Quantitative Aspects of Transplacental Tumor Induction with Ethylnitrosourea in Rats

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SUMMARY

Dose-response relationships of transplacental tumor induction with the resorptive carcinogen, ethylnitrosourea, were investigated in Sprague-Dawley and Fischer rats. Neuroectodermal tumors were produced in offspring of all rats exposed to a single dose of ethylnitrosourea, 1, 5, 20, or 50-mg/kg, near the end of gestation. The incidence of experimentally induced neoplasms was directly proportional to the dose of carcinogen, while mean survival times were inversely related to exposure. The location of tumors varied with the dose of carcinogen and strain of rat. Brain tumors were the cause of death for 69% of the Fischer rats exposed to ethylnitrosourea, 50 mg/kg, whereas only 27% of similarly exposed Sprague-Dawley rats died of brain tumors. A significantly longer survival time was demonstrated for rats that died of gliomas compared with those that died of neurinomas or ependymomas. This investigation demonstrates that the age at which an animal develops neoplasia following transplacental exposure to a carcinogen depends on the level of exposure and the tumor type. This suggests that tumors of adults, as well as of children, may be due to transplacental exposure to carcinogenic agents.

INTRODUCTION

Much of the recently expanded interest in environmental pathology stems from the demonstration that many chemicals are capable of inducing tumors at sites distant to their application, i.e., resorptive carcinogens. Among the most interesting examples are the resorptive N-nitroso compounds. The initial recognition of the carcinogenic properties of this group of compounds is credited to Magee and Barnes (22), who demonstrated that hepatic carcinomas could be induced in rats following the feeding of dimethylnitrosamine. More recently, Druckrey et al. (10) published a compendium of 65 N-nitroso carcinogens, most of which are organ specific. One of the most intriguing of these compounds is the acylalkylnitrosamide, ENU. ENU has been shown to produce tumors of many organs when administered as a single dose to adult rats. Its ability to induce neuroectodermal tumors in 100% of the offspring following a single transplacental exposure has stimulated its use in experimental neurooncology.

The exquisite sensitivity of the rat fetal nervous system to ENU was first demonstrated in papers by Druckrey et al. (8), Ivankovic and Druckrey (16), and Ivankovic et al. (17) and has subsequently been confirmed by others (12, 14, 19, 20, 34). These studies have demonstrated that as little as 2% of the adult 50% lethal dose resulted in a 63% incidence of neuroectodermal tumors. In addition to its carcinogenic properties, ENU has been shown to be a potent teratogen (11, 16, 33). This may allow future studies into the perplexing problem of neoplastic predisposition of hamartomas and teratological syndromes.

The purpose of this communication is to evaluate the effects of various doses of ENU on the incidence, location, and type of neuroectodermal tumors induced transplacentally in rats. Exposures to the carcinogen ranged from less than 0.5% of the adult 50% lethal dose to twice that necessary to induce neoplasms in 100% of the offspring. In most previous investigations ENU was administered to pregnant rats on the 15th day of gestation. Since it had been demonstrated that the susceptibility of the fetus to this carcinogen was greatest at the end of gestation (16), we chose to study the dose-response relationships to ENU following transplacental exposure of the fetus on the 20th day of gestation. Sprague-Dawley and Fischer rats were used to evaluate potential strain differences in the expression of neoplastic transformation induced by ENU.

MATERIALS AND METHODS

Two to four pregnant Sprague-Dawley (CD) rats (Charles River, Wilmington, Mass.) were inoculated with ENU, 1, 5, 20, or 50 mg/kg, via the lateral tail vein on the 20th day of gestation. Three additional Fischer (CDF) rats were inoculated with ENU, 50 mg/kg, on the 20th day of gestation. The ENU (from Professor H. Druckrey, Freiburg, Germany) was freshly prepared by dissolving 10 mg/ml in sterile 0.9% NaCl solution and adjusting the pH to 4.5 with crystalline ascorbic acid. Approximately equal numbers of male and female offspring were weaned at 28 days of age and housed in individual cages in isolated rooms. All animals were fed autoclaved Purina Lab Chow 5010 C and water ad libitum. Rats were examined daily and weighed weekly throughout the experimental period. Progressive neurological signs and weight loss were used to select animals for closer observation.

All experimental rats were subjected to a complete necropsy.
immediately after death. Brains and spinal cords were fixed in Cajal's bromformol solution, while nonneural tissues were fixed in 10% buffered formalin. All lesions, serial blocks of brain, and selected segments of spinal cord were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination. Wilder's reticulin, Masson's trichrome, cresyl violet, and phosphotungstic acid-hematoxylin were utilized as supplemental histochemical procedures. In addition to light microscopic studies, tissues from selected moribund rats were prepared for electron microscopy according to previously published methods (20).

Survival times were recorded as the time from birth to death. The significance of differences in survival times between Sprague-Dawley rats exposed to different doses was evaluated with a 1-way analysis of variance and the method of least significant difference for mean separation. Average survival times were also calculated for tumor types of each dose and analyzed similarly. When multiple tumors occurred, the cause of death was attributed to the neoplasm that caused the most severe impairment of vital function. Survival time differences between strains and sexes exposed to the same dose were evaluated with a 2-tailed Student's t test.

RESULTS

All doses of ENU used in this study were capable of inducing neoplasms of the rat nervous system following transplacental exposure. The incidence of neuroectodermal tumors was directly related to the level of exposure, while an inverse relationship was evident for mean survival time (Table 1). The incidence of extraneural tumors decreased sharply with the shortening of the mean survival time. The frequency of multiple neuroectodermal tumors per animal increased with increasing doses of ENU.

Significant differences in mean survival times were demonstrated between Sprague-Dawley rats exposed to ENU, 1 and 5 mg/kg ($p < 0.005$), 5 and 20 mg/kg ($p < 0.005$), and 20 and 50 mg/kg ($p < 0.05$). Fischer rats exposed to ENU, 50 mg/kg, had increased survival times when compared with Sprague-Dawley rats exposed to the same level of carcinogen ($p < 0.01$). In addition to strain differences, the sex of the animal was found to influence mean survival times. Males of all dose levels had shorter mean survival times than females, but the differences were statistically significant only in the rats exposed to ENU, 20 mg/kg ($p < 0.05$).

All tumors are tabulated according to location in Table 2. The data include tumors that resulted in the death of the animal, incidental findings at necropsy, and tumors only detected microscopically (i.e., microtumors). While the number of tumors in all portions of the nervous system increased with greater exposure to carcinogen, those of the brain demonstrated the most marked increase. Most rats exposed to 50 mg/kg had multiple brain tumors at various stages of development.

A summary of the neuroectodermal and extraneural tumor types that developed in rats exposed transplacentally to ENU is given in Table 3. Neuroectodermal neoplasms comprised 87% of the tumors, and over 70% of these occurred in the central nervous system. Most gliomas were relatively well differentiated, while most neurinomas were highly anaplastic.
Neoplastic oligodendrocytes represented the predominant cell type of small microtumors. With increasing tumor size, astrocytic elements became mixed with the oligodendrocytes. These mixed gliomas underwent a transition from being primarily isomorphic in early stages to becoming increasingly pleomorphic in late stages of growth.

Ependymomas, primarily occurring in the spinal cord, comprised an important group of tumors in the Sprague-Dawley rats receiving ENU, 50 mg/kg, and to a lesser degree ENU, 20 mg/kg. This type of neoplasm did not occur in the spinal cord of Sprague-Dawley rats exposed to ENU, 1 or 5 mg/kg, or in Fischer rats exposed to ENU, 50 mg/kg. With this exception, dose of carcinogen had little effect on the morphology of tumors of the nervous system.

The majority of extraneural tumors occurred in the group of rats exposed to ENU, 1 mg/kg. Over one-half of these were incidental findings at necropsy upon termination of the experiment 2 years after exposure to the carcinogen. Extraneural tumors were subdivided into 2 groups: those likely to be spontaneous and those likely to be induced with ENU. Most pituitary adenomas, the adrenocortical carcinoma, pheochromocytoma, and one-half of the mammary tumors (fibroadenomas) were considered spontaneous neoplasms, since their incidence corresponded to that expected for similar aged control rats. Mammary carcinomas, renal mesenchymal tumors, leukemias, and scattered malignant tumors of other organs probably represent ENU-induced tumors.

The location of the tumors responsible for the death of each animal are summarized in Table 4. Dose-response relationships are readily apparent. Over one-half of the animals exposed to ENU, 1 mg/kg, and one-fourth of the rats exposed to ENU, 5 mg/kg, did not die of neoplastic disease during the 2 years of observation. All rats receiving 20 or 50 mg/kg died of neoplastic disease. Over 30% of the rats exposed to ENU, 1 mg/kg, died of extraneural tumors, whereas less than 5% of the animals exposed to higher levels of carcinogens died from neoplasms of nonneural tissues. The incidence of death due to peripheral nerve tumors increased proportionately with the logarithm of the dose. Spinal cord tumors increased dramatically in Sprague-Dawley rats exposed to 20 and 50 mg/kg, while the percentage of animals dying from brain tumors decreased at 50 mg/kg. Marked differences existed between Sprague-Dawley and Fischer rats. Brain tumors were responsible for 69% of the deaths of Fischer rats, more than twice that of Sprague-Dawley rats receiving the same dose of carcinogen. Death due to spinal cord and peripheral nerve tumors was much less common in Fischer rats.

Mean survival times were evaluated for animals of each group with gliomas, ependymomas, or neurinomas that caused death.
either death or severe neurological dysfunction (Table 5). Rats bearing gliomas had significantly longer survival times than those bearing ependymomas \((p < 0.10\) at 20 mg/kg; \(p < 0.01\) at 50 mg/kg) or neurinomas \([p < 0.02\) at 20 mg/kg, \(p < 0.025\) at 50 mg/kg (Sprague-Dawley), and \(p < 0.05\) at 50 mg/kg (Fischer)]. The level of carcinogen exposure influenced mean survival times within each group in a manner similar to the overall survival times expressed in Table 1. No significant differences existed between gliomas or neurinomas of Sprague-Dawley and Fischer rats exposed to ENU, 50 mg/kg.

**DISCUSSION**

The importance of transplacental exposure as a route of carcinogenesis is becoming apparent. This route was first discovered in 1947 by Larsen (21), who produced lung tumors in mice transplacentally exposed to urethan. Subsequently, oncogenesis following transplacental exposure has been demonstrated experimentally for diethylnitrosamine (25), ENU (8), cycasin (28), dimethylsulfate (1), 1,2-diethylhydrazine and azo- and azoxyethane (9), benz(a)pyrene (3), methylnitrosourea (33), dimethylbenzanthracene (4), and methylcholanthrene (32). The potential human hazard associated with transplacental exposure to carcinogens has been recently emphasized with the demonstration of an increased incidence of vaginal cancer in adolescents whose mothers had been treated with stilbesterol during pregnancy (24).

Several investigators have suggested that the transplacental route of exposure may be responsible for many childhood neoplasms (2, 24). Transplacental exposure may also result in tumors that appear in adults. The results of this investigation clearly point out that the age at which an animal develops neoplasia following transplacental exposure to a carcinogen depends on the level of exposure.

The unique sensitivity of the rat fetal nervous system to the carcinogenic effects of ENU is readily apparent. A dose of 1 mg/kg was innocuous to the pregnant female, yet it induced tumors of the nervous system in over 12% of the offspring. Higher doses increased the incidence to 100% and still caused no significant lesions in the mother. When our data were analyzed according to the method of Ivanovic and Drucker (16) for linear regression, a straight-line function was evident. Thus the equation \(dt^n = \text{constant}\), where \(d\) is dose and \(t\) is time, has an \(n\) of 2.53 for Sprague-Dawley rats exposed transplacentally on the 20th day of gestation. In addition to the dose-response relationships regarding latency periods, increased numbers of neuroectodermal tumors per rat were evident with increasing levels of exposure to ENU. Much of the tumor multiplicity seen in rats exposed to ENU, 50 mg/kg, was due to increased numbers of microtumors. These usually occurred in the brain of an animal with at least 1 grossly demonstrable neoplasm.

Studies on the occurrence of spontaneous neoplasms in Sprague-Dawley rats have demonstrated an exponential increase in tumor incidence with increasing age (26). The prominent increase in extraneural tumors that was demonstrated in rats receiving the 2 lowest doses of carcinogen correlates with the expected increase due to their lengthened life-span. While the designation of spontaneous versus induced neoplasms is open to question for any given tumor, generalizations can be drawn from the vast number of rats that have been evaluated for their natural tumor incidence (6, 18, 27, 31). The pituitary adenomas, benign mammary tumors, and adrenal gland neoplasms were considered spontaneous since their incidence corresponded to that reported for control animals. The neoplasms arising in the kidney of rats exposed to ENU closely resembled renal mesenchymal tumors induced with dimethyl-nitrosamine (15, 23).

The degree of differentiation of tumors of the nervous system did not appear to be influenced by the dose of carcinogen. Most gliomas were well differentiated, while neurinomas were anaplastic. Sprague-Dawley rats proved to be particularly susceptible to the development of spinal cord ependymomas when exposed to the 2 higher doses of carcinogen. The predilection for peripheral nerve tumors (7, 29) is again evident with this route of exposure.

Differences in survival time demonstrated for gliomas, ependymomas, and neurinomas correspond to previous studies on the sequential development of these neoplasms (30). When BD-IX rats were exposed to ENU, 50 mg/kg, on the 22nd day of gestation, early neoplastic changes were detected in the spinal roots, trigeminal nerves, and spinal cord of the offspring as early as 1 to 2 months of age. Similar microscopic changes were not evident in the brain until 4 months of age. Early neoplastic proliferation apparently starts at different periods for different tissues; however, the period from initial detection of morphological transformation to death is similar for all 3 tumor types. The difference in median survival times between Sprague-Dawley and Fischer rats can therefore be explained on

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### Table 5

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Strain</th>
<th>Glioma</th>
<th>Ependymoma</th>
<th>Neurinoma</th>
</tr>
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<tbody>
<tr>
<td>1 mg/kg</td>
<td>SD</td>
<td>408 ± 86 (2)</td>
<td>472 (1)</td>
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<tr>
<td>5 mg/kg</td>
<td>SD</td>
<td>447 ± 145 (8)</td>
<td>346 ± 160 (9)</td>
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<td>20 mg/kg</td>
<td>SD</td>
<td>348 ± 111 (8)</td>
<td>234 ± 89 (3)</td>
<td>228 ± 68 (8)</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>SD</td>
<td>252 ± 66 (7)</td>
<td>179 ± 51 (9)</td>
<td>195 ± 55 (13)</td>
</tr>
<tr>
<td>50 mg/kg</td>
<td>Fischer</td>
<td>274 ± 47 (18)</td>
<td>206 ± 58 (6)</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.D. Numbers in parentheses, number of tumors.
the basis of the much higher incidence of gliomas in the latter. The molecular interactions responsible for the induction of neoplastic transformation by ENU remain unknown. Goth and Rajewsky (13) have recently demonstrated similar degrees of ethylation of nucleic acids by ENU in fetal and adults rats. Levels of ethylated 7-guanine from liver DNA were approximately twice those of brain. Since hepatic tumors have not been induced with ENU and the oncogenic sensitivity of the fetus is much greater than that of the adult, there appears to be no correlation between ethylation of 7-guanine and oncogenic specificity. It is possible, however, that the importance of this mechanism depends on the capacity of various tissues for repair of ethylated 7-guanine. Interactions with other molecules such as histones, acidic nuclear proteins, etc., have not been evaluated with ENU. When HeLa cells were exposed to the closely related carcinogen, methyl nitrosourea, significant levels of histone methylation and acetylation were demonstrated (5). The importance of alterations of this genetic regulatory component for neoplastic transformation remains unknown.

REFERENCES

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