Z. Krebsforsch. 89, 273—295 (1977)

Zeitschrift für Krebsforschung und Klinische Onkologie
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Phenotypic Properties of Neoplastic Cell Lines Developed from Fetal Rat Brain Cells in Culture After Exposure to Ethylnitrosourea in vivo* **

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Summary. We have recently reported that fetal BD IX-rat brain cells (FBC), transferred to long-term culture after a transplacental pulse of EtNU on the 18th day of gestation, undergo neoplastic transformation in vitro ("BT-cell lines"). Tumors developed upon s.c. reimplantation of BT-cells into baby BD IX-rats, appeared histologically as neurinoma-, glioma- or glioblastoma-like, and frequently as pleiomorphic neoplasms. In spite of a more atypic cellular morphology, these tumors grossly resembled the different types of neuroectodermal rat neoplasms induced by EtNU in vivo. Like the neoplastic cell culture lines derived from EtNU-induced, neuroectodermal BD IX-rat tumors ("V-cell lines"), the BT-lines contained multipolar glia-like cells, but also flat cells with fewer and shorter cytoplasmic processes, and occasionally giant cells. Both the V- and BT-lines showed different levels of aneuploidy. They contained multiple subpopulations of cells, as reflected, e.g., by plurimodal pulse-cytophotometric DNA distributions. All lines contained, to varying degrees, the nervous system-specific protein S-100, a "marker" not yet expressed in FBC. There was no indication of more than borderline neurotransmitter activity, suggesting that proliferating (precursor) cells of glial lineages may preferentially undergo malignant transformation after exposure to EtNU during this stage of brain development.

* This is communication No. 14 on a research program devoted to molecular and cellular mechanisms in pulse-carcinogenesis by N-ethyl-N-nitrosourea in the rat nervous system

** Research supported by the Deutsche Forschungsgemeinschaft (Ra 119/5-6; SFB 51/E-13), the C. Bohnewand-Foundation and by the Norwegian Cancer Society

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Abbreviations. EtNU, N-ethyl-N-nitrosourea; i.v., intravenous; s.c., subcutaneous; FBC, fetal BD IX-rat brain cells; PBS, phosphate buffered saline; BSA, bovine serum albumin.

Zusammenfassung. Gehirnzellen der fetalen (18. Tag der Gravidität) BD-IX-Ratte, nach einem transplacentaren N-Äthyl-N-Nitrosoharnstoff (ÄNH)-Puls in vivo in ein Langzeit-Zellkultursystem überführt, durchlaufen den Prozeß der neoplastischen Transformation in vitro (BT-Zellinien). Die nach s.c. Rückimplantation von BT-Zellen in neugeborene BD IX-Ratten entstandenen Tumoren (histologisch vom Neurinom-, Gliom- oder Glioblastomtyp, häufig auch pleomorph) waren insgesamt den verschiedenen Arten neuroectodermaler Neoplasmen vergleichbar, die bei BD IX-Ratten nach ÄNH-Applikation in vivo beobachtet werden. Ebenso wie Zellkultur-Linien (V-Zellinien), die von ÄNH-induzierten Tumoren des Nervensystems der BD IX-Ratte abgeleitet wurden, enthielten die BT-Linien multipolare, gliaähnliche Zellen, neben wenigen Riesenzellen aber auch flache Zellen mit vergleichsweise wenigen, kürzeren cytoplasmatischen Fortsätzen. Die V- und BT-Linien waren verschiedengradig aneuploid und aus multiplen Subpopulationen von Zellen zusammengesetzt, widergespiegelt u.a. durch plurimodale impulscytophotometrische DNS-Verteilungen. Alle Linien enthielten das vorwiegend gliaspezifische „Marker"-Protein S-100, welches im fetalen Gehirn noch nicht exprimiert ist. Eine nennenswerte Neurotransmitter-Aktivität wurde nicht gefunden. Nach Applikation von ÄNH während der perinatalen Phase der Gehirnentwicklung werden offenbar vorwiegend proliferative Zellen glialer (Vorläufer-) Populationen neoplastisch transformiert.

Introduction

We have recently described an "in vivo-in vitro-system" (Laerum and Rajewsky, 1975) where fetal (18th day of gestation) BD IX-rat brain cells (FBC) undergo neoplastic transformation in vitro, after a transplacental pulse of the ethylating carcinogen N-ethyl-N-nitrosourea (EtNU) (Ivankovic and Druckrey, 1968) in vivo and subsequent transfer to long-term cell culture. The cultured FBC became tumorigenic (as assayed by reimplantation into isogeneic hosts), after an average time interval similar to the median time ($t_{50} \sim 200$ days) until death with neuroectodermal (Wechsler et al., 1969) neoplasms, in the offspring of pregnant BD IX-rats treated transplacentally with the same dose of EtNU (75 µg/g body wt.) at the same stage of fetal development (Goth and Rajewsky, 1974; Laerum and Rajewsky, 1975). The exceptionally high carcinogenicity of EtNU in the fetal and early postnatal rat brain (Druckrey et al., 1970) suggests a relationship between the probability of neoplastic transformation on the one hand, and the proliferative activity and differentiated state of the target cells on the other (Rajewsky, 1972; Goth and Rajewsky, 1974; Laerum and Rajewsky, 1975).

The FBC population initially exposed to EtNU in vivo, contains cells of both glial and neuronal lineages at different stages of differentiation, with a probable predominance of glial precursors (Langmann et al., 1971; Laerum and Rajewsky, 1975). Therefore, this system may give information on (i) the interference of neoplastic transformation with the process of differentiation for specific phenotypic properties of glial or neuronal cells, (ii) the persistence or reappearance, after neoplastic conversion, of phenotypic "markers" characteristic of FBC, and (iii)

the comparative histologic patterns of solid tumors obtained upon reimplantation into isogeneic hosts of FBC transformed in culture, and those of the corresponding autochthonous neuroectodermal neoplasms.

The present report is concerned with some of the properties of neoplastic cell lines developed from FBC in vitro (cellular morphology; proliferative characteristics; karotypes; neurotransmitter enzymes; nervous system-specific S-100 protein), and with the histologic appearance of the corresponding tumors obtained by reimplantation of these cells into baby BD IX-rats.

Materials and Methods

Animals

Rats of the inbred strain BD IX (Druckrey, 1971) were used. The 1st day after conception was counted as day 1 of gestation.

Cell Culture Lines Derived from Neuroectodermal Tumors Induced by EtNU in vivo ("V-Lines")

These lines originate from neuroectodermal tumors developed in the offspring of pregnant BD IX-rats after a single i.v. pulse of 25 µg of EtNU/g body weight on the 18th day of gestation (Goth and Rajewsky, 1974; Laerum and Rajewsky, 1975). The histoligic appearance and local distributon of EtNU-induced BD IX-rat tumors (mainly mixed gliomas, astrocytomas, oligodendrogliomas, glioblastomas, and ependymomas of the brain; neurinomas of the N. trigeminus, the plexus lumbosacralis and brachialis, and of peripheral nerves) have been described (Ivankovic and Druckrey, 1968; Wechsler et al., 1969). From these tumors, V-lines were established in monolayer or suspension culture, as well as transplantation tumors passaged in BD IX-rats either s.c. or in the ascitic form, in part by alternating in vitro-in vivo passages. Cloned sublines were obtained by picking single cell-derived colonies (Hanke and Rajewsky, 1975; Laerum and Rajewsky, 1975) from semi-solid (0.15%) agar medium (Macpherson and Montagner, 1964). The designations and histological types of the original neuroectodermal tumors are listed in Table 1.

Neoplastic Cell Lines Developed from FBC in Culture After Exposure to EtNU in vivo ("BT-Lines")

The procedure for establishment and maintenance of FBC cultures have been described (Laerum and Rajewsky, 1975). Briefly, FBC suspensions were obtained from the brains of BD IX-rat fetuses excised at 20—90 h after an i.v. transplacental pulse of 75 µg of EtNU/g on the 18th day of gestation, and transferred to cell culture (1—2 × 10^6 viable celles/100 mm plastic dish or 250 ml plastic culture flask, respectively; Falcon Plastics, Oxnard, CA, USA). After a characteristic sequence of phenotypic alterations (including morphological changes, increased proliferation rate, formation of "piled-up" foci, reduction of substrate adhesion, and acquisition of the ability to form colonies in semi-solid (0.15%) agar medium, the cells finally became tumorigenic after ~ 200 days (as assayed by reimplantation into baby BD IX-rats; Laerum and Rajewsky 1975). Seven "par-

Table 1. Characteristics of cultured neurogenic cell lines (V-lines) derived from autochthonous neuroectodermal tumors, induced by a transplacental pulse of EtNU to fetal (18th day of gestation) BD IX-rats

Designation of tumour	Origin	Histological appearance	Cellular morphology in monolayer culture	Giant cells	S−100 protein[d]
GV 1	Right brain hemisphere	Mixed glioma[a] Neurinoma-like, with glial elements[b]	Small bi- or tripolar, spindle-like cells with long cytoplasmic processes	−	+
TV 1	N. trigeminus	Neurinoma I−II[c]	Small, mainly tripolar cells with long cytoplasmic processes, and large flat, often binucleate cells	(+)	±
NV 1	Pl. lumbo-sacralis	Neurinoma I−II[b]	Mainly large, flat cells with short processes. Few tripolar cells. Many piled-up foci	+	±

[a] Original tumor
[b] 2nd transplant passage
[c] 1st transplant passage
[d] By indirect immune fluorescence (see Material and Methods). +, most or all cells positive. ±, positive and negative cells

ental" malignant neurogenic cell lines (BT1C-BT7C; see Table 2) were obtained by this procedure. All BT-lines could also be maintained as transplantation tumors ("BT culture-derived tumors") in BD IX-rats.

Cell Culture Conditions

Modified Eagle-Dulbecco medium (Frank et al., 1972), supplemented with 10% inactivated bovine serum, was used. The cultures were kept at 37° C under a humidified atmosphere of 5% CO_2 in air, and the medium was renewed at 3 day-intervals. For passaging, cultures were harvested by trypsinization with 0.25% trypsin in Ca^{++}- and Mg^{++}-free PBS, and diluted at 1:10 to 1:20. Prior to staining with hematoxylin and eosin, cell cultures were washed twice with PBS at 37° C, and fixed in ethanol/acetic acid (3:1).

Transplantation and Histologic Analysis of Tumors

Neuroectodermal tumors developed in vivo at typical sites (i.e., brain, N. trigeminus, plexus lumbosacralis; see Table 1) were serially passaged in BD IX-rats, either in the solid form by s.c. transplantation of tissue explants ($\sim 1\ mm^3$), or (in the case of the GV1A2-tumor) in the ascitic form by i.p. passage of $\sim 10^6$ cells/animal. To assay for tumorigenicity, cultured FBC (1—4 $\times 10^6$ cells/animal in 0.1 ml of culture medium) were injected s.c. into 5—10 day-old BD IX-rats. Tumors of 1—2 g were excised and passaged in adult BD IX-rats. Tumor specimens were fixed in buffered 4% formalin. Microtome sections (5 µm) were stained with hematoxylin and eosin, Nissl, or Wilder silver stain.

Table 2. Characteristics of "parental" neoplastic cell lines developed from FBC in monolayer culture after exposure to EtNU in vivo (BT-lines). The two BT4C-derived tumors (a, b) were produced by cells from the 10th and 13th culture passage, respectively. S − 100 protein (by indirect immune fluorescence; see Material and Methods): +, most or all cells positive; + −, positive and negative cells

Designation of cell line	Cell culture lines				Tumors obtained after s.c. reimplantation of cultured cells into isogeneic hosts		
	Cellular morphology			Expression of S − 100 protein	in vivo transplant passage No.	Histological appearance	Giant cells present
	Glia-like cells	Flat cells	Giant cells				
BT1C	+	+	+	+ −		Anaplastic tumors:	
					1	Astroblastoma-like	+
					2	Glioblastoma-like	+
					9	Glioblastoma-like	+
BT2C	+	+	+	+ −	3	Pleiomorphic tumor	+
BT3C	+	−	+	+	1	Astroblastoma-like	−
BT4C	+	+	+	+ −		Pleiomorphic tumors:	
					1	(a) "Round-cell" type	+
					1	(b) Neurinoma-like	−
BT5C	+	+	+	+ −		Pleiomorphic tumors:	
					1	Glioblastoma-like	+
					4	Mixed glioma-like	+
BT6C	+	+	+	+ −		Pleiomorphic tumors:	
					1 − 3	"Round-cell" type	+
					5	Astroblastoma-like	+
					10	"Mixed" type	+
BT7C	+	+	+	+	1 − 2	Malignant neurinoma I − II	−

Pulse-Cytophotometric Measurement of DNA Content/Cell, and Chromosome Analysis

For pulse-cytophotometric analysis of the relative DNA content/cell, 0.5—5.0×10^5 ethanol-fixed cells/sample were treated with RNase (1 mg/ml, 37° C, 1 h), pepsin (2 mg/ml of 0.2% HC1, 37° C, 15 min), and their DNA stained with ethidium bromide (10 μg/ml of Tris buffer, pH 7.5), as previously described (Hanke and Rajewsky,1975; Laerum and Hansteen, 1975). All chemicals were obtained from Serva GmbH (Heidelberg, Germany). Relative fluorescence intensity/cell (proportional to relative cellular DNA content) was measured in an ICP 11 pulse-cytophotometer (PHYWE AG, Göttingen, Germany).

For chromosome analyses, log-phase cell cultures were incubated with colcemide (Ciba-Geigy AG, Basel, Switzerland; 3 μg/ml of culture medium, 1 h), harvested by trypsinization, washed in Hanks' balanced salt solution, centrifuged, and resuspended in 75 mM KC1 for 8—10 min (Laerum and Hansteen, 1975). After fixation in Carnoy's fixative on microscope slides, chromosomes were stained by the Giemsa banding technique (Summer et al., 1971), and ~ 100 mitotic cells/sample were analyzed according to Levan's nomenclature for G-bands in rat chromosomes (Levan, 1974).

S-100 Protein Assay

For production of specific antiserum, the predominantly glia-specific (Cicero et al., 1970) S-100 protein was prepared from beef brain and further purified as described by Moore (1965). All buffers contained 2.5 mM EDTA and 0.1 mM 2-mercaptoethanol. The purity of the protein was controlled both by disc electrophoresis and electrophoresis in a continuous buffer system (Plescia et al., 1964; Haglid and Stavrou, 1973). A single precipitation band was obtained when the purified potein was subjected to double diffusion against its antiserum in 1% agar. Antiserum was prepared using a complex of S-100 protein with methylated BSA as antigen (Haglid and Stavrou 1973). One ml of 0.15 M NaCl containing 1 mg of purified S-100 protein, was added to 0.1 ml of 1% (w/v) BSA. The resulting flocculent suspension was emulsified with an equal volume of complete Freund's adjuvant and injected into the toe-pads and hind subcutis of albino rabbits, 4 times at weekly intervals. Ten weeks after the last injection, a single i.v. injection was given as a booster, and blood for antiserum preparation was collected 10 days later.

The presence of S-100 protein in cultured log-phase V- and BT-cells was assayed by indirect immune fluorescence. Glass coverslips (20 × 20 mm) were placed into plastic dishes, and removed when covered with a sufficient number of cells, either during log-phase proliferation or at confluence. The coverslips were washed twice with PBS, fixed in acetone for 30 sec at 4° C, dried and stored at −20° C. To avoid nonspecific fluorescence, the anti-S-100 serum was absorbed with acetone-treated liver powder (Hijmans et al., 1969) prior to use. After a 5 min wash in PBS, the coverslips were covered with antiserum (at dilutions of 1:2—1:8 in PBS) for 30 min, and washed 3 times for 5 min in PBS (pH 7.1; 4° C). Goat anti-rabbit globulin-γ-globulin conjugated with fluorescein isothiocyanate (Flow Labs., Irvine, Scotland) was then applied to the cells for 30 min. Excess antibody was removed by repeated washing with PBS. The coverslips were mounted in buffered glycerol, and the cells examined in a Zeiss UV fluorescence microscope. Control cells treated as described above (except for exposure to anti-S-100-serum) were run in parallel, as well as cells exposed to anti-S-100-serum repeatedly absorbed with the antigen, to an unrelated hyperimmune serum (rabbit anti-human IgG), to preimmune serum, or to normal rabbit serum (dilutions 1:2—1:20). These controls were negative.

Glial Fibrillary Acid (GFA) Protein

The brain-specific GFA protein, a major constituent of neuroglial fibers (Dahl and Bignami, 1973), was assayed by indirect immune fluorescence (courtesy of A. Bignami).

Myelin Basic Protein

Following acid extraction of cell or tissue homogenates, the myelin basic protein (Kies, 1965) was assayed as recently described (Sundarraj et al., 1975).

Neurotransmitter Synthesis

Either during log-phase proliferation or at confluence, cells were removed from petri dishes with a rubber policeman, washed twice in PBS, and homogenized.

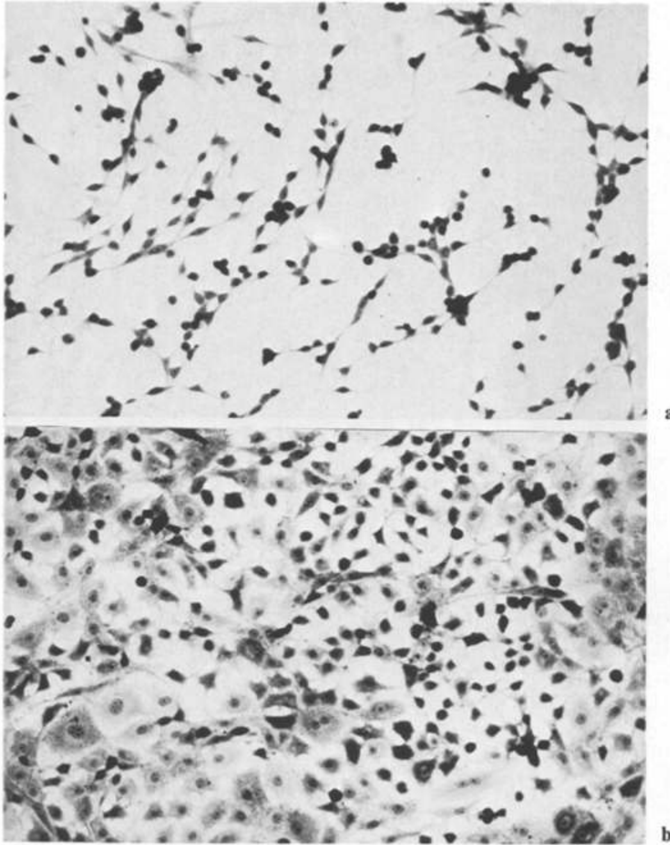


Fig. 1a and b. Cellular morphology in neoplastic neurogenic cell culture lines (V-lines) derived from different EtNU-induced neuroectodermal tumors of the BDIX-rat. **a** Mixed glioma-derived line GV1C, consisting mainly of small bi- or tripolar cells. **b** Trigeminal neurinoma-derived line TV1C, containing both small bi- or tripolar cells, and larger flat epithelioid cells. Hematoxylin-eosin stain. Magnification × 470

Mouse brain, as well as two non-neurogenic cell lines in monolayer culture [BICR/M1R_K rat mammary tumor cells (Rajewsky and Grüneisen, 1972); and 3T3 Balb/c mouse embryo fibroblasts], were used as controls. Protein determinations were made by the method of Lowry et al. (1951).

Acetylcholine esterase (AChE; EC 3.1.1.7) activity was measured according to Hall (1973). Choline acetyltransferase (ChAc; EC 2.3.1.6.) activity was determined as described by Giller and Schwartz (1968). The activity of L-glutamate descarboxylase (GAD; EC 4.1.15), catalyzing the α-decarboxylation of L-glutamic acid to form the inhibitory transmitter γ-aminobutyric acid (Roberts et al., 1976), was measured in cell lines GV1C and BT6C (courtesy of J. Storm-Mathisen). Final concentrations in the assay mixture were 2.6 mM DL-[1-^{14}C] glutamic acid (25 mCi/mM; The Radiochemical Centre, Amersham, England), 10.2 mM sodium-L-glutamate, 50 mM sodium phosphate buffer (pH 6.5), 0.1 mM pyridoxal phos-

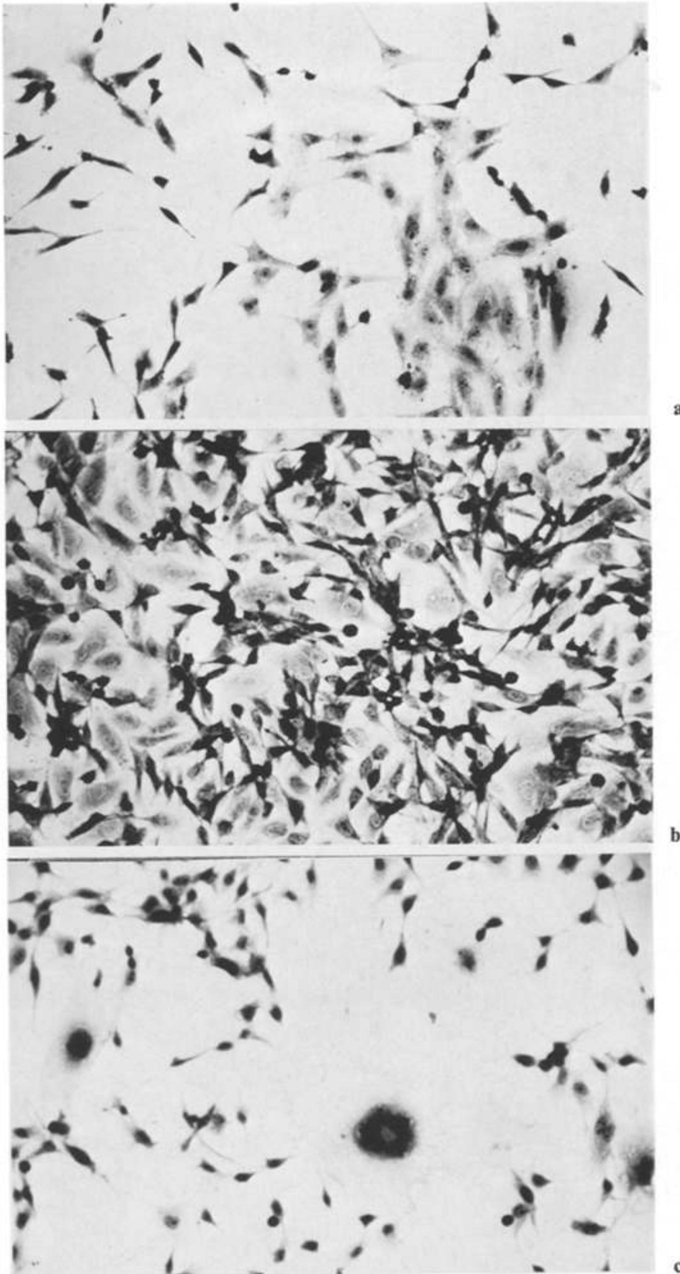
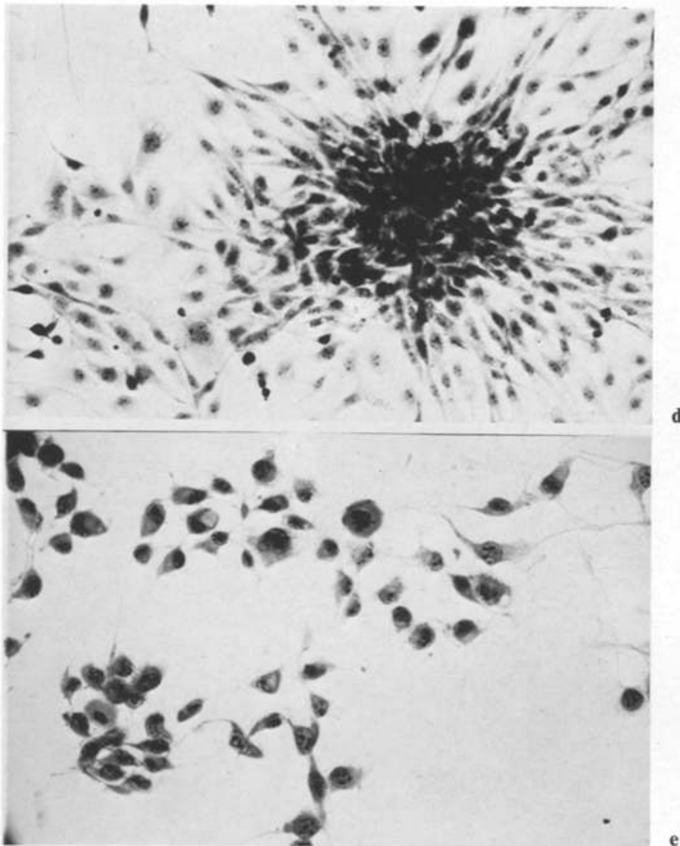


Fig. 2a—e. Cellular morphology in neoplastic neurogenic cell culture lines (BT-lines), derived from BD IX-rat FBC transformed in culture after exposure to EtNU in vivo. Cell lines BT2C (**a**) and BT6C (**b**) consist of mixed populations of both small glia-like, and larger flat epithelioid cells. **c** Cell line BT5C, containing both small glia-like cells, and giant cells. **d** Cell line BT5C, with typical „piled-up" focus. **e** Cell line BT3C, consisting of larger (approximately hexaploid; see Table 4) glia-like cells. Hematoxylin-eosin stain. Magnification × 470



phate, 1 mM dithiothreitol, and 1.5 mg/ml Triton-X-100. Incubation was at 37° C for 60 min. Under these conditions, interference by the supposedly non-neuronal GAD activity, "GAD-II", is minimized (Storm-Mathisen and Fonnum, 1971; Roberts et al., 1976).

gs-Interspecies Viral Antigens

Cell lines NV1C, GV1C, BT3C, and BT5C2 (a BT5C subline), were analyzed serologically in the Ouchterlony test, for the presence of gs-interspecies RNA tumor virus antigens as described by Schäfer et al. (1973) (courtesy of W. Schäfer).

Results

Morphologial Appearance of Cells in Culture

Cell Lines Derived from Autochthonous, EtNU-Induced Neuroectodermal Tumors ("V-Lines")

As indicated in Table 1 and Fig. 1, three main cell types were observed in the V-lines grown in monolayer culture: small bi- or tripolar glia-like cells with long

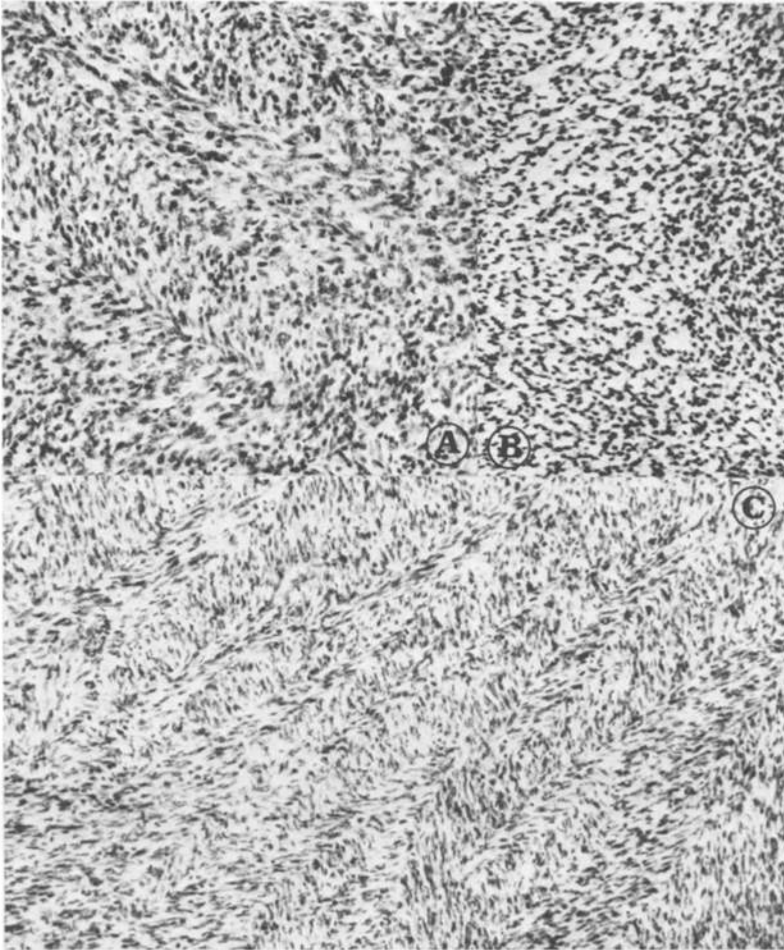


Fig. 3a—c. Typical histologic appearance of neuroectodermal BD IX-rat tumors induced by a transplacental pulse of EtNU in vivo, and carried by s.c. transplantation in BD IX-hosts. **a** and **b** Mixed glioma (GV1), 2nd transplant passage. **c** Peripheral neurinoma (NV1), 38th transplant passage. Hematoxylin-eosin stain. Magnification ×60

cytoplasmic processes; larger flat cells with shorter processes, sometimes with an epithelioid appearance; and few giant cells. GV1C-cultures derived from the mixed glioma GV1, almost exclusively contained cells of the glia-like type; TV1C-cultures derived from the trigeminal neurinoma TV1, showed a mixture of all cell types; and NV1C-cultures established from the peripheral neurinoma NV1 mostly contained flat cells.

Parental (i.e., Non-Cloned) Neoplastic Cell Lines Developed from FBC in Culture After Exposure to EtNU in vivo ("BT-Lines")

The morphological appearance of the BT-cells was generally similar to that observed in the V-lines: glia-like cells, flat cells and some giant cells. While a mixture

of these different cell types was usually present, the majority of the cells always had a glia-like appearance (Table 2). All BT-cultures characteristically exhibited multiple denser areas, frequently with piled-up cells (Table 2; Fig. 2). In confluent cultures, glia-like cells were often seen on an underlayer of flat cells (Fig. 2b). Glia-like cells of an unusually large size were observed in the parental line BT3C which mainly consists of hexaploid cells (Table 4; Fig. 2e).

Cloned V- and BT-Sublines

Cloned sublines established from single V- or BT-cell colonies in semisolid agar medium, exhibited a uniform cellular morphology characteristic of the respective cellular subtypes observed in the corresponding parental lines. Although the morphology of the cloned lines was remarkably stable over prolonged periods of culture, the glia-like lines showed a tendency towards a gradual reduction in length and number/cell of cytoplasmic processes.

Histologic Appearance of Autochthonous and BT-Culture-Derived Tumors

Autochthonous and Transplanted EtNU-Induced Neuroectodermal Tumors

The histologic patterns of the three selected tumors are described in Table 1. On further s.c. passaging, the neurinoma TV1 and NV1 retained their characteristic histologic pattern, with some variation between a high degree of cellularity and the formation of multilocular, fluid-filled cysts (Fig. 3). The mixed glioma GV1, however, gradually shifted to a more "neurinoma-like" appearance during later animal passages.

Tumors Grown in Baby BD IX-Rats from Neoplastic FBC Transformed in Culture After Exposure to EtNU in vivo ("BT Culture-Derived Tumors")

As assayed by s.c. reimplantation into baby BD IX-rats, FBC transferred to long-term culture alfter a pulse of 75 µg of EtNU/g to fetal BD IX-rats, became tumorigenic after a mean period of 199 ± 28 days (S.E.) culture passages (Laerum and Rajewsky, 1975; Table 2). Cells from earlier culture passages did not produce tumors during an observation period of 1 year. All tumors could be further passaged in BD IX-hosts. The cultured BT-lines retained their tumorigenicity also at later passages (tested for up to 2 years). At the first positive reimplantation the mean latency period from s.c. injection of cells until the first tumors became palpable was 48 ± 13 days (S.E.); however, intervals of up to 10 months were occasionally recorded (Laerum and Rajewsky, 1975). Latency intervals decreased somewhat during subsequent transplant passages (see Table 3), and were similar or slightly longer than those recorded for the transplanted, autochthonous neuroectodermal tumors.

In spite of an overall resemblance to autochthonous EtNU-induced tumors (Table 1; Fig. 3; compare Benda et al., 1971), the histologic appearance and cellular phenotypes of the BT-culture-derived tumors were more heterogenous (Table 2; Fig. 4), sometimes varying between different transplant passages. Malignant BT-cells gave rise to three main types of tumors: Neurinoma-like, glioma- or glioblastoma-like, and undifferentiated, pleiomorphic neoplasms. Silver-positive

Table 3. Transplantation characteristics of autochthonous EtNU-induced neuroectodermal tumors and neoplastic neurogenic cell culture lines (BT-lines). See Materials and Methods for experimental details

Origin	Designation of tumors/ cell lines	Time from initial EtNU-pulse until observation of tumor		Duration of 1st animal passage (days)	Average duration of passages 2 – 5 (days ± S.E.)
		Age of animal (days)	Culture period in vitro plus first animal passage (days)		
	Tumors				
Autochthonous,	GV 1	211		78	25±6
EtNU-induced	TV 1	225		52	28±3
neuroectodermal	NV 1	291		33	30±2
tumors					
Mean (±S.E.)		242±25		54±13	28±1
FBC trans-	*BT-lines*				
formed in	BT 1		273	46	n.m. [a]
culture after	BT 2		385	61	21±3
exposure to	BT 3		190	16	n.m. [a]
EtNU	BT 4		259	40	n.m. [a]
in vivo	BT 5		116	31	n.m. [a]
	BT 6		279	118	34±4
	BT 7		228	26	48±7
Mean (±S.E.)			247±31	48±13	34±6

[a] n.m., not measured (due to freeze storage of early passage material)

fibrils were rarely observed. With the exception of neurinoma-like tumors, histologic patterns did not permit a firm diagnosis; the designations "glioma-" or "glioblastoma-like" must, therefore, be considered with caution.

Chrosome Analyses and Pulse-Cytophotometric DNA Distributions of V- and BT-Cell Lines in Culture

The pulse-cytophotometric data on the distributions of cellular DNA content in ethidium bromide-stained cells are summarized in Table 4 and Fig. 5 (see also Fig. 6). The three V-lines analyzed exhibited approximately diploid G_1-DNA values. However, the corresponding chromosome analyses indicated additional mitotic subpopulations with near-tetraploid karyotypes. The GV1C-line contained a high proportion (~ 50%) of mitotic cells with a trisomy for chromosome No. 4. About 4% of the mitotic cells were tetraploid. The TV1C-line was bimodal, with ~ 40% of the mitotic cells exhibiting an apparently diploid karyotype, and ~ 60% with ~ 80 chromosomes; some cells carried an additional marker on chromosome No. 4, other cells showed a reduction in chromosome numbers, down to values $\leqq$ 25. The sublines NV1Cb and NV1Cc were derived from the parental NV1C line (with a near-tetraploid overall chromosome mode) by cloning in semisolid (0.15%) agar medium. The modal G_1-DNA content of the NV1Cc-line approximates a diploid value (Fig. 6). Chromosome analysis, however, indicated the presence of mitotic cells with modes of 59 (~ 8% of the cells) and 78

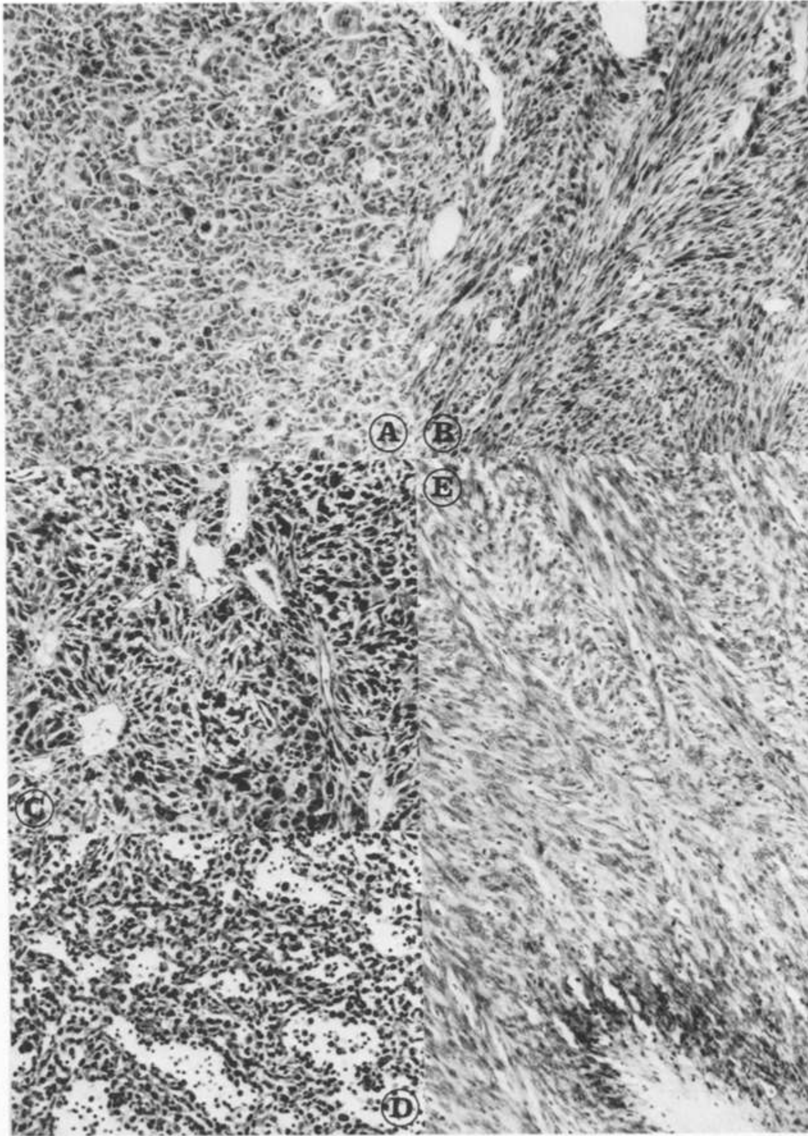


Fig. 4a—e. Varying histological appearance of tumors obtained upon s.c. reimplantation of BD IX-rat FBC transformed in culture (BT-lines) into baby BD IX-rats. **a** BT1C-derived tumor, 1st animal passage (pleiomorphic appearance). **b** BT1C-derived tumor, 9th passage (neurinoma-like). **c** BT5C-derived tumor, 4th passage (astroblastoma-like). **d** BT6C-derived tumor, 5th passage (pseudopapillary growth; astroblastoma-like). **e** BT7C-derived tumor, 1st passage (neurinoma). Except for **e**, none of the diagnostic evaluations given are unequivocal. Hematoxylin-eosin stain. Magnification ×60

Table 4. Modal chromosome numbers, and distributions of DNA content/cell (as measured by pulse-cytophotometry of ethidium bromide-stained cells) of neoplastic neurogenic cell lines (V- and BT-lines). Rat lymphocytes (euploid; modal G_1-DNA value, 2.0) were used for calibration of the pulse-cytophotometer. See text for further details

Cell line	Modal chromosome number	(range)	Ploidy	Fraction of mitotic cells/modal range (%)	Modal G_1−DNA value (relative units)	Coefficient of variation (%)	Fraction of cells per modal G_1−DNA value (%)
V-lines:							
GV1C	42	(38– 44)	(2)	34.3			
	43	(38– 44)	(~2)	51.7	2.0	± 7.8	
	84	(81– 86)	(4)	4.0			
TV1C	42	(25– 48)	(2)	40.2	1.7	± 8.1	
	80	(60– 84)	(3.6)	59.8			
NV1Cc	39	(34– 42)	(1.8)	66.0	2.0	± 9.8	
(cloned	59	(56– 67)	(2.8)	7.5			
subline)	78	(74– 96)	(3.7)	26.5			
BT-lines:							
BT1C	46	(38– 53)	(2.2)	94.5	2.0	±13.1	
	93	(85– 94)	(4.4)	5.5			
BT2C		(31– 60)		12.0			
		(71– 91)		18.0			
	99	(98–130)	(4.7)	58.0	4.2	± 8.6	
		(140–262)		12.0			
BT3C		(45– 85)		15.6	2.0	±23.9	4
	116	(95–145)	(5.5)	57.8	6.4	± 9.4	43
		(145–181)		4.7			
		(200–266)		21.9			
BT4C	65	(52– 80)	(3.1)	50.4	3.0	±14.2	
	125	(94–139)	(6)	46.1			
		(205–242)		3.5			
BT5C	44	(16– 49)	(2.1)	9.8	2.0	± 9.9	11
	69	(50– 82)	(3.3)	74.6	3.4	± 9.7	33
		(95–161)		15.6			
BT6C	42	(38– 56)	(2)	7.0	1.8	±10.9	11
	70	(63– 95)	(3.3)	88.0	3.1	±10.2	72
		(105–148)		5.0			
BT7C	50	(41– 53)	(2.4)	14.6			
	63	(54– 64)	(3)	75.0	2.6	±11.2	
		(75–146)		10.4			

(~27%) chromosomes, in addition to a major mode of 39 (~66%). No changes in the modal G_1-DNA values of the NV1Cc and other cloned sublines (Fig. 6) of the parental neurogenic lines were observed over periods of $\leqq 90$ successive culture passages (Hanke and Rajewsky, 1975).

Plurimodal DNA distributions were recorded for the lines BT3C, BT5C, and BT6C, while for the remaining BT-lines (BT1C, BT2C, BT4C, BT7C) the distributions were not obviously plurimodal (Table 4 and Fig. 5). Only line BT1C had a near-diploid modal chromosome number of 46 (Table 4). All BT-lines contained

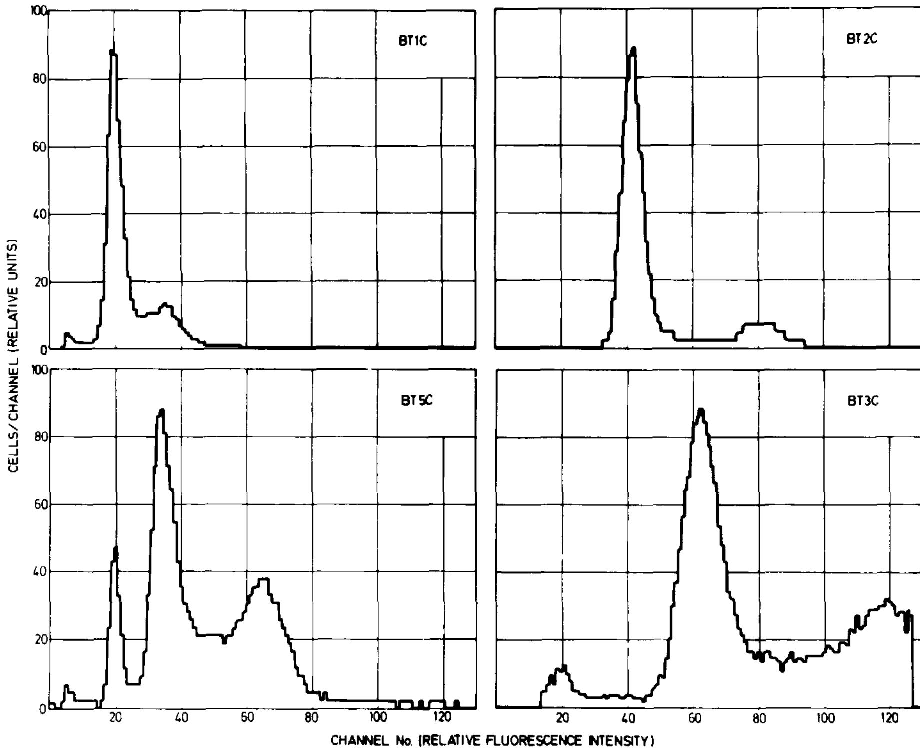


Fig. 5. Pulse-cytophotometric DNA distributions of 4 parental neurogenic BT-lines in culture. All measurements were performed on 1—2 × 10^5 ethidium bromide-stained, log-phase cells. Log-phase BD IX-rat embryo cells in secondary culture [euploid; relative model G_1-DNA value (channel No.), 20.0], or the approximately diploid line GV1C were used for calibration of the pulse-cytophotometer. Note the apparently unimodal distribution of lines BT1C and BT2C, and the plurimodal distributions of lines BT5C and BT3C

different subpopulations of mitotic cells with a hypo- or hyper-diploid chromosome complement, up to hexaploid values (lines BT3C and BT4C; Table 4). Chromosome breaks and marker chromosomes were observed in all BT-lines. The lines BT3C and BT5C contained up to 14 and 15 marker chromosomes/mitotic cell, respectively.

S-100 Protein

Two of the three parental V-lines (GV1C and TV1C) expressed the S-100 protein, as indicated by positive immune fluorescence. The cells of the peripheral neurinoma-derived line NV1C were only weakly fluorescent (Table 1). The mixed glioma-derived line GV1C was characterized by a particularly high fluorescence intensity in most of the cells (Fig. 7).

All BT-Lines contained clearly S-100 protein positive cells; however, only in one of these lines (BT3C) all cells showed a strong fluorescence (Fig. 7a). The other BT-lines also contained varying proportions of S-100 negative cells, some of which

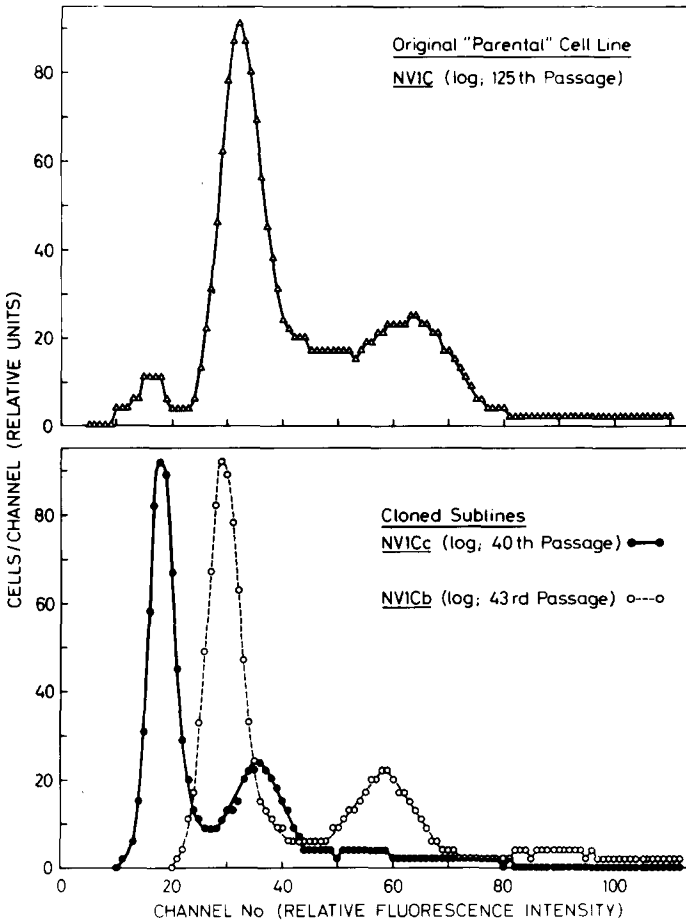


Fig. 6. Pulse-cytophotometric DNA distributions of the "parental" line NV1C (125th culture passage) and of two NV1C-derived, cloned sublines (NV1Cb, NV1Cc; Hanke and Rajewsky, 1975) in their 43rd and 40th culture passage, respectively. Measurements were performed on $5\text{-}10 \times 10^4$ ethidium bromide-stained, log-phase cells. Secondary log-phase BD IX-rat embryo cells [euploid; relative modal G_1-DNA value (channel-No.), 16.0] were used for instrument calibration. Note the unimodal distribution of the cloned sublines, as opposed to the bi- or plurimodal appearance of the parental line

with a glia-like phenotype. Many of the glia-like cells, however, as well as many flat, epithelioid cells, were strongly fluorescent. Cells in mitosis as well as giant cells generally showed a weakly positive fluorescence. The intensity of fluorescence varied considerably between cells. In a given cell, the highest fluorescence intensity was usually observed over the perikaryon; it was lower over the remaining cytoplasm, and almost absent over the nuclei (see Fig. 7). When untreated control FBC (18th day of gestation) were transferred to monolayer culture, these cells gave no S-100 fluorescence during the first few days. Some weakly fluorescent cells began to appear on day 7, but could no longer be detected one day after trypsinization and passaging of the primary cultures on day 11.

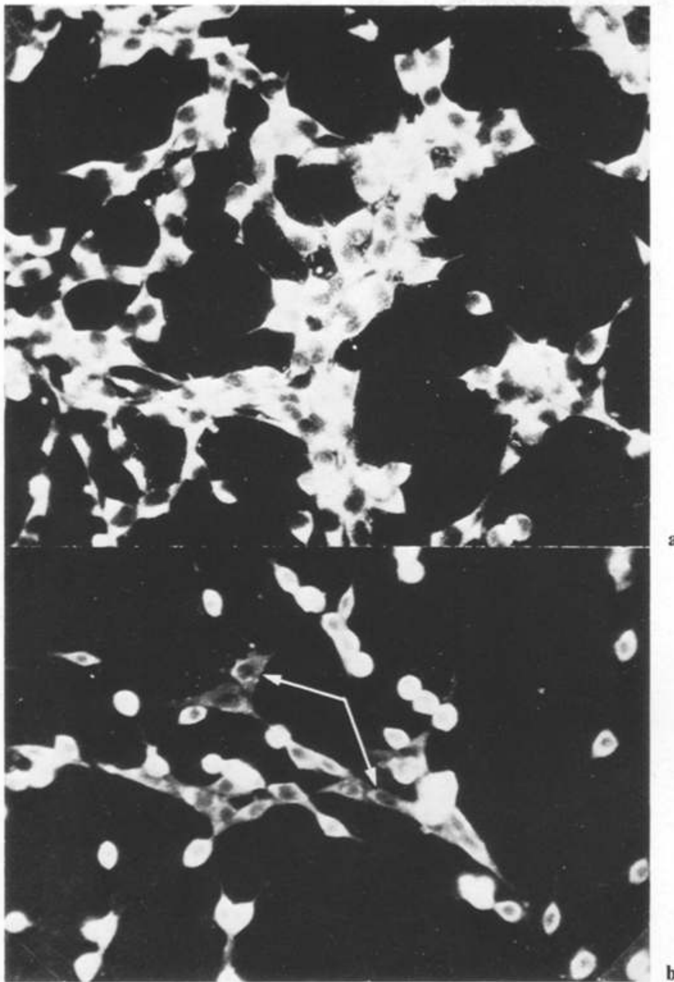


Fig. 7 a and b. Indirect immune fluorescence assay for S-100 protein. **a** Cell line BT3C. Strong fluorescence in all cells. **b** Cell line GV1C. Scattered, strongly fluorescent cells; but note arrows indicating weakly fluorescent perinuclear halos. Magnification × 470

GFA Protein and Myelin Basic Protein

Both the glial fibrillary acidic (GFA) protein and the myelin basic protein could not be detected in any of the V- and BT-monolayer cultures.

Neurotransmitters

To test for the expression of neuronal properties in the neoplastic neurogenic cell lines, these were analyzed for their neurotransmitter activity. No elevated ChAc activity could be detected in any of these lines (Table 5). The level of ChAc activity was $< 1\%$ of that of brain, and identical with that of a non-neurogenic, rat

Table 5. Acetylcholine esterase (AChE) and choline acetyltransferase (ChAc) activity in neoplastic neurogenic cell culture lines. Values for brain and non-neurogenic cell lines are given for comparison

Designation of cell line	AChE activity[a]		ChAc activity[b] confluent cultures
	log-phase cultures	confluent cultures	
V-lines:			
GV1C	0.020	0.095	0.021
TV1C	0.096	0.098	0.036
NV1C	0.015	0.003	0.017
BT-lines:			
BT1C	0.053	0.173	0.049
BT2C	0.125	0.103	0.053
BT3C	0.045	0.004	0.015
BT4C	0.438	0.031	0.023
BT5C	0.007	0.006	0.017
BT6C	0.481	0.636	0.052
BT7C	0.323	0.225	0.037
BICR/M1R_K[c]	0.012	0.010	0.033
3T3[d]		0.030	
Mouse brain	6.01[e]		41.0[f]

[a] AChE activity expressed as μmoles of acetylcholine hydrolyzed/(hour × mg protein of cell homogenate)
[b] ChAc activity expressed as μmoles of acetylcholine synthesized/(hour × mg protein of cell homogetate)
[c] Rat mammary tumor-derived cell culture line BICR/M1R_K
[d] Balb/c mouse embryo fibroblast-derived cell culture line
[e] 4.01 (Amano et al., 1972); 6.96 (Augusti-Tocco and Sato, 1969). Rat brain: 14.4 (Rieger and Vigny, 1976)
[f] 33.0 (Amano et al., 1972); 48.6 (Augusti-Tocco and Sato, 1969). Rat brain: 60.0 (Rossier et al., 1973)

mammary tumor-derived cell line (BICR/M1R_K). Similarly, no AChE activity was found in the V-lines. Again the values were of the same magnitude as in the BICR/M1R_K-cells and in 3T3 mouse fibroblasts (Table 5). The BT-lines, assayed both during log-phase proliferation and at confluency, also gave essentially negative results with regard to AChE activity, with levels similar to those of the non-neurogenic control lines (Table 5). However, three of the BT-lines (BT4C, BT6C, BT7C) exhibited somewhat higher AChE levels than the control cultures, and the AChE activity of confluent BT6C cells amounted to ~ 10% of the value for mouse brain. It cannot, therefore, be excluded that the latter lines may contain subpopulations of cells with elevated AChE activity. Two neurogenic lines (GV1C, BT6C) were assayed for the presence of GAD. While rat cerebral cortex had a GAD activity level of 312 nmoles per hour per mg protein, the corresponding values for the neurogenic lines were below the sensitivity limit of the assay, i.e., $< 7\%$ of the activity in rat cortex.

gs-Interspecies Viral Antigens

No evidence was found for the expression of gs-interspecies antigens characteristic of C-type RNA viruses, when selected V- (NV1C; GV1C) and BT-lines (BT3C; BT5C2, a subline of BT5C) were subjected to serological analysis in the Ouchterlony test.

Discussion

In the fetal and newborn rat, the tumorigenic effect of EtNU is highly specific for the central and peripheral nervous system (Ivankovic and Druckrey, 1968; Druckrey et al., 1970; Goth and Rajewsky, 1974). Both the histological appearance of the resulting neuroectodermal neoplasms, and the cellular morphology and biochemical characteristics of cell culture lines derived from such tumors, are in most cases reminiscent of the phenotypic properties of glial or Schwann cells (Druckrey et al., 1970; Benda et al., 1971; Schubert et al., 1974; Laerum and Rajewsky, 1975). The present data on neoplastic cell lines derived from 3 neuroectodermal BD IX-rat tumors induced by EtNU in vivo (V-lines), and developed from FBC in culture after an EtNU-pulse in vivo (BT-lines), are in agreement with these oberservations. Thus, all V- and BT-lines expressed the predominantly glia-specific acidic protein S-100, though in varying proportions of cells and with differing activity per cell. In the V-lines, the strongest activity was found in the mixed glioma-derived GV1C-cells, followed by the trigeminal neurinoma-derived TV1C-cells, and the peripheral neurinoma-derived NV1C-cells (Table 1). None of the V-lines exhibited significant neurotransmitter activities characteristic of cholinergic or GABA-dependent neuronal synapses (Wilson et al., 1972; Schubert et al., 1974). In the BT-lines, the possible presence of subpopulations with elevated AChE activity could not be excluded, since 3 of these lines showed AChE values ranging between 5—10% of the value for brain (Tab. 5). Furthermore recent measurements in this laboratory failed to demonstrate electrical membrane excitability in both V- and BT-cells (Laerum et al., 1976).

In accordance with observations indicating that neurinomas may contain both S-100 positive and negative subpopulations of cells (Pfeiffer and Wechsler, 1972), we found that typically shaped glia-like cells were sometimes S-100 negative. However, these cells might also have originated from cells of an ependymal or fibroblast type, both known to be S-100 negative (Hyden and Rönnbäck, 1975). On the other hand, the S-100 protein was also expressed by cells whose morphological appearance differed from the morphological phenotype of astrocytes or oligodendrocytes. Multinuclear giant cells, too, frequently gave a faintly positive fluorescence, suggesting their possible origin from S-100 positive cells (Stavrou et al. 1974).

The S-100 protein is not detectable in the fetal rat brain, but gradually appears postnatally (Hershman et al., 1971). This was also reflected in the present experimental system, where FBC were S-100 negative during the early period after transfer to monolayer culture. Preliminary observations (Haugen, Å., Laerum, O. D., unpublished results) indicate that in S-100 negative, secondary FBC cultures, morphological differentiation of flat, epithelioid (glial precursor) cells (Laerum

and Rajewsky, 1975) to glia-like cells with long cytoplasmic processes (as inducible by medium conditioned with extracts of adult rat brain; Lim et al., 1973), is accompanied by a concomitant expression of S-100 protein. Since all BT-lines contained S-100 positive cells, differentiation for expression of this marker protein, analogous to the situation in normal brain, occurred in these cultures without interference by the process of neoplastic transformation.

Although the patental neoplastic BT-populations appeared heterogenous with regard to their composition, they always contained fractions of cells with glia-like morphology, thus generally resembling the V-lines (Table 1; Fig. 2). Other authors have described similar cellular phenotypes in cultures established from neurogenic neoplasms (Pontén and Macintyre, 1968; Manuelidis, 1969; Benda et al., 1971; Pfeiffer and Wechsler, 1972).

None of the present neoplastic neurogenic lines in monolayer culture gave a positive reaction for the GFA protein (Dahl and Bignami, 1973). However, it has recently been shown (Bissell et al., 1974) that rat C-6 glioma cells which were GFA-negative in monolayer culture, expressed the GFA protein when grown on a three-dimensional sponge matrix apparently favoring phenotypic differentiation.

Karyotyping and pulse-cytophotometric analyses of V- and BT-lines indicated varying degress of aneuploidy, with the appearance of marker chromosomes, loss of chromosomes and/or endoreduplication, and chromosome aberrations, breaks and rearrangements (Laerum and Hansteen, 1975). The heterogenous composition of the parental cell lines was in many cases reflected by plurimodal DNA distributions. The karyotypic heterogeneity of the BT-lines appeared to be considerably greater than that of the V-lines, possibly reflecting the absence of host-mediated selection pressure under cell culture conditions. In contrast, the corresponding cloned sublines showed a remarkable stability of their modal G_1-DNA values over prolonged periods of culture.

In terms of a direct observation of phenotypic alterations associated with neoplastic transformation, in vivo studies present considerable difficulties. The present in vivo-in vitro method used to develop the BT-lines, has been established to help overcome this difficulty . Some of the tumors resulting from reimplantation of FBC that had undergone neoplastic transformation in culture after a pulse of EtNU in vivo, exhibited histological patterns strongly resembling neuroectodermal neoplasms induced by EtNU in vivo, i.e., neurinomas and various types of gliomas (Table 2; Figs. 3 and 4). On the other hand, some of the glioblastoma-like or pleiomorphic histologic pictures observed after inoculation of BT-cells into baby BD IX-rats, are not commonly seen in EtNU-induced, autochthonous tumors. The histologic appearance of BT-cell-derived tumors, carried by transplantation in BD IX-rats showed noticable passage to passage variation, possibly due to host-mediated selection processes. Interestingly, tumors grown from BT-cells in immune-deficient, nude mice did not exhibit significant shifts in their respective histologic patterns (Mørk, S., Laerum, O.D., Rajewsky, M.F.; unpublished observations).

Both neurons and glial cells are believed to be of common neuroectodermal origin (see Langman et al., 1971). Therefore, cells expressing both glial and/or neuronal properties could, in principle, be expected in EtNU-induced neurogenic tumors. There is, however, little evidence (but see Schubert et al., 1974) for the

occurrence of neoplastic cell clones with neuronal traits under the present experimental conditions. This suggests a particularly high probability of neoplastic transformation for (precursor) cells of glial cell lineages at certain "high-risk stages" of differentiation during rat brain development (Laerum and Rajewsky, 1975). On the other hand, a predominance of neoplastic clones with a glia-like cellular phenotype could also be the result of a numerical excess of glial over neuronal precursor cells present in the target FBC population at the time of the EtNU-pulse.

Acknowledgements. O.D.L. and M.F.R. gratefully acknowledge a Research Training Felloship and a Travel Fellowship, respectively, both awarded by the International Agency for Research on Cancer, Lyon, France.

We are greatly indebted to K.Hanke, Max-Planck-Institut für Virusforschung, Tübingen, Germany, and to I.L.Hansteen, Institute of Pathology (Sect. B.), University of Oslo, Rikshospitalet, Oslo, Norway, for their collaboration in the pulse-cytophotometric and chromosome analyses, respectively; to Dr. J.H. Schwartz, Department of Microbiology, New York University School of Medicine, N.Y., USA, for his help with the choline acetyltransferase assays; to Dr. J.Storm-Mathisen, Departement of Toxicology, Defence Research Establishment, Kjeller, Norway, for the GAD determinations; to Dr. A.Bignami, Department of Pathology, Stanford University School of Medicine, Palo Alto, CA, USA, for the GFA assays; and to Prof. W.Schäfer, Max-Planck-Institut für Virusforschung, Tübingen, Germany, for the gs-interspecies viral antigen assays. Miss B.Fjell, M.Holmemo, I.Klein, and Mr. A.Wälder have provided valuable technical assistance.

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Received December 15, 1976; accepted March 16, 1977