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Neural Differentiation in the OTT-6050 Mouse Teratoma

Production of a Tumor Fraction Restricted to Stem Cells and Neural Cells After Centrifugal Elutriation

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Summary. Dissociation and centrifugal elutriation procedures were applied to subcutaneous transplants of the OTT-6050 mouse teratoma line in order to enrich the neuroepithelial cells. One of the resultant cell fractions, designated IB-21, was then implanted beneath the renal capsule of syngeneic mice and rebanked every 3 to 6 weeks for a total of 58 passages over 5 years. Sequential passages resulted in a tumor restricted to stem cells and neural cells (neuroblasts and glial cells). The primitive neural cells lost the ability to form rosettes after the early transplants. Subcutaneous or intracerebral transplantation of these tumors evinced their capacity for further neuroepithelial differentiation, with the demonstration of astrocytes and occasional mature synapse-forming neurons. Conversion of the tumor to the ascitic form resulted in unorganized clusters of neoplastic cells in contrast to the highly structured embryoid bodies that are characteristic of the parent OTT-6050 line.

The absence of non-neural cells in the IB-21 tumor fraction and its ability to demonstrate divergent neural differentiation suggest that a transplantable neural-determined cell population exists in the OTT-6050 mouse teratoma.

Key words: Mouse teratoma – Centrifugal elutriation – Neuroepithelial enrichment

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Introduction

The solid implants, in syngeneic hosts, of the mouse OTT-6050 transplantable teratoma line have provided useful populations of neoplastic cells capable of differentiating along derivatives of the three principal germ layers (Amano and Hagiwara 1976; Ishikawa and Hagiwara 1977; Jakob et al. 1973; Jami and Ritz 1974; McBurney 1976; Nicolas et al. 1976; Stevens 1970; VandenBerg et al. 1975). Previous work (Herman et al. 1975; VandenBerg et al. 1975) has detailed in these implants the various stages of divergent neuroepithelial differentiation, which span the entire range of neural cytogenesis from embryonal to mature cells of both neuronal and glial lineage. It has also been shown that the multipotential stem cells of the OTT-6050 line likewise exhibit divergent neural differentiation and maturation when grown in vitro (see Herman and VandenBerg 1978; VandenBerg et al. 1976). The neuroepithelial component of the OTT-6050 line has therefore been suggested as a suitable system for the study of central neoplastic neuroepithelial differentiation. However, the utility of this system is limited by the constant, though variable, presence of differentiated non-neural cells.

In an attempt to obtain tumor cell fractions that would be consistently characterized by an enrichment of the neural cell populations, we have applied successive cell dissociation and centrifugal elutriation procedures to the subcutaneous transplants of the OTT-6050 line, followed by implantation of the resulting cell suspensions into various tissues of syngeneic hosts. The studies to be reported (present study; Erdelyi et al. 1981; VandenBerg et al. 1981) describe the characteristics of two tumor fractions recovered from these maneuvers and their behavior in the course of their sequential passages into the various tissues. One of these two tumor fractions, designated as IB-21, was characterized by being restricted to the persistence of stem cells and neural cells only, and is the subject of this first paper.

Materials and Methods

Tumor Line and Animals

Solid tumors of the mouse transplantable teratoma line OTT-6050 (Stevens 1970) were serially transplanted subcutaneously in 129/J hosts as previously described (VandenBerg et al. 1975) and used in the 22nd transplant generation as the starting tissue for the cell separations at 2,000 RPM as described below. (All solid tumor generations had been carried in our laboratory and were originally obtained from incidental subcutaneous implants of the OTT-6050 embryoid bodies.) Syngeneic hosts for the initial implantation and serial transplantations of the renal tumors were 129/J female mice, usually 6–12 weeks old, but ranging from 4–14 weeks. Syngeneic hosts for the subcutaneous and intracerebral tumors were 129/J male and female mice, usually 4–12 weeks old, but ranging, in a few instances, up to 24 weeks.

Tumor Dissociation

The following steps were performed at 22° C unless otherwise specified. Animals bearing solid subcutaneous flank implants were killed by cervical dislocation, the tumors dissected free, minced into 1–2 mm^3 pieces and rinsed in calcium magnesium-free phosphate buffered saline (CMF-PBS) using 5-min centrifugations ($150 \times g$, R_{max}). The finely-minced tissue was then resuspended (20 ml/g wet tumor tissue) in CMF-PBS supplemented with 0.1% (w/v) trypsin (1:250) (GIBCO, Grand Island Biological Company, Grand Island, NY 14072) and 5.0 mM disodium ethylene diamine

tetraacetate (disodium salt EDTA) (Sigma Chemical Co., St. Louis, Mo. 63172) and incubated without agitation for 18 min at 37° C.

Following incubation, the suspension was supplemented with 5.0 mM $MgCl_2$ and centrifuged (5 min, 176 × g, R_{max}). The tissue pellet was then rinsed with elutriation medium supplemented with trypsin soybean inhibitor (Sigma Chemical Co.) to inhibit 8.4 mg trypsin. The rinsed 176 × g pellet (5 min) was then resuspended in elutriation medium (pH 7.31–7.32 at 24° C) supplemented with 5.0 mM $MgCl_2$ and DNase I (875 Kunitz units/ml). The tissue suspension was then dissociated to single cells by a series of repeated filtrations at 30 μ and triturations through precisely defined orifices as detailed below.

Following an initial sieving through a 180 μ stainless steel screen, the suspension was triturated 5 times (0.2 mm orifice), then filtered through double 30 μ pore nylon screens mounted in a Swinnex apparatus. The filtrate was centrifuged at 176 × g (5 min) and resuspended in the elutriation medium supplemented with $MgCl_2$ and DNase by trituration 4 times (0.3 mm orifice) followed by filtration at 30 μ. After incubation at 22° C for 10 min, the cell suspension was supplemented with fresh elutriation medium (1:1) and with unfiltered fetal calf serum (Rehatuin™, Reheis Chemical Co., Kankakee, Ill. 60901) to a final concentration of 1% (v/v) and incubated in the dark at 37° C for 30 min.

Following incubation, the cells were transported to the centrifugation facilities (10–15 min at 22° C) at Beckman Instruments, Inc. (Spinco Division, Palo Alto, Calif. 94304).

Buffered solutions for cell dissociation, centrifugal elutriation and agar embedding were formulated as follows: (1) CMF-PBS: 137 mM NaCl, 4 mM KCl, 0.514 mM Na_2HPO_4, 0.147 mM KH_2PO_4, 6.32 mM glucose, titrated to pH 7.34 at 22° C, 289 mOsmol/Kg H_2O; and (2) elutriation medium: 111 mM NaCl, 5.36 mM KCl, 0.98 mM $MgCl_2$, 0.10 mM $CaCl_2$, 17.8 mM Na_2HPO_4, 2.24 mM NaH_2PO_4, 6.32 mM glucose, Minimal Eagle Medium (MEM) essential amino acids (GIBCO) × 1 concentration, MEM vitamins (GIBCO) × 1 concentration, titrated to 7.40–7.42 at 24° C or to 7.40–7.42 at 8° C, 299–302 mOsmol/Kg H_2O.

Centrifugal Elutriation Procedure

The cells were resuspended in elutriation medium by centrifugation (6 min, 176 × g), trituration (0.3 mm orifice) and filtration through a 30 μ pore nylon screen and maintained at either 22° C or on ice until loaded into the JE-6 elutriation rotor (Beckman Instruments, Inc.). The elutriation chamber was sterilized by flushing with 70% (v/v) ethanol followed first by a rinse with isotonic saline, then by elutriation medium (pH 7.4 at 8° C). The rotor system was then loaded with elutriation medium (pH 7.4 at 8° C) and the cell suspension injected into the system through double 30 μ pore filters in a Swinnex apparatus. The cell suspension was then elutriated into cell populations of different sedimentation velocities by altering the flow rate through the rotor (chamber temperature 10° C) and 150 ml was collected at each flow rate.

The following flow rates and rotor speeds were used, since they provided smaller tumor cells. At 2,000 RPM: 4 ml/min, 6 ml/min, 10 ml/min, 11 ml/min (IB-8), 12 ml/min (IB-9) (VandenBerg et al. 1981), 13 ml/min, and 14 ml/min. Each 150 ml fraction was centrifuged (213 × g, R_{max} 13 min) and resuspended in 1.5 ml of elutriation medium supplemented with 18% (v/v) unfiltered fetal calf serum. A small sample was removed for phase microscopy and the remainder maintained at 37° C until used for implantation.

Further elutriation was performed with a cell suspension derived from a subcutaneous tumor of the unfractionated OTT-6050 in the 30th transplant generation and a 5th sequential renal transplant of IB-9 (VandenBerg et al. 1981). At a rotor speed of 3,000 RPM, the flow rates were 10 ml/min, 12 ml/min, 13 ml/min, 18 ml/min, and 29 ml/min. *IB-21* was obtained from the renal implant of one fraction (29 ml/min) at 3,000 RPM; none of the other fractions obtained at this RPM led to the development of a tumor.

Implantation of Cell Suspensions

Each fraction was centrifuged (176 × g, 5 min), and the pellet was resuspended in 0.02–0.03 ml elutriation buffer (without amino acids and vitamins). This suspension was mixed rapidly with an equal volume of 1% agar (w/v) in elutriation buffer (maintained at 40° C) and rapidly cooled to 22° C. The cell-agar was then immediately implanted beneath the renal capsule as described below in detail or subcutaneously over the abdomen of syngeneic hosts.

Table 1. IB-21 transplantations

Transplantation site	Sets[a] of animals implanted	Number of serial transplantations per set	Source of tumor
Renal	1	58	Subcutaneous, OTT-6050 Renal, IB-9[b]
Subcutaneous	3	10, 22, 17	Renal, IB-21
Intracerebral	3	12, 7, 7	Renal, IB-21
Intracerebral	3	None [Primary implants only]	Renal, IB-21
Intraperitoneal	3	3, 3, 3	Renal, IB-21 (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

[b] For details see VandenBerg et al. (1981)

Controls to check the viability and the effects of the dissociation and implantation on the tumor cells were the following: 1) small undissociated tumor fragments from unelutriated OTT-6050 transplants which were implanted beneath the renal capsule, 2) dissociated cell populations of unelutriated OTT-6050 transplants which were embedded in agar and implanted beneath the renal capsule and 3) unelutriated residual cells from the elutriator rotor chamber after centrifugation which were agar-embedded and implanted beneath the renal capsule.

Transplantation of Elutriation-Derived Tumors

Tumors which arose from the implantations described above were serially transplanted either beneath the renal capsule, subcutaneously in the flanks, or intracerebrally. The numbers of transplants according to site are summarized in Table 1.

1. Renal Transplants. One to three tumor fragments, each measuring 0.5–1 mm^3, were serially transplanted to new recipients every $2^1/_2$–6 weeks for a total of 58 passages over a period of 5 years. From the 26th passage onwards we always used two or three tumor pieces; the use of less material often led to poor or no growth.

The methods are detailed as follows. All instruments used were carefully kept away from detergents and other cytotoxic solutions except 70% ethanol. The instruments for tumor removal and dissection were soaked in 70% alcohol, rinsed, boiled in deionized water and dry-heat sterilized. Oil-free scalpel blades were wiped well with 70% ethanol rinsed in deionized water and then dry-heat sterilized. Instruments for tumor implantation were rinsed with deionized water, air-dried, cleaned with an argon plasma flux (Plasmod Generator, Tegal Corporation, Richmond, Calif. 94804) and then sterilized by ultraviolet light.

After the hood and working surfaces were prepared, the tumor was rapidly removed from the host following deep anesthesia with intraperitoneal Diabutal (sodium pentobarbital, Diamond Laboratories, Inc., DesMoines, Iowa 50304) and then rapidly trimmed in room temperature Hank's 1× balanced salt solution (BSS) (GIBCO) supplemented with MEM essential amino acids (50× concentrated, GIBCO; 0.5–0.65 ml/125 ml BSS) in order to help maintain a physiologic pH. To make the choice of all implants as consistent as possible, S.R.V. or M.M.H. always removed and trimmed the tumor and selected pieces for implantation. The criterium for selection was optimal tissue viability by inspection under a dissecting microscope; the best pieces were grey and translucent. The pieces were kept moist in BSS and in dim light.

The recipients were moderately deeply anesthetized with intraperitoneal Diabutal, supplemented with ether just before beginning the skin incision; the ether was administered well away from the surgical area.

After a nick in the kidney capsule was made with a cataract knife, the tumor was pushed beneath the capsule with a fine glass needle which had a smooth closed tip, while the edge of the capsule was lifted with delicate Dumont forceps. Then several tumor pieces were distributed over the lateral convexity of the kidney, while avoiding trauma and bleeding in the renal parenchyma along the needle track. After tumor inoculation, the kidney was retuned to its subperitoneal location, the fascia of the posterior muscles brought into apposition, and the skin closed with several metal staples. Four recipients could be inoculated within one and one-half hours after termination of the host. If further time was required, a second tumor-bearing animal was terminated.

2. Subcutaneous Transplants. Two pieces of tumor, each measuring 16–25 mm^3, were implanted in both flanks and transplanted every 14–30 days. The 3 sets of animals were implanted with tumor from the 21st, 26th, and 37th generation of the IB-21 renal transplants and almost all developed tumors. The methods have been detailed elsewhere (VandenBerg et al. 1975).

3. Intracerebral Transplants. Usually one (occasionally two) tumor fragments, each measuring 0.3–0.4 mm^3, were placed in the right frontal cortex immediately below the meninges. The 3 sets of animals (a total of 6 animals) were initially implanted with tumor from the 26th, 37th and 38th generation of the IB-21 renal transplants. The tumors were serially transplanted every 17–30 days depending on when symptoms such as lethargy, hyperactivity, weight loss and/or seizures developed. Only four animals out of a total of 73 did not develop a tumor.

For the implantation, we used anesthesia similar to that described for the renal implants. The instruments and blades were treated as described above for tumor removal and dissection. A small bone flap was made over the right frontal skull using a dental drill; it was important to avoid pressure on the skull, since this caused increased intracerebral pressure and often death. A small nick was made in the meninges and the tumor implant placed 1–2 mm beneath the meninges using a 27-gauge needle. The bone flap was then reseated in the skull and the skin incision stapled shut. The bone flap was necessary to prevent growth of tumor outside the skull. Some of the staples were left in place for 7–10 days in order to provide support for healing of the bone defect.

In addition to the serial transplants, 17 single-passaged intracerebral implants of IB-21 were studied (Table 1). They were performed in 3 groups: 5 recipients (24th transplant generation of the IB-21 renal transplants); 6 (25th generation); and 6 (27th). Only one recipient did not develop a tumor. The implantation techniques are described above.

4. Production of Ascitic Tumors. Solid IB-21 renally transplanted tumors (at the 36th, 38th and 39th generations) were transformed to ascitic form by mechanical dissociation and intraperitoneal injection as follows. Tumor-bearing animals were killed by cervical dislocation and 1–2 g of tumor tissue was dissected free of gross renal tissue. Tumors were rinsed and dissociated in room temperature Dulbecco's CMF-PBS (GIBCO) which was supplemented to a final glucose concentration of 150–200 mg%. The tissue was minced into approximately 1 mm^3 fragments, sieved through a 180 μ pore, then a 74 μ pore, stainless steel screen and gently triturated through a 5 ml standard bore pipette to give a suspension of single cells and small cell clusters. The suspension was centrifuged at 500 × g for 5 min, resuspended, and injected intraperitoneally into 2–3 syngeneic males or females (0.5 ml/mouse) (ages 8–10 weeks) to initiate each of the three experiments. After abdominal distension developed (3 to 8 weeks), the abdominal cavity was gently flushed with Dulbecco's PBS (GIBCO) and aspirated for cytologic examination and serial intraperitoneal transplantation. The tumors were serially transplanted three times into one or two recipients for each experiment (see Table 1). Cytology with routine hematoxylin and eosin staining was performed on 95% (v/v) ethanol-fixed smears and 3 μ and/or 5 μ nucleopore filtrates.

As a *control*, a solid subcutaneous implant of the ascitic form of OTT-6050 was dissociated, initially implanted into 2 females (age 8 weeks), and was similarly serially transplanted and studied.

Morphology

Light Microscopy. The renal tumors were monitored by light microscopy at each bank from the 9th passage onwards and in the majority of passages before that; all subcutaneous and brain tumors were examined by light microscopy at each bank. Histology was performed on the visceral organs of all ascitic tumor recipients including the OTT-6050 control.

Tumors were fixed with 10% phosphate-buffered formalin using either immersion (fixative at room temperature) (used for most of the renal and subcutaneous tumors) or gravity perfusion for 10 min via a left ventricle cardiac cannula (fixative at 34–37° C) (used for most of the intracerebral tumors). Perfusion with fixative was preceded by a ten second flush with phosphate-buffered normal saline at 34–37° C. Special stains used included Nissl, a modification of Bielschowsky's silver method for axons applied to frozen sections (Horten and Rubinstein 1976), Lapham's phloxine-fast green-gallocyanin (PFG) (Lapham et al. 1964) and iron hematoxylin-van Gieson.

Electron Microscopy. The tumors were fixed by immersion in chilled 3.5% cacodylate-buffered glutaraldehyde (pH 6.9–7.1, 530–550 mOsm) or, for the intracerebral implants, by gravity-fed intracardiac perfusion with 3% glutaraldehyde – 1% paraformaldehyde (pH 7.2) at 34–37° C. Following intracardiac perfusion, pieces of the tumor were trimmed into 1 mm^3 pieces and transferred to 3.5% glutaraldehyde, as above. The remainder of the tumor and brain were transferred to 10% buffered formalin and processed for light microscopy. Methods for further processing and examination by electron microscopy are given elsewhere (Sipe et al. 1973).

Immunohistochemistry. Immunocytochemical detection of GFA protein and myelin basic protein (MBP) using a peroxidase-antiperoxidase method was performed on selected tissue sections from tumors in the kidney, brain and subcutaneous tissue (see Pappas, 1981 for methods used). The immune and control sera for GFA protein were diluted 1:250 and for MBP, 1:500.

Results

Elutriation

All three types of controls designed to test cell viability and the effects of the dissociation and centrifugation procedures yielded teratomas typical of OTT-6050. No specific effects of dissociation, or of centrifugation without elutriation, or of renal subcapsular implantation were found on the resultant tumor morphology.

All fractions collected from the elutriator contained dissociated cell populations, with the exception of fractions with no cells collected at a flow velocity of 13 ml/min and 14 ml/min at 2,000 RPM. The elutriated fractions (4 ml/min, 2,000 RPM; 12–13 ml/min at 3,000 RPM) for cells of the lowest sedimentation velocity often contained cells with thin tapering processes characteristic of differentiating neuroepithelial cells (Fig. 1; compare with Fig. 11).

Eight cell fractions elutriated at 2,000 RPM were obtained; of these, only two developed tumors, and only after renal implantation. No subcutaneous implants yielded tumors. These fractions were contiguous at flow rates of 11 ml/min and 12 ml/min and were designated IB-8 and IB-9, respectively. Of the eight fractions elutriated at 3,000 RPM, only one, at 29 ml/min, developed a tumor after renal implantation (*IB-21*).

The initial tumors of IB-8 and IB-9 were remarkable for the abundance of melanin grossly and microscopically, in association with significant amounts of primitive neuroepithelium. The tumor IB-9 will be described in detail in subsequent reports (Erdelyi et al. 1981; VandenBerg et al. 1981). The initial growth of *IB-21* had only rare foci of melanin but a predominant primitive neural component. *IB-21* was maintained as serial renal implants for 58 passages. Macroscopically the tumor was grey and translucent and contained numerous foci

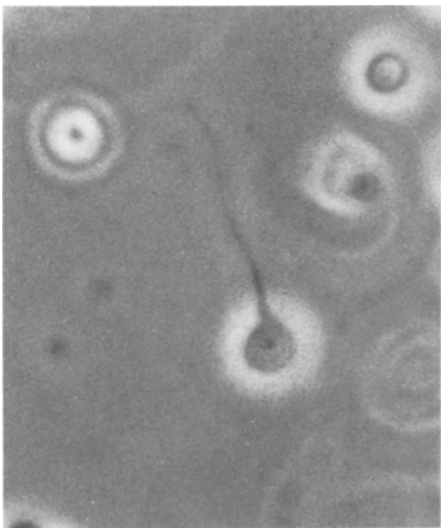


Fig. 1. Cell with long tapering process from a dissociated cell fraction elutriated at 4 ml/min (2,000 RPM). Compare with Fig. 11. Phase contrast (×860)

of hemorrhage and necrosis; it extensively invaded the kidney, but distant metastases were not found. Its morphologic features were as follows.

Morphology of the Renal Transplants

In the *early passages* (through the twentieth passage, i.e., during the first two years) there were very rare small foci of melanin in a predominantly primitive neural component admixed with stem cells. The undifferentiantly stem cells, which had frequent mitotic figures, were morphologically indistinguishable from those of the parent OTT-6050 tumor (Herman et al. 1975; VandenBerg et al. 1975) and of another elutriated fraction derived from OTT-6050, IB-9 (VandenBerg et al. 1981). Very rare foci of cartilage and other non-neural teratomatous components were also found in the very early passages, and then disappeared.

A significant proportion of medullary and ependymoblastic rosettes, similar to those described elsewhere (Herman et al. 1975; VandenBerg et al. 1975) comprised the primitive neural component. In addition, small foci of maturing neuroblasts and glia were sometimes present.

In the *later passages* (20th through the 58th, i.e., during the last three years the tumor was carried), the rosettes were no longer present. Cell elements were of two types only, both showing frequent mitotic figures: undifferentiated stem cells (Figs. 2 and 3) and small neural cells of predominantly primitive character (Figs. 2–4). In contrast to stem cells, the neural cells had elongated nuclei and stout polarized cytoplasmic processes (Fig. 4). Discrete, more-differentiated neural areas whose cells demonstrated GFA protein-positive processes (Fig. 5) were also found in some tumors. The spectrum of the predominantly primitive neural cell populations remained stable from the 20th to the last (58th) passage.

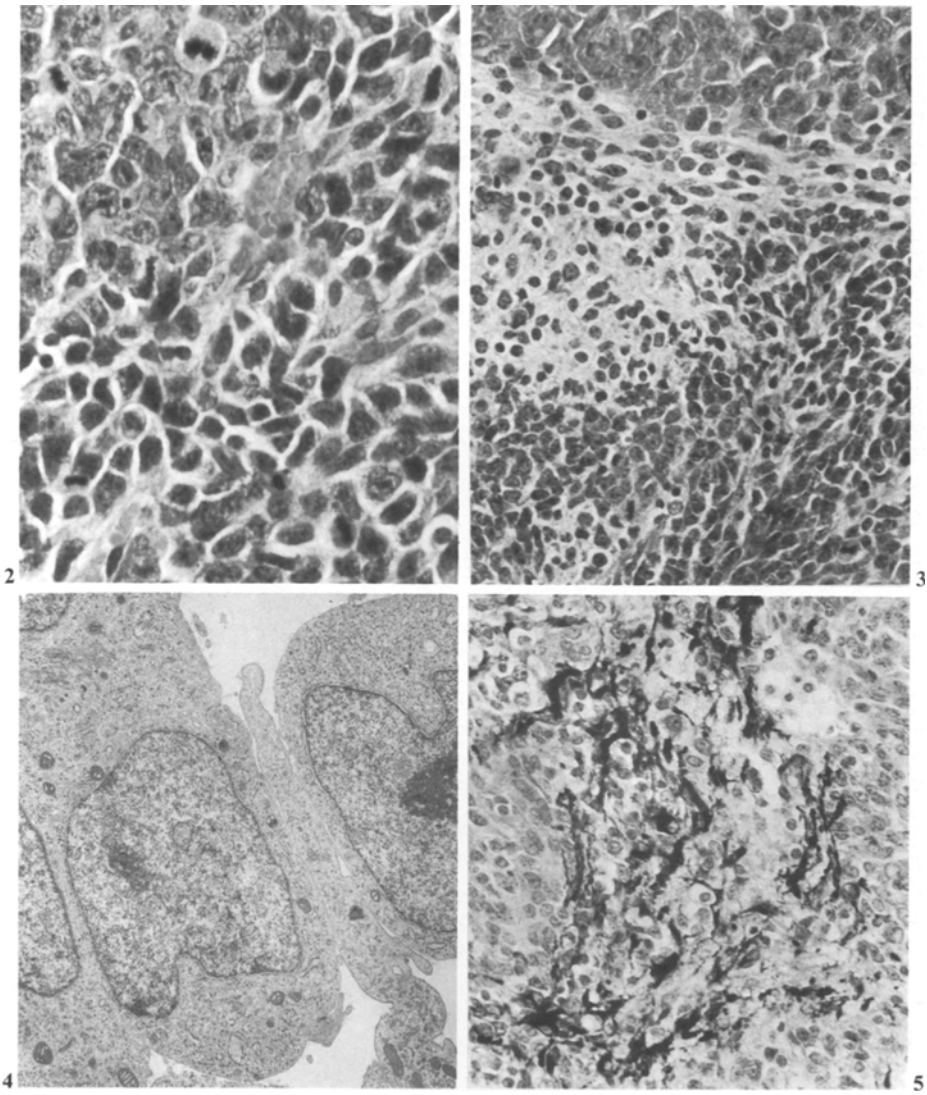


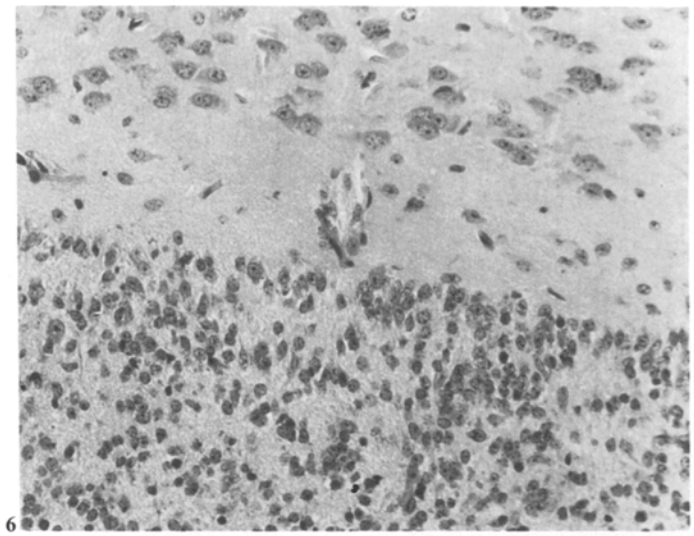
Fig. 2. 33rd renal passage. A typical field of stem cells (upper left corner) adjacent to primitive neuroepithelial cells, which are smaller and have darker, more compact nuclei. Note mitoses in both populations. Hematoxylin-eosin (×480)

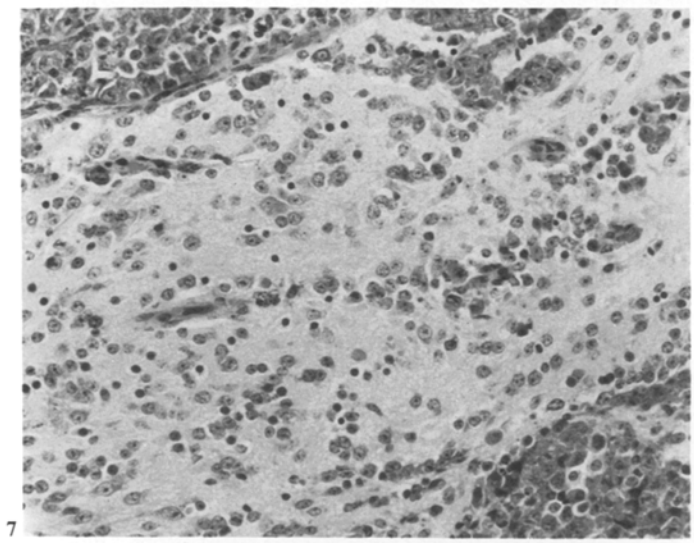
Fig. 3. 33rd renal passage. A different field from the same tumor as in Fig. 2. Stem cells (top), less compactly arranged differentiating neural cells (center) and primitive neural cells (bottom). Hematoxylin-eosin (×300)

Fig. 4. 28th renal passage. Electron micrograph of three primitive neuroepithelial cells showing their polarized cytoplasm and paucity of specialized organelles (×5,200)

Fig. 5. 17th renal passage. GFA protein immunoperoxidase preparation, 1:250 dilution, counterstained with hematoxylin. Numerous positive cell processes are found in areas of differentiating neural cells (×300)

Figs. 2–5 reduced approx. 15%





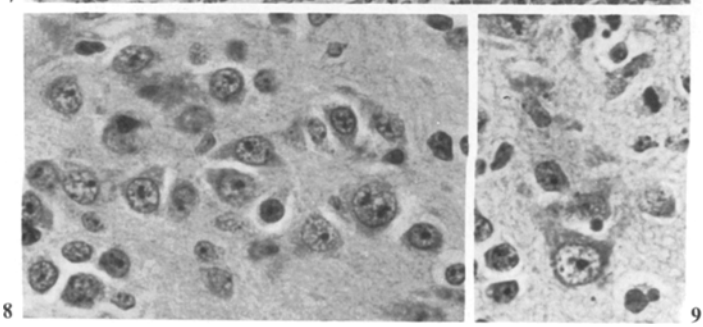


Fig. 6. Intracerebral tumor, 10th passage. Differentiating neural cells (bottom) abutting on normal cortex. Hematoxylin-eosin (×180)

Fig. 7. Subcutaneous tumor, 22nd passage. Maturing tumor neuropil flanked by foci of more primitive, compactly arranged cells. Hematoxylin-eosin (×190)

Fig. 8. Subcutaneous tumor, 26th passage. Field of neurons and maturing neuroblasts. Hematoxylin-eosin (×480)

Fig. 9. Intracerebral tumor, 8th passage. Mature neuron. Note mitotic cell (top right). Hematoxylin-eosin (×480)

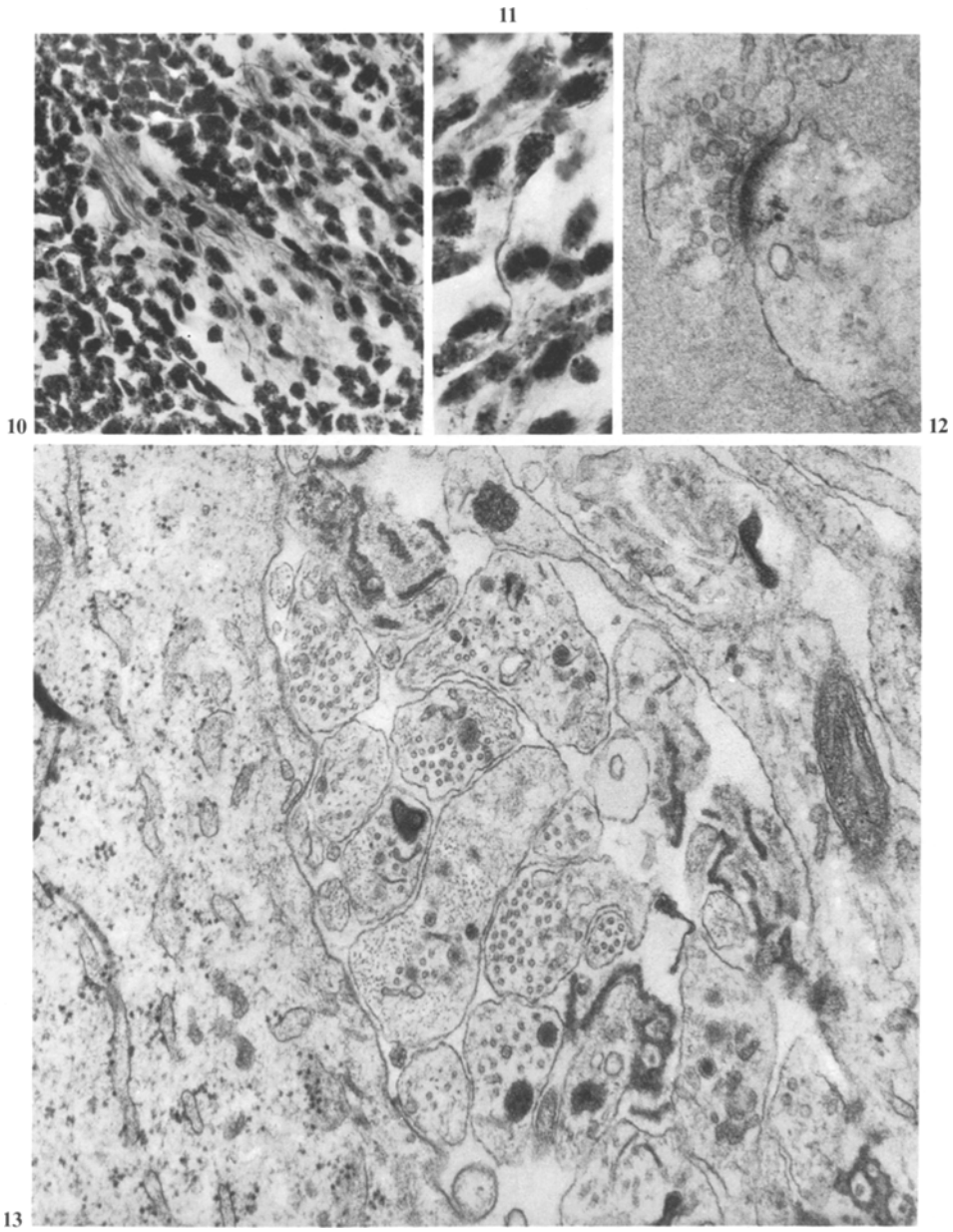


Fig. 10. Subcutaneous tumor, 25th passage. Field of neuroblasts with arrays of delicate neurites. Bielschowsky's silver impregnation for axons, frozen section (× 400)

Fig. 11. As Fig. 10, a different field. Neuroblast with a long apical neurite. Compare with Fig. 1. Bielschowsky's method (× 800)

Fig. 12. Intracerebral tumor, 4th passage. Well-developed synapse (× 53,600)

Fig. 13. Intracerebral tumor, 1st passage. Well-developed neuropil consisting of numerous neurites containing microtubules and/or filaments adjacent to the cell body of a neuron (left field) (× 34,300)

Figs. 10–13 reduced approx. 15%

Subcutaneous and Intracerebral Transplants Derived from Sequential Renal Transplants

Subsequent transplants of the renal tumors to subcutaneous and intracerebral sites usually produced larger proportions of differentiating neuroblasts (Figs. 6–8), mature ganglion cells (Figs. 8 and 9), and astrocytes. Silver-positive processes were found by the Bielschowsky method in the maturing neuroblastic areas (Figs. 10, 11). Electron microscopy confirmed the presence of synapses (Fig. 12) and neurites (Fig. 13). Variable numbers of stem cells and primitive neural cells persisted in these extrarenal sites.

Cells with the light microscopic characteristics of oligodendrocytes were noted. They were negative for myelin basic protein by immunoperoxidase. No myelin was found by electron microscopy. As previously reported, myelin, as determined morphologically and chemically (by immunoradiometric assay), was likewise absent from the parent OTT-6050 tumor (Herman et al. 1975; Ludwin et al. 1976; VandenBerg et al. 1975).

Proximity of the transplants to the brain or to the subcutaneous space did not appear to have any effect on the degree of differentiation of neural cells, nor were there significant differences in cell types or maturation between the superficial and the more deeply placed intracerebral tumors, or between the single passaged or sequentially passaged transplants.

Ascitic Conversion

Three separate trials of conversion to ascitic form yielded transplantable ascitic tumor cells in 10 of 14 recipients. In additon, autopsy of the tumor-bearing animals revealed numerous intraperitoneal implants with variable renal, adrenal and hepatic metastases and very occasional pulmonary seeding. One trial with a primary subcutaneous implant of OTT-6050 also yielded a transplantable ascitic tumor, confirming previous observations (Stevens 1970).

The ascitic form of IB-21 consistently contrasted markedly with that of the control OTT-6050 parent tumor, even after three sequential intraperitoneal passages. IB-21 contained many multi-sized, unorganized cell clusters in addition to single tumor cells, and lacked the typical highly-organized embryoid bodies characteristic of the ascitic form of OTT-6050. The unstructured cell clusters of IB-21 were composed mostly of undifferentiated cells, while the intraperitoneal solid implants and visceral implants resembled the tumors before their conversion to the ascitic form. The cytology of the ascitic tumor cells of IB-21 was similar to that illustrated for IB-9, against which the typical embryoid bodies obtained with OTT-6050 are shown for comparison (VandenBerg et al. 1981).

Discussion

Centrifugal elutriation of the OTT-6050 solid teratoma followed by renal implantation produced two different transplantable tumors, both of which were distinct from the parent tumors. IB-21, described in this report, differed significantly by its restriction to undifferentiated stem cells and neural cells, and by its

inability to form ascitic embryoid bodies. The morphologic characteristics were maintained during a period of 5 years over a course of 58 sequential transplantations. Neuroepithelial differentiation of a usually primitive character was maintained during the renal transplantations. Subcutaneous or intracerebral transplants derived from the renal implants evinced an enhanced capacity for a wide spectrum of neuroepithelial differentiation, with the demonstration of mature astrocytes and synapse-forming neurons.

Sedimentation velocity was the sole basis for separating cells using elutriation with centripetal flow. Density, diameter and surface membrane projections of the viable cells were important factors that determined sedimentation velocity. Phase microscopy showed that the elutriated fractions had a larger proportion of cells with smaller diameters, cytoplasmic processes and similar cytology, but considerable heterogeneity remained. The results of the teratoma elutriation, while not unexpected, could not be predicted. The most neuroepithelial-appearing fractions, which had the lowest sedimentation velocities and which contained many cells with processes, never produced tumors. The fractions which developed transplantable tumors and which contained cell populations of low to moderate sedimentation velocities (0.08–0.14 mm/min at 1 g), were more heterogenous in their cell diameter and morphology. In the three fractions (IB-8, IB-9, IB-21) which produced tumors, the slowest sedimenting cells were part of IB-21. Heterogeneity, associated with an enrichment of neural cells, has likewise been described in rat embryonic cerebral cells that were dissociated and subjected to centrifugal elutriation (Schengrund and Repman 1979).

The reasons for the apparent differences in the spectrum of neuroepithelial maturation between renal and extrarenal sites are not clear. Tumor vascularization, size, and duration of implant growth, in addition to as yet uncharacterized tissue-specific factors, may be important in maintaining the extensive population of mitotically active primitive neural cells in the renal implants. The control OTT-6050 tumors implanted beneath the renal capsule demonstrated, however, that the renal environment in the absence of previous elutriation failed to produce the distinct morphologic features of IB-21. The fact that neural maturation was similar in the subcutaneous and intracerebral implants of IB-21 and in the solid tumors of the unelutriated OTT-6050 indicates that there was no specific extrarenal influence on neuroepithelial differentiation.

The absence of a non-neural teratomatous component in IB-21 offers analogies with a teratocarcinoma reported previously in C3H/H mice (Damjanov et al. 1973), in which differentiation was restricted solely to neuroectodermal derivatives. The two tumors share a number of similarities. Both originally arose from renal subcapsular implants and contained undifferentiated stem cells that were indistinguishable from the multipotential stem cells of teratomas. When converted into an ascitic form, neither developed embryoid bodies. This finding might suggest a possible relationship between the inability to form embryoid bodies and the absence of divergent somatic differentiation from undifferentiated stem cells, but cloning experiments have shown that such a relationship is not constant (Kahan and Ephrussi 1970). Indeed, in our studies, conversion of a solid OTT-6050 tumor to ascitic form initially yielded either the typical embryoid bodies or only single tumor cells with large necrotic cell

clusters. In either case, the solid visceral tumor implants resulting from the intraperitoneal spread of the ascitic form were teratomas resembling OTT-6050. Ultrastructural evidence for myelin in both the IB-21 and the C3H/H tumors was lacking (Tresman and Evans 1975) and no myelin basic protein was found in IB-21.

There are, however, several differences between the two tumors showing restricted neuroepithelial differentiation. Aside from their occurrence in different strains of mice, the C3H/H strain-tumor was serially transplanted subcutaneously while IB-21 was maintained by intrarenal banking. The loss of teratomatous elements occurred during the early subcutaneous passaging of the C3H/H tumor, while the loss in IB-21 occurred after elutriation of OTT-6050 tumors followed by sequential renal transplantation. The neural cells in IB-21 frequently demonstrated mitotic figures and lost the ability to form rosettes. The C3H/H neuroectodermal cells formed rosettes, and only the undifferentiated stem cells showed significant numbers of mitotic figures.

The absence of non-neural differentiation in IB-21, its capacity for divergent neural differentiation, and the presence of abundant mitotic primitive neural cells suggest that a transplantable neural-determined cell population exists in the OTT-6050 teratoma. Such a transplantable, and presumably tumorigenic, population enhances the applicability of this tumor as a system to study the neoplastic neuroepithelial differentiation of human embryonal CNS tumors.

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