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Molecular and Cellular Mechanisms in Nervous System-specific Carcinogenesis by N-Ethyl-N-nitrosourea

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Abstract: A single pulse of N-ethyl-N-nitrosourea (ENU), applied to BDIX rats during the perinatal age, specifically results in a high incidence of neuroectodermal neoplasms in the central and peripheral nervous system (NS). The pronounced sensitivity of the developing NS suggests a dependence of the carcinogenic effect on the proliferative and/or differentiative state of the target cells at the time of the ENU pulse. The specificity of ENU for the NS cannot be due to tissue variations in the degree of carcinogen-cell interactions, since the reactive, electrophilic ethyl cation is produced by rapid, nonenzymatic decomposition of ENU indiscriminately in all tissues. Correspondingly, the initial molar fractions of ethylated purine bases are similar in the DNA of "high-risk" (perinatal brain) and "low-risk" tissues (*e.g.*, liver; adult brain). However, while the respective half lives in DNA of N^7-ethylguanine and N^3-ethyladenine show only minor differences for both types of tissues, the mutagenic ethylation product O^6-ethylguanine is removed from brain DNA very much more slowly than from the DNA of other tissues. Together with their high rate of DNA replication during the perinatal age, the incapacity of rat brain cells for enzymatic elimination of O^6-alkylguanine from their DNA could account for an increased probability of neoplastic conversion, and hence for the NS specificity of ENU in the rat.

Dissociated fetal (18th day of gestation) BDIX-rat brain cells (FBC), transferred to long-term cell culture at 20–90 hr after a transplacental pulse of ENU (75 μg/g body weight), contrary to untreated FBC became tumorigenic after *ca.* 200 days (as assayed by reimplantation into baby BDIX rats). The multiclonal

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proliferation of neoplastic neurogenic cells was preceded by a characteristic sequence of phenotypic alterations of the cultured FBC. This "*in vivo-in vitro* system" may represent a model for analysis of the unclarified interval between primary carcinogen-cell interaction and the onset of malignant growth on the one hand, and for characterization of the type and differentiated state of the particular FBC that undergo neoplastic transformation by ENU on the other.

One of the few signposts in the present labyrinth of approaches to the problem of carcinogenesis remains the notion that direct interactions of carcinogenic chemicals with informational macromolecules, and with genetic material in particular, can apparently be necessary prerequisites for initiation of the process of neoplastic transformation (*1–3*). There is evidence indicating a mostly covalent binding to DNA of many carcinogens, or rather of their reactive, generally electrophilic metabolic derivatives ("ultimate carcinogens") (*1, 2*). Furthermore, most carcinogens prove to be mutagenic when tested in appropriate microbial and eukaryote systems (*4*). Extensive analyses, however, of both the metabolic activation of oncogenic agents (*5*) as well as the physicochemical nature of their primary interactions with various cellular constituents (*2, 3*) have thus far failed to provide unequivocal correlations between the extent and types of initial reactions on the one hand, and the carcinogenic effect on the other.

Until recently, surprisingly little attention has been given to the question whether specific phenotypic properties of cells coming into play after the initial carcinogen-cell interaction might perhaps have an equally important influence on the probability of neoplastic transformation. Of particular relevance in this context may be the rate of target cell proliferation, since repeated rounds of DNA replication and cell division seem to be required for the "fixation" and phenotypic expression of carcinogen-induced genome alterations (*3, 6–10*) and the capacity of target cells to eliminate and correctly replace (repair) carcinogen-modified, potentially mutagenic molecular structures in their DNA (*11–20*). This would shift the emphasis towards the differentiative state and functional behavior of target cells and, accordingly, require characterization of possibly small subpopulations of "high-risk cells" contained in the target tissues with their generally complex cellular composition. A lead in this direction may be provided by the pronounced tissue specificity of the tumorigenic effect of certain chemical carcinogens in those cases where this specificity is not a "secondary" one, *i.e.*, not due to tissue differences in the activity of enzymes required for the formation of their ultimate reactive forms (*18, 19*).

Nervous System (NS)-specific Carcinogenic Effect of ENU in the Rat

A "model carcinogen" that fulfils the above condition is the ethylating agent N-ethyl-N-nitrosourea (ENU) (*21*). Alkylation of nucleic acid constituents in relation to mutagenesis and carcinogenesis has recently received much attention (*2–5, 22, 23*). Under *in vivo* conditions, ENU decomposes heterolytically (*i.e.*, without

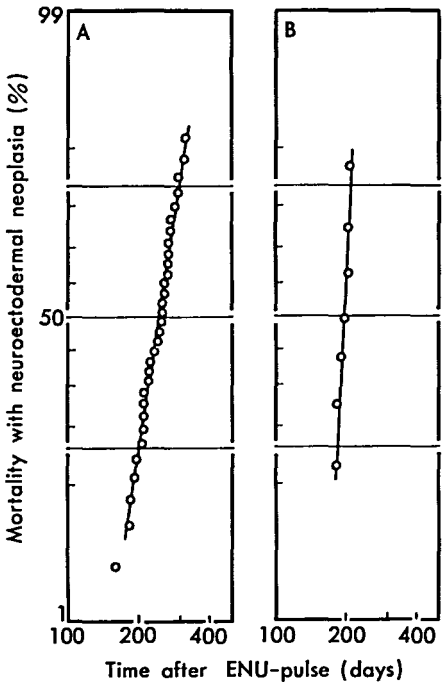


FIG. 1. Mortality with neuroectodermal tumors in the offspring of BDIX rats treated with a single, transplacental intraperitoneal (i.p.) injection of either 25 or 75 mg of ENU/kg body wt., on the 18th day of gestation. Each point represents one animal. Note normal distribution of times until death with tumors. Horizontal lines (probits) indicate 1 standard deviation of the T_{50} values, respectively (probability grid). Υ represents the percentage of animals with macroscopically detectable neoplasms (*27*). A: ENU, 25 mg/kg; T_{50}, 240 days±19% (S.D.); Υ, 89%. B: ENU, 75 mg/kg; T_{50}, 195 days±8% (S.D.); Υ, 89%. The corresponding values for BDIX rats treated with a single i.p. injection of 75 mg of ENU/kg body wt. at the age of 10 days, were T_{50}=291 days±22% (S.D.), and Υ=95%, respectively (*18, 19*).

enzyme involvement) with a half life of $t_{1/2} \leqq 8$ min (*24*). The ultimate reactant, an electrophilic ethyl cation, is thus produced indiscriminately in all tissues. In spite of this fact, a single pulse of ENU, when applied to rats during the perinatal age, specifically results in a very high incidence of neuroectodermal neoplasms in the central and peripheral NS (*21, 25, 26*), after a dose-dependent "latency period" (Fig. 1) (*18, 19, 27*). The panel of ENU-induced rat tumors encompasses mixed glioma-, astrocytoma-, oligodendroglioma-, glioblastoma-, and ependymoma-like neoplasms in the brain; and neurinoma- or Schwannoma-like tumors in the peripheral NS (*21, 26, 27*). The particular sensitivity of the developing NS suggests that the carcinogenic effect may be related to the proliferative and/or differentiative state of the target cells at the time of the ENU pulse (*18, 19*). In the rat NS, the perinatal age is characterized by the presence of highly proliferative matrices, particularly in the subependymal area of the brain (Fig. 2) (*18, 19, 28, 29*).

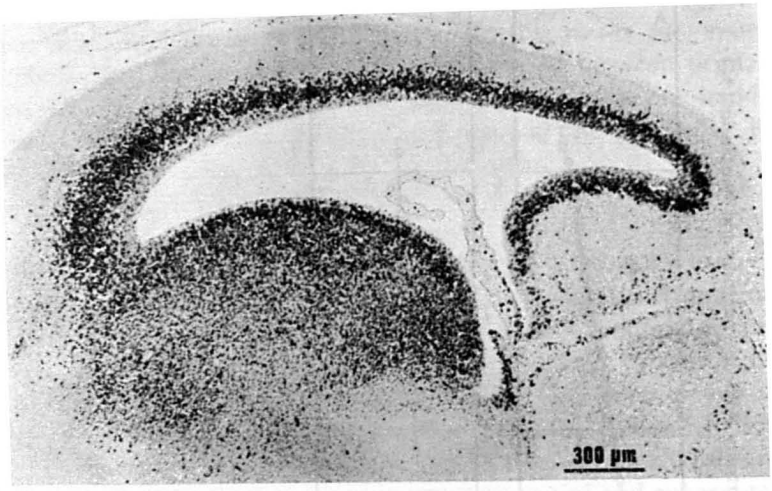


FIG. 2. High DNA-synthetic activity in the subependymal proliferative matrix of the fetal (18th day of gestation) BDIX-rat brain. Feulgen-stained sagittal section. Autoradiogram (Ilford K5 enulsion; exposure time, 15 days) prepared after a transplacental pulse of thymidine-methyl-^{3}H (spec. act., 15 Ci/mmole; 3 μCi/g body wt.). ^{3}H-labeled, DNA-synthesizing nuclei: black spots (*19*).

Molecular Mechanisms

1. Initial degree of DNA ethylation by ENU

As a result of their equal exposure to the ENU-derived ethyl cation, the initial (at 1 hr after the ENU pulse) extent of base ethylation in the DNA of both "high-risk" and "low-risk" tissues could be expected to be of similar magnitude. To test this assumption, [1-^{14}C]-ENU (specific activity, 5.72 Ci/mole; Farbwerke Hoechst AG, Frankfurt/Main, Germany) was applied to fetal (18th day of gestation), 10-day-old, and adult rats of the BDIX strain (*30*) *in vivo*, and DNA was then isolated from brain, liver, and other pooled tissues with a modified Kirby method (*24*). After mild hydrolysis of DNA in 0.1 N HCl at 37°C for 20 hr (*31*), and addition of nonradioactive ethylated purine bases as "markers," radiochromatography was performed on Sephadex G-10 (Fig. 3), with 0.05 M ammonium formate buffer, pH 6.8 (*32*). The molar fractions of the ethylated bases were calculated from the integral ^{14}C-activity of the corresponding chromatographic peaks, considering their specific ^{14}C-activity as identical with that of the [1-^{14}C]-ENU applied. The amounts of guanine (G) and adenine (A) were derived from the UV-extinction coefficients, at neutral pH, of $\varepsilon_{260}=7{,}200$ for G, and $\varepsilon_{260}=13{,}300$ for A (*33*).

The initial (1 hr after injection of 75 μg of [1-^{14}C]-ENU/g) molar fractions of N^{7}-ethylguanine (N^{7}-EtG/G), O^{6}-ethylguanine (O^{6}-EtG/G) and N^{3}-ethyladenine (N^{3}-EtA/A) in the DNA of "high risk" (fetal and 10-day-old brain) and "low risk" (*e.g.*, liver; adult brain) tissues were indeed similar ($\sim$ 1–2 $\times 10^{-5}$) (*18*, *19*). The relative initial frequencies of these ethylation products in DNA were $\sim$ 55% (N^{7}-EtG), $\sim$ 28% (O^{6}-EtG), and $\sim$ 17% (N^{3}-EtA), respectively. The same relative

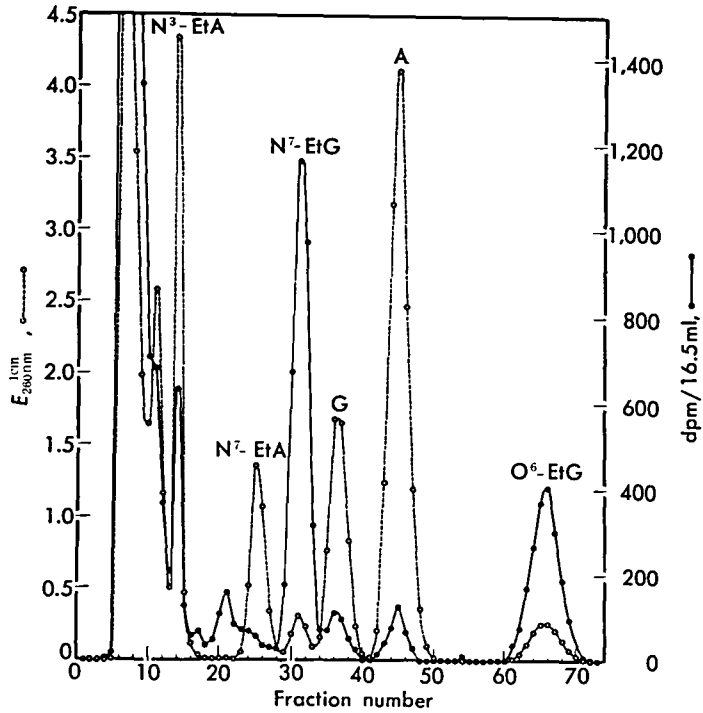


FIG. 3. Separation of bases from brain DNA by radiochromatography on Sephadex G-10, at 4 hr after ethylation *in vivo* by an i.p. pulse of 75 μg of [1-^{14}C]-ENU/g body wt. to 10-day-old BDIX rats. Nonradioactive N³-EtA, N⁷-EtG, and O⁶-EtG were added as "markers" (*18*, *19*).

base ethylation frequencies were found after incubation of rat liver DNA with [1-^{14}C]-ENU for 1 hr at 37°C in 25 mM potassium phosphate buffer, pH 7.2 (*19*). Therefore, the initial degree of purine base ethylation in DNA *per se* does not provide an explanation for the NS specificity of the carcinogenic effect. It was, however, of interest to find that O⁶-EtG is a major product of ethylation by ENU *in vivo* and *in vitro*. Among the different possible substitutions of purine bases in DNA, O⁶-alkylguanine is particularly likely to cause miscoding and anomalous base-pairing during subsequent DNA replication, and represents a mutagenic and thus potentially carcinogenic event (*23*, *34*, *35*). Formation of O⁶-alkylguanine in DNA after *in vivo* administration of alkylating N-nitroso carcinogens primarily of the "S_N1-type" (*36*) has recently been reported by several authors (*18*, *19*, *31*, *37–40*). The relative yield of O⁶-EtG obtained in the present analyses was *ca.* 4 times higher than the corresponding O⁶-methylguanine value reported for the less carcinogenic (*41*) methylating homologue of ENU, N-methyl-N-nitrosourea (MNU) (*39*). Furthermore, the same molar fractions of N⁷-EtG/G, O⁶-EtG/G and N³-EtA/A were obtained in fetal DNA, when ENU was administered during temporary reduction of the rate of fetal DNA replication to ~ 1% of the control value by hydroxyurea (500 μg/g body weight, injected 1 hr prior to the ENU pulse) (*19*). Correspondingly, significant differences in the degree of base ethylation were

neither observed in fetal or 10-day-old *versus* adult rat tissues nor in liver DNA, when the ENU pulse was placed into the "prereplicative phase" (at 11 hr) *versus* the phase of maximum DNA replication (at 24 hr) after partial hepatectomy (*19*). With respect to O^6-G, this indicates that ethylation is not restricted to a situation where this position is not "protected" by hydrogen bonding (*i.e.*, replicating DNA).

In the present analyses of DNA ethylated by ENU *in vivo* or *in vitro*, only 20–30% of the total ^{14}C-activity in the Sephadex G-10 radiochromatograms was due to ethylated purine bases (*18*, *19*). Most of the remaining ^{14}C-activity eluted immediately after the void volume (Fig. 3). Although this "early peak" (*19*) includes ethylated pyrimidine nucleotides (not investigated in this study), there is evidence suggesting that its major component are phosphotriesters formed by reaction of the ENU-derived ethyl cation with the phosphodiester groups of DNA (*19*, *42–45*). These appear to be relatively stable in DNA (*40*), and could cause alterations of conformation (*46*) and molecular interactions (*47*), possibly resulting in a reduced susceptibility of DNA to enzymatic hydrolysis (*43*). Phosphotriester formation in DNA has recently been proposed to be a potentially important molecular event in the process of carcinogenesis by alkylating agents (*23*, *45*).

2. Elimination rates of ethylated purine bases from the DNA of "high-risk" and "low-risk" rat tissues

Since cellular repair processes for structurally modified DNA involve the removal of altered bases from DNA (*11–20*), the tissue-specific carcinogenic effect of ENU could possibly result from tissue differences in the elimination rates of potentially mutagenic ethylated bases from DNA. Measurements were, therefore, performed over a period of 240 hr following the ENU pulse of the elimination rates

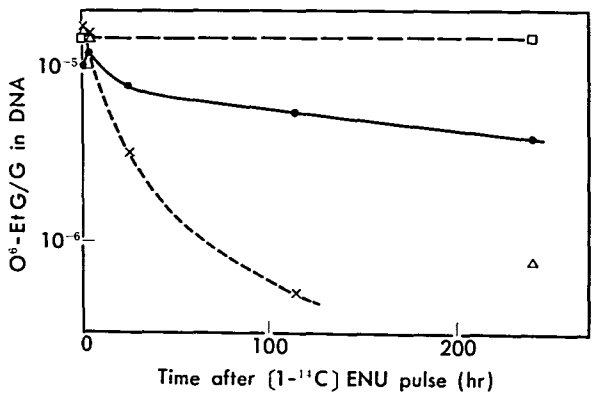


FIG. 4. Kinetics of the elimination of O^6-EtG from the DNA of different tissues of 10-day-old BDIX rats *in vivo*. Molar fraction of O^6-EtG/G in DNA of brain, liver, and other pooled tissues (intestine, kidney, lung, muscle, spleen), as a function of time after an i.p. pulse of 75 μg of [1-^{14}C]-ENU/g body wt. For comparison: O^6-EtG/G in the DNA of pooled tissues (isolated at 4 hr after an [1-^{14}C]-ENU pulse *in vivo*, and dissolved in 25 mM potassium phosphate buffer, 0.02% sodium azide, pH 7.2), incubated *in vitro* at 37°C for 240 hr (*18*). □ *in vitro*; ● brain; × liver; △ other tissues pooled.

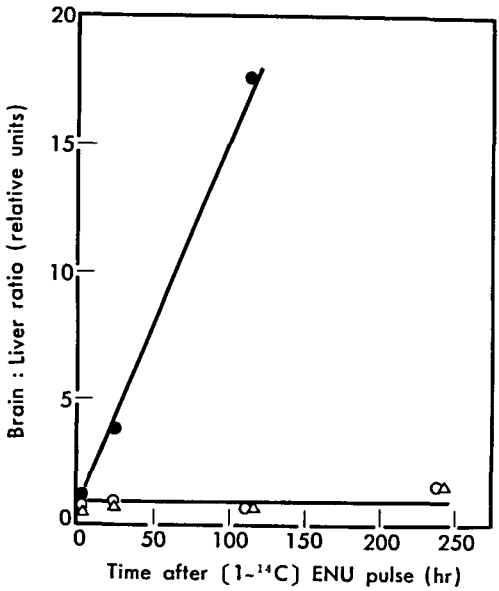


FIG. 5. Brain-liver ratio for the molar contents in DNA of O⁶-EtG/G (●) and N⁷-EtG/G (○), respectively, and for the "early peak" specific ^{14}C-activity (expressed as dpm/mg of input DNA ; compare Fig. 3) in the Sephadex G-10 radiochromatograms (△ Goth and Rajewsky, unpublished results), as a function of time after an i.p. pulse of 75 μg of [1-^{14}C]-ENU/g body wt. to 10-day-old BDIX rats. Initial values at 1 hr after the carcinogen pulse, normalized to 1.0 (*18, 19*).

of N⁷-EtG, O⁶-EtG and N³-EtA from the DNA of different tissues of 10-day-old BDIX rats (*17–19*). The data obtained by these analyses revealed a remarkable difference between brain on the one hand, and liver and a number of other "low-risk" tissues on the other. O⁶-EtG disappeared from brain DNA very much more slowly ($t_{1/2} \sim 229$ hr) than from liver DNA ($t_{1/2} \sim 36$ hr) or from the DNA of several other (pooled) tissues (kidney, lung, spleen, intestine, muscle; $t_{1/2} \sim 54$ hr); and also more slowly than N⁷-EtG and N³-EtA whose respective half lives were similar in brain and liver DNA (Figs. 4 and 5, Table 1). Equally similar for brain and liver DNA were the half lives ($t_{1/2} \sim 115$ hr and 99 hr, respectively) of the integral ^{14}C-activity of the "early peaks" (mainly attributed to ethylphosphotriesters) (*19, 23, 45*) in the Sephadex G-10 radiochromatograms (Fig. 5). Incubation for 240 hr *in vitro* of DNA previously ethylated by ENU *in vivo*, showed complete stability of O⁶-EtG in DNA, while the half lives of N⁷-EtG and N³-EtA in DNA were $t_{1/2} \sim 225$ hr and $t_{1/2} \sim 33$ hr, respectively (Table 1) (*19*). In view of the mutagenic potential of guanine-O⁶ alkylation (*22, 23, 34, 35*), it appears conceivable that the selective persistence of O⁶-EtG in brain DNA, together with the high rate of DNA replication and cell division at this developmental stage of the rat NS, could increase the probability of malignant transformation. This, in turn, might provide an explanation for the NS specificity of the carcinogenic effect of ENU. In the same context, it remains to be investigated whether, and in which way, the presence in DNA of

TABLE 1. Half Lives (hr) of Ethylated Purine Bases in the DNA of Different Tissues, after an Intraperitoneal Pulse of [1-^{14}C]-ENU to 10-day-old BDIX Rats (*18–20*)

Tissue	*In vivo*/*in vitro*	N^3-EtA	N^7-EtG	O^6-EtG
Brain	*In vivo*	16[a]	89[b]	229[b]
Liver	*In vivo*	12[a]	64[b]	36[c]
Other tissues (pooled)	*In vivo*	Not measured	60[d]	54[d]
DNA (ethylated *in vivo*)	*In vitro*[e]	33[f]	225[f]	Stable[f]

[a] Observation period: 1–25 hr after [1-^{14}C]-ENU pulse. [b] Observation period: 25–240 hr after [1-^{14}C]-ENU pulse. [c] Observation period: 25–114 hr after [1-^{14}C]-ENU pulse. [d] Based on measurements at 1, 4, and 240 hr after [1-^{14}C]-ENU pulse. [e] 25 mM potassium phosphate buffer (0.02% sodium azide); pH 7.2; 37°C. [f] Observation period: 1–240 hr after the beginning of incubation.

alkylated purines such as O^6-EtG could also impair interactions of DNA with regulatory proteins involved in the control of gene expression (*48–50*).

The observed differences in the elimination rates from DNA of O^6-EtG are unlikely to result from differential dilution of this ethylation product in DNA due to different rates of DNA replication and cell division in the respective tissues. If this were the case, the elimination rates, *e.g.*, of N^7-EtG from brain *versus* liver DNA, should differ by a factor similar to that found for the half lives of O^6-EtG, unless large differences existed in these tissues with regard to intranuclear conditions for the hydrolysis of glycosidic linkages leading to release of N^7-EtG from DNA. Rather, the data strongly argue for the existence of a specific enzymatic mechanism for the recognition and elimination of O^6-alkylguanine from DNA, which is either lacking or substantially less effective in rat brain (*18, 19*). Such a mechanism could basically operate according to the scheme of excision repair (*11*), involving specific enzymes for recognition and elimination of modified bases, and subsequent repair of the resulting apurinic sites. A mammalian enzyme selectively releasing O^6-alkylguanine from DNA has thus far not been identified. However, recent data from several laboratories (*51–56*) suggest that this may soon be the case.

Selective persistence in the DNA of rat brain has recently also been demonstrated for O^6-methylguanine (O^6-MeG), when the predominantly NS-specific carcinogen MNU was applied over a period of 35 days (*57, 58*). Since kidney tumors are known to develop occasionally after single high doses of MNU (*59*), it is of interest that a low degree of accumulation of O^6-MeG was also found in kidney DNA. Extending these analyses to the dialkylnitrosamines, it was further shown (*60, 61*) that after a single high dose of 20 μg of dimethylnitrosamine (DMN)/g to adult rats (which induces tumors of the kidney but not of the liver) (*62*), O^6-MeG is much more slowly eliminated from kidney DNA, than after a low (not kidney-tumorigenic) dose (2.5 μg of DMN/g) or from liver DNA after either dose. This suggests that the enzyme system responsible for the elimination of O^6-alkylguanine from DNA, either has only a limited capacity or can be inhibited by high doses of the alkylating carcinogen.

Neoplastic Transformation of Fetal Rat Brain Cells in Culture after Exposure to ENU in Vivo

The above data have been obtained on whole tissues; *i.e.*, they do not provide information on the differentiative and proliferative properties of the particular (precursor?) brain cells undergoing neoplastic transformation after exposure to ENU. Furthermore, the interval between primary carcinogen-cell interaction and the onset of clonal tumor growth remains a largely unclarified phase in the process of carcinogenesis. Yet this period may involve a characteristic sequence of phenotypic and functional alterations of the presumptive cancer cells. Investigation of these problems *in vivo* is hampered by the complex composition of intact tissues, together with the fact that apparently only a minor fraction of their constituent cells undergo the changes ultimately resulting in expression of a "malignant phenotype." On the other hand, cultured cells as target populations for oncogenic agents (*3*, *63*) have obvious limitations as substitutes for the highly controlled cell systems *in vivo* with their distinct subcompartments of proliferating and differentiating cells (*27*). Furthermore, by the capacity for "unlimited" proliferation acquired by established cell cultures, part of the process of carcinogenesis might be anticipated.

We have recently shown (*27*) that these limitations of *in vitro* systems may in part be circumvented if a tissue-specific carcinogen (ENU) is administered *in vivo*, prior to transfer of the respective target cells (fetal BDIX-rat brain cells; FBC) into long-term cell culture. This "*in vivo-in vitro* system" (Fig. 6) combines several favorable features: (1) The target cells are exposed to the carcinogen under physiological conditions *in vivo*. (2) The sequence of events subsequently monitored *in vitro*, occurs in a cell population derived from the very cell system giving rise to neuroectodermal tumors *in vivo* after an ENU pulse at the perinatal age. (3) Fetal rat cells appear to undergo "spontaneous" neoplastic transformation in culture less frequently than embryonic cells from other rodent species (*64*). (4) With the aid of different markers (*65–69*), the resulting neoplastic cell lines can be analyzed for phenotypic expression of NS-specific properties. (5) Under standardized conditions, the system may be applicable to assay the transformation frequency of preselected subpopulations of FBC.

Single cell suspensions of FBC, transferred to long-term monolayer cell culture at 20–90 hr after a pulse of 75 μg of ENU/g body weight administered on the 18th day of gestation, became tumorigenic after $\sim$ 200 days (as assayed by reimplantation into baby BDIX rats) (*27*, *70*). This time interval is similar to the average period of time ($\sim$ 195 days) until death with neuroectodermal tumors, in the offspring of BDIX rats injected with the same dose of ENU at the same stage of gestation (*19*, *27*). Acquisition of tumorigenicity in culture was preceded by a characteristic sequence of phenotypic alterations, termed "Stages I–IV" (Figs. 6 and 7; Table 2). During early primary culture (Stage I), both ENU and untreated control cultures exhibited stationary glia-like cells on a growing layer of flat, epithelioid (possibly glial precursors) (*27*, *70*) and few fibroblastoid cells. Stage II ($\sim$ 10th–40th day) was characterized by a constant proportion of glia-like cells in the ENU cultures, and their gradual disappearance in the controls. During Stage III ($\sim$ 40th–100th day), slowly-proliferating glia-like cells in the ENU cultures formed

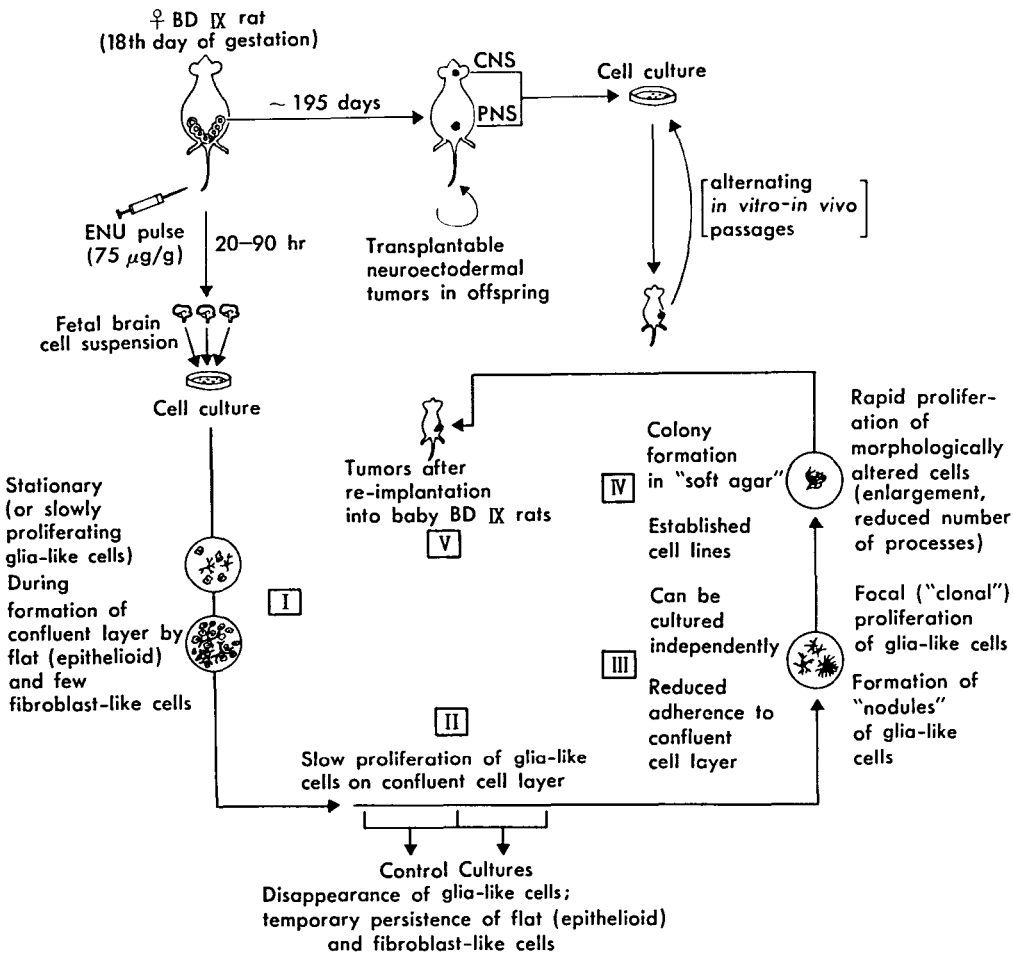
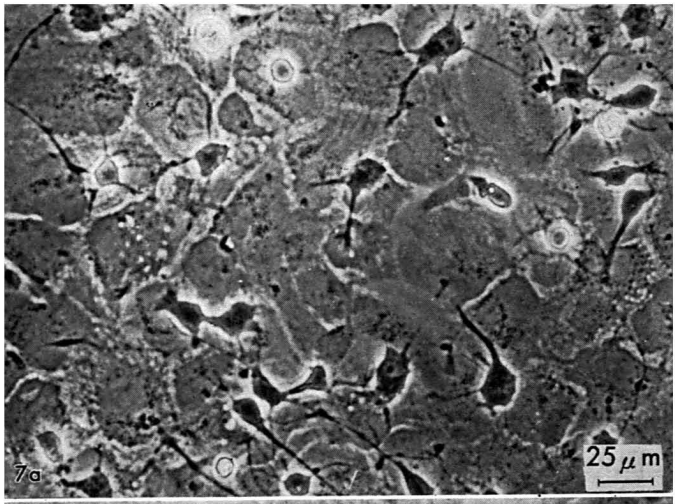


FIG. 6. Diagrammatic representation of the *in vivo-in vitro* system for neoplastic transformation of fetal BDIX-rat brain cells by ENU. CNS, central nervous system ; PNS, peripheral nervous system (*27*).

→ FIG. 7 (a-f). Phenotypic alterations of FBC (18th day of gestation) during neoplastic transformation in culture, after a transplacental pulse of 75 μg of ENU/g body wt. *in vivo* (phase contrast micrographs) (*27*). (a) Secondary FBC (untreated control ; 19th day in culture). Glia-like cells on dense layer of flat, epithelioid and few fibroblast-like cells. (b) Later appearance of FBC culture shown in (a) ; 3rd culture passage. Glia-like cells have disappeared, but the flat epithelioid cell layer persists for sometimes up to 10 months (!). (c) Primary culture (8th day) of FBC exposed to ENU *in vivo*. Multiple glia-like cells on dense layer of flat, epithelioid and few fibroblast-like cells (Stage II, see Fig. 6). (d) FBC exposed to ENU *in vivo* (4th culture passage). Multifocal proliferation of glia-like cells with long cytoplasmic processes. Piled-up foci (Stage III, see Fig. 6). (e) Glia-like cells shown in (d), after separation from underlying cell layer and further subculture (advanced Stage III, see Fig. 6). Strong resemblance to astrocytes ; note apparent intercellular communication. (f) FBC exposed to ENU *in vivo* (7th culture passage). Rapid, disordered proliferation of "morphologically transformed" cells (shorter and fewer cytoplasmic processes per cell ; Stage IV, see Fig. 6).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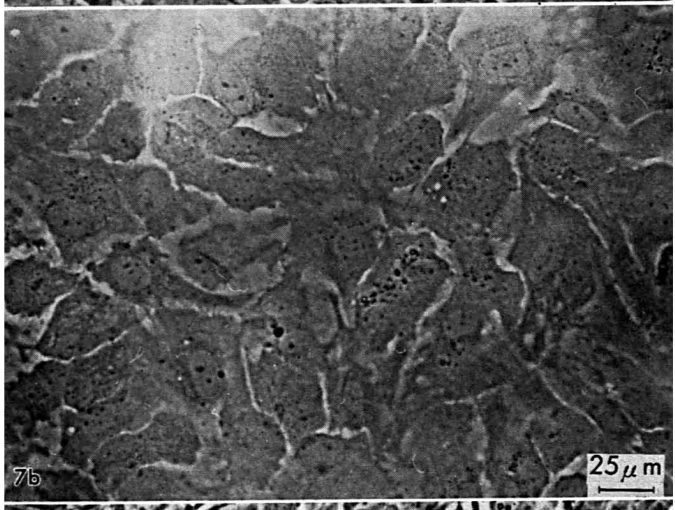
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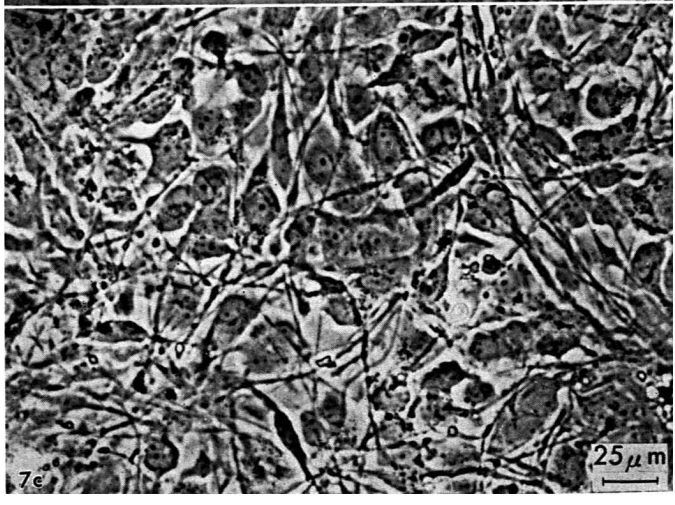
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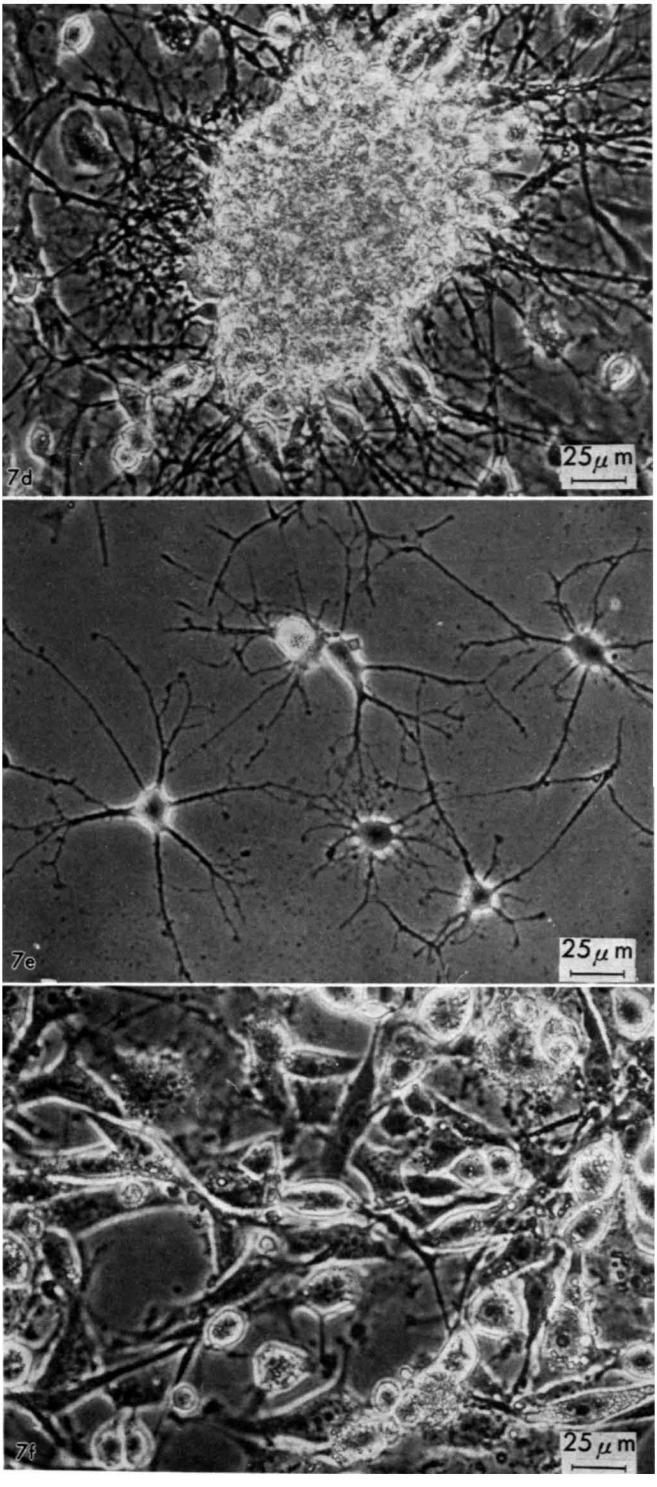
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TABLE 2. *In Vivo-in Vitro* System for Neoplastic Transformation of Fetal (18th Day of Gestation) BDIX-rat Brain Cells in Culture, after Exposure to ENU *in Vivo*

	t_M[a]		t_A[b]		t_T[c]	
	Days	Passage number	Days	Passage number	Days	Passage number
Mean (±S.E.)	98 (±20)	4 (±0.7)	138 (±19)	6 (±2)	199 (±28)	12 (±2)

Average time intervals between transplacental ENU pulse (75 μg of ENU/g body wt.) and first observation of " morphological transformation " (t_M), ability to form colonies in semi-solid agar medium (t_A), and tumorigenicity upon reimplantation into isogeneic hosts (t_T). Mean values (±S.E.) for 7 independent sets of experiments. Cell suspensions from 6–10 pooled fetal brains per experiment (≧10 separate cultures; 1–2×10^6 viable cells per 100 mm Falcon plastic dish or 250 ml plastic flask; Eagle-Dulbecco medium, 10% inactivated bovine serum; gassed with 5% CO_2 in humidified air) (*27*). [a] Time interval between ENU pulse *in vivo* and first observation of morphological transformation. [b] Time interval between ENU pulse *in vivo* and first observation of ability to form colonies in semisolid agar medium. Initial cloning efficiency of 10^4 viable cells seeded into 30 ml of 0.15% agar medium, 0.9± 0.3% (S.E.). [c] Time interval between ENU pulse *in vivo* and first observation of tumorigenicity. The time interval from subcutaneous reimplantation of 10^6 cells into baby BDIX rats, until the first tumors became palpable, was 48±13 days (S.E.). However, much longer latency intervals (≦10 months) were occasionally recorded.

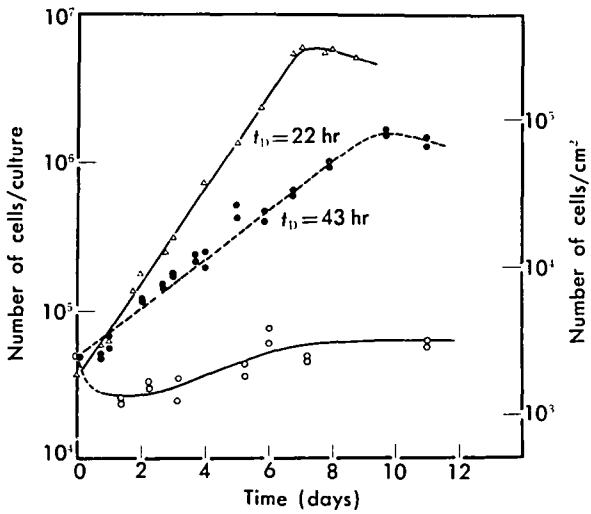

FIG. 8. Growth curves for the (neoplastic) neurogenic cell line BT5C during culture passages 8 (Stage IV ; see Fig. 6) and 25 (Stage V ; Fig. 6), respectively. Population doubling times (t_D) and maximum cell densities were t_D=43 hr (8×10^4 cells/cm²) during the 8th passage, and t_D=22 hr (30×10^4 cells/cm²) during the 25th passage, respectively. A curve for secondary FBC (19th day of gestation ; untreated control) is given for comparison (*27*). △ BT5C (25th passage) ; ● BT5C (8th passage) ; ○ fetal brain cells.

"piled-up" foci. These could be removed and cultured separately. Transition to Stage IV (*ca.* 100th–200th day) was marked by proliferation of morphologically transformed cells, which formed colonies in semi-solid agar, and finally became tumorigenic (Stage V; see Fig. 8 for comparison of proliferation rates during Stages IV and V, and Table 2 for average time intervals between the different

stages). The solid tumors developed upon subcutaneous reimplantation of the resulting neoplastic neurogenic cell lines ("BT lines") (*70*) into baby BDIX rats, appeared histologically as neurinoma-, glioma-, or glioblastoma-like, and frequently pleiomorphic neoplasms. Although exhibiting a more atypical cellular morphology, these tumors resembled the different types of neuroectodermal rat neoplasms induced by ENU *in vivo*. Like several neurogenic cell culture lines ("V lines") (*70*) derived from ENU-induced, neuroectodermal BDIX-rat tumors (*27, 70–73*), the BT lines contained multipolar, glia-like cells, but also flatter cells with shorter and fewer cytoplasmic processes, and occasionally giant cells. The pluriclonal "parental" BT and V lines exhibited different degrees of aneuploidy, and contained multiple subpopulations of cells, as reflected by plurimodal DNA distributions recorded by pulse-cytophotometry (Fig. 9) (*70, 74, 75*). All parental lines and their (cloned) sublines (total number >30) expressed, to a varying extent, the NS-specific "mark-

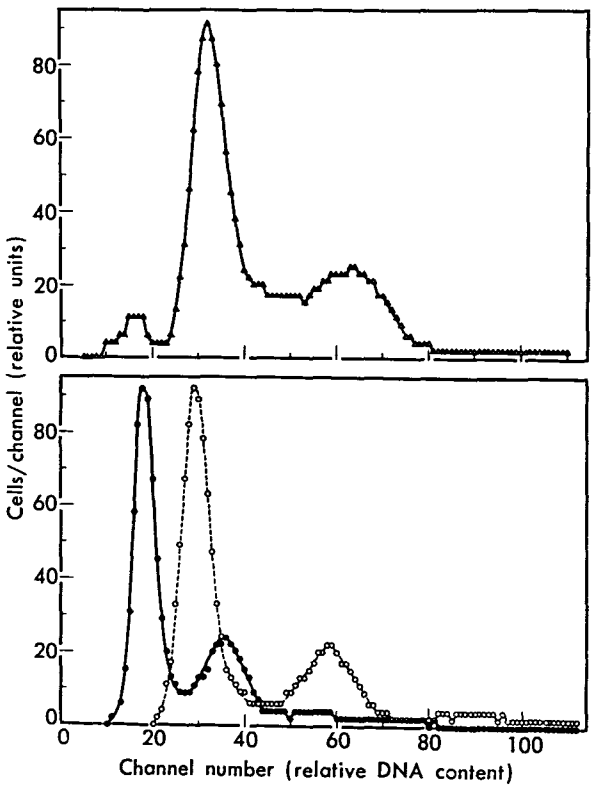

FIG. 9. Pulse-cytophotometric DNA distributions of the original ("parental") neoplastic, neurogenic "V line" (*27, 70*) NV1C (125th culture passage ; above), and of 2 NV1C-derived, cloned sublines (NV1Cb, ○ ; and NV1Cc, ●) in their 43rd and 40th culture passage, respectively (below). Data for $5–10 \times 10^4$ ethidium bromide-stained, log-phase cells, respectively. Euploid log-phase BDIX-rat embryo cells had a relative modal G_1-DNA value corresponding to channel No. 16. Pulse-cytophotometer ICP 11 (PHYWE AG, Göttingen, Germany). Note the unimodal DNA distributions of the cloned sublines, as opposed to the plurimodal distribution of the parental line (*70, 74, 75*).

er" protein S-100, which is virtually absent in FBC (*65, 70*). There was no indication of more than borderline neurotransmitter activity (acetylcholinesterase, choline acetyltransferase, L-glutamate decarboxylase I) (*67, 70*), nor has electrical membrane excitability thus far been detected (*76*). Serological analyses in the Ouchterlony test, of selected BT and V lines gave no evidence for the presence of group-specific(gs) interspecies oncornaviral antigens (by courtesy of Prof. W. Schäfer of this institute) (*27, 70, 77*).

To this date, this "*in vivo-in vitro* system" has not permitted subpopulations of probable "high-risk" FBC to be defined with certainty. Yet, the glia-like morphology of the particular cells that underwent "morphological transformation" and the demonstration, in the resulting neoplastic cell lines, of the predominantly glial S-100 marker protein (with the lack of evidence for expression of neuronal properties) (*27, 70, 76*), make glia cells, or rather their (proliferative) precursors potential candidates (*18, 27, 70*). The latter would be in accordance with the assumption that the risk of malignant transformation may vary with the stage of the target cells in their differentiative pathway at the time of interaction with a carcinogen (*9, 18, 19, 27*). For example, the probability of a genetic "fixation" of carcinogen-induced modifications of DNA may depend on the number of rounds of DNA replication that target cells would still (be programmed to?) undergo, before reaching a terminally differentiated, nonproliferative state (with much reduced risk of neoplastic conversion).

Neuroglia and neurons are, however, believed to have common neuroepithelial precursors (*78*); and immature cells of neuronal lineages were certainly not absent from the present FBC cultures initially. Therefore, neoplastic clones expressing both glial and/or neuronal properties might, in principle, have been expected to develop. However, during rat brain development, and on an overall scale, neurons precede the glial cell populations with respect to proliferation and differentiation (*28*). Hence, at the developmental stage chosen for the ENU pulse (18th day of gestation), the precursor compartments may predominantly contain glial precursors. This, in turn, might explain the prevalence of neoplastic cells with phenotypic traits of glial cells. It may, however, be noted, that some neoplastic cell lines expressing neuron-like properties have recently been derived from neuroectodermal BDIX-rat tumors induced by application of ENU on the 15th day of gestation (*73*).

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