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Nervous-system-specific Carcinogenesis by Ethylnitrosourea in the Rat: Molecular and Cellular Aspects

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Present evidence suggests that the process of neoplastic transformation can be initiated by direct structural alterations of the genetic material of target cells. Correspondingly, there is a strong correlation between oncogenicity and mutagenicity for almost all carcinogens tested (McCann et al. 1975; Montesano et al. 1976). Covalent binding to DNA has been shown for the electrophilic, ultimate metabolic derivatives of a large number of chemical carcinogens (Miller and Miller 1974). Many aromatic carcinogens are, in addition, capable of stacking interactions with DNA (Ames et al. 1972). On the other hand, extensive studies on both the metabolic activation of oncogenic agents (Magee 1974) and the physical chemistry of their primary interactions with cellular constituents have thus far failed to establish a specific, common type of "oncogenic" reaction at the molecular level (Heidelberger 1975). Instead, it appears that a variety of initial molecular alterations caused by different types of carcinogenic agents may share the property of increasing the probability for expression of a "neoplastic phenotype."

The probability of neoplastic transformation, however, is unlikely to be a simple function of the type and degree of primary carcinogen–cell interaction. Specific phenotypic properties of the target cells at the time of exposure to a carcinogen, i.e., their state of differentiation and proliferation, may be equally important determinants (Rajewsky 1972; Goth and Rajewsky 1974a,b; Rajewsky et al. 1976). Particularly relevant in this context are the rate of target-cell proliferation, since repeated rounds of DNA replication and cell division seem to be required for the "fixation" of carcinogen-induced genome alterations (Sachs 1966; Rajewsky 1967, 1972; Kakunaga 1974),

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and the capacity of target cells to eliminate and replace (repair) carcinogen-modified, potentially mutagenic molecular structures in DNA (see Roberts et al. 1968; Cleaver 1973; Goth and Rajewsky 1974a,b; van Lancker 1974; Cleaver and Bootsma 1975). This requires characterization of the state of differentiation and functional behavior of the target cells and possibly an analysis of subpopulations of "high-risk" cells contained in the target tissues with their usually complex cellular composition.

A lead in the search for cellular determinants favoring neoplastic transformation may be provided by the pronounced tissue specificity of the oncogenic effect of certain carcinogens which do not require enzymatic metabolic activation, i.e., in cases where this specificity cannot be due to tissue differences in the activity of enzymes involved in the formation of the ultimate reactants (Goth and Rajewsky 1974a,b). A carcinogen that fulfills this condition is the ethylating agent *N*-ethyl-*N*-nitrosourea (EtNU) (Ivankovic and Druckrey 1968). Alkylation of nucleic acid constituents by *N*-nitroso compounds in relation to mutagenesis and carcinogenesis has received considerable attention recently (see Druckrey et al. 1967; Lawley 1974; Singer 1975).

Nervous-system-specific Carcinogenicity of EtNU in the Rat

Under in vivo conditions, EtNU undergoes nonenzymatic, heterolytic decomposition with a half-life of $t_{½} \leq 8$ minutes (Goth and Rajewsky 1972). The ultimate reactant, an ethyl cation, is thus produced indiscriminately in all tissues. In spite of this, a single pulse of EtNU applied to rats during the perinatal age specifically results in a very high incidence of neuroectodermal neoplasms in the central and peripheral nervous systems (NS) (Ivankovic and Druckrey 1968; Wechsler et al. 1969) after a strain-dependent (Druckrey et al. 1970a) and dose-dependent "latency time" (Table 1) (Goth and Rajewsky 1974a,b; Laerum and Rajewsky 1975). During the prenatal and early postnatal age period, when the tumorigenic effect of EtNU is maximal, the developing rat NS contains highly proliferative matrices, e.g., the subependymal area of the brain (Altman 1969; Bosch et al. 1972; Goth and Rajewsky 1974a,b). With increasing age and maturation of the NS, the sensitivity towards EtNU subsides drastically (Druckrey et al. 1970b; see Table 1 and Fig. 1). This indicates that the carcinogenic effect also depends on the presence at the time of the carcinogen pulse of (precursor type) target cells with particular proliferative and/or differentiative properties (Goth and Rajewsky 1974a,b). The spectrum of neuroectodermal rat tumors induced by EtNU encompasses mixed glioma-, astrocytoma-, oligodendroglioma-, glioblastoma-, and ependymoma-like neoplasms in the brain and neurinoma- or Schwannoma-like tumors in the peripheral NS (Ivankovic and Druckrey 1968; Wechsler et al. 1969; Laerum and Rajewsky 1975; Swenberg et al. 1975).

Initial Degree of DNA Ethylation by EtNU

The initial extent of base ethylation in DNA would be expected to be of similar magnitude in both "high-risk" and "'low-risk" tissues due to their

Table 1
Pulse Carcinogenesis by EtNU in the Central and Peripheral Nervous Systems of the BD IX Rat: Dose–Response Relationships

BD IX rats		*Dose of EtNU (μg/g body wt.)*	*Additional conditions*	*Animals with macroscopic neuroectodermal tumors (%)*	T_{50}[a] *(days ± S.D.)*
age	*number*				
Fetus, 18th day of gestation	73	25 (i.v.)[b]	—	86	240 ± 20%
Fetus, 18th day of gestation	36	25 (i.v.)[b]	fostered on untreated mothers	89	240 ± 19%
Fetus, 18th day of gestation	74	25 (i.v.)[b]	250 μg of HU[c]/g 1 hr prior to EtNU pulse	80	240 ± 20%
Fetus, 18th day of gestation	9	75 (i.v.)[b]	—	89	195 ± 8%
10 days	58	75 (i.p.)	—	95	291 ± 22%
10 days	22	75 (i.p.)	500 μg of HU[c]/g 1 hr prior to EtNU pulse	91	330 ± 18%

Data from Goth and Rajewsky (1974a,b), Laerum and Rajewsky (1975), and M. F. Rajewsky (unpubl.).
[a] T_{50} is the median time until death with neuroectodermal neoplasms.
[b] Transplacentally.
[c] HU = hydroxyurea.

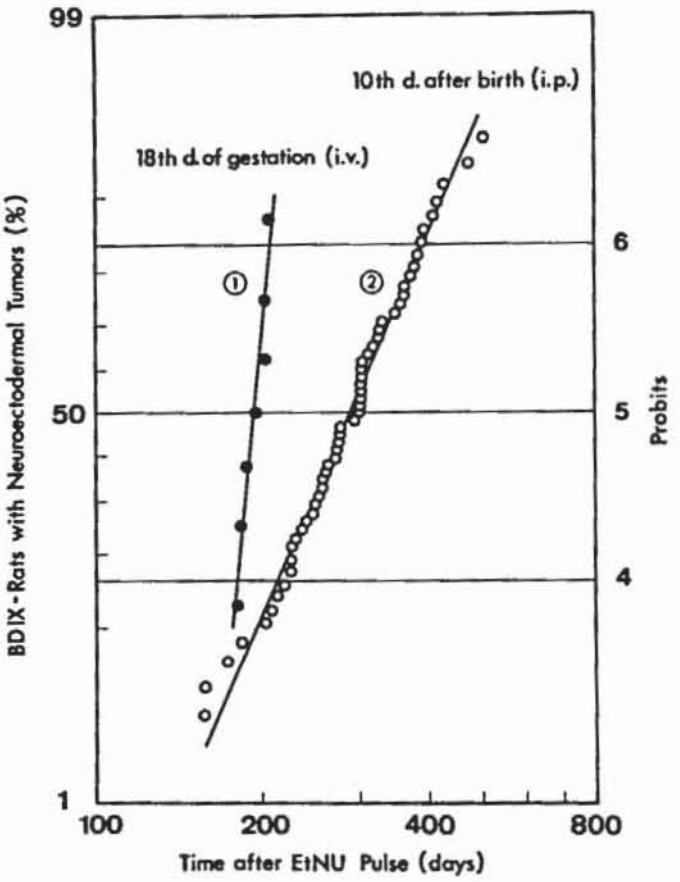

Figure 1

Mortality with neuroectodermal tumors in the offspring of BD IX rats exposed transplacentally to a single dose of 75 μg of EtNU/g body weight administered to pregnant females by i.v. injection on the 18th day of gestation (*group 1*) and in BD IX rats treated i.p. with the same dose of EtNU at the age of 10 days (*group 2*). Each point represents one animal. Note the normal distribution of times until death with tumors. Horizontal lines (probits) indicate one standard deviation of the T_{50} values. Y is the percentage of animals with macroscopically detectable neoplasms. T_{50} is the median time until death with neuroectodermal neoplasia (cf. Table 1). *Group 1:* $Y = 89\%$, $T_{50} = 195$ days $\pm$ 8% (S.D.); *group 2:* $Y = 95\%$, $T_{50} = 291$ days $\pm$ 22% (S.D.).

equal exposure to the EtNU-derived ethyl cation. This was tested by intravenous (i.v.), transplacental (t.p.), or intraperitoneal (i.p.) application of [1-^{14}C]EtNU (sp. act. = 5.7 Ci/mole; Farbwerke Hoechst AG, Frankfurt/Main, Germany) to fetal (18th day of gestation), 10-day-old, and adult rats of the BD IX strain, as well as to 10-day-old rats of the BD IV strain, respectively. The genotypes and phenotypes of the inbred BD strains have been described elsewhere (Druckrey 1971). DNA was isolated from brain, liver, and other pooled tissues with a modified Kirby method (Goth and Rajewsky 1974a,b). After mild hydrolysis of DNA in 0.1 N HCl at 37°C for 20 hours (Lawley and Thatcher 1970) and addition of nonradioactive ethylated purine bases as markers, radiochromatography was carried out on Sephadex G-10 (Fig. 2) with 0.05 M ammonium formate buffer, pH 6.8 (Lawley and Shah 1972). The molar fractions of ethylated bases were calculated from the in-

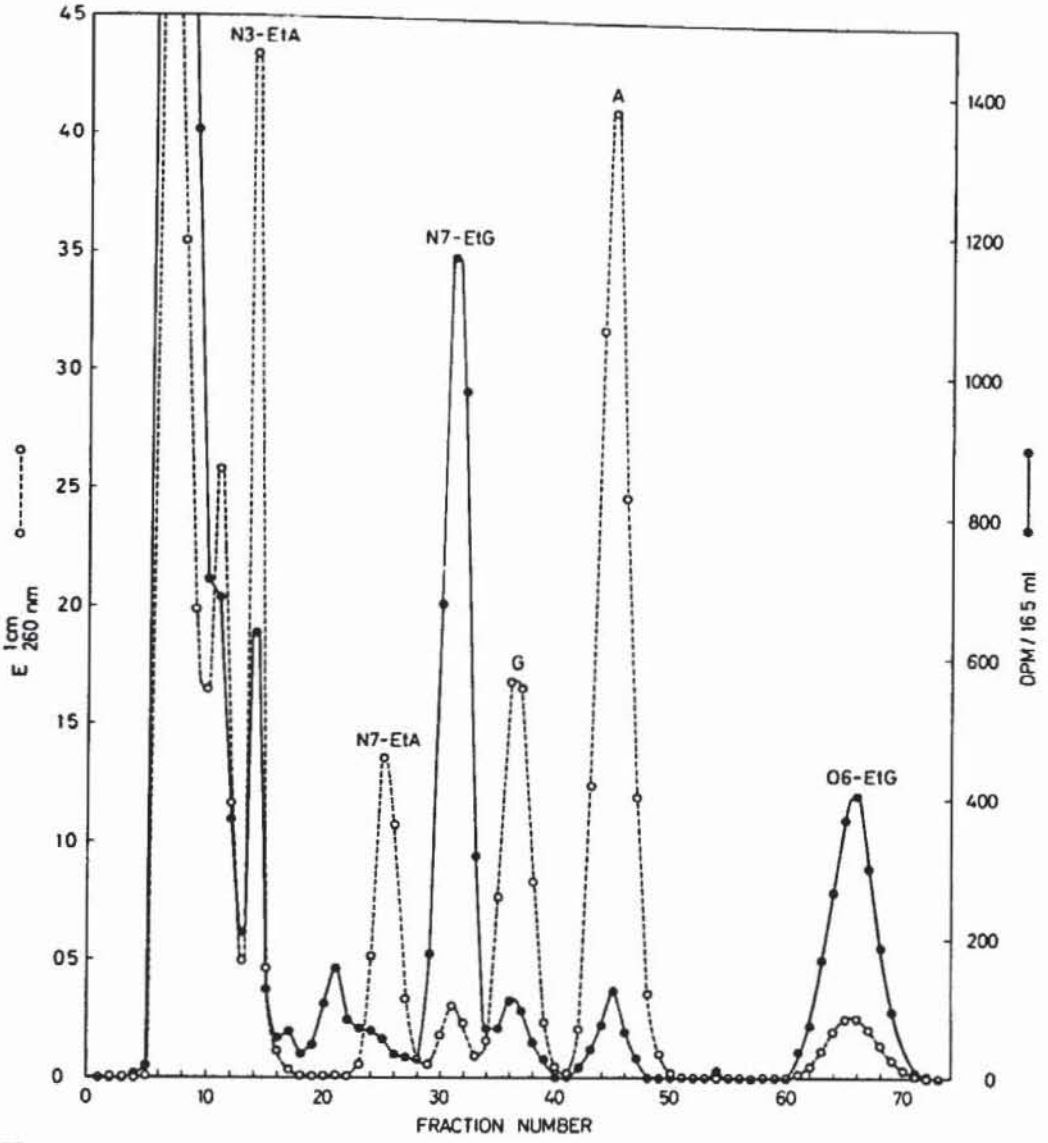

Figure 2
Separation of bases from brain DNA by radiochromatography on Sephadex G-10 at 4 hr after ethylation in vivo by an i.p. pulse of 75 μg of [1-^{14}C]EtNU/g to 10-day-old BD IX rats. Nonradioactive N^3-EtG, N^7-EtG, and O^6-EtG were added as markers. (Reprinted, with permission, from Goth and Rajewsky 1974a.)

tegral ^{14}C-activity of the corresponding chromatographic peaks, considering their specific ^{14}C-activity as identical with that of the [1-^{14}C]EtNU used. The amounts of guanine (G) and adenine (A) were derived from the ultraviolet (UV)-extinction values of the respective peaks and based on molar extinction coefficients, at neutral pH, of $\varepsilon_{260} = 7200$ for G and $\varepsilon_{260} = 13{,}300$ for A (Chargaff and Davidson 1955).

As expected, the initial (1 hr after injection of 75 μg of [1-^{14}C]EtNU/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 (liver and adult brain) tissues were found to be similar ($\sim 1\text{–}2 \times 10^{-5}$) (Goth and Rajewsky 1974a,b). The relative initial frequencies of these ethylation products in DNA were ~55% (N^7-EtG), ~28% (O^6-EtG), and ~17% (N^3-EtA), respectively. Similar rela-

tive values were obtained after incubation of rat liver DNA with [1-^{14}C]EtNU for 1 hour at 37°C in 25 mM potassium phosphate buffer, pH 7.2 (Goth and Rajewsky 1974b). Therefore, the initial degree of purine base ethylation in DNA per se does not explain the NS specificity of the carcinogenic effect.

Only 20–30% of the total ^{14}C-activity in the Sephadex G-10 radiochromatograms was due to ethylated purine bases. Most of the remaining ^{14}C-activity eluted immediately after the void volume (Fig. 2). This "early peak" includes ethylated pyrimidine nucleotides. However, its main component is probably phosphotriesters formed by reaction of the EtNU-derived ethyl cation with the phosphodiester groups of DNA (Rhaese and Freese 1969; Bannon and Verly 1972; Goth and Rajewsky 1974b; Sun and Singer 1975). Phosphotriesters are relatively stable in DNA (O'Connor et al. 1973). Their presence could lead to alterations of molecular interactions (Miller et al. 1974), possibly including a reduced susceptibility of DNA to enzymatic hydrolysis. The possible importance of phosphotriesters as promutagenic and potentially carcinogenic structural DNA modifications has recently been stressed (Singer 1975; Sun and Singer 1975).

Ethylation of Guanine-O^6 in DNA

The above analyses have shown that the extranuclear oxygen-6 atom of guanine in DNA is a major site of ethylation by EtNU. Among the various base substitutions in DNA, O^6-alkylguanine in particular represents a promutagenic and thus potentially carcinogenic alteration because of its high probability of miscoding and anomalous base-pairing during subsequent DNA replication (Loveless 1969; Gerchman and Ludlum 1973). Formation of O^6-alkylguanine in DNA after in vivo administration of alkylating *N*-nitroso carcinogens has been reported by a number of authors over the last several years (Lawley and Thatcher 1970; Frei 1971; Craddock 1973; Kleihues and Magee 1973; O'Connor et al. 1973; Goth and Rajewsky 1974a,b; Kleihues and Margison 1974; Magee et al. 1975; Margison and Kleihues 1975; Nicoll et al. 1975). The relative ability of the members of this class of carcinogens to alkylate the O^6 of guanine in DNA increases with their reactivity according to Ingold's S_N1 mechanism (Ingold 1953) and shows a positive correlation with their tumorigenicity (Goth and Rajewsky 1974b; Lawley 1974). For example, the relative yield of O^6-EtG obtained in the present analyses was about four times higher than the corresponding O^6-methylguanine value reported for the less carcinogenic (Druckrey et al. 1973) methylating homolog of EtNU, *N*-methyl-*N*-nitrosourea (MeNU) (Kleihues and Magee 1973).

No significant changes were observed in the molar fractions of N^7-EtG/G, 0^6-EtG/G, and N^3-EtA/A in fetal DNA when the EtNU pulse was administered during temporary reduction of the rate of DNA replication to ~1% of the control value by hydroxyurea (500 μg/g body wt., injected 1 hr prior to the EtNU pulse) (Goth and Rajewsky 1974b). Similarly, the degree of base ethylation in DNA was of the same magnitude in fetal or 10-day-old vs. adult rat tissues and in liver DNA when the EtNU pulse was placed into the "prereplicative phase" (at 11 hr) vs. the phase of maximum DNA replication (at 24 hr) after partial hepatectomy (Goth and Rajewsky 1974b). Correspondingly, the median times (T_{50}) until death with neuro-

ectodermal neoplasms and the tumor incidence were not significantly changed when EtNU was administered to fetal or 10-day-old BD IX rats during temporary inhibition of DNA synthesis by hydroxyurea (Table 1) (Goth and Rajewsky 1974b). This indicates that O^6-ethylation of guanine in DNA is not restricted to a situation where the extranuclear oxygen atom is "unprotected" by hydrogen bonding (i.e., replicating DNA).

Elimination of Ethylated Purine Bases from DNA (Repair)

Cellular repair processes for structurally modified DNA involve the elimination of altered bases from DNA. The rate of elimination from DNA of promutagenic alkylated bases such as O^6-alkylguanine must influence, for example, the frequency of GC-AT transition mutations during DNA replication. Elimination rates could, in principle, vary for different tissues, different cell types within a given tissue, and different states of differentiation and proliferation of a particular cell lineage. They could therefore represent an important factor in determining the tissue or cell specificity of the carcinogenic effect. Therefore, measurements of the elimination rates of N^7-EtG, O^6-EtG, and N^3-EtA from the DNA of different tissues of 10-day-old BD IX rats were performed over a period of 240 hours following the EtNU pulse (Goth and Rajewsky 1974a,b). These analyses revealed one remarkable difference between brain on the one hand and liver and a number of other low-risk tissues on the other. O^6-EtG disappeared from brain DNA very much more slowly ($t_{1/2} \sim 229$ hr) than from liver DNA ($t_{1/2} \sim 26$ hr; see Table 2) 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 N^7-EtG and N^3-EtA whose respective half-lives were similar in brain and liver DNA (Table 2; Figs. 3 and 4). Equally similar for brain and liver DNA were the half-lives ($t_{1/2} \sim 115$

Table 2
Approximate Half-lives ($t_{1/2}$) of Ethylated Purine Bases in the DNA of Different Tissues after an i.p. Pulse of [1-^{14}C]EtNU to 10-Day-old BD IX Rats

Tissue	*Elimination kinetics*	*Half-life* $t_{1/2}$ *(hr)*		
		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]	26[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]

$t_{1/2}$ values obtained for EtNU-ethylated DNA incubated in vitro are given for comparison (Goth and Rajewsky 1974a,b; L. Ya. Lomakina and M. F. Rajewsky, unpubl.).

[a] Time interval analyzed: 1–25 hr after [1-^{14}C]EtNU pulse.

[b] Time interval analyzed: 25–240 hr after [1-^{14}C]EtNU pulse.

[c] Time interval analyzed: 25–75 hr after [1-^{14}C]EtNU pulse. $t_{1/2}$ values for 1–25-hr and 75–114-hr time intervals: 10 hr and 49 hr, respectively.

[d] Based on measurements at 1, 4, and 240 hr after [1-^{14}C]EtNU pulse.

[e] 25 mM potassium phosphate buffer (0.02% sodium azide), pH 7.2, 37°C.

[f] Time interval analyzed: 1–240 hr after beginning of incubation.

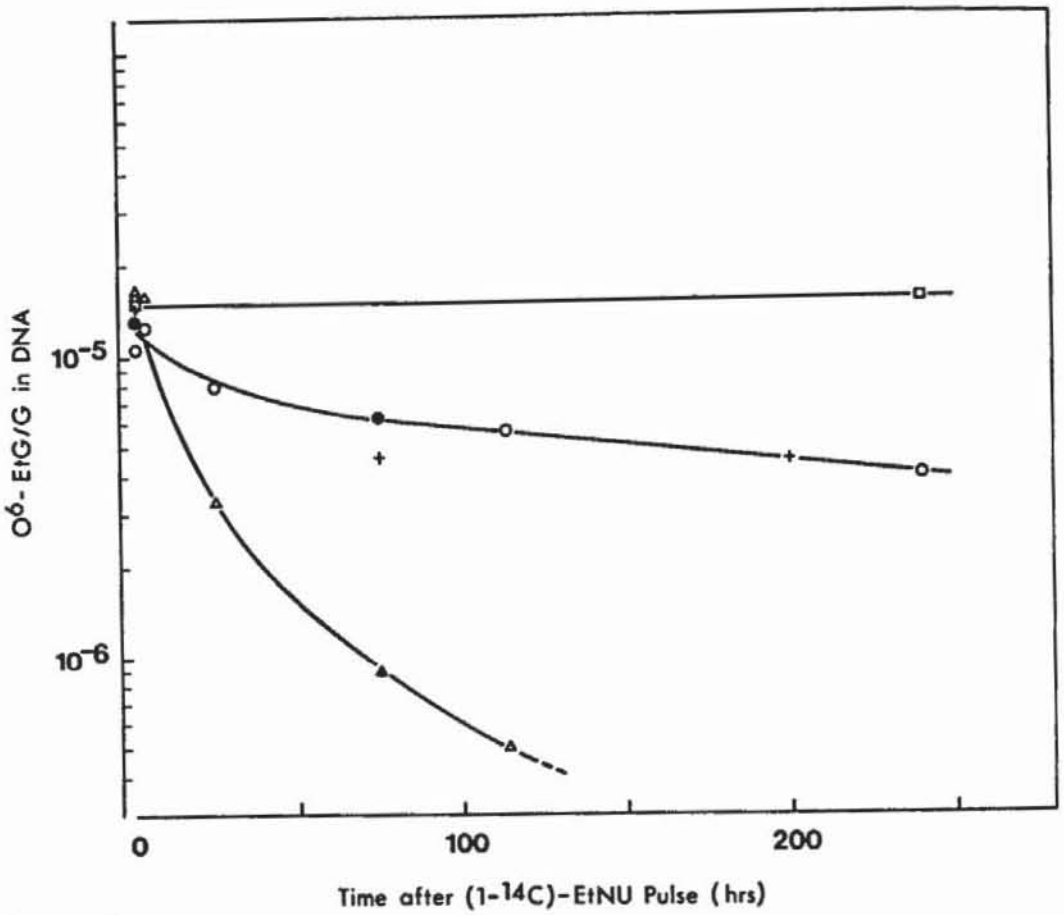

Figure 3
Kinetics of the elimination of O^6-EtG from DNA of brain and liver of 10-day-old BD IX and BD IV rats. Molar fractions of O^6-EtG/G in DNA of brain and liver are given as a function of time after an i.p. pulse of 75μg of [1-^{14}C]EtNU/g. Values for DNA isolated from pooled tissues at 4 hr after a [1-^{14}C]EtNU pulse in vivo and incubated in 25 mM potassium phosphate buffer, 0.02% sodium azide, pH 7.2, at 37°C for 240 hr in vitro are given for comparison (Goth and Rajewsky 1974a,b; L. Ya. Lomakina and M. F. Rajewsky, unpubl.). (o, •) Brain, BD IX rat; (△, ▲) liver, BD IX rat; (+) brain, BD IV rat; (□) DNA incubated in vitro.

hr and ~ 99 hr, respectively) of the integral ^{14}C-activity of the "early peaks" (mainly attributed to ethylphosphotriesters (Goth and Rajewsky 1974b; Singer 1975; Sun and Singer 1975) in the Sephadex G-10 radiochromatograms (Fig. 4). For comparison, incubation at 37°C for 240 hours of DNA previously ethylated by EtNU in vivo showed complete stability of O^6=EtG in DNA, whereas the in vitro half-lives of N^7-EtG and N^3-EtA in DNA were ~ 225 hours and ~ 33 hours, respectively (Table 2; Fig. 3) (Goth and Rajewsky 1974b). The observed selective persistence of O^6-EtG in brain DNA, together with the high rate of DNA replication and cell division in the developing NS of the rat, could be responsible for an increased probability of malignant transformation in this tissue and could thus provide an explanation for the NS specificity of the carcinogenic effect of EtNU.

Application of a pulse of 75 μg of EtNU/g to the fetal or newborn rat causes a temporary reduction of DNA synthesis in the NS (Goth and Rajewsky 1974b; Bosch and Ebels 1976), probably accompanied by a re-

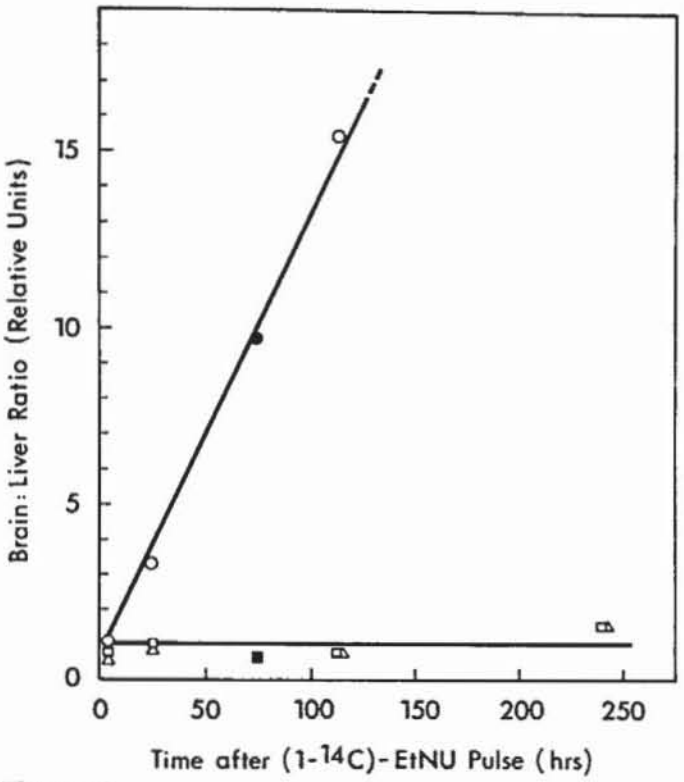

Figure 4

Brain-to-liver ratio for the molar contents in DNA of O^6-EtG (o, •) and N^7-EtG (□, ▪), respectively, and for the specific ^{14}C-radioactivity (dpm/mg input DNA) of the "early peak" in the Sephadex G-10 radiochromatograms (△) (R. Goth and M. F. Rajewsky, unpubl.) as a function of time after an i.p. pulse of 75 μg of [1-^{14}C]EtNU/g to 10-day-old BD IX rats. Initial values are those at 1 hr after the carcinogen pulse, normalized to 1.0 (Goth and Rajewsky 1974a,b; L. Ya. Lomakina and M. F. Rajewsky, unpubl.).

duced influx of cycling cells into the S phase. In principle, this could counteract the effect of a reduced elimination rate of O^6-alkylguanine from DNA, in terms of the probability of anomalous base-pairing during DNA replication. However, as has been demonstrated recently in 8-day-old Wag rats by means of [^{3}H]thymidine labeling and autoradiography (Bosch and Ebels 1976), cells resume DNA synthesis at 36 hours in the subependymal layer of the brain, and at 12 hours in the trigeminal nerve, following a pulse of 100 μg of EtNU/g body weight. These delay periods are well below the half-life value for O^6-EtG in brain DNA of the 10-day-old BD IX rat (~ 229 hr; Table 2).

As shown in Figure 3, O^6-EtG was equally persistent in brain DNA of 10-day-old BD IX and BD IV rats. According to Druckrey et al. (1970a), the median time (T_{50}) until death with neuroectodermal neoplasms after a dose of 50 μg of EtNU/g on the 15th day of gestation was approximately three times longer (with a somewhat reduced tumor incidence) in BD IV than in BD IX rats. On the other hand, no data are as yet available on the tumorigenicity of EtNU in 10-day-old BD IV rats and on the relative frequencies of high-risk target cells present in the NS of 15th-day BD IV vs. BD IX fetuses. Nonetheless, comparative analyses in genetically defined

strains with a differential response to EtNU (Searle and Jones 1976) may provide important information on host factors influencing the process of carcinogenesis during the period following the initial carcinogen–target-cell interactions.

It might be argued that the observed tissue differences in the elimination rates from DNA of O^6-EtG could result mainly from differential dilution of this promutagenic product in DNA, due to different rates of DNA replication in the respective tissues. However, if this were the case, the elimination rates, for example, of N^7-EtG from brain vs. liver DNA should differ by a factor similar to that found for the half-lives of O^6-EtG, unless unexpected variations existed in these tissues regarding intranuclear conditions for hydrolysis of glycosidic linkages in DNA. Rather, the data argue strongly for the existence of a specific enzymatic mechanism for elimination of O^6-alkylguanine from DNA which is either lacking or substantially less effective in rat brain. Such a mechanism basically could operate analogously to the scheme of excision repair, involving (a) specific enzyme(s) for recognition and removal of the modified bases and (b) 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 (Kirtikar and Goldthwait 1974; Maher et al. 1974; Verly 1975; Laval 1976; Lindahl 1976) suggest that this may soon be the case.

Selective persistence of O^6-methylguanine (O^6-MeG) in the DNA of rat brain was also demonstrated recently when the predominantly NS-specific carcinogen MeNU was applied to young-adult BD IX rats over a period of 35 days (Kleihues and Margison 1974; Margison and Kleihues 1975). Since it is known that kidney tumors occasionally develop after single high doses of MeNU (Druckrey et al. 1967), it is of interest that a low degree of accumulation of O^6-MeG also was seen in kidney DNA. Extending these analyses to the dialkylnitrosamines, it was further found (Nicoll et al. 1975; Magee et al. 1975) 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 [Swann and Magee 1968]), O^6-MeG is much more slowly eliminated from kidney DNA than after a low (non–kidney-tumorigenic) dose (2.5 μg DMN/g). More recently, Kleihues and Margison (1976) have found that the excision repair system for O^6-MeG in rat liver apparently can be exhausted by sublethal doses of MeNU and related alkylating carcinogens and requires several days for recovery.

DNA–Protein Interactions

Various investigators have pointed out that besides direct mutational effects on DNA, interference of carcinogens with molecular systems controlling gene transcription could lead equally to quasipermanent alterations in the readout of DNA (Pitot and Heidelberger 1963; Tsanev and Sendov 1971; Miller and Miller 1974; Heidelberger 1975). In this context, the interactions of regulatory chromosomal proteins (CP) with DNA require particular attention. Investigations regarding this point are still very much in their infancy. After a t.p. pulse of 75 μg of EtNU/g to the 18th-day BD IX rat fetus, the rate of [^{14}C]leucine incorporation into histones F2a1, F2a2, and F3 and into 13 in-

dividual nonhistone CP of the brain (separated by electrophoresis in 15% and 10% polyacrylamide gels) showed a biphasic response, with a common second maximum as late as 15 days after the EtNU pulse (Biessmann and Rajewsky 1975, 1976). A first peak of nonhistone CP synthesis appeared at 24 hours, i.e., at a time when histone synthesis (and DNA synthesis; Goth and Rajewsky 1974b) had not yet recovered from an initial depression. Chromatin dissociation and reassociation experiments following incorporation of radioactive leucine into brain CP at 120 hours after a pulse of 75 μg of EtNU/g to 10-day-old BD IX rats indicated an increased rate of CP synthesis and an increased affinity of the newly synthesized CP for brain DNA isolated from both control and EtNU-treated BD IX rats (Augenlicht et al. 1975). The significance of these observations in relation to the process of carcinogenesis by EtNU remains to be evaluated.

Malignant Transformation of Fetal Rat Brain Cells in Culture after Exposure to EtNU In Vivo

The above analyses on whole tissues do not provide information on the differentiative and proliferative properties of the particular (precursor) brain cells that undergo neoplastic transformation after exposure to EtNU. Furthermore, the interval between the initial carcinogen–cell interaction and the onset of clonal tumor growth represents an unclarified phase in the process of carcinogenesis, which may involve a sequence of characteristic phenotypic and functional alterations of the presumptive cancer cells. The obvious difficulties in analyzing these phenomena in intact tissues may be circumvented in part by the use of appropriate cell-culture systems. We have therefore developed an "in vivo–in vitro" system where the tissue-specific carcinogen EtNU is administered in vivo prior to transfer of the respective target cells "at risk" (fetal BD IX rat brain cells, FBC) to long-term cell culture (Laerum and Rajewsky 1975). This procedure 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 that would give rise to neuroectodermal tumors in vivo after an EtNU 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 (Sanford 1967). (4) With the aid of different markers (Herschman et al. 1971; Eng et al. 1971; Wilson et al. 1972; Laerum et al. 1976, 1977a), the resulting neoplastic cell lines can be analyzed for the phenotypic expression of NS-specific properties. (5) Under standardized conditions, the system may become applicable for assaying the transformation frequencies of preselected subpopulations of FBC.

Dissociated FBC, transferred to long-term monolayer cell culture at 20–90 hours after a t.p. pulse of 75 μg of EtNU/g body weight on the 18th day of gestation, became tumorigenic after about 200 days (as assayed by reimplantation into baby BD IX rats; Table 3) (Laerum and Rajewsky 1975). This time interval is of the same order as the median time until death with neuroectodermal tumors (T_{50} = 195 days; Table 1) in the offspring of BD IX rats injected with the same dose of EtNU at the same stage of gesta-

Table 3
Neoplastic Transformation of Fetal (18th Day of Gestation) BD IX Rat Brain Cells in Culture after a t.p. Pulse of 75 μg of EtNU/g In Vivo

Time interval	*Days*	*Passage number*
t_M[a]	98 (±20)	4 (±0.7)
t_A[b]	138 (±19)	6 (±2)
t_T[c]	199 (±28)	12 (±2)

Mean values (±S.E.) are given for seven independent sets of experiments. Cell suspensions from 6–10 pooled fetal brains, corresponding to ≥10 separate primary monolayer cultures (1–2 × 10^6 cells/culture), were used per experiment. Untreated control cultures died out after 121 ± 39 days (S.E.) and ≤4 culture passages. (Data from Laerum and Rajewsky 1975.)

[a] Time interval between EtNU pulse in vivo and first observation of morphological transformation.

[b] Time interval between EtNU pulse in vivo and first observation of colony formation in semisolid agar medium. Initial cloning efficiency for 10^4 viable cells/30 ml 0.15% agar medium, 0.9 ± 0.3% (S.E.).

[c] Time interval between EtNU pulse in vivo and first observation of tumorigenicity in isogenic hosts. The average time interval from subcutaneous (s.c.) reimplantation of 10^6 cells into baby BD IX rats until the first tumors became palpable was 48 ± 13 days (S.E.). Occasionally, much longer latency intervals (≤10 months) were recorded.

tion (Goth and Rajewsky 1974b; Laerum and Rajewsky 1975). Acquisition of tumorigenicity in culture was preceded by a sequence of characteristic phenotypic changes, termed "stages I–V" (Fig. 5; Table 3). During stage I (early primary culture), both EtNU and untreated control cultures contained stationary glia-like cells on a growing layer of flat, epithelioid cells (possibly glial precursors) and a few fibroblastoid cells. Stage II (~10th–40th day) was characterized by a constant proportion of glia-like cells in the EtNU cultures and their gradual disappearance in the controls. During stage III (~40th–100th day), slowly proliferating glia-like cells in the EtNU cultures formed piled-up "nodules" which could be removed and cultured separately. The transition to stage IV (~100th–200th day) was marked by proliferation of "morphologically transformed" cells (enlarged cell bodies; reduced length and number per cell of cytoplasmic processes). These formed *colonies in semisolid* agar and finally became tumorigenic, as assayed by subcutaneous (s.c.) reimplantation into baby BD IX rats (stage V; see Table 3 for average time intervals between different stages).

The neoplastic neurogenic cell-culture lines (BT lines) developed in this in vivo–in vitro system have been analyzed with regard to some of their phenotypic properties (Laerum et al. 1977b). Like several neurogenic cell-culture lines (V lines) derived from EtNU-induced, neuroectodermal BD IX rat tumors (Laerum and Rajewsky 1975), the BT lines contained

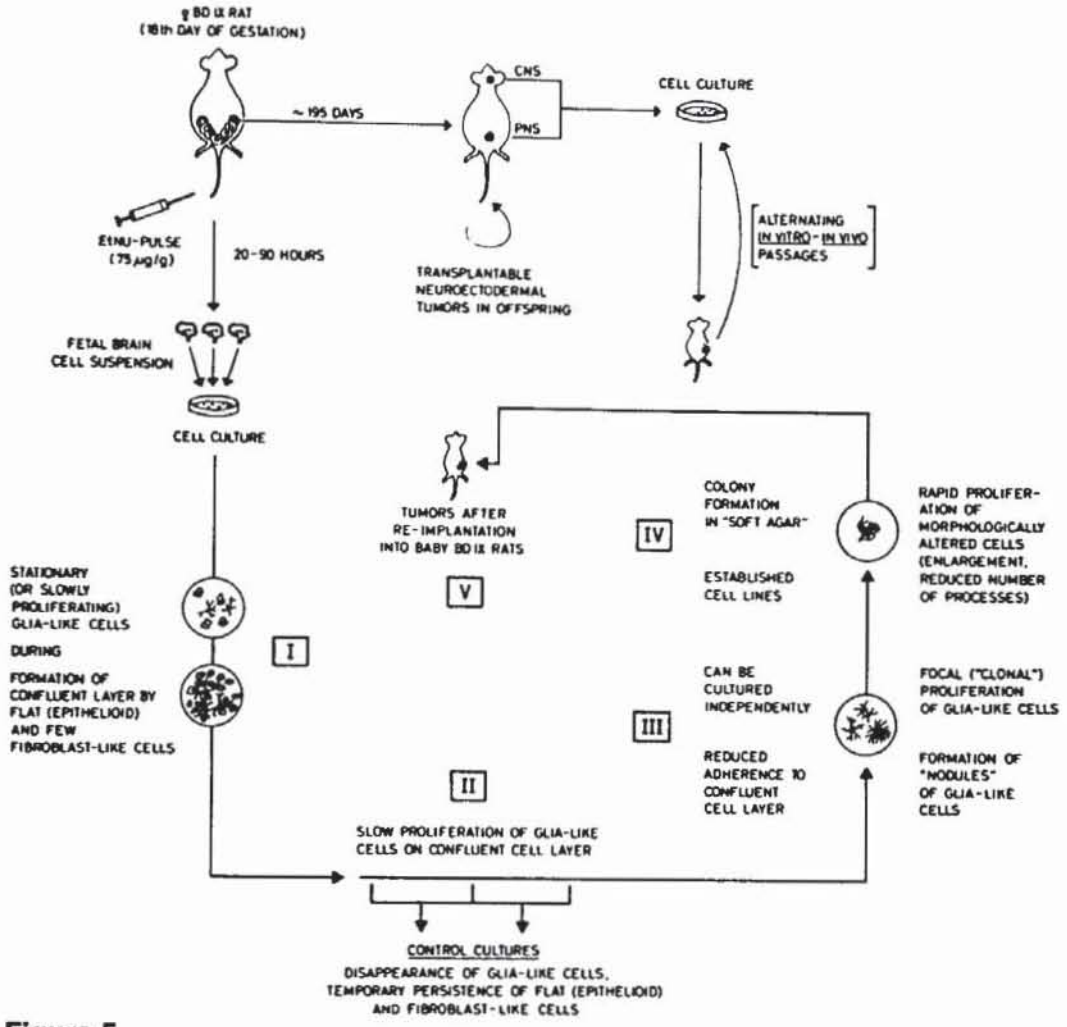

Figure 5
Diagrammatic representation of the in vivo–in vitro system for neoplastic transformation of fetal BD IX rat brain cells in culture, after t.p. exposure to EtNU in vivo. CNS indicates central nervous system; PNS indicates peripheral nervous system. (Reprinted, with permission, from Laerum and Rajewsky 1975.)

multipolar, glia-like cells, but also flatter cells with shorter and fewer cytoplasmic processes, and occasionally giant cells. As would be expected, the multiclonal "parental" BT and V lines contained multiple subpopulations of cells with different degrees of aneuploidy (Hanke and Rajewsky 1975; Laerum and Hansteen 1975). This was also reflected by plurimodal DNA distributions recorded by pulse-cytophotometry (Laerum and Hansteen 1975; Hanke and Rajewsky 1975; Rajewsky et al. 1976). As assayed by indirect immune fluorescence, all parental lines and their (cloned) sublines (total number > 30) contained the NS-specific marker protein S-100, but to a varying degree and in varying proportions of cells (Laerum and Rajewsky 1975; Laerum et al. 1977b). This predominantly glia-specific protein is not yet expressed in FBC (Herschman et al. 1971). There was no indication of more than borderline neurotransmitter activity (acetylcholinesterase, choline acetyltransferase, L-glutamate decarboxylase), nor has electrical membrane excitability been detected (Laerum et al. 1976). Serologic analyses by

the Ouchterlony test of selected BT and V lines gave no evidence for the presence of group-specific (gs) interspecies oncornaviral antigens (Schäfer et al. 1973; by courtesy of Prof. W. Schäfer, Max-Planck-Institut für Virusforschung, Tübingen, Germany).

The solid, transplantable tumors developed upon s.c. reimplantation of the parental BT lines appeared histologically as neurinoma-, glioma-, or glioblastoma-like and frequently pleiomorphic neoplasms. Although exhibiting a more atypical cellular morphology, they grossly resembled the different types of neuroectodermal rat neoplasms induced by EtNU in vivo (Wechsler et al. 1969).

The present in vivo–in vitro system has not yet permitted subpopulations of probable high-risk FBC to be defined. However, 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) (Laerum and Rajewsky 1975; Laerum et al. 1976) make proliferative glial precursors potential candidates (Goth and Rajewsky 1974a,b; Laerum and Rajewsky 1975). This would be in accordance with the assumption that the risk of neoplastic conversion may vary with the stage of the target cells in their differentiative pathway (from the stem cell to the mature, nonproliferative "end-cell") at the time of interaction with a carcinogen (Rajewsky 1972; Goth and Rajewsky 1974a,b; Laerum and Rajewsky 1975; Cairns 1975; Rajewsky et al. 1976).

It should be noted, however, that neuroglia and neurons are believed to have common neuroepithelial precursors (see Langman et al. 1971). Immature cells of neuronal lineages were certainly not absent from the present FBC cultures initially. Therefore, neoplastic clones expressing glial and/or neuronal properties might, in principle, have been expected to develop. However, during rat brain development and on an overall scale, the neuronal system precedes the glial cell populations with respect to proliferation and maturation (see Altman 1969). Hence, at the developmental stage of the NS chosen for the EtNU pulse (18th day of gestation), the precursor compartments may contain predominantly glial precursors. This in turn might explain the prevalence of neoplastic cells with glial phenotypic traits. Interestingly, some neoplastic cell lines also expressing neuron-like properties have recently been derived from neuroectodermal BD IX rat tumors induced by application of EtNU on the 15th day of gestation (Schubert et al. 1974).

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