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Neural Differentiation in the OTT-6050 Mouse Teratoma

Production of a Tumor Fraction Showing Melanogenesis in Neuroepithelial Cells After Centrifugal Elutriation

Scott R. VandenBerg, J. Ronald Hess, Mary M. Herman, Stephen J. DeArmond, Meredith Halks-Miller, and Lucien J. Rubinstein

Department of Pathology (Neuropathology), Stanford University School of Medicine, Stanford, California 94305, USA

Summary. Dissociation and elutriation procedures were applied to the OTT-6050 mouse teratoma line carried in subcutaneous implants in 129/J mice in order to enrich the differentiating neuroepithelial cells. Subsequent renal subcapsular implantation of one of the resultant cell fractions (IB-9) in syngeneic mice led to the constant production of macroscopically pigmented tumors which, in addition to undifferentiated stem cells, contained primitive neuroepithelial populations composed of medullary epithelium, neuroblasts, and numerous ependymoblastic rosettes. Melanin pigment, confirmed by the presence of melanosomes and premelanosomes, was found in medullary epithelium and other primitive neural cells. The tumors preserved their characteristics through 65 sequential transplants over a period of $5^1/_3$ years. The pigment was maintained in vitro for up to 3 months in an organ culture system.

Subcutaneous or intracerebral transplantation of the renal tumors of the IB-9 fraction accentuated the capacity of these primitive cells towards further neuroepithelial differentiation into mature synapse-forming neurons, and was associated with a decrease in primitive neuroepithelium and an absence or a marked decrease of melanin. Return of the tumor to the kidney resulted in the reappearance of melanin after one to three passages, again associated with the presence of primitive neuroepithelium.

The recognition of melanin pigment in the OTT-6050 mouse teratoma transplants could be a useful marker for the successful selection of primitive neuroepithelial cell populations in this experimental tumor system. These populations may help to study the relationship between melanin production and certain types of primitive neuroectodermal tumors in man.

Offprint requests to: Dr. Mary M. Herman, Division of Neuropathology, Department of Pathology, University of Virginia School of Medicine, Charlottesville, Virginia 22908, USA

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Introduction

A previous report (VandenBerg et al. 1981) described the production, following the application of cellular dissociation and centrifugal elutriation to the solid transplants of the OTT-6050 mouse teratoma line, of a tumor fraction, designated IB-21, which was restricted to stem cells and neural cells only. The present report describes the morphologic features of another tumor fraction, IB-9, resulting from the subsequent renal subcapsular implantation of another cell fraction obtained by centrifugal elutriation (see Materials and Methods in VandenBerg et al. 1981, and Discussion below). In sequential renal passages, this fraction demonstrated melanosomal melanin in association with a predominantly primitive neuroepithelial cell population. Subsequent subcutaneous or intracerebral transplantation of the intrarenally maintained tumor fraction was associated with a marked decrease or absence of melanin, and return of the tumor to the kidney resulted in its reappearance. These findings are relevant to the association, increasingly documented but still poorly understood, that appears to exist between melanogenesis and certain types of human central neuroepithelial neoplasms.

Materials and Methods

Tumor Line and Animals, Tumor Dissociation, Centrifugal Elutriation, and Implantations as described in VandenBerg et al. (1981).

Transplantation of Elutriation-Derived Tumors. Tumors arising from the elutriation-derived implantations were serially transplanted either beneath the renal capsule, subcutaneously in the flank, or intracerebrally. The numbers of transplants according to site are summarized in Table 1. Methods and ages, sex and numbers of recipients used per set were similar to those previously described (VandenBerg et al. 1981); almost all recipients developed tumors.

Table 1. IB-9 transplantations

Transplantation site	Sets[a] of animals implanted	Number of serial transplantations per set	Source of tumor
Renal	1	65	Subcutaneous, OTT-6050
Subcutaneous	3	7, 11, 12	Renal, IB-9
Intracerebral	3	20, 7, 7	Renal, IB-9
Intracerebral	6	None [Primary implants only]	Renal, IB-9
Renal reimplant	5	6 for all	Intracerebral, IB-9
Renal reimplant	2	7, 5	Subcutaneous, IB-9
Intraperitoneal	3	3, 3, 3	Renal, IB-9 (dissociated)

[a] Each set was composed of 2–6 recipient animals implanted from a separate donor at the same transplant generation. The sets differed according to the transplant generation from which the transplanted tumors were derived

A total of 65 sequential *renal* transplants were performed over a period of $5^1/_3$ years; pigmented grey translucent fragments were always selected for implantation. Three sets of animals were implanted *subcutaneously*, using similarly pigmented fragments from the 42nd, 43rd, and 44th generation of the IB-9 renal transplants to initiate serial transplants. Three sets of animals were implanted *intracerebrally* for serial transplant studies, using similarly pigmented fragments from the 34th, 42nd and 43rd generation of the IB-9 renal transplants as starting material. In addition, six sets of *single-passaged intracerebral* implantations of IB-9 were performed, using similarly pigmented fragments from the 25th, 26th, 28th, 29th, 30th or 33rd generation of the IB-9 renal tumors, on a total of 28 recipients (2 from the 25th generation, 6 from the 26th, 3 from the 28th, 6 from the 29th, 6 from the 30th, and 5 from the 33rd respectively); only 3 did not develop a tumor. *Ascitic* tumors, originating at the 36th, 41st, and 43rd generations of the solid IB-9 renal transplants, were obtained by dissociating the solid tumors as for IB-21 (VandenBerg et al. 1981).

The following experiments were performed (see also Table 1) to test the return of the melanin pigment, which disappeared in extrarenal sites after 1–3 passages. In one experiment, tumors from two different renal generations were carried subcutaneously in the flank for 2 and 3 passages and were then returned to the kidney using small pieces as for the other renal implants; they were carried in the kidney for 7 and 5 passages respectively. In another experiment, tumors grown intracerebrally for 1, 2, 12, 13 or 18 passages were also returned to the kidney, where they were carried for 6 passages; the intracerebral tumors were initiated with renal tumors from 3 different generations. In both experiments, two recipients were used for each tumor passage.

Morphology and Immunohistochemistry. These were similar to those for IB-21. In addition, for melanin histochemistry the following procedures were used on paraffin-embedded sections:

1) 1.7% (w/v) $AgNO_3$ acetate-buffered (0.2 M) solution, pH 4.0 at 22° C for 1 min 15 s to 2 min 15 s staining (Lillie 1965);

2) Lillie's diamine silver technique (Pearse 1972); and

3) Masson-Fontana ammoniacal silver nitrate technique (Pearse 1972).

Positive controls were paraffin-embedded human skin and substantia nigra. For bleaching of the melanin granules, 10% (v/v) H_2O_2 was used at 22° C for 12 h.

In vitro Studies in an Organ Culture System. Macroscopically pigmented grey translucent tissue fragments were explanted from serial subcapsular renal transplants of IB-9 onto sponge foam (Ferrosan, Copenhagen) by methods previously described (Rubinstein et al. 1973). The nutrient medium (which contained 50 units/ml of penicillin G) and the conditions of incubation were similar to those used previously (VandenBerg et al. 1976). The medium was freshly thawed and replaced daily. Minimal levels of room and fluorescent illumination were used while the cultures were out of the incubator. Methods for light (Rubinstein et al. 1973) and electron microscopy (Herman et al. 1975) have been detailed.

Results

Morphology. IB-9 was developed from implanting, in the kidney of one animal, the fraction obtained at a flow rate of 12 ml/min during elutriation at 2,000 RPM. The *renal implants* had the following features. Macroscopically they were grey, soft and focally pigmented. They extensively invaded the kidney and contained foci of hemorrhage and necrosis. Distant metastases were not found.

Microscopically, the tumors contained a predominance of stem cells and primitive neuroepithelial cells, a feature that was maintained throughout the 65 sequential implants. The stem cells were morphologically similar to those described in the parent OTT-6050 tumor and in IB-21. The abundant neuroepi-

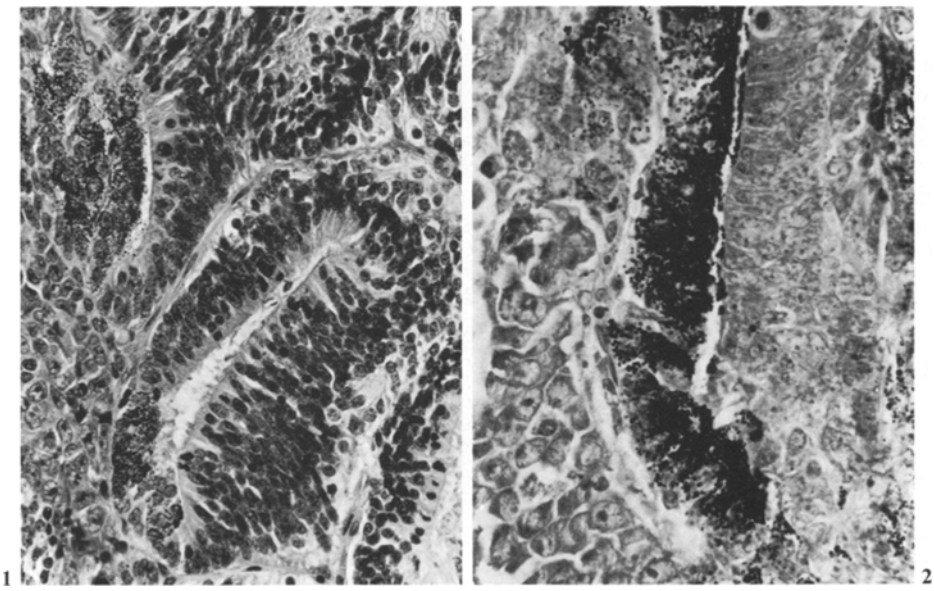

Fig. 1. Renal tumor. 36th passage. Melanin pigment (upper left) in primitive medulloepithelial rosettes. Note the apical mitoses. H and E ×300

Fig. 2. Renal tumor, 6th passage. Dense melanin pigment in primitive neural cells composing a medulloepithelial rosette. Lillie's diamine silver with neutral red counterstain. ×415
Figs. reduced approx. 15%

thelium demonstrated primitive medullary rosettes (Figs. 1 and 2) containing many mitotic figures (Fig. 1). Significant amounts of melanin were found throughout all serial passages, accompanied by some periodic fluctuations. It was virtually always associated with primitive neuroepithelial cells, especially in areas of medullary epithelium (Figs. 1 and 2). Its biochemical nature was confirmed by Lillie's diamine silver method (Fig. 2) and by the Masson-Fontana method; it disappeared following bleaching with H_2O_2.

Ependymoblastic rosettes (Fig. 3) as well as transitional forms (Herman et al. 1975) were abundant. They often merged at their periphery with fields of small neuroblasts. Their fine structural appearance, as demonstrated in Figs. 4 and 5 from the first intracerebral passage of a renal tumor, confirmed the presence of early ependymal features.

Electron microscopic evidence of early neuroepithelial differentiation from stem cells consisted of nuclear elongation, delicate clumping and margination of the nuclear chromatin, and cytoplasmic polarization (Figs. 6 and 7). Numerous small immature apical junctions were present in the primitive medullary rosettes (Fig. 6). Other similar-appearing cells, not forming a luminal epithelium, had elongated interdigitating processes (Fig. 8). Junctional complexes were often found between their apices (Fig. 9). Melanosomes and premelanosomes were prominent in these cells (Figs. 9 and 10). They were also often present in cells forming epithelial ribbons (Fig. 7), thus concurring with the light microscopy findings (Fig. 2). Melanosomes and premelanosomes were identified in culture

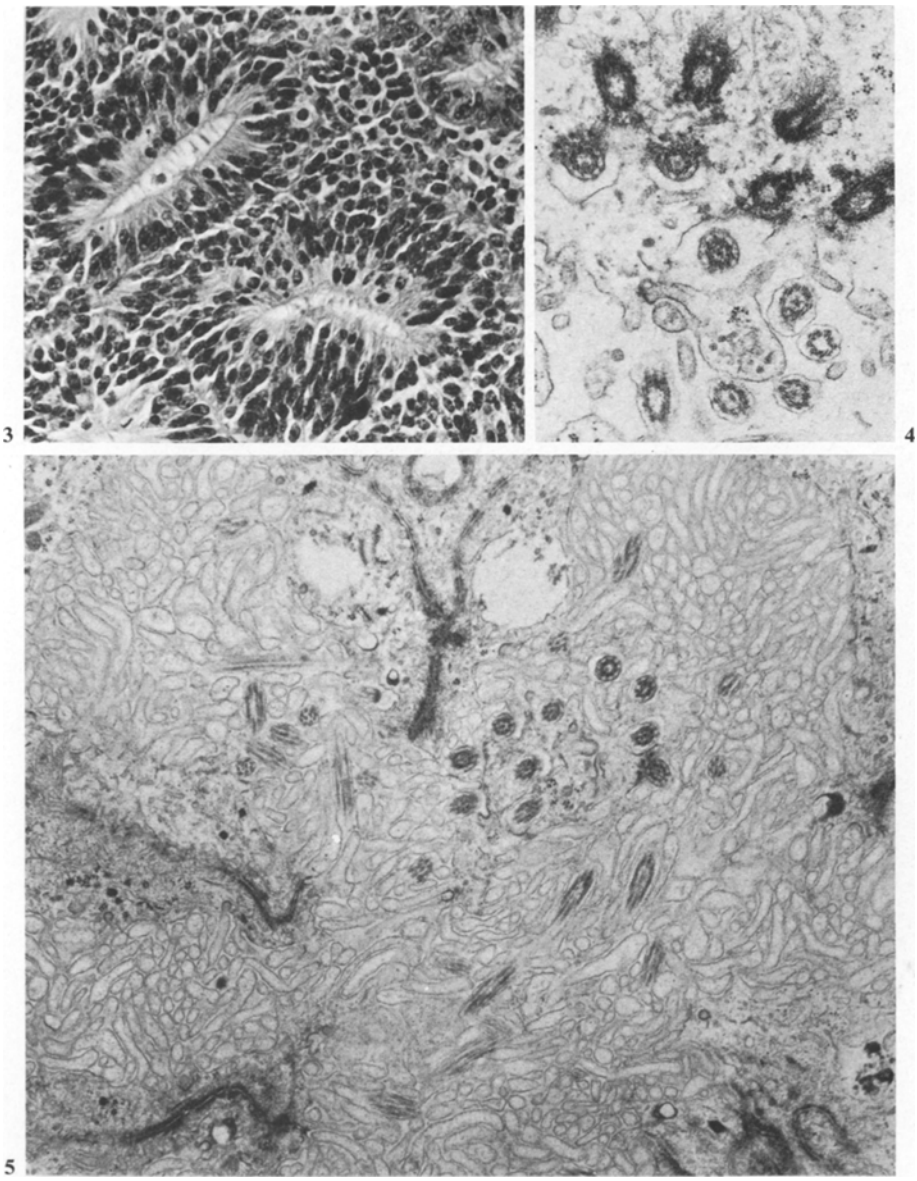

Fig. 3. Renal tumor, 38th passage. Three ependymoblastic rosettes with apical mitosis and cilia; the rosettes are surrounded by primitive neural cells. H and E × 300

Fig. 4. Intracerebral tumor, first passage. Electron microscopic counterpart of the lumenal surface of an ependymoblastic rosette, showing numerous basal centrioles and cilia. Uranyl acetate-lead citrate stained. × 19,600

Fig. 5. Same animal as Fig. 4, different field. Apical surface of several adjacent cells composing an ependymoblastic rosette. There are several lengthy intercellular junctions and numerous cilia and microvilli; similarly stained. × 11,400

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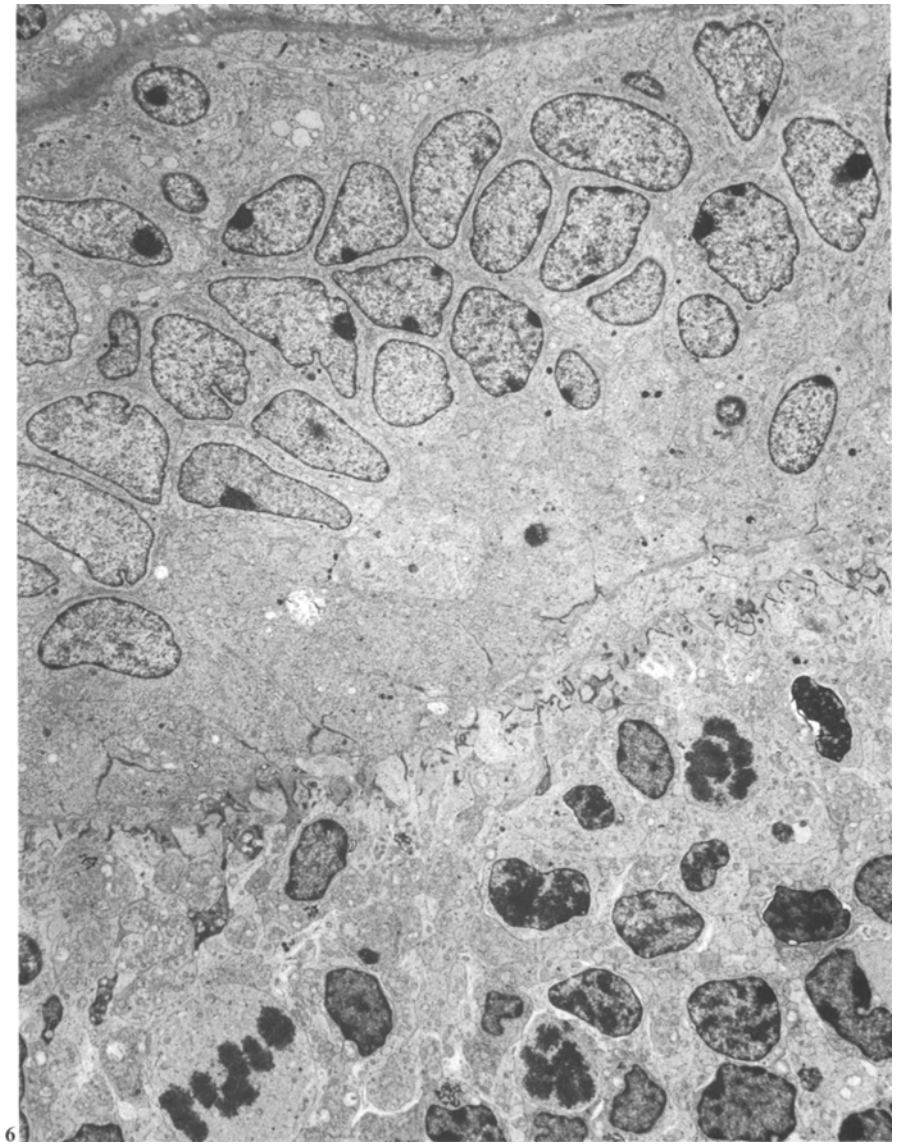


Fig. 6. Renal tumor, 10th passage. Medulloepithelial rosette with numerous apical junctions (lower center). Primitive neuroepithelial cells with elongated nuclei and variably polarized cytoplasm (top) are apposed to smaller maturing neural cells (bottom) with several mitotic figures. Larger cells (top) show numerous convoluted cytoplasmic tags (similar to cells in Fig. 8) where they abut on the external limiting membrane. ×2,100

up to 90 days in vitro (Fig. 10 inset and Fig. 11). Melanin pigment was not found in ependymoblasts or in maturing neuroblasts or glia.

In sequential intrarenal transplants the neural population, which remained predominantly primitive, occasionally contained small foci with a neuropil-like matrix. A small number of mature neurons were present (Fig. 12). No myelin

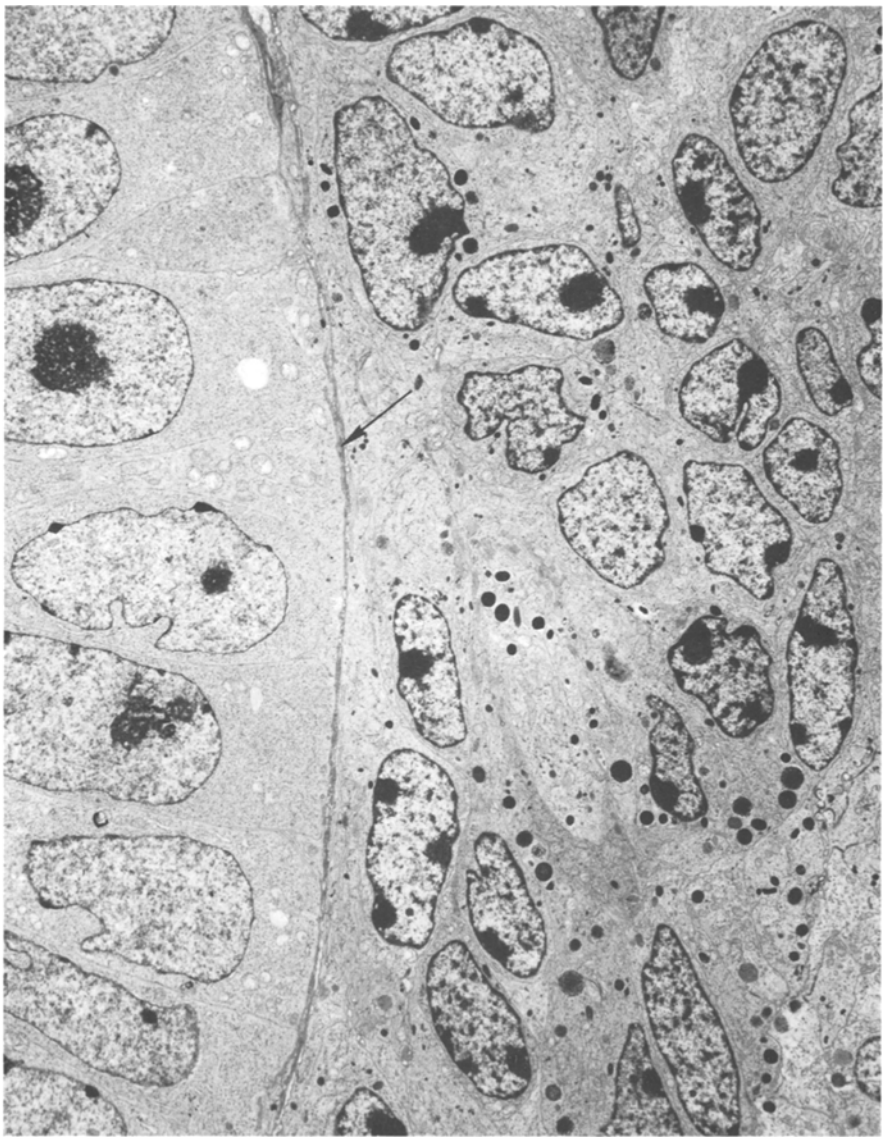


Fig. 7. Same animal as Fig. 6, different field. Primitive neuroepithelial cells (left) with large nuclei and polarized cytoplasm abutting on an external limiting membrane (arrow). Melanin-containing smaller cells (center and right), many with infolded cytoplasm as in Fig. 8, whose apices show numerous junctions (right lower corner). The apical junctions are similar to those in Fig. 9. ×2,400

was identified by conventional light microscopy, by immunoperoxidase staining for myelin basic protein, or by electron microscopy.

Transplantation of these tumors to *subcutaneous* and *intracerebral sites* resulted in a markedly higher proportion of mature neurons (Fig. 13) and astrocytes, and a much lower proportion of medullary and ependymoblastic (Figs. 4

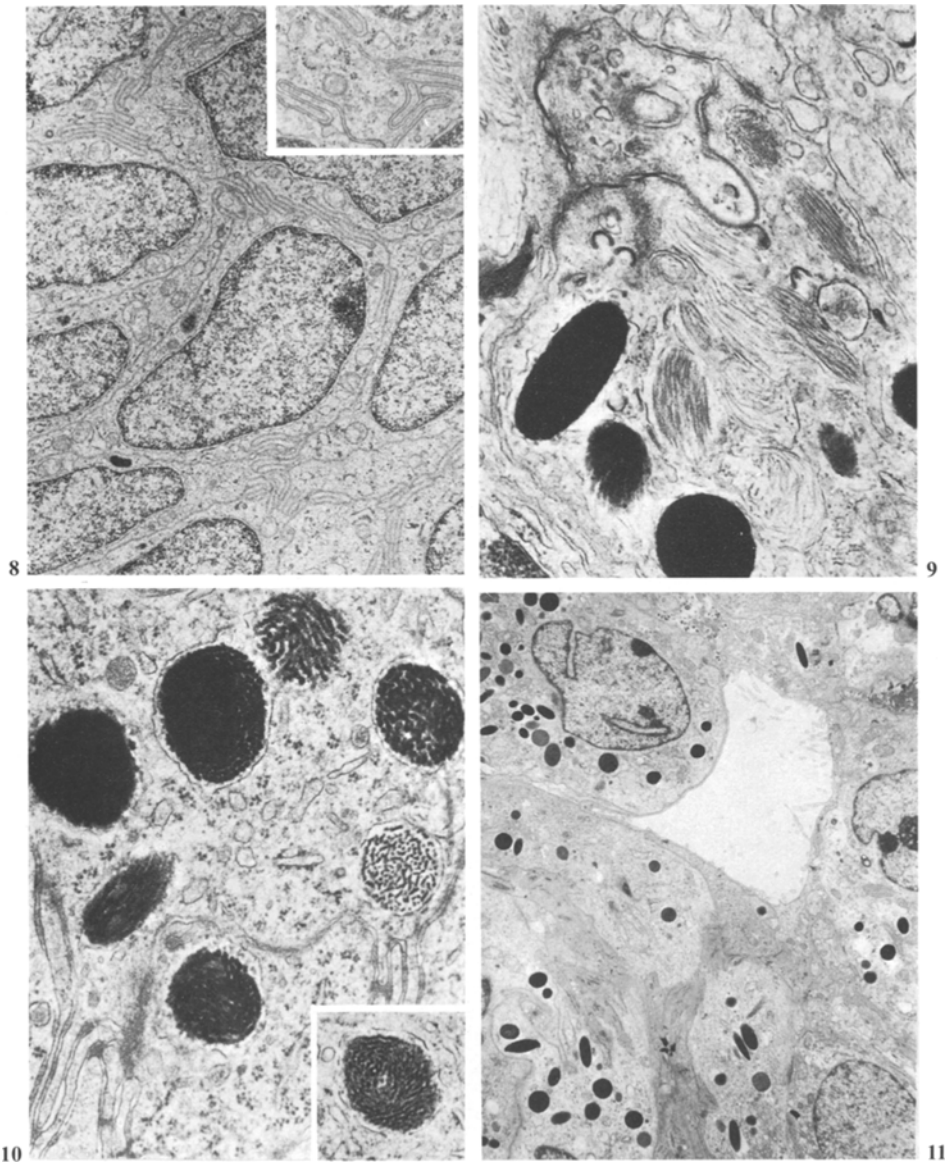


Fig. 8. Same animal. Primitive neuroepithelial cells with numerous cytoplasmic infoldings. ×7,000. Inset is a detail of the infoldings. ×16,400

Fig. 9. Same animal. Melanosomes and premelanosomes in apical cytoplasm, which is joined by several junctional complexes. ×25,200

Fig. 10. Renal tumor, 5th passage. Cells similar to those in Figs. 8 and 9, containing melanosomes and premelanosomes. ×27,800. Inset – Detail of melanosome at 90 days in vitro. ×24,500

Fig. 11. 90 days in vitro (tumor obtained from the 9th renal passage) showing numerous melanin-bearing cells surrounding a lumen. ×2,800

Figs. reduced 25%

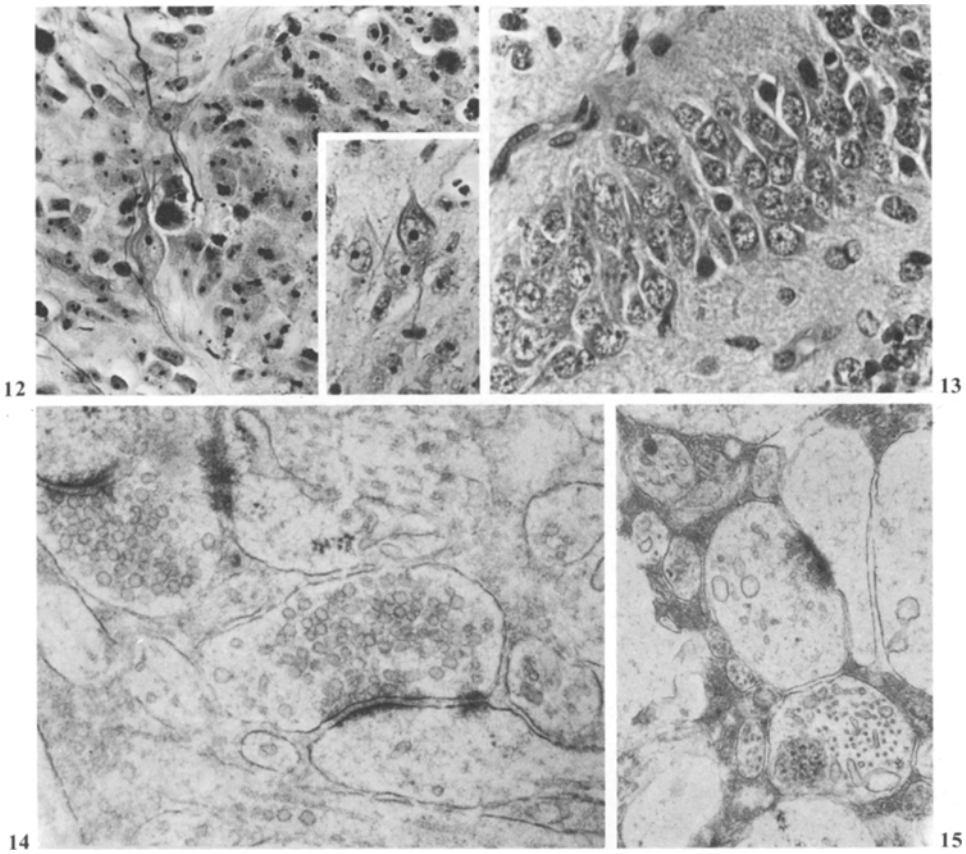


Fig. 12. Renal tumor, 38th passage. Focus of neuroblasts and (center) a neuron and axon. Bielschowsky's silver preparation for axons, frozen section. ×425. Inset – Intracerebral tumor, first passage, mature neuron. Bielschowsky's method. ×480

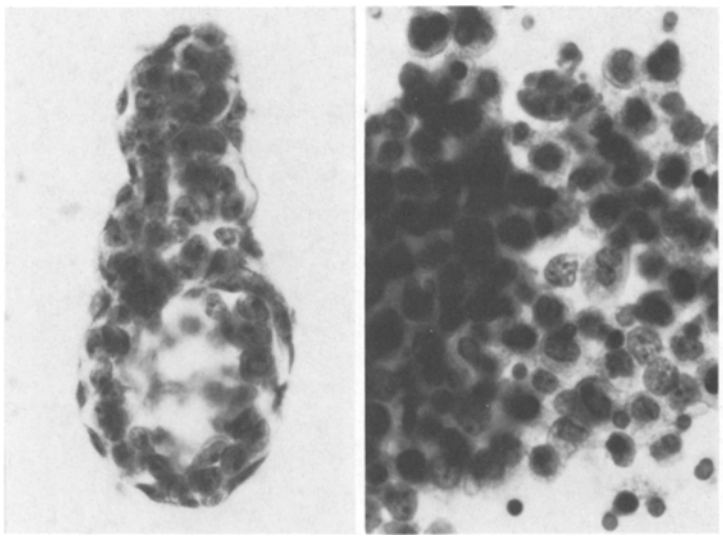
Fig. 13. Subcutaneous tumor, 8th passage. Compact array of mature neurons and adjacent neuropil. H and E ×450

Fig. 14. Intracerebral tumor, first passage, a different series of animals than Fig. 13. Two well-developed synapses. ×40,500

Fig. 15. Subcutaneous tumors, 8th passage. A synapse and several neurites of various diameters. ×21,500

Figs. reduced approx. 20%

and 5) rosettes. Rosettes were frequent only in the early intracerebral passages. Features of neuronal maturation included the development of neurites (Figs. 12 inset, 14 and 15) and of well-developed synapses (Figs. 14 and 15) with clear-centered and dense-core vesicles. Myelin was absent. Astrocytes, positive for GFA-protein, were similar to those illustrated in IB-21 (VandenBerg et al. 1981). Proximity of the transplants to the brain or to the subcutaneous space did not appear to have any effect on the degree of neural differentiation. There were no consistent differences between the superficial and the more deeply placed intracerebral tumors, or between the single-passaged and the sequentially-passaged transplants.



16 17

Fig. 16. Typical embryoid body (EB) obtained from the dissociation of a solid OTT-6050 tumor implant (81st intraperitoneal passage of EB's in our laboratory) which grew along the needle track following intraperitoneal inoculation of EB's. 5 μ filtered preparation, H and E ×370

Fig. 17. Disorganized cell clusters obtained from the dissociation of IB-9 at the 44th intrarenal passage. Prepared and stained as Fig. 16. ×480

In the extrarenal sites melanin-containing cells were considerably decreased after the second transplantation. Pigment was reduced to very rare and tiny macroscopically-visible spots, and to scanty microscopic foci. Melanosomal organelles were not encountered in the tissue surveyed by electron microscopy. This marked decrease, or loss, of melanin correlated with the great decrease in the number of primitive neural rosettes.

Return of the tumors to the renal subcapsular site resulted after two or more sequential passages in the return of a higher proportion of primitive neural rosettes. Grossly detectable melanotic foci increased to amounts often approximating those in the tumors exclusively transplanted in the kidney. Return of the pigment occurred after as many as 18 extrarenal passages.

Regardless of the site of implantation, there was a consistent reduction of non-neural elements when compared to the solid parent OTT-6050 tumor. Low proportions of non-neural elements, including endodermal epithelium, cartilage, primitive mesenchyme, rhabdomyoblasts and squamous pearls continued, however, to be present in some of the tumors. Mesenchymal elements were more conspicuous in the renal and subcutaneous transplants than in the intracerebral transplants.

Ascitic Conversion

Unorganized cell clusters, similar to those in IB-21, were found in the smears and filtrates. They were strikingly different from the well-organized embryoid

bodies obtained when the parent OTT-6050 tumor was dissociated and processed by the same methods (Fig. 16). The unstructured cell clusters obtained from IB-9 by filtering the ascitic fluid (Fig. 17) occasionally contained pigment after the first intraperitoneal passage. The solid intraperitoneal implants of IB-9 and IB-21 obtained concurrently with the ascitic cell clusters resembled the tumors before their conversion to the ascitic form: in IB-9 the implants maintained their predominant neuroepithelial components (including neural rosettes) and occasional melanotic foli, whereas in IB-21 the implants were restricted to stem cells and neural cells. In the parent OTT-6050 tumors the solid implants showed the full range of teratomatous elements, as previously described (VandenBerg et al. 1975).

Discussion

Centrifugal elutriation of dissociated OTT-6050 teratomas produced two different transplantable tumors from separate fractions of tumor cells (VandenBerg et al. 1981). The fraction described in this report, IB-9, differed from the original OTT-6050 tumor by its morphologic characteristics, maintained over more than five years through 65 sequential renal subcapsular transplants, and by its inability to form ascitic embryoid bodies. It differed from the previously described IB-21 tumor fraction (VandenBerg et al. 1981) by: 1) a different spectrum of neuroepithelial differentiation, 2) the development of melanin in areas of primitive central neuroepithelium, and 3) the persistence of low numbers of differentiated non-neural cells.

IB-9 and IB-21 were derived from cell populations that were similar in sedimentation velocity, but separated at different rotor speeds. The 3,000 RPM rotor speed was used to achieve a higher resolution of sedimentation velocity per ml/min flow. In terms of sedimentation velocities at $1 \times g$, the cells of IB-9 had a range of 0.13 to 0.14 mm/min, and those of IB-21 had a range of 0.08 to slightly less than 0.14 mm/min. Most of the cell populations in IB-9 therefore tended to sediment like the fastest component of IB-21. It is unclear whether the higher proportion of these cells in IB-9 accounted for the melanin-containing cell population or for the teratomatous components. The overlap between the fractions was considerable, especially with respect to the faster sedimenting cells. There was no precise way to demonstrate any relationship between sedimentation velocity and the cell types obtained.

The significant amount of melanin present while IB-9 was serially transplanted in the kidney, and not in subcutaneous or intracerebral sites, suggests that melanogenesis may partly be dependent on the capacity of the renal environment to maintain a necessary number of primitive neuroepithelial cells. Renal subcapsular implantation produced tumors with extensive populations of primitive neural cells in both the IB-9 and IB-21 tumors (VandenBerg et al. 1981). The concomitant reduction of primitive neural cell populations and the disappearance of melanotic cells in the subcutaneous or intracerebral sites is consistent with this hypothesis.

Maintenance of any primitive neural cell population by the renal environment, however, is not by itself sufficient for melanin expression, as serial renal

transplantation for two years failed to preserve or augment the rare microscopic pigmented foci initially present in IB-21 (VandenBerg et al. 1981). The regulation of melanin biosynthesis is complex (Pawelek et al. 1980). Adenosine 3′,5′-monophosphate (cAMP) has been implicated as the second messenger in tyrosinase stimulation (Fuller and Hadley 1979; White et al. 1979), and dibutyryl-cAMP has been shown to increase in vitro melanogenesis in a human melanotic neuroectodermal tumor (Dehner et al. 1979). Another presumptive effect of cAMP may be the prevention of melanosomal degradation (Takeuchi and Kajishima 1976). The kidney has relatively high cAMP levels, as shown in the rat, but so also has the brain (Kimura et al. 1974). A simple mechanism based solely on tissue cAMP is therefore unlikely. Renal tissue shows approximately 170% of the guanosine 3′,5′-monophosphate (cGMP) level of the cerebrum (Kimura et al. 1974). However, while cGMP has been implicated in other regulatory systems (Greengard 1978), its regulatory role in melanogenesis is unknown. The renal parenchyma is also capable of synthesizing several prostaglandins, PGA_2, PGE_2, and $PGF_2\alpha$ (Lee and Attallah 1975), but it is not known whether these substances play any role in neurogenesis and, particularly, melanogenesis.

Melanogenesis in primitive neuroepithelium associated with various stages of melanosomal maturation was maintained in vitro for approximately three months in the absence of renal tissue. This is consistent with the observation, in embryonic chick retinal pigment epithelium (Israel et al. 1980), that different culture media may significantly affect melanogenesis: modified Eagle's medium (MEM) has been shown to promote melanogenesis with the appearance of membrane binding sites for retinol and retinoic acid. The nutrient medium in the present study was similar to the MEM formula of basic salts and essential amino acids. In addition, it contained other possible modulating factors such as unfiltered fetal calf serum and hydrocortisone (Abramowitz and Chavin 1979). Various nutritional factors suspected to favor embryonic melanogensis in vitro could therefore play a role in the renal effect on IB-9.

Melanin developing in pre-melanosomes and associated with melanosomes occurs in the course of melanocyte differentiation from the neural crest. It also occurs, in central neurocytogenesis, in the course of retinal differentiation from a neural tube-ependymal analogue (Mund and Rodrigues 1979), and in pineal differentiation (Dooling et al. 1977; Møller 1974). Since the 129/J mouse is of an albino strain, the presence of melanotic neuroepithelium in these experiments is very unlikely to be due to retina-like differentiation. Kastin et al. (1976) found no melanin associated with albino rat retina, whereas melanin was present in other parts of the central nervous system in both albino and non-albino rats. The argument, however, may not strictly apply to neoplastic neuroepithelium. The melanosomal nature of the transient pineal melanin is not well-characterized (Møller 1974), but the pigmented primitive neuroepithelium in IB-9 was arranged in epithelial ribbons rather similar to those of the fetal pineal gland (Dooling et al. 1977). This morphologic resemblance, and the transient expression of melanin in the pineal gland during the early stages of its development, present an analogy with the evolution of melanin in IB-9. It suggests that melanin production could be an expression of early differentiation in neoplastic neuroepithelial cells.

The phenomenon of melanogenesis in human neurogenic tumors is poorly understood (Russell and Rubinstein 1977). Melanin formation has been described in neurogenic neoplasms of both central and peripheral origin (see Erdelyi et al. 1981 for discussion), and melanosomal melanin has been ultrastructurally documented in both types of neoplasm (Boesel et al. 1978; McCloskey et al. 1976; Mennemeyer et al. 1979; Palacios 1980; Spence et al. 1976). The association of melanosomal melanin with two embryonal neurogenic neoplasms in the human, the cerebellar medulloblastoma (Boesel et al. 1978) and the melanotic neuroectodermal tumor of infancy (see Palacios 1980 for recent review), and its presence in the primitive neuroepithelium in IB-9 suggest that melanosomal melanin may be a useful marker for selecting certain primitive neuroepithelial cell populations in the OTT-6050 teratoma. These populations may serve as an experimental system to study the relationship between melanin production and neural differentiation in primitive human neuroectodermal neoplasms.

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