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# High Susceptibility of Analbuminemic Rats to Neurogenic Tumor Induction by Transplacental Administration of N-Ethyl-N-nitrosourea

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**The susceptibilities of Nagase analbuminemic rats (NAR) and control Sprague-Dawley rats (SDR) to N-ethyl-N-nitrosourea (ENU) were compared. In Experiment I, the rats were given daily subcutaneous injections of 10 mg/kg of ENU for a week from 4 weeks of age. In Experiment II, mother rats were given a single subcutaneous injection of 60 mg/kg of ENU on day 17 of pregnancy and tumor development in their offspring was examined. In Experiment I, the incidence of neurogenic tumors was slightly, but not significantly, higher in NAR than in control rats. In Experiment II, the incidence of total tumors including neurogenic tumors was significantly higher in NAR (40/43, 93.0%) than in SDR (13/61, 21.3%). NAR showed particularly high susceptibility to induction of neurogenic tumors (34/43, 79.1%) and renal tumors (15/43, 34.9%). In an attempt to elucidate the underlying mechanisms of the increased susceptibility of NAR to ENU, $O^6$-ethylguanine, a major premutagenic ethylated DNA adduct, was quantitated in fetal brain DNA of NAR and SDR after a pulse exposure to 60 mg/kg ENU. No significant difference in the initial formation or subsequent repair of $O^6$-ethylguanine was observed in the two strains, indicating that abnormality at some later stage(s) of chemical carcinogenesis may lead to the increased susceptibility of NAR to induction of neurogenic tumors.**

Key words: Analbuminemic rats — N-Ethyl-N-nitrosourea — Transplacental administration — Neurogenic tumor

Nagase analbuminemic rats (NAR)[6] were established from a stock of Sprague-Dawley rats (SDR) and are characterized by an extraordinarily low level of serum albumin and hyperlipidemia.[1] Serum albumin is a transporter of many endogenous and exogenous substances such as bile acids, hormones, toxins, and probably carcinogens.[2,3] Various characters of NAR have been examined, including their susceptibilities to various carcinogens. The finding of high susceptibilities of NAR to certain carcinogens[4–7] has generated interest in the possible use of NAR as models in studies on carcinogenesis.

Since Ivankovic and Druckrey reported the selective induction of neurogenic tumors in rats by transplacental administration of N-ethyl-N-nitrosourea (ENU), ENU has been widely used as a selective carcinogenic agent for induction of neurogenic tumors after transplacental exposure.[8–10] This compound is characterized as a pulse carcinogen, because of its rapid heterolytic decomposition under physiological conditions. Its movement across the placental barrier is unrelated to serum transporting or carcinogen metabolizing systems. This treatment with ENU by this route may be particularly useful in studying molecular and cellular aspects associated with the process of malignant transformation. As one of a series of experiments on carcinogenesis in various organs of NAR, in the present study we examined the neurocarcinogenic effect of ENU injected subcutaneously into young (4-week-old) and pregnant animals.

When cells are exposed to ENU, more than ten different DNA ethylation products are formed,[11] among which $O^6$-ethylguanine ($O^6$-EtG) is considered to be a major premutagenic lesion.[12] A close correlation between the amount of $O^6$-EtG in DNA of target cell populations and the probability of malignant transformation has been demonstrated *in vitro*[13] as well as *in vivo*.[14] We, therefore, quantitated $O^6$-EtG in fetal brain DNA after the application of ENU to obtain an insight into possible mechanisms of the altered susceptibility of NAR to ENU.

## MATERIALS AND METHODS

**Chemicals** ENU was purchased from Nakarai Chemical, Ltd., Kyoto (purity >99.9%). DNase I, phosphodiesterase, and alkaline phosphatase were obtained from

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[6] Abbreviations used: NAR, Nagase analbuminemic rats; SDR, Sprague-Dawley rats; ENU, N-ethyl-N-nitrosourea; $O^6$-EtG, $O^6$-ethylguanine; $O^4$-EtT, $O^4$-ethylthymine.

Boehringer Mannheim (Mannheim, Germany). All other reagents were of analytical grade.

**Animals** Pregnant female and 4-week-old male SDR were purchased from CLEA Japan Inc., Tokyo, and pregnant female and 4-week-old male NAR were bred in the Sasaki Institute. During the experimental period, the rats were housed 4 or 5 to a plastic cage with wood chips for bedding in a conventional animal room with air conditioning (23±2°C and 55±5% relative humidity) and with a 12-h light period from 7 AM to 7 PM. They were maintained on commercial diet (CE-2: CLEA Japan Inc., Tokyo) and tap water *ad libitum*.

**Experimental procedure**

Experiment I: Groups of 20 male SDR and NAR, initially 4 weeks old, were given daily subcutaneous injections of ENU (10 mg/kg body wt) for a week.

Experiment II: Groups of 6 pregnant SDR and NAR were given a single subcutaneous injection of ENU (60 mg/kg body wt.) on day 17 of pregnancy. All surviving offspring were weaned at 4 weeks old, divided into family groups, and housed as described.

In both experiments, the observation period lasted until experimental week 51, when all surviving animals were killed. During the experiment, moribund or dead rats were autopsied and examined for tumors in all organs and tissues including the nervous system. The brains were bisected transversely and fixed in Lille-buffered formalin. Other tissues with grossly visible lesions were also fixed. The tissues were dehydrated, embedded in paraffin, sectioned at 4 $\mu$m thickness, and stained with hematoxylin and eosin (H&E). Tumor types were determined by histopathologic examination.

**Quantitation of $O^6$-EtG and $O^4$-EtT** Fetal brains were excised 1 and 24 h after subcutaneous injection of 60 mg/kg ENU, and DNA was isolated by a conventional phenol/chloroform extraction method as described by Maniatis *et al.*[15] The DNA was then hydrolyzed enzymatically to 2′-deoxynucleosides by serial treatments with DNase I (100 $\mu$g/ml), phosphodiesterase (100 U/ml), and alkaline phosphatase (100 $\mu$g/ml) as described by Muller and Rajewsky.[16] Aliquots were analyzed by HPLC under similar conditions to those reported by Huh and Rajewsky,[17] except that an ODS-80TM column (Tosoh, Tokyo) was used for quantitation of normal 2′-deoxynucleosides, and preparations were monitored for possible contamination with RNA. Peak areas of adenosine were always less than a few percent of those of 2′-deoxyadenosine, indicating negligible contamination of the DNA preparations with RNA. The DNA hydrolyzates were injected into an HPLC system equipped with a 4-mm×250-mm reverse-phase column (Li-Chrospher 100 RP-18(e), 5 $\mu$m; Merck, Darmstadt, Germany), and eluted at 40°C with a linear concentration gradient of methanol of 10 to 20% for 20 min, and then 20 to 50% for 15 min in 0.1 *N* ammonium acetate, pH 5.0. The flow rate was 1 ml/min. The fractions corresponding to $O^6$-ethyl-2′-deoxyguanosine ($O^6$-EtdGuo, retention time; 27.5 min) and $O^4$-ethyl-2′-deoxythymidine ($O^4$-EtdThd, retention time; 35 min) were collected, dried under reduced pressure, and quantitated by competitive radioimmunoassay[16] using anti[$O^6$-EtdGuo] monoclonal antibody ER-6[18] and anti[$O^4$-EtdThd] monoclonal antibody ER-01,[19] respectively. The antibodies and tritiated tracers for both ethylated nucleosides were kindly provided by Dr. M. F. Rajewsky, University of Essen, Germany.

**Statistical comparisons** The statistical significance of differences in tumor incidences was evaluated by a modification of Fisher's exact probability test.

## RESULTS

**Experiment I** Some tumors including neurogenic tumors were found in both groups, although at low incidences. There was no significant difference between the total numbers of tumors in the two groups (Table I). The incidence of neurogenic tumors was slightly, but not significantly, higher in NAR (2/19, 10.5%) than in control rats (SDR) (1/18, 5.6%). Other tumors were observed in the intestine, spleen, and subcutis of NAR, and in the subcutis of SDR.

**Experiment II** The organ locations and incidences of tumors in the two strains are summarized in Table II. The weaning rate of NAR (46/65, 71%) was slightly lower than that of SDR (62/73, 85%). The total incidences of tumors were significantly higher in NAR (males, 90%; females, 95.7%) than in SDR (males,

Table I. Sites and Incidences of Tumors in Experiment I

| | NAR ♂ | SDR ♂ |
|---|---|---|
| Initial number | 20 | 20 |
| Effective number | 19 | 18 |
| Body weight (g) | | |
| Initial | 105±14[a] | 131±10 |
| Final | 516±38 | 715±55 |
| Incidence of total tumors (%)[b] | 4/19 (21.1) | 2/18 (11.1) |
| Localization and incidence of tumors (%)[b] | | |
| Nervous system | | |
| Brain | 2/19 (10.5) | 1/18 (5.6) |
| Extraneural organs | | |
| Intestine | 2/19 (10.5) | |
| Spleen | 3/19 (15.8) | |
| Subcutis | 2/19 (10.5) | 1/18 (5.6) |

*a*) Mean±SD.
*b*) Numbers in parentheses, percentages of rats with tumors.

Table II. Sites and Incidences of Tumors in Experiment II

| | NAR | | SDR | |
|---|---|---|---|---|
| | ♂ | ♀ | ♂ | ♀ |
| No. of mothers treated | 6 | | 6 | |
| No. of offspring | | | | |
| Delivered | 36 | 29 | 45 | 28 |
| Weaned | 22 | 24 | 36 | 26 |
| Effective | 20 | 23 | 35 | 26 |
| Incidence of total tumors (%)[a] | 18/20 (90)[c] | 22/23 (95.7)[c] | 8/35 (22.9) | 5/26 (19.2) |
| Localization and incidence of tumors (%)[a] | | | | |
| Nervous system | | | | |
| Brain | 7/20 (35) | 8/23 (34.8) | 6/35 (17.1) | 4/26 (15.4) |
| Spinal cord | 7/20 (35)[c] | 12/23 (52.2)[c] | | |
| Extraneural organs | | | | |
| Kidney | 7/20 (35)[b] | 8/23 (34.8)[b] | 2/35 (5.7) | 1/26 (3.8) |
| Intestine | 3/20 (15) | 2/23 (8.7) | | |
| Spleen | 1/20 (5) | 3/23 (13) | 1/35 (2.9) | |

*a*) Numbers in parentheses are percentages of rats with tumors.
*b, c*) Significantly different from the value for the control group (SDR): *b*) $P<0.05$; *c*) $P<0.001$.

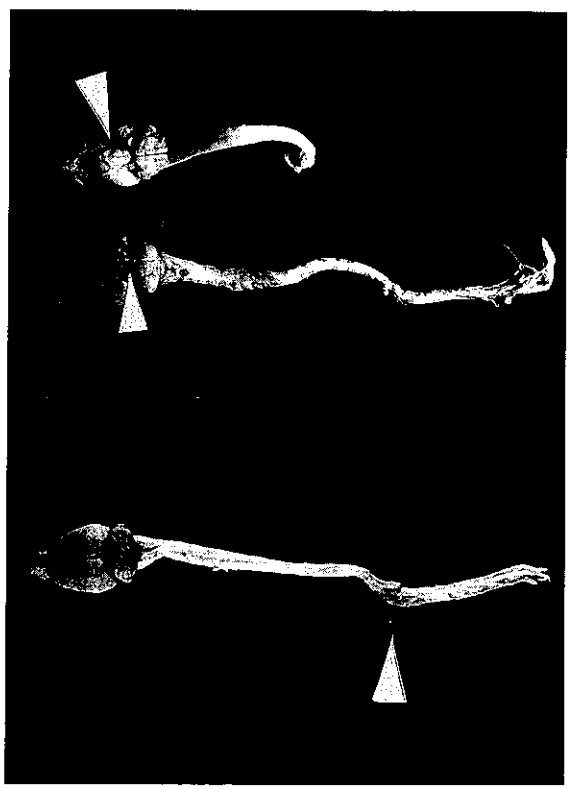

Fig. 1. Macroscopic appearance of neurogenic tumors observed in NAR treated with ENU (Experiment II). Neurogenic tumors were observed in the cerebrum and spinal cord (arrowheads).

22.9%; females, 19.2%, $P<0.001$). NAR showed particularly high susceptibility to induction of neurogenic tumors (34/43, 79.1%). These neurogenic tumors were mostly located in the brain and spinal cord, and were often associated with clinical signs of paralysis. Non-neurogenic tumors in NAR were found in the kidney, intestine, and spleen, though at low incidences. NAR also had a significantly higher incidence of renal tumors than SDR ($P<0.05$).

**Histological classification of ENU-induced tumors** The neurogenic tumors were mostly found in the cerebrum, including the meninges, and in the spinal cord, as shown in Fig. 1. These lesions could be classified into oligodendrogliomas, meningiomas, and mixed gliomas, as summarized in Table III. Oligodendrogliomas were the most common in both strains, followed by meningiomas and mixed gliomas (Fig. 2). In the spinal cord, only oligodendrogliomas were found. No tumors of the peripheral nervous system were found in either experiment. The tumors in extraneural organs were nephroblastoma of the kidney, adenoma of the intestine, cavernous hemangioma of the spleen, and fibroma of the subcutis (Fig. 3). Nephroblastomas were found only in Experiment II.

**Quantitation of alkylated DNA adducts** $O^6$-EtG in fetal brain DNA was quantitated 1 h after the injection of 60 mg/kg ENU by competitive radioimmunoassay. The molar ratio of $O^6$-EtG to guanine was $1.51\pm0.22$ in NAR and $1.42\pm0.46$ in SDR. We then compared the rates of elimination of $O^6$-EtG from cellular DNA in a

Table III. Histological Classification of Tumors in Experiments I and II

| | Experiment I | | Experiment II | | | |
|---|---|---|---|---|---|---|
| | NAR♂ | SDR ♂ | NAR ♂ | NAR ♀ | SDR ♂ | SDR ♀ |
| Neurogenic tumors | | | | | | |
| Oligodendroglioma | 2/19 (10.5)[a] | 1/18 (5.6) | 12/20 (60) | 18/23 (78.3) | 5/35 (14.3) | 4/26 (15.4) |
| Meningioma | | | 2/20 (10) | 1/23 (4.3) | 1/35 (2.9) | |
| Mixed glioma | | | | 1/23 (4.3) | | |
| Non-neurogenic tumors | | | | | | |
| Nephroblastoma (kidney) | | | 7/20 (35) | 8/23 (34.8) | 2/35 (5.7) | 1/26 (3.8) |
| Adenoma (intestine) | 2/19 (10.5) | | 3/20 (15) | 2/23 (8.7) | | |
| Hemangioma (spleen) | 3/19 (15.8) | | 1/20 (5) | 3/23 (13) | 1/35 (2.9) | |
| Fibroma (subcutis) | 2/19 (10.5) | 1/18 (5.6) | | | | |

*a*) Numbers in parentheses are percentages of rats with tumors.

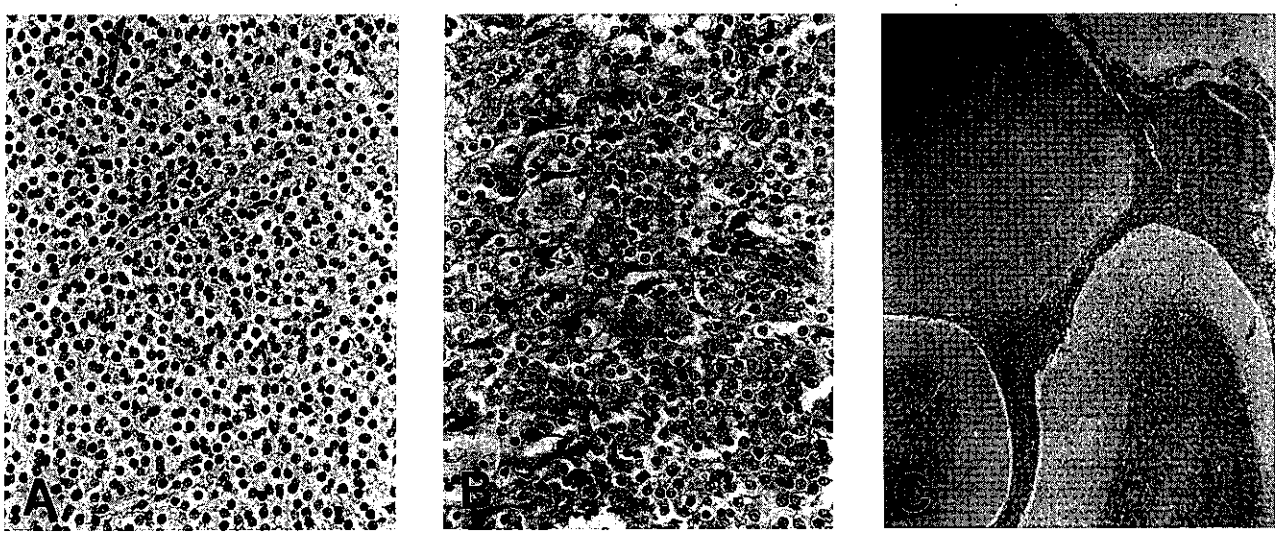


Fig. 2. Histological appearances of an oligodendroglioma (A), mixed glioma (B), and meningioma (C) in NAR treated with ENU (Experiment II). H&E, A; ×200, B; ×200, C; ×40

period of 24 h after application of ENU. To exclude possible bias due to DNA replication during the observation period, we expressed the content of $O^6$-EtG as a molar ratio to $O^4$-ethylthymine ($O^4$-EtT), which is known not to be repaired appreciably within 24 h in any mammalian tissues thus far examined, including rat brain.[17,20] In NAR, the molar $O^6$-EtG/$O^4$-EtT ratios 1 h and 24 h after the injection of ENU were 7.6 and 8.8, respectively. The corresponding values for SDR were 7.8 and 7.8. These results indicate negligible elimination of $O^6$-EtG from fetal rat brain cells, consistent with reports by others.[14]

## DISCUSSION

In Experiment I, there was no significant difference in the incidences of neurogenic tumors in NAR and controls, although their number in NAR was nearly twice that in SDR. On the other hand, in Experiment II, the incidence of neurogenic tumors was significantly higher in NAR than in SDR. These results show high susceptibility of NAR to induction of neurogenic tumor by transplacental administration of ENU. The present study also showed that NAR were susceptible to induction of nephroblastomas by transplacental administration of ENU. As reviewed by Maekawa and Mitsumori,[21] the incidence, location and type of chemically induced

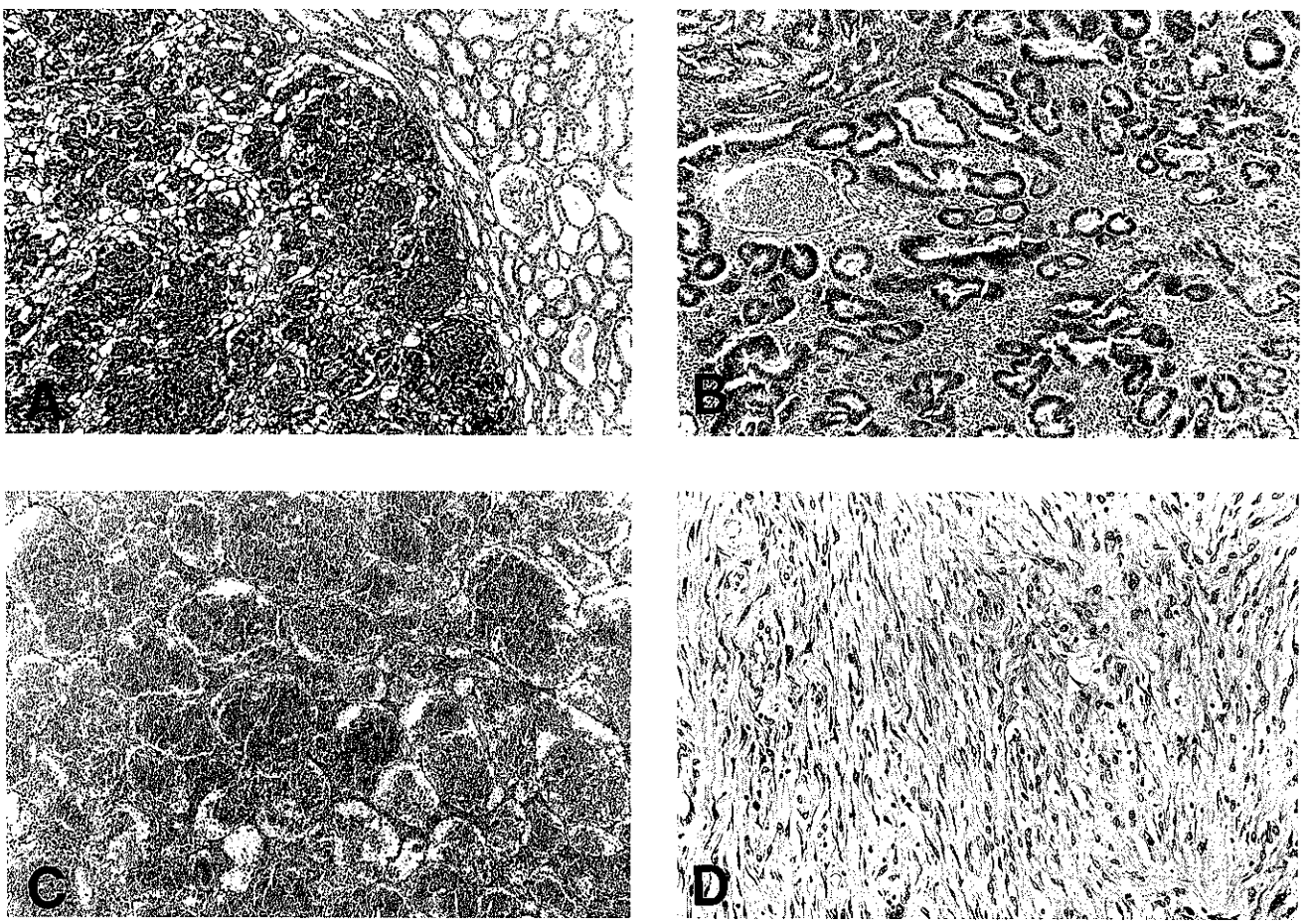


Fig. 3. Histological appearances of nephroblastoma in the kidney (A), adenoma in the intestine (B), cavernous hemangioma in the spleen (C), and fibroma in the subcutis (D) of NAR treated with ENU (Experiment II). H&E, A; ×100, B; ×100, C; ×100, D; ×200.

neurogenic tumors are influenced by the route of administration and host factors such as the age and strain of rats. Susceptibility of experimental antimals to carcinogenesis in the nervous system is known to be age-dependent,[21] fetal and newborn animals being the most sensitive to neurocarcinogens such as ENU. The nervous system was less susceptible to carcinogenesis in Experiment I than in Experiment II. The present results are consistent with those in studies by others.

NAR are a mutant strain established from a stock of SDR and are characterized by serum albumin deficiency and hyperlipidemia. The cause of analbuminemia is a seven-base-pair deletion in an intron of the albumin gene, which results in blocking of the process of m-RNA maturation from m-RNA precursors.[22] Previous experiments indicated that NAR show high susceptibility to induction of renal tumors by N-dimethylnitrosamine,[4] stomach cancer by N-methyl-N′-nitro-N-nitrosoguanidine,[5] and bladder cancer by N-butyl-N-(4-hydroxybutyl)nitrosamine.[6,7] NAR also show abnormalities of physiological parameters, e.g., increases in serum globulin, lipids, and urea nitrogen, and decreases in serum tryptophan, bile acid, prolactin, testosterone and bilirubin. The difference in tumor-promoting activity in NAR and control rats may be due to these abnormalities.

Formation and subsequent repair of $O^6$-EtG in DNA and the mitotic activity of a given target cell population are the most critical parameters determining the probability of neoplastic transformation by ENU in the initial stage of chemical carcinogenesis. The high incidence of neurogenic tumors induced by treatment with ENU in the late gestational and early neonatal stages of develop-

ment reflects active stage-specific proliferation of the target cells. It is unlikely, however, that neuronal cells proliferate more actively in NAR than in SDR in the perinatal stage, because no significant difference has been observed in the development of the brain in NAR and SDR.

We tested for a possible difference in the initial ethylation level or in repair efficiency of the premutagenic alkylated DNA adducts in the two strains. As described in the "Results," however, we found that formation and subsequent repair of $O^6$-EtG in the fetal brain cells were similar in the two strains, indicating that some later stage(s) of carcinogenesis, e.g., intrinsic phenotypic instability of cells, tumor promotion, or sensitivity of primitive malignant cells to immune systems, may be altered in NAR. Thus neurogenic tumor induction in NAR by ENU provides an appropriate experimental system for studies on later steps in the multi-step process of chemical carcinogenesis. The increased susceptibility of NAR to tumor induction should also be applicable in developing a more sensitive test system for evaluation of putative carcinogens.

The present study showed that NAR were susceptible to induction of not only neurogenic tumors but also nephroblastomas by transplacental administration of ENU.

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