusion of crude extract against antiserum revealed a single arc of precipitation. Further properties of this antiserum will be described elsewhere (Raese et al. 1981).

Immunotitration. Tissues were prepared as described under enzymatic assays. 50 µl of the supernatants were incubated for 2 h at 30° C with different amounts of the antiserum against tyrosine hydroxylase. The teratoma-derived tumors and brain extracts were assayed at pH 6. The B16 melanoma was assayed at both pH 6 and pH 7. After incubation, the samples were centrifuged at 12,000 g for 5 min and aliquots of the supernatants were assayed for tyrosine hydroxylase activity.

Indirect Immunofluorescence. IB-9 renal transplants were frozen to −80° C and stored for approximately 1 week prior to transverse sectioning at −20° C. Sections were cut at 7 to 8 µ and placed on gelatin-coated slides for examination with the antiserum against tyrosine hydroxylase using an indirect immunofluorescence technique. They were then dried at room temperature for approximately 3 to 5 min followed by fixation in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on ice for ten minutes. This was followed by a rinse in 0.01 M phosphate buffer with 0.3% (v/v) Triton X-100. Sections were then covered with either immune or control serum diluted 1:20 and incubated for 1 h at 37° C. Following rinsing in the above PBS-Triton X-100 buffer, the sections were covered with FITC-labeled antirabbit IgG, diluted 1:40, and incubated for one hour at 37° C. Following rinsing in the PBS-Triton X-100 buffer, the sections were mounted with 33% glycerol in 0.01 M phosphate buffer (pH 7.4) and observed with a Zeiss photomicroscope III equipped with a IIIRS epifluorescent condenser and a 455–490 nm exciter filter, and a FT510 chromatic splitter and LP 520 barrier filter.

Results

The results of the enzymatic assays for tyrosine hydroxylase (TH) activity performed under standard conditions on the pigmented (IB-9) fraction of the OTT-6050 teratoma line, compared to those obtained in nonpigmented OTT-6050 teratoma-derived tumors, the B16 pigmented mouse melanoma line and adult 129/J whole mouse brain, are shown in Table 1. These assays, performed on 11 tumors of the IB-9 fraction, resulted in their separation into two groups based on their enzyme activity in standard conditions. Two tumors forming group I showed very low activity (25% above the blank); the nine other tumors, constituting group II, showed an enzyme activity amounting to approximately two-thirds of that present in the nonpigmented teratoma-derived tumors and approximately 20% of that present in the adult mouse whole brains.

The low level of apparent TH activity in the B16 mouse melanoma line is subsequently shown in Table 2 and in Fig. 1 to be due entirely to tyrosinase activity.

Table 2 shows the results from variations of the assay conditions in an attempt to differentiate the $^{14}CO_2$ produced by TH and tyrosinase activities. The combination of the TH inhibitor 3-iodotyrosine, the variation in pH, and the presence of DL-DOPA (which stimulates tyrosinase as a cofactor and inhibits TH) were the criteria permitting the clearest distinction between the two enzymatic activities, using the pigmented melanoma line as the standard for tyrosinase activity and the 129/J whole mouse brain as the standard for TH activity.

Although, as shown in Table 1, group I of the IB-9 tumors had very low TH activity, it could not be inhibited by the highest concentration of 3-iodotyrosine (which inhibited TH activity in whole brain extract). At the pH optimum for tyrosinase, the activity in group I of the IB-9 tumor was increased to 185%, reinforcing the suggestion that the low level of enzyme activity demonstrated was due to tyrosinase. However, 1 mmol DL-DOPA at pH 6 was not able to stimulate activity in that group.

Both group II of the IB-9 tumors and the nonpigmented teratoma-derived tumors showed predominantly TH activity, as detected by variations of the assay conditions. The results, using the tyrosinase pH optimum of 7.0 and

Table 1. Tyrosine hydroxylase activity in IB-9 as compared to nonpigmented teratoma-derived tumors, mouse melanoma and brain

IB-9 Group I (2 tumors)	IB-9 Group II (9 tumors)	Mouse non-pigmented teratoma-derived tumors (see Materials) (9 tumors)	Mouse B16 melanoma (6 tumors)	Adult 129/J whole brain (4 brains)
0.77 (±0.16)	6.22 (±1.14)	9.12 (±1.42)	1.15 (±0.283)	31.6 (±1.46)

Tyrosine hydroxylase activity, expressed as $nM^{14}CO_2/g/h$ (±standard error), was assayed under standard conditions. Samples run in duplicate

Table 2. Variation of assay conditions to differentiate tyrosine hydroxylase from tyrosinase activity (Values are expressed as a percentage of the tyrosine hydroxylase activity as assayed under standard conditions and shown in Table 1)

	Tyrosine hydroxylase inhibition	Tyrosinase inhibition		Tyrosinase stimulation		Tyrosine hydroxylase inhibition + tyrosinase stimulation	
	pH 6.0 + 3-iodo-tyrosine (0.7 μM) ((1.4 μM)) [7.0 μM] [[1.0 mM]]	pH 6.0 + 1 mM thiourea	pH 6.0 + 1 M diethyl-dithio-carbamate (+1 mM Cu^{+2})	pH 7.0	pH 7.0 + 1 mM DL-DOPA	pH 6.0 + 1 mM DL-DOPA	pH 7.0 + 1 mM DL-DOPA + 0.7 μM 3-iodo-tyrosinase
IB-9 Group I	[[111]]	–	–	185	–	80	–
IB-9 Group II	(47) ((49)) [23] [[9]]	105	23 (53)	68	21	40	20
Non-pigmented teratoma-derived tumors	(45) ((35)) [5] [[0]]	112	32 (72)	38	11	31	8
Melanoma line (B16)	(54) ((127)) [122] [[49]]	45	20 (690)	185	1,750	652	1,649
129/J whole brains	(40) ((30)) [0] [[0]]	108	30 (59)	23	4	31	3

the addition of the DL-DOPA cofactor, suggest a low background of tyrosinase activity in the IB-9 tumors.

Figure 1 illustrates the inhibition of TH activity as assayed under standard conditions following the addition of antiserum against TH to the reaction mixture. Both in the IB-9 tumors and in the nonpigmented teratoma-derived tumors, the TH activity was inhibited by the immune serum. This is in contrast to the lack of effect by the immune serum on the activity of the enzyme in the B16 melanoma line at both pH 6 and 7.

Results of TH Activity by Immunofluorescence. Localization of TH activity by *indirect immunofluorescence* on frozen sections of the IB-9 tumors showed activity in low numbers of single cells or in small clusters of cells in moderately cellular fields. Some of the positive cells showed broad cytoplasmic processes and were in cell populations adjacent to medullary-like epithelium (Fig. 2A

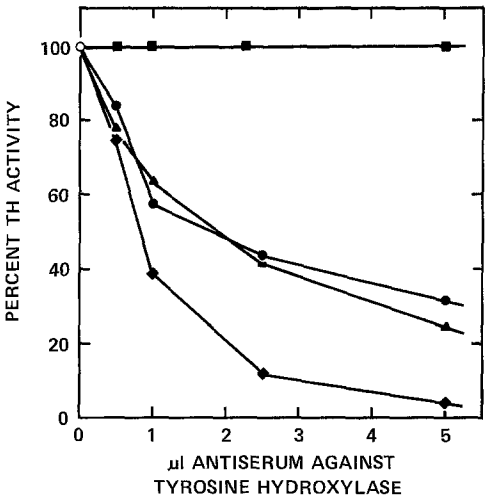

Fig. 1. Titration curves for mouse brain (◆), IB-9 Group II (●), non-pigmented teratoma-derived tumors (▲) and pigmented B16 melanoma (■). Tissue extracts were prepared as described under enzymatic assays, and incubated for 2 h at 30° C with 0, 1, 2, 3, 4 and 5 µl of antiserum. Tyrosine hydroxylase activity was measured as described under Methods. The percentage shown is comparative only to each respective tissue since absolute values differ significantly (see Table 1)

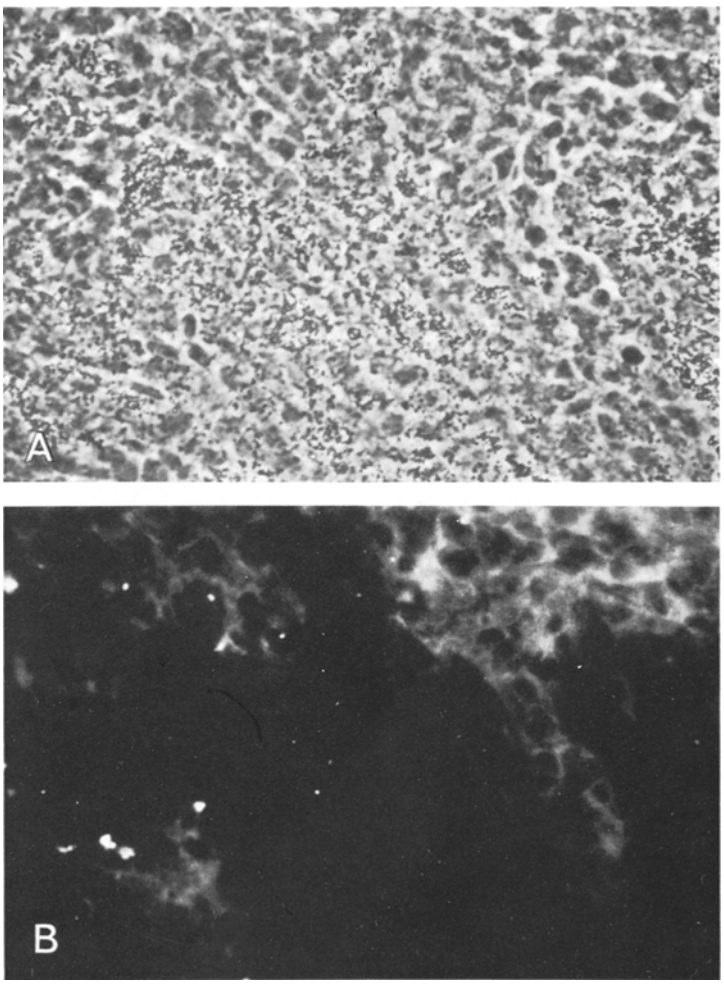

Fig. 2A, B. Frozen section (8 µm) processed for indirect immunofluorescence with TH antiserum (see Methods). **A** Phase contrast illumination demonstrates the well-defined clusters of non-pigmented cells and pigmented cells with distinct granules. ×435. **B** Immunofluorescence of the identical field as shown in A. The pigmented cell clusters show no labelling with TH antiserum. Only the non-pigmented cells are labelled. ×435

and B). Clusters of cells with abundant melanin granules were also located adjacent to and within the medullary-like epithelium (Fig. 2A). As a comparison of Fig. 2A and B makes clear, none of the cells with abundant pigment granules showed any immunofluorescence when exposed to antiserum against TH. This feature was a consistent finding in all the IB-9 tumors examined.

Discussion

The enzymatic assay of tyrosine hydroxylation activity, which is measured by quantitating the release of $^{14}CO_2$ from the decarboxylation of the hydroxylation product 3,4-dihydroxyphenylalanine (DOPA), does not permit a distinction between TH and tyrosinase activity unless the reaction conditions are modified. This is because tyrosinase shares with TH the ability to catalyze the hydroxylation of tyrosine. However, a distinction can be made to a variable extent after stimulation and/or inhibition of each enzyme in the conditions listed in Table 2. No single criterium of enzyme stimulation or inhibition is sufficient by itself to distinguish the two enzymes unequivocally, but meaningful results are obtained if (a) several criteria are used, and the results considered collectively; and (b) adequate materials are employed as standards for the activity of each enzyme in the different conditions of assay. The two conditions were fulfilled in the present study and permitted us to make a distinction in the tyrosine hydroxylation activity mediated by the two enzymes.

Variations in TH and tyrosinase enzyme activity were, however, to be expected between different tumor samples originating from the teratoma because of biologic variations in the material (Orenberg et al. 1976). These may reflect variations in the extent and degree of neuroepithelial differentiation in different fragments; the amount of pigmented and nonpigmented cellular elements in the samples; and observer selection, which in our material favored the more melanotic areas for biochemical assays because of their gross pigmentation. Paradoxically, cells containing large numbers of mature melanosomes have been reported to have low tyrosinase activity. This is presumably due to tyrosinase inactivation during the multi-step process of melanin formation (Seiji and Fitzpatrick 1961). An early step of melanin formation, the DOPA-tyrosinase reaction, can experimentally be uncoupled from the subsequent steps of melanin pigment formation, and tyrosinase has been shown to be inactivated in the course of melanogenesis as long as the DOPA-tyrosinase reaction continues to take place, even if no melanin pigment is formed (Seiji et al. 1978).

With the above qualifications, our data then suggest that in the TH assays performed under conditions in which tyrosinase was stimulated and TH inhibited, low levels of tyrosinase activity were present in groups I and II of the IB-9 pigmented tumor fraction. Although the low total activity in the samples makes the results difficult to interpret, the data in Table 2 suggest that in group I the enzyme activity present at a low level was almost certainly entirely tyrosinase. The only inconsistency in this conclusion (i.e., the inability of 1 mM DL-DOPA to stimulate this low activity) could partly be explained by the inhibitory effect of the lower pH. Even the 1 mM DOPA stimulation of tyrosinase in the B16 melanoma shows, at pH 6, only 37% of the stimulation present

at pH 7. By contrast, the enzyme activity in group II was apparently largely due to TH activity, with, presumably, a low background of tyrosinase activity as well.

The difference in the antiserum inhibition curves between the teratoma-derived tumors and the 129/J brain (Fig. 1) most likely reflected two factors. The first was the significant difference in absolute TH activity between the teratoma-derived tumors and the brain, as shown in Table 1. Thus, a decrease to approximately 30% in the tumors represented about the same level of activity as did 9% in the brain tissues. The second factor may have been that the TH in the neoplastic neural cells of the teratoma was structurally slightly different from that in the brain.

Both the enzymatic assays (Table 2) and the antibody inhibition studies (Fig. 1) therefore indicate that TH predominated over tyrosinase activity in all the teratoma-derived tumors. The data in Table 1 and our previous work on subcutaneously transplanted nonpigmented OTT-6050 teratomas (Orenberg et al. 1976) show that the level of TH activity in the various tumor samples is independent of the presence or the extent of melanin pigment. In fact, as shown in Table 1, nonpigmented teratoma-derived tumors were richer in TH activity than the tumors in either group of the IB-9 tumor fraction.

The absence of any direct relationship between pigment formation and TH was confirmed by the localization, by immunofluorescence, of TH to cells of the IB-9 fraction that did not contain the pigment, whereas the pigmented cells showed no TH. When these findings are taken in conjunction with the presence by electron microscopy of melanosomes and premelanosomes in the neuroepithelial areas of the IB-9 tumor fraction (VandenBerg et al. 1981), they become entirely consistent with the view that melanosomal differentiation in the primitive central neuroepithelial cells of the IB-9 fraction is to be accounted for by tyrosinase activity only, despite the paradoxical observation that only very low tyrosinase activity could be demonstrated in the pigmented tumors and that TH activity predominated over tyrosinase activity in all the teratoma-derived neoplasms. We conclude therefore that in this system melanosomal melanin formation as an expression of cellular differentiation in primitive neoplastic neuroepithelial cells proceeds through a synthetic pathway presumably similar to that which causes the formation of melanosomal melanin in neural-crest derived cells, and is unrelated to metabolic pathways involved in catecholamine synthesis and degradation. At this stage of differentiation, the expression of catecholamine synthesis via tyrosine hydroxylase in neuroepithelial cells, and of melanin pigment via tyrosinase, are probably mutually exclusive.

The melanotic neuroectodermal tumor of infancy provides an analogous example of a human neoplasm in which melanosomal melanin and catecholamine synthesis seem paradoxically associated. These tumors, as shown by electron microscopy (Dehner et al. 1979; Hayward et al. 1969; Ishikawa et al. 1967; Misugi et al. 1965; Neustein 1967; Nozicka and Spacek 1978; Palacios 1980; Taira et al. 1978; Zajtchuk et al. 1978), are composed of at least two distinct cell types: pigmented cells containing melanosomal melanin, and a nonpigmented cell type often showing neuroblastic or, less frequently, undifferentiated features. No dense-core vesicles, presumed to be associated with catecholamines, have been described within the melanotic tumor cells. In the reports (Dehner et al. 1976, 1979) describing a case with elevated urinary catecholamine metabolites (vanillymandellic acid and homovanillic acid), TH, dopamine-β-hydroxylase and dense-core vesicles were strictly

localized to the neuroblastic cells. Thus, in a human tumor derived from neuroectodermal precursors, melanosomal melanin and catecholamine synthesis also seem to be mutually exclusive within the same cells.

The interest of our findings also lies in the additional information they provide on the relationship, increasingly documented but still poorly understood, between melanogenesis and human neurogenic tumors (Russell and Rubinstein 1977). Melanin formation has been reported in various neoplasms of central neuroepithelial origin (Boesel et al. 1978; Boesel and Suhan 1979; Hahn et al. 1976a; Herrick and Rubinstein 1979; Lana-Peixoto et al. 1977; McCloskey et al. 1976; Russell and Rubinstein 1977; Sung et al. 1973). However, it occurs more often in neurogenic tumors presumed to be derived from the neural crest (Hahn et al. 1976b; Mennemeyer et al. 1979; Mullins 1980; Palacios 1980; Spence et al. 1976). In several examples of either derivation (Boesel et al. 1978; McCloskey et al. 1976; Mennemeyer et al. 1979; Palacios 1980; Spence et al. 1976), the melanin has been shown to be melanosomal. In others (Boesel and Suhan 1979; Hahn et al. 1976b; Mullins 1980), melanin has been shown to consist in, or be closely related to, neuromelanin, the fine structural features of which are quite distinct from those of melanosomal melanin (Bagnara et al. 1979; Barden 1975; Marsden 1969). Neuromelanin in these melanotic neoplasms has been interpreted as a byproduct of catecholamine metabolism either via lysosomal enzyme systems or as a nonenzymatic phenomenon resulting from the pseudoperoxidation of lipofuscin (Barden 1969, 1975; Boesel and Suhan 1979; Mann and Yates 1974) or from the auto-oxidation of catecholamines by molecular oxygen (Graham 1978, 1979). It is increasingly apparent from published reports that, as the experimental tumor in this study illustrates, pigmented neurogenic neoplasms may contain either melanosomal melanin or a pigment identified as neuromelanin or a related substance, seemingly regardless of the cytogenesis of the pigment-bearing cells.

The pigment found in association with the primitive neuroepithelial cells in our case was entirely melanosomal and never showed the morphologic features of neuromelanin. Moreover, melanosomes and premelanosomes were never associated, in all the samples we examined, with the presence of either dense-core vesicles or autophagic vacuoles in the same cells. These observations therefore preclude the consideration of neuromelanin and its complex potential interrelationships with the biogenic amines that have been the object of other studies concerned with the appearance of melanin in the CNS (Ule et al. 1979).

Regarding the widely endorsed association of melanosomal melanin with cells originating from the neural crest, we are, admittedly, unable to exclude the theoretical possibility that those primitive neuroepithelial cells in the IB-9 tumor in which melanogenesis was demonstrable would, in a subsequent stage of their development, have differentiated into neural crest elements. However, we have no evidence to support such a speculation. We failed to find any topographic relationship between melanin-bearing cells and differentiated cells known to originate from the neural crest. We therefore think it reasonable, on the collective basis of the morphologic and biochemical data we have reported, to interpret the melanin-containing cells as evidence that neoplastic neuroepithelium of the CNS type is capable of melanosomal melanin formation. Such a conclusion is supported by the demonstration of melanosomal melanin

in a human cerebellar medulloblastoma (Boesel et al. 1978), a tumor whose central neuroepithelial origin is not in doubt.

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