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Neural Differentiation in the OTT-6050 Mouse Teratoma: Effects of Intracerebral Environment on the Neural Differentiation of Embryoid Bodies

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Summary. Small embryoid bodies (EB's) from the OTT-6050 transplantable mouse teratoma, obtained by gravity filtration through a 74 μ mesh, were injected into the right cerebral hemisphere of syngeneic newborn or adult mice of both sexes in order to produce differentiating teratomas after a single passage. In subsequent experiments, two solid tumors resulting from two different EB-implants into the brains of adult hosts were used to initiate sequential tumors and were carried intracerebrally in adult mice for 12 and 18 passages respectively. The animals were sacrificed when signs of increased intracranial pressure developed. Survival times were as follows: single passages in adult mice: mean, 35 days; single passages in neonatal mice: mean, 19 days; sequential passages in adult mice: mean, 25 days. Multipotential stem cells accounted for $^1/_2$ to $^3/_4$ of the cells in all tumors. Primitive neural cells, ependymoblastic rosettes, neuroblasts and glia were present in all; stem cells, primitive neural cells and rosettes decreased proportionately as the more differentiated neural populations became prominent. Mature ganglion cells were found only in the sequentially passaged tumors and in tumors maintained for more than one month after a single passage in adult mice. Synapses were noted in the most differentiated areas. Neuroblasts were infrequent in tumors developing in neonatal hosts, and mature ganglion cells were absent. Glial fibrillary acidic protein was present by the 24th day in tumors obtained in adult hosts after single passage and in sequential passages.

Both in the OTT-6050-derived tumor fractions IB-9 and IB-21, previously reported, and in the EB-derived tumors described in the present study the cerebral microenvironment did not appear to have unique properties favoring neural differentiation and maturation, since similar neural features were

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found in their subcutaneous counterparts. The findings reported suggest that any accentuation of neuroepithelial differentiation elicited by injecting EB's either intracerebrally or subcutaneously is apparently directly related to the total time of in vivo maintenance of the tumor and therefore presumably to the length of time necessary for such maturation to occur.

Key words: Mouse teratoma – Embryoid bodies – Cerebral transplantion – Neural differentiation

Introduction

Previous reports have described the results of successive cellular dissociation, centrifugal elutriation, and serial transplantation into syngeneic hosts, of fractions derived from solid implants of the OTT-6050 mouse transplantable teratoma line, in an attempt to obtain tumor fractions with an enriched neuroepithelial cell population. Two transplantable tumor fractions were obtained by these means: one, labelled IB-21, in which differentiation was restricted to pluripotential neuroepithelial cell elements (VandenBerg et al. 1981 a); and another, designated IB-9, which was characterized by melanogenesis that appeared to be site-related, i.e., melanin pigment was maintained in the renal subcapsular environment, but lost in subcutaneous and intracerebral implantation. The loss of melanin pigment was associated with increased differentiation and maturation of the neuroepithelial elements (VandenBerg et al. 1981 b).

These results at first suggested the possibility that the site of implantation might play a major role in modulating the direction of neural differentiation or the degree of maturation in the teratoma implants. However, the validity of such a hypothesis was questionable because the starting material for these various implantation experiments was composed of cells that had been preselected by the dissociation and elutriation procedures. The hypothesis therefore needed to be tested by using as primary material a cell population that had not been preselected in this manner but had retained its range of multipotentiality. For this purpose, we selected the embryoid bodies (EB's) of the OTT-6050 mouse teratoma line (Stevens 1970), since it is well recognized that these bodies, within the size range used in our experiments, are composed largely if not entirely of multipotential stem cells and trophectoderm (Gearhart and Mintz 1974). The trophectoderm has also been termed proximal endoderm or visceral yolk sac endoderm (see Pierce 1980, for review).

The present report describes the tumor implants resulting from the intracerebral injection of embryoid bodies and their subsequent intracerebral transplantation in syngeneic hosts. Very small (microliter) quantities of EB's were used as the starting material.

Materials and Methods

Materials. Embryoid bodies, the ascitic form of the transplantable mouse teratoma line OTT-6050, were originally obtained from Dr. L.C. Stevens at the Jackson Laboratory, Bar Harbor, Maine, in January, 1973, and have been carried in our laboratory by serial intraperitoneal transplantation

every 16–24 days into syngeneic 5- to 10-week old 129/J female mice as previously described (VandenBerg et al. 1975). The EB's used in the present report were obtained from passages 65, 67, 69 or 74 through 76 in our laboratory.

EB-bearing donors with abdominal distention were sacrificed by cervical dislocation. Then, 2.5 ml of Dulbecco's phosphate buffered saline (PBS) (Grand Island Biological Co., Grand Island, N.Y.) with 300 mg% glucose were injected intraperitoneally with a 20 gauge needle followed by immediate gentle aspiration. The ascitic fluid from 2 to 3 animals was pooled occasionally to obtain a sufficient quantity of EB's. The EB-containing ascitic fluid was gravity-filtered through a double-layered nylon screen with 74 μ pores and the EB's allowed to sediment by gravity. They were then rinsed two or three times by resuspension in PBS-glucose solution. Implantation into host brains was usually begun 30 min after harvesting and was not performed after two hours.

Adult recipients were 4 to 10 week old 129/J female and male mice. Neonatal 129/J female and male recipients were implanted within 24 h of birth.

Brain Implantation for Single Passages. Adults were anesthetized by the intraperitoneal injection of Diabutal (sodium pentobarbital, Diamond Laboratories, Inc., Des Moines, Iowa 50304) supplemented with ether. The scalp was incised and retracted and a 2 to 3 mm square bone flap was made with a dental drill about 1.5 mm caudal to the coronal suture and 1.5 mm to the right of the sagittal suture. The head was secured with a Kopf (model DKI-900) small animal stereotaxic instrument. Micropipettes to inject the EB's into the brain were made from glass microelectrode tubing (O.D. = 1.0 mm; I.D. = 0.480 mm). An inside tip diameter of 100 μ was obtained by stretching the tube in a microelectrode puller and shaping the resulting tip with No. 600 emery paper. The micropipette was filled by first drawing the sedimented EB's into a P.E. 100 polyethylene tube attached to a 0.2 ml Gilmont micrometer syringe. The micropipette was then filled from the top and the previously filtered EB's were allowed to compact by sedimentation in the micropipette for 5 to 10 min. This permitted a rough quantitation of the number of EB's implanted according to the volume of fluid injected. The pipette was then lowered stereotactically to 2 mm below the cortical surface, about 1.5 to 2 mm to the right of and posterior to the bregma. The bodies were slowly injected into the brain parenchyma (15–30 s) in quantities varying from 0.5 to 5 μl. Following withdrawal of the pipette, the bone flap was replaced and the skin closed with metal staples.

Twenty-two adult mice (15 were female) were injected with 0.5 to 5 μl of filtered EB's (from the 65th, 67th and 69th intraperitoneal passages) in five different experiments (see Table 1). Only 3 of the 22 implants failed to grow, indicating an 86% success rate. Animals were sacrificed when symptoms of increased intracranial pressure became evident. These included lethargy, weight loss, irritability and seizures.

To study the early events in EB differentiation, 12 adult female mice received right cerebral hemisphere implants of 2 μl of filtered EB's from the 74th intraperitoneal passage and were then sacrificed at 5, 10, 15 and 18 days after implantation. The animals varied in age (4, $6^1/_2$ and 10 weeks of age), but each age was represented equally at all time points of the tumor growth.

To compare the EB differentiation in hosts of different ages, twenty neonatal mice of both sexes were given 2 μl injections of filtered EB's (from the 74–76th intraperitoneal passage) into the right cerebral hemisphere. To inoculate the neonates, the reservoir of the Gilmont micrometer syringe was filled with EB's. The desired volume was then implanted in the brain 1 to 2 mm below the surface of the skin with a 27-gauge stainless steel needle inserted through the scalp overlying the anterior fontanelle.

Sequential Intracerebral Transplantation. Two series of sequential transplants were initiated by injecting 2 μl of two populations of EB's selected by different filtration methods. In Group A, EB's were obtained from the 67th intraperitoneal passage. Those chosen for implantation were the ones that remained on top of the two 74 μ nylon screens during gravity sedimentation. The EB's were collected by reverse flushing through the filters with PBS-glucose solution. For Group B, EB's were obtained from the 69th intraperitoneal passage and gravity filtered through one 74 μ and one 53 μ nylon screen. EB's in the resulting filtrate were used for implantation. Group A was, therefore, presumably initiated with larger embryoid bodies than Group B. However, smears of the filtrate and sediment remaining on the screens did not reveal a difference in the size of EB's from the two series. Many EB's in the filtrate were elongated and narrow, suggesting that

they penetrated the filter by deformation. The larger EB's from both populations had an average width of 63 μ and a length of 96 μ.

To initiate the sequential transplantations, 2 μl of EB's from Group A or B were injected into the right cerebral hemisphere of 2 adult female mice. For the sequential passaging of Group A, the donor animal was sacrificed after 32 days of EB growth, and for Group B after 29 days. The resultant solid tumors were removed from the donor mice and diced into 0.5 to 1 mm^3 fragments in Hank's balanced salt solution with supplemented MEM amino acids (VandenBerg et al. 1981 a). Translucent gray tumor fragments were selected for transplantation. A bone flap was made in adult recipients as described above. A tumor fragment was placed on the tip of a 27 gauge needle and was pushed through the pia to a depth of about 1 mm in the brain in 2 or 3 recipients. Most often the fragment remained in the brain with the initial insertion of the needle; however, at times several insertions of the needle were required to seat the fragment. The neoplasm was allowed to grow until signs of increased intracranial pressure developed. For the next sequential transplant, one animal was used as a source of the tumor and the remaining one or two animals used for histology. For sequential bank A, the tumor was transplanted a total of 12 passages, for B, 18 passages. Adult mice of both sexes were used.

Estimation of Approximate Tumor Volume. The volume was approximated by multiplying $\pi/6$ by the product of the 3 principal diameters (width, length and height) of the neoplasm as measured at the time of brain removal (Steel 1977).

Histology and Electron Microscopy of the Brain Implants. Unless stated otherwise, animals were sacrificed when symptoms of increased intracranial pressure became evident. A complete autopsy was performed on each mouse. For *light microscopy,* the tissues were fixed with phosphate-buffered neutral 10% formalin at room temperature by gravity-fed cardiac perfusion for 10 minutes followed by post-fixation in a similar formalin solution. A few animals were fixed by immersion in similar fixative. Stains included hematoxylin and eosin, Mallory's phosphotungstic acid hematoxylin (PTAH), cresyl echt violet for Nissl substance, periodic acid Schiff (PAS), reticulin, and Bielschowsky's silver method for axons (see VandenBerg et al. 1975 for methods used).

Immunoperoxidase Studies. Sections of paraffin-embedded tissue from all groups of animals were stained for glial fibrillary acidic (GFA) protein (Eng and Rubinstein 1978) by the Sternberger peroxidase-antiperoxidase method (Sternberger et al. 1970) as modified by Taylor and Burns (1974).

Estimation of Proportions of Cell Types. For each animal, 2 or 3 histological sections were taken randomly through the neoplasm. The proportions of stem cells and neuroepithelial cells were estimated by visual inspection at low microscopic powers and averaged for the 2 or 3 sections to obtain a score for each tumor. Scoring was done by two independent observers.

For *electron microscopy* the animals were perfused as follows. An initial ten-second perfusion with phosphate-buffered normal saline at 37° C was followed immediately by a 10-min perfusion with 3.5% glutaraldehyde in 0.06 M cacodylate buffer (pH 7.0 and 550 mosm/kg H_2O) at 34–37° C. The animal was then placed at 4° C for 1 hour, after which the brain was removed, the tumor dissected out, trimmed into 1 mm^3 fragments, and immersed in cold glutaraldehyde fixative (as above) for 3 to 12 hours. The tissues were subsequently washed in cacodylate buffer with sucrose (pH 7.2 and 340 mosm), osmicated, dehydrated through graded alcohols, and embedded in Epon 812. Numerous thick sections were performed and areas of interest were selected for thin sections; the latter were stained on the grid and examined with methods used previously (Sipe et al. 1973). One single-passaged adult animal and six sequentially-passaged adults from Groups A and B (passages 11, 12, 14 and 16) were studied.

Results

Early Evolution of Single-Passaged Intracerebral EB Implants in Adults. In general, the embryoid bodies were evenly dispersed throughout the micropipette track, although they tended to accumulate subpially at the entrance of the track. By the *fifth post-implant day* (Fig. 1), many mitoses were present in both

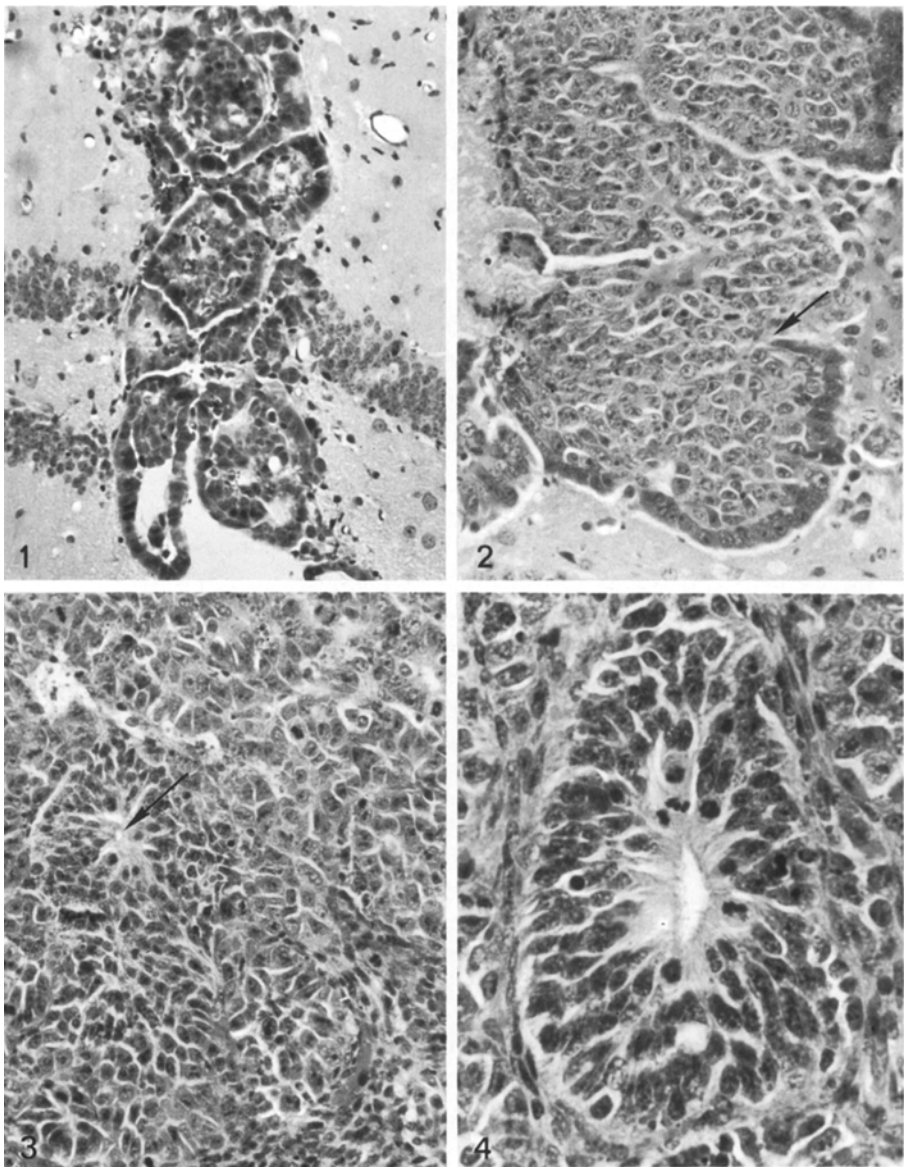
the inner stem cell mass and the outer layer of trophectoderm. The increased mitotic activity was associated with a notable increase in stem cells, while the trophectoderm became sinuous and in some areas formed tube-like structures containing homogeneous eosinophilic material. Pools of this dense material, which, in mouse teratomas, has been immunologically compared to the embryonal Reichert's membrane (Pierce 1980) and shown in the F9 and OCI551 teratoma-derived cell lines to consist of multiple extracellular matrix glycoproteins (Wartiovaara et al. 1980), were present in all tumors at all stages of growth. In many of the older tumors, it formed large lakes. In some areas the stem cells appeared to rupture through the trophectoderm. By the *10th day* the stem cell masses were only partially covered by trophectoderm, and some of them seemed to merge, forming larger confluent lobular masses. The largest growth occurred subpially and indented the brain. Delicate blood vessels penetrated the tumor from the adjacent brain and pia. By the *15th day*, large confluent masses of stem cells were formed and were only partially bordered by trophectoderm (Fig. 2). Primitive neuroepithelial cells, usually forming sheets (Fig. 3) and occasionally rosettes, were identified at 18 days. Their cytoplasm was sparser and more elongated, and their nuclei smaller and more compact than those of stem cells, as previously described (VandenBerg et al. 1975; Herman et al. 1975). Distinction of these cells from stem cells has been confirmed by the demonstration of a neuroepithelial-associated antigen on their cytoplasmic membranes (VandenBerg et al. 1977). Ependymoblastic rosettes were frequently seen.

Features of Tumors Obtained After Single Passage of EB's in Adult Brain. Table 1 lists the tumor volumes obtained. No correlation was found between tumor volume on the one hand and the size of the EB inoculum or the time interval between implantation and the appearance of intracranial symptoms on the other hand. However, signs of increased intracranial pressure tended to occur sooner with larger volumes of inoculum (see table). The sex of the animals did not appear to influence tumor growth or differentiation.

The tumors usually formed a single mass with borders that were sharply demarcated from the surrounding brain. The margins tended to displace the surrounding brain; however, after one month brain invasion was demonstrated with increasing frequency. The invading zones at the periphery of the tumor were composed of relatively more differentiated neuroepithelial elements such as neuroblasts, occasional mature ganglion cells and astrocytes, and in general neuroepithelial differentiation and maturation were more pronounced in the lower half of the tumor implant, i.e., that facing the host brain. This feature was not seen in immediate proximity to the pia.

Sixteen primary implants were examined in detail by light microscopy and tabulated as shown in Table 1. The other five successful implants were examined in less detail and not tabulated because portions of them were used for either sequential transplantation or electron microscopy.

The tumors that were maintained longer showed a relative decrease in the total number of stem cells and a corresponding relative increase in the number of neuroepithelial cells. On the average, three-fourths of the tumor volume was composed of stem cells in 8 tumors examined between 3 to 4 weeks after



Figs. 1–4. Differentiation of embryoid bodies (EB's) in single passages in adult mice. All figures reduced 15%

Fig. 1. 5 days after implantation. EB's in the cerebral micropipette tract showing outer layer of trophectoderm and inner core of stem cells. H and E ×215

Fig. 2. 15 days. Confluent masses of stem cells. Partial loss of trophectoderm (*arrow*). H and E ×100

Fig. 3. 18 days. Primitive neuroepithelial cells (*lower left*) including a primitive rosette (*arrow*), adjacent to stem cells (*upper right*). H and E ×430

Fig. 4. 24 days. High magnification of an ependymoblastic rosette similar to that in Fig. 3 showing multilayered polarized cells with apical mitoses. A few stem cells in upper right corner. H and E ×530

Table 1. Intracerebral growth and differentiation of embryoid bodies

Animals analyzed in detail	µl E.B. (number of animals injected)	Number of animals in each group	Postimplant duration		Tumor volume		% Stem Cells	Primitive N.E. Cells	Proportion of tumors showing neuroepithelial differentiation			
			Range (days)	Mean (days)	Mean (mm^3)	Std Dev (mm^3)			Medullary epithelium	Ependymoblastic rosettes	Neuroblasts and glial cells	Mature ganglion cells
I. Adult single passage 74 µ filtrate	5 (3) 3 (3) 2 (1) 0.8 (1)	8	21–29	25	172 (107	184 68)[a]	3/4	8/8	2/8	7/8	4/8	0/8
	2 (5) 0.5 (3)	8	30–77	45	124 (141	91 85)[b]	1/2	8/8	0/8	7/8	8/8	2/8
II. Adult Sequential												
A. 74 µ Non-Filtered Residue	2	16	19–36	25	112	70	<1/2	16/16	7/16	13/16	16/16	6/16
B. 53 µ Filtrate	2	27	21–31	25	106	99	1/2	27/27	2/27	22/27	25/27	7/27
III. Neonatal single passage 74µ Filtrate	2	20	17–23	19	101	78	>3/4	17/20	9/20	14/20	3/20	0/20

[a] Excluding high value of 628 mm^3
[b] Excluding low value of 5.0 mm^3

implantation. In contrast one-half of the tumor volume was composed of stem cells in 8 tumors maintained for more than 4 weeks. Eighty to 90% of the differentiating cell population was neuroepithelial, the remainder being non-neural.

The majority of the neuroepithelial cells were primitive. These cells were present in all 16 tumors. Neuroepithelial areas included numerous ependymoblastic rosettes (Fig. 4) (found after 3 weeks in 14 of the 16 neoplasms) and occasional medullary rosettes (Fig. 5) (found in 2 of the 16 tumors at 3–4 weeks). More differentiated neural elements formed a minor component of the primary growths. These elements included numerous neuroblasts, sometimes arranged in Homer-Wright rosettes (which occurred after 3 weeks in 12 of the 16 tumors) and rare ependymal rosettes (which occurred in only one of the 16 tumors, at 9 weeks). A small nest of neuroblasts was found at 3–4 weeks in two other tumors. Occasional mature ganglion cells were identified only in two tumors, at 5–6 weeks. The zones of neuroblastic differentiation became more extensive in the older tumors (after one month). Electron microscopy confirmed many of these findings.

Immunoperoxidase staining for GFA protein was performed on tumors at 6 time points (3–5 weeks and 11 weeks). Positivity for the protein was present after $3^1/_2$ weeks and in all older neoplasms. The positivity was often lacy and delicate in the younger tumors. In older tumors, the GFA protein-containing cells had thicker processes that stained intensely. Positive cells were usually found in regions of the neuropil containing neuroblasts and ganglion cells, but an occasional positive cell was present amidst the stem cells.

Growth of Single-Passaged EB's in Neonatal Mouse Brain. Signs of increased intracranial pressure occurred earliest in these animals, which were all terminated by day 23 (Table 1). The tumors had numerous foci of necrosis and usually invaded the brain. They were of the same size as in adult mice when maintained for the same length of time. Several tumor masses were often present, and tumor was commonly found in the ventricular system. Unattached EB's were usually present next to solid tumor when the latter invaded the ventricular system. Features of tumor growth and neural differentiation in the neonate were similar to those in the primary tumors in the adult after the same period of growth in the brain. As in the adult, most of the tumor was composed of stem cells. The neural tissue was almost entirely primitive (Table 1) and was found as early as $2^1/_2$ weeks after implantation. Neuroblasts, when present, were very sparse. Two of 20 tumors showed differentiation to skin (keratinizing squamous epithelium with hair follicles) and 7 of 20 showed cartilagenous differentiation. With regard to the latter, an interesting association, identified in several tumors, was the proximity of neuropithelial elements to cartilage. Trophectoderm (most commonly), mesenchyme, epithelial cysts and nonkeratinized squamous epithelium were also found. The presence of GFA protein in tumor cells could not be verified in 3 neoplasms $2^1/_2$–3 weeks after implantation. One animal had numerous lung metastases after $2^1/_2$ weeks.

Sequential Transplantation of Solid Intracerebral Tumor in Adult Mouse Brain. Only one animal in Group A and one in Group B failed to develop a tumor.

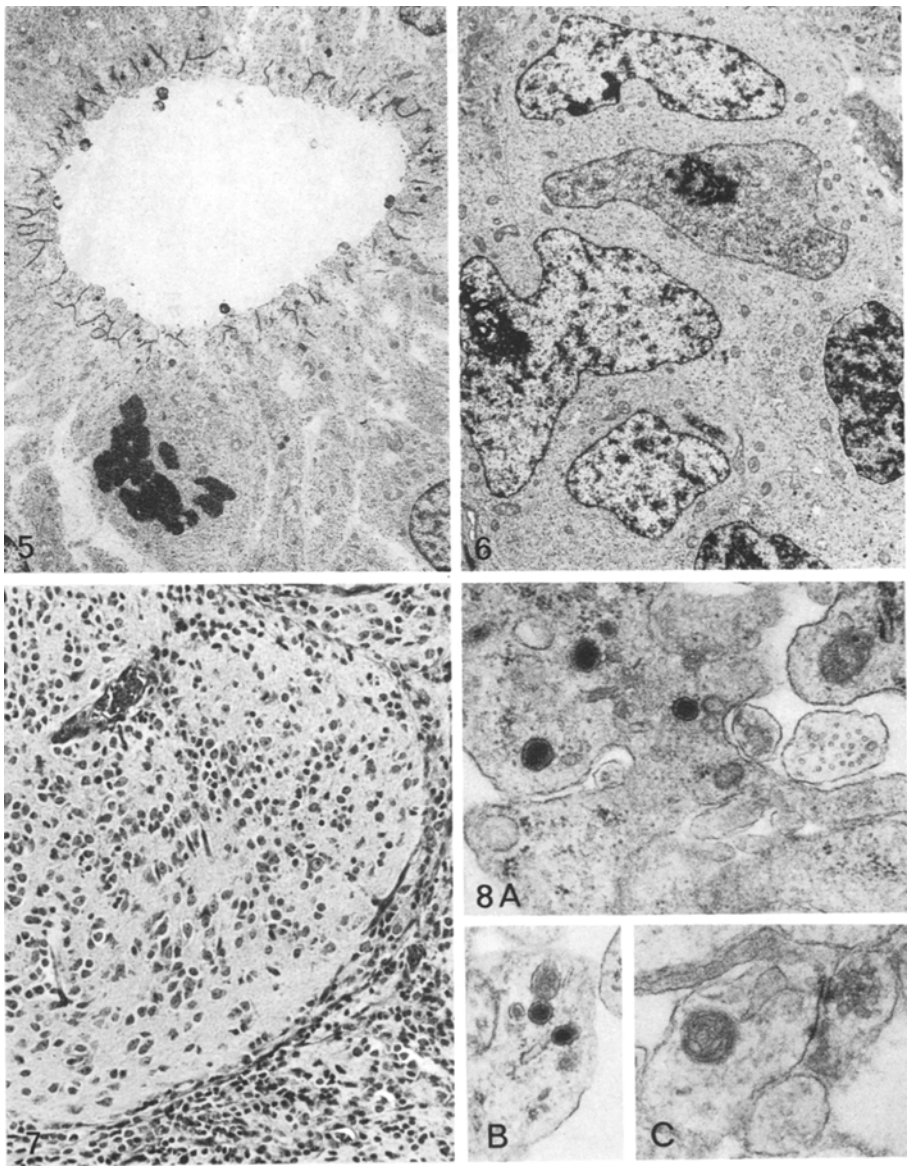


Fig. 5. Single passage in adult mouse after 31 days. Medulloepithelial rosette of polarized cells, with numerous apical junctions and one apical mitotic figure. ×2500

Fig. 6. Sequential intracerebral implantation, Group A, 11th passage. Primitive neuroepithelial cells with elongated nuclei. ×3100

Fig. 7. Sequential intracerebral implantation, Group A, 12th passage. Mature neuropil containing ganglion cells. Differentiating neuroblasts are present in right lower corner. H and E ×170

Fig. 8a–c. Sequential intracerebral implantation, Group A, 11th and 12th passages. Electron microscopic features of differentiated neuropil including neurites, dense-core vesicles (measuring 1,200 to 1,400 Å in inner diameter and 1,800–2,000 Å in outer diameter) (**a** and **b**) and a well-developed synapse (**c**). **a** ×28,700 **b** ×20,000 **c** 33,300
All figures reduced 15%

The tumor volumes were approximately the same as those recorded after single passage of the EB's. However, the duration of tumor growth after sequential intracerebral transplantation was sometimes appreciably shorter (Table 1). Sixteen of the tumors from Group A and 27 from Group B were analyzed in detail by light microscopy. The rest of the tumors were monitored by light microscopy but not analyzed in detail since they were used as donor tissues or for electron microscopy. Again, the sex of the animals did not appreciably influence the results.

Sequential transplants of the solid tumor differed from the single-passaged growth of the EB's in two ways. First, the proportion of the tumor volume occupied by stem cells was smaller: the decrease became apparent after the fourth to sixth passage in both groups. On the average, stem cells occupied $^1/_2$ of the tumor volume during the early half of the passages in both Groups A and B, and $^1/_4$ of the tumor volume during the latter half of Group A. Second, although 90% of the non-stem cell tumor mass was neural (as in the single passages), mature neuroepithelial elements, especially ganglion cells, were more numerous and occupied a larger volume of the tumor (Table 1). The volume occupied by medullary and ependymoblastic rosettes was proportionately decreased. Groups of primitive neural cells (Fig. 5) were, however, still present. Ganglion cells, distributed sparsely in an abundant neuropil, occurred either singly or in small clusters (Fig. 7). Neuronal differentiation was confirmed by electron microscopy, which revealed synapses and neurites with dense-core vesicles in the most mature areas (Fig. 8). As with the tumors obtained after single passage of EB's, the lower half of the implant (that facing the brain) demonstrated more neural differentiation and maturation than the upper half.

Differentiation towards astrocytes was confirmed by positive staining for GFA protein in 4 tumors from Group A and in 3 tumors from Group B, ranging in age from $2^1/_2$ to 4 weeks. The GFA-positivity of the tumor astrocytes tended to be delicate and lacy while reactive astrocytes adjacent to the tumor in the host brain contained broader, strongly staining processes that often penetrated the tumor along blood vessels. Intracytoplasmic filaments consistent with glial filaments were not found by electron microscopy.

The range of non-neural elements was similar to that described above. In addition, keratinized skin was present in two tumors of Group B but was not found in any of Group A. Cartilage was found in 4 of 16 tumors in Group A and in 4 of 23 tumors in Group B. Melanin pigment was occasionally seen microscopically in association with medullary epithelium.

Growth of Single- and Sequentially-Passaged Subcutaneous Implants. The spectrum of neural differentiation in solid flank implants derived from EB growth along the needle track and in visceral implants has been described (VandenBerg et al. 1975; Herman et al. 1975). Since then the tumor has been carried subcutaneously in the flank for up to 61 passages with similar findings, i.e., an array of neural differentiation similar to that reported in the present study.

Discussion

The susceptibility of stem cells of the OTT-6050 teratoma line to be influenced by their environment has been demonstrated by Mintz and Illmensee (1975)

and Mintz et al. (1975). They found that exposure of single stem cells derived from small EB's to a blastocyst environment could overcome their potential for malignant behavior, with the production of normal chimera offspring. However, virtually no information is at present available on the influence of a mature tissue environment on the differentiating potential of this line of EB's.

With the object of Studying the effects of the microenvironment on differentiation of the OTT-6050-derived EB's, Hagiwara and his colleagues (Amano and Hagiwara 1976; Ishikawa 1979; Ishikawa and Hagiwara 1977) injected EB's into the tail vein of mice and obtained almost exclusively pulmonary tumors. Neural tissue was found in the lung tumors after fifteen days (Ishikawa and Hagiwara 1977), which is closely similar to our findings following intracerebral injection, i.e., after 17 days in the neonate and 18 days in the adult.

The range of neural differentiation and the degree of maturation of the EB-derived tumors in the brain reported in this study were similar to those previously found in EB-derived visceral and subcutaneous implants (VandenBerg et al. 1975; Herman et al. 1975). On the other hand, subsequent experiments on two tumor fractions derived from the OTT-6050 teratoma, namely IB-9 and IB-21, suggested that the neuroepithelial cell populations showed a wider and more abundant spectrum of neural maturation in the brain and the subcutaneous tissue when compared to the implants of the same fractions in the kidney (VandenBerg et al. 1981 a, b). No notable differences in neural maturation were found on comparing the intracerebral and the subcutaneous implants obtained with those tumor fractions. At present we have no knowledge of any site-related mechanisms that may be involved in the selection of neoplastic differentiation: the IB-21 and IB-9 tumor fractions were obtained following procedures that were designed to preselect the tumor cells so as to enrich their neuroepithelial components, but we are unable to ascertain which, or to what extent, undifferentiated and/or differentiating cell populations were involved so as to modify the spectrum of maturation in the different sites. In any event, while we have no data on the extent of differentiation and maturation that would result from the implantation of EB's beneath the renal capsule of syngeneic hosts, a comparison between the spectrum of differentiation obtained with the EB-derived tumors after intracerebral and subcutaneous implantation, and that resulting from the intracerebral and subcutaneous implantation of the IB-9 and IB-21 fractions appears relevant.

Intracerebral tumors obtained from IB-21 were composed predominantly of differentiating neuroblasts, glial and stem-like cells; neural rosettes and non-neural elements were not found (VandenBerg et al. 1981 a). In comparison, the EB-derived tumors contained a number of neural rosettes and a small proportion of differentiated non-neural elements. When contrasted to the IB-9 intracerebral implants (VandenBerg et al. 1981 b), the EB-derived tumors showed a relatively greater proportion of non-neural elements, particularly cartilage, mesenchyme and epithelium of various types, and a lesser proportion of primitive neural rosettes; the latter were often the predominant component in the early intracerebral transplants of IB-9, but decreased with sequential passages.

When compared to the subcutaneous implants of the IB-21 and IB-9 fractions (VandenBerg et al. 1981 a, b), the EB-derived subcutaneous tumors showed a very similar spectrum of differentiation and degree of maturation of their neural

component, the only difference being that the EB-derived and IB-9 tumors, like their cerebral counterparts, contained a number of non-neural elements, whereas the IB-21-derived tumors were restricted to stem cells and differentiating neural cells.

The comparisons that have been drawn in the above paragraphs suggest that any differences in neural maturation between the EB-derived tumors on the one hand and the IB-21 and IB-9-derived tumors on the other are best explained by the fact that the EB cells have retained a wider spectrum of multipotential differentiation than the preselected cell populations from which the IB-21 and IB-9 fractions were derived.

How are we then to explain the apparent accentuation of neuroepithelial differentiation elicited by injecting EB's intracerebrally and subcutaneously in our experiments? In cerebral implants, mature ganglion cells appeared only after one month of single passage in the adult, or in the second or third passage in the sequentially transplanted tumors. Neuroblastic differentiation was sparse in the neonate (present in only 15% of the tumors), was present in only half of the primary-passaged tumors maintained under a month in the adult, in all single-passaged tumors maintained for more than a month in the adult, and in most of the sequentially transplanted tumors. We conclude, therefore, that neuroblastic differentiation and ganglionic maturation in this system require either a minimum growth of a month in the case of the single-passaged tumors, or sequential transplantation, i.e., sufficient time has to accrue in vivo for neuronal maturation to occur. So far, we can draw such a conclusion only from our intracerebral implantation experiments. We have no comparable time-related data from subcutaneous implantations. In any event, we found that an intracerebral environment did not eliminate non-neural elements: these were present in all tumor groups.

A finding warranting brief discussion concerns the tendency of the neural cells to be better differentiated in the cerebral implants in areas adjacent to the host brain. It is possible that peripheral differentiation was favored by, or contingent on, contact and attachment of the mitotically active tumor cells to brain parenchyma at the edge of the implants prior to migration and invasion of the host by these cells. In support of this hypothesis is the well-established observation (Gearhart and Mintz 1974; Hall et al. 1975; Levine et al. 1974; Teresky et al. 1974) that small EB's of this line require attachment in order to differentiate into their full spectrum. A similar tendency to exhibit the most pronounced degree of differentiation has been observed in the migrating outgrowth zones of explanted small EB's grown in monolayer (Hall et al. 1975; Lehman et al. 1974; Nicolas et al. 1975; Sherman 1975; Teresky et al. 1974; VandenBerg et al. 1976) or in an organ culture system (DeArmond in preparation; Herman and VandenBerg 1978). We see no reason to attribute the greater degree of neural differentiation in certain areas of the intracerebral implants directly to their immediate proximity to the host brain.

In summary, our results do not support the hypothesis that neural differentiation and maturation from the EB's of the OTT-6050 mouse teratoma are specifically favored by the cerebral microenvironment. Any apparent in vivo accentuation of neuroepithelial differentiation elicited by injecting EB's either intracerebrally or subcutaneously is presumably related to the accrued time of in vivo maintenance of the tumor and therefore to the length of time necessary for such maturation to occur.

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