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EXPERIMENTAL NEUROONCOGENESIS USING
RESORPTIVE NITROSOUREAS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By


* * * * *

The Ohio State University
1970

Approved by

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Adviser
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>11</td>
</tr>
<tr>
<td>VITA</td>
<td>iii</td>
</tr>
<tr>
<td>TABLES</td>
<td>v</td>
</tr>
<tr>
<td>ILLUSTRATIONS</td>
<td>vi</td>
</tr>
<tr>
<td>Chapter</td>
<td></td>
</tr>
<tr>
<td>I. INDUCTION OF TUMORS OF THE NERVOUS SYSTEM IN RATS WITH INTRAVENOUS METHYLNICOTROUREA</td>
<td>1</td>
</tr>
<tr>
<td>- Introduction</td>
<td>1</td>
</tr>
<tr>
<td>- Materials and Methods</td>
<td>3</td>
</tr>
<tr>
<td>- Results</td>
<td>7</td>
</tr>
<tr>
<td>- Discussion</td>
<td>49</td>
</tr>
<tr>
<td>- Summary</td>
<td>54</td>
</tr>
<tr>
<td>II. TRANSPLACENTAL PRODUCTION OF NEOPLASMS OF THE NERVOUS SYSTEM IN RATS WITH ETHYLNITROSOUREA</td>
<td>55</td>
</tr>
<tr>
<td>- Introduction</td>
<td>55</td>
</tr>
<tr>
<td>- Materials and Methods</td>
<td>56</td>
</tr>
<tr>
<td>- Results</td>
<td>59</td>
</tr>
<tr>
<td>- Discussion</td>
<td>97</td>
</tr>
<tr>
<td>- Summary</td>
<td>100</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>102</td>
</tr>
</tbody>
</table>
TABLES

Table | Page
--- | ---
1. Survival time and average number of tumors of adult rats treated with MNU. | 7
2. Number, location, and size of 54 tumors in adult rats given intravenous MNU. | 8
3. Incidence and classification of 57 methylnitrosourea-induced tumors of the nervous system. | 9
4. Enzyme histochemical activity of MNU-induced neurogenic tumors. | 48
5. Survival time and average number of tumors in offspring of CD rats treated with 50 mg/kg ENU on the 20th day of gestation. | 59
6. Number, location and size of 109 tumors in offspring of CD rats treated with 50 mg/kg ENU on the 20th day of gestation. | 60
7. Incidence and classification of 102 ENU-induced tumors of the nervous system. | 61
FIGURES

Table of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Gliopendymoma in the left cerebral hemisphere of a male CD rat with a survival time of 257 days.</td>
</tr>
<tr>
<td>2.</td>
<td>Early neoplastic proliferation of periventricular glial cells. Mitosis (arrow), lateral ventricle (V). Hematoxylin and eosin.</td>
</tr>
<tr>
<td>3.</td>
<td>Periphery of an oligodendroglioma with prominent vascular proliferation and scattered astrocytes (arrows). Hematoxylin and eosin.</td>
</tr>
<tr>
<td>4.</td>
<td>Neoplastic oligodendrocytes with round nuclei, patchy chromatino and few cytoplasmic organelles.</td>
</tr>
<tr>
<td>5.</td>
<td>Poorly differentiated astrocyte in an anaplastic glioma. The cytoplasm contains a few glial filaments (f), scattered microtubules (MT) and dense-core vesicles (arrows).</td>
</tr>
<tr>
<td>6.</td>
<td>Ependymomatous portion of a gliopendymoma. Neoplastic cells form rosettes (R) and are in mitosis (arrows). Hematoxylin and eosin.</td>
</tr>
<tr>
<td>7.</td>
<td>Poorly differentiated cell in mitosis in a gliopendymoma. The centriole, spindle (S) and a desmosome (D) are visible in the mitotic cell. An astrocytic process (A) is in the adjacent tissue.</td>
</tr>
<tr>
<td>8.</td>
<td>Neoplastic ependyma (E) line the lateral ventricle (V) and form giant cells (arrows). Hematoxylin and eosin.</td>
</tr>
<tr>
<td>9.</td>
<td>Poorly differentiated ependymal cell with a smooth cytoplasmic membrane and an eccentric nucleus (N). Moderately electron-dense material is present in the extracellular space (E).</td>
</tr>
<tr>
<td>10.</td>
<td>Microvilli-like projections (MV) and terminal bars (T) in an anaplastic ependymoma.</td>
</tr>
<tr>
<td>11.</td>
<td>Neoplastic cells of a gliosarcoma are surrounded by reticulin fibers. Wilder's reticulin stain.</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>12. Sarcoma cells are within the perivascular space. Glial basement membrane (BM).</td>
<td>39</td>
</tr>
<tr>
<td>13. Elongate processes (P) of neoplastic cells of a neurinoma form long interdigitations. Toluidine blue.</td>
<td>41</td>
</tr>
<tr>
<td>14. Neoplastic Schwann cell with a basement membrane (arrow) and cytoplasmic filaments (f). Collagen fibers (C) are closely associated with the tumor cell.</td>
<td>44</td>
</tr>
<tr>
<td>15. Many cell processes (P) interdigitate with those of a neoplastic Schwann cell which is surrounded by a basement membrane (BM).</td>
<td>46</td>
</tr>
<tr>
<td>16. Early neoplastic proliferation of oligodendrocytes in packets and in satellite position around neurons (S). Hematoxylin and eosin.</td>
<td>64</td>
</tr>
<tr>
<td>17. A mixed glioma microtumor is adjacent to the lateral ventricle (V). Hematoxylin and eosin.</td>
<td>67</td>
</tr>
<tr>
<td>18. Neoplastic oligodendrocytes with moderate numbers of organelles and few processes.</td>
<td>70</td>
</tr>
<tr>
<td>19. Many glial filaments (f) comprise a large portion of the cytoplasm of a neoplastic astrocyte.</td>
<td>72</td>
</tr>
<tr>
<td>20. Cerebellar glioependymoma consisting of oligodendroglial (ol) and ependymal (E) regions. The neoplastic ependyma form cords and rosettes (r). Mitosis (arrow). Hematoxylin and eosin.</td>
<td>74</td>
</tr>
<tr>
<td>21. Anaplastic ependymoma of the spinal cord contain cysts (c) and many blood vessels (BV). Hematoxylin and eosin.</td>
<td>77</td>
</tr>
<tr>
<td>22. Cells of an anaplastic ependymoma form long columns and packets. Many fine cell processes extend into the extracellular space (ECS). Toluidine blue.</td>
<td>79</td>
</tr>
<tr>
<td>23. Anaplastic ependymoma cells with large oval nuclei, a thin rim of cytoplasm and many fine processes (P).</td>
<td>81</td>
</tr>
<tr>
<td>24. A centriole (Ce) is present in the cytoplasm of a neoplastic ependymal cell.</td>
<td>83</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>25.</td>
<td>Prominent desmosomes (D) connect several neoplastic ependymal cells forming microcysts (Cy).</td>
</tr>
<tr>
<td>26.</td>
<td>Meningioma composed of small spindle cells wrapped around each other forming whorls. The cytoplasmic border is indistinctly outlined. Hematoxylin and eosin.</td>
</tr>
<tr>
<td>27.</td>
<td>Neoplastic Schwann cells have invaded the trigeminal nerve up to its junction with the brain (arrows). CNS invasion is restricted to perivascular spaces. Hematoxylin and eosin.</td>
</tr>
<tr>
<td>28.</td>
<td>Poorly differentiated Schwann cell has incorporated a non-myelinated axon (A) and has a partial basement membrane (arrow).</td>
</tr>
<tr>
<td>29.</td>
<td>A short segment of basement membrane (arrow) is opposed to a neoplastic Schwann cell.</td>
</tr>
</tbody>
</table>
CHAPTER I

INDUCTION OF TUMORS OF THE NERVOUS SYSTEM

IN RATS WITH INTRAVENOUS METHYLNITROSOUREA

Introduction

The etiology and pathogenesis of tumors of the nervous system of man and animals are unknown. An ideal tumor model for neurooncological research should (a) produce a high incidence of neoplasms in the brain, spinal cord and peripheral nerves, (b) be highly reproducible, (c) induce autochthonous tumors with morphology and predilection sites similar to that of naturally occurring tumors of man and animals, (d) utilize natural pathways for exposure of target organs, and (e) be induced by compounds or agents to which man and animals have potential exposure through environmental contact.

Brain tumors have been produced experimentally by oncogenic viruses (3), chemical carcinogens (27), and ionizing radiation (7). To assure organ specificity, most of these agents had to be applied locally. The resorptive carcinogens with neural specificity introduced by Druckrey and coworkers (8) fulfill the tumor model criteria to a higher degree than previous models. Repeated oral or intravenous administration of methylnitrosourea (MNU) to adult BD IX rats resulted in tumors of the brain, spinal cord and peripheral nervous systems in over 90% of the animals. Tumors of other organs were rare. The neurooncogenic
effect of MNU has not been restricted to one species, as brain tumors have also been induced in rabbits (14) and in a dog (15).

Nitroso compounds may offer a serious carcinogenic hazard to man since they are common environmental pollutants (16) and are utilized in many industries (9). Methylnitrosourea, the carcinogen employed in this study, is itself commonly used by chemists to generate diazo-methane.

The classification of MNU-induced tumors presents some difficulties. The tumors have been described as gliomas, medulloblastomas, gliosarcomas, ependymonas, malignant neurinomas, malignant ganglionicomas, adventitial cell sarcomas, microgliomas and meningiomas (15) (22) (24). Discrepancies in interpretation of tumor cell types have been in part, responsible for the divergence of diagnoses. Only limited work by techniques other than light microscopy has been applied to the MNU model. Georgsson et al. (10) studied six neurinomas by electron microscopy and concluded that the tumor cells were probably of Schwann cell origin. No reports have been published on the ultrastructure of MNU-induced gliomas. Likewise, only a limited number of tumors have been characterized by enzyme histochemistry (17) (20) (21).

The objectives of the present investigation were to produce neuro-ectodermal tumors in Fischer and Sprague-Dawley rats by weekly intravenous administration of methylnitrosourea and to characterize these tumors with light microscopy, electron microscopy and enzyme histochemistry. The two strains of rats were chosen because previous investigators had noted strain differences in susceptibility (15) and
because they represented inbred and random-bred animals commercially available as specific-pathogen-free stock in the United States.

**Materials and Methods**

Eleven (8 male, 3 female) 9-week-old SPF CD (Sprague-Dawley) and seven 9-week-old CDF (Fischer) male rats were injected intravenously with 5 mg/kg of methylnitrosourea for 36 weeks, a total dose of 180 mg/kg. Solutions of MNU were freshly prepared for each administration by dissolving 2.5 mg/ml in sterile saline and adjusting the final pH to 4.5 with ascorbic acid. The crystalline MNU was stored in a desiccator at -20°C when not in use and was periodically examined for stability by calculating the logarithm of the coefficient of extinction (log e) according to the standards reported by Druckrey (9).

Experimental animals were fed autoclaved Purina Lab Chow 5010C and water *ad libitum*. Rats were examined daily and weighed weekly throughout the duration of the experiment. Progressing neurologic signs and weight loss were utilized to select animals for euthanasia. Animals selected for electron microscopy were deeply anesthetized with Metophane (Pitman-Moore, Indianapolis, Ind.) and perfused via the ascending aorta with either 3% glutaraldehyde or cacodylate buffer (pH 7.4) followed by a 4% paraformaldehyde-2.5% glutaraldehyde solution for 10 minutes at 150 mm. Hg. Tissue specimens were diced into one mm. cubes and immersed in the same fixative for one hour. Rats selected for both histochemistry and electron microscopy were deeply anesthetized with Metophane while the tumor was excised. Tissues for electron microscopy were immediately diced in 3% glutaraldehyde or 1% osmic acid.
and fixed for one hour. Samples for enzyme histochemistry were immedi-
ately placed in liquid nitrogen and cold formol-calcium. All
experimental animals were subjected to a complete necropsy. Brains
and spinal cords were fixed in Cajal's brom-formol solution, while non-
neural tissues were fixed in 10% buffered formalin.

Samples for electron microscopy were post-fixed for one hour in
buffered 1% osmic acid, dehydrated and embedded in Maraglas. Sections
1 μ thick stained with toluidine blue were utilized in selecting areas
for electron microscopy. Thin sections were cut with glass knives on
a Porter-Blum ultramicrotome, mounted on copper grids, stained with
uranyl acetate and lead citrate, and examined with a Philips EM-200
electron microscope.

Tissues for enzyme histochemistry that had been frozen in liquid
nitrogen were stored in a -70°C freezer. Formol-calcium fixed tissues
were transferred to gum sucrose medium at 4°C after 18-24 hours of
fixation. Frozen sections, 8 μ thick and mounted on warmed coverslips,
were made from all unfixed and formalin-fixed tissues. Coverslips
coated with gelatin were used for formalin-fixed tissues. Alternate
serial sections were stained with hematoxylin and eosin for orienta-
tion.

The activities of the coenzyme-linked dehydrogenases, glucose-6-
phosphate (G6PDH) and lactate (LDH) were determined on cold acetone-
fixed tissue by a method modified by Hess et al. (13), using nitro BT
as the tetrazolium salt. The respiratory inhibitor was omitted in the
LDH reaction and 0.01 M sodium fluoride was used in G6PDH. The osmo-
ularity of the solutions was maintained at 0.43 M by addition of sucrose
to the incubating mediums. Control tissue sections were incubated without the addition of substrate to the medium.

The diaphorases, TPNH and DPNH, were investigated by the method of Scarpelli et al. (18). Cytochrome oxidase (CO) activity was determined on cold acetone fixed tissue by the method of Burstone (5). Control sections were placed in a substrate mixture containing potassium cyanide which blocked the respiratory pathway. The activity of adenosine triphosphatase (ATPase) was demonstrated by the method of Wachstein et al. (25). Control sections were incubated without substrate.

Alkaline phosphatase (ALP), acid phosphatase (ACP), beta-glucuronidase (BG) and free esterase (EST) were demonstrated in formalin-fixed tissues with simultaneous coupling azo-dye techniques. A modified method of Burstone (4) utilizing a ammediol buffer at pH 9.2 was used to determine ALP activity. Naphthol AS-B1 phosphate (disodium salt) was used as substrate, and fast blue RR as the azo-dye. The method of Barka and Anderson (2) was used to determine the activity of ACP. Naphthol AS-TR phosphoric acid (Na salt) was used as the substrate, and hexazonium pararosanilin as the azo-dye. The method of Hayashi et al. (12) was employed for measuring BG activity and the method of Burstone (6) was utilized for determining free esterase. Control sections were incubated under identical conditions without substrate.

Sections of rat liver or kidney mounted on the same coverslips were incubated in each substrate mixture as positive controls.

Sections were mounted on glass slides with glycerine-jelly and rimmed with clear nail lacquer except BG, ACP and H&E sections which
were dehydrated, cleared and mounted in picolyte.

All lesions, serial blocks of brain, and six selected segments of spinal cord were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Wilder's reticulin, Masson's trichrome, cresyl violet and phosphotungstic acid-hematoxylin were utilized as additional histochemical or staining procedures.
Results

The survival times and average number of tumors per rat are summarized in Table 1. The mean survival time, i.e. the time between the first injection of the carcinogen and death, was 278 days. The shortest survival time was 235 days (after a total dose of 170 mg/kg MNU), while the longest period was 374 days. The mean survival time for Sprague-Dawley (CD) males was significantly shorter (P<0.05) than that of Fischer males (CDF), although the number of tumors per rat was not significantly different.

Table 1. Survival time and average number of tumors of adult rats treated with MNU.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Number of Rats</th>
<th>Survival Time Mean</th>
<th>Survival Time Range</th>
<th>Number of Tumors/Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>M</td>
<td>8</td>
<td>266*</td>
<td>250-314</td>
<td>3.9</td>
</tr>
<tr>
<td>CD</td>
<td>F</td>
<td>3</td>
<td>291</td>
<td>235-374</td>
<td>1.7</td>
</tr>
<tr>
<td>CDF</td>
<td>M</td>
<td>7</td>
<td>286*</td>
<td>254-321</td>
<td>2.6</td>
</tr>
<tr>
<td>TOTALS</td>
<td></td>
<td>18</td>
<td>278</td>
<td>235-374</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* Significant difference (P<0.05) using Student's t-test.

All treated animals developed neoplasms and, except for one which died from a massive squamous cell carcinoma of the salivary duct, all had neoplasms of the nervous system. Because of the multiple occurrence of neurogenic tumors, ranging from 1 to 8 tumors per rat with a
mean of 3.0, a total of 54 neoplasms were encountered. Of these, only
two tumors developed in extra-neural locations. The distribution of
the MNU-induced tumors is shown in Table 2.

Table 2. Number, location and size of 54 tumors in adult rats treated
intravenously with MNU.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sex</th>
<th>Gross</th>
<th>Micro</th>
<th>E.N.P.*</th>
<th>Spinal Cord</th>
<th>Peripheral Nerves</th>
<th>Other</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD</td>
<td>M</td>
<td>11</td>
<td>6</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>CD</td>
<td>F</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>CDF</td>
<td>M</td>
<td>5</td>
<td>6</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>20</td>
<td>12</td>
<td>11</td>
<td>2</td>
<td>7</td>
<td>2</td>
<td>54</td>
</tr>
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</table>

* Early neoplastic proliferation.

The incidence of neurogenic tumors and a descriptive classification
based on the predominant cell type(s) are given in Table 3.
Tumors described as "anaplastic" had undergone marked cellular de-
differentiation.
Table 3. Incidence and classification of 52 methylnitrosourea-induced tumors of the nervous system.

<table>
<thead>
<tr>
<th>I. Tumors of the Central Nervous System</th>
<th>45</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Early neoplastic proliferation</td>
<td>(11)</td>
</tr>
<tr>
<td>B. Oligodendroglioma</td>
<td>(8)</td>
</tr>
<tr>
<td>C. Mixed glioma</td>
<td>(9)</td>
</tr>
<tr>
<td>D. Anaplastic glioma</td>
<td>(9)</td>
</tr>
<tr>
<td>E. Anaplastic glioblastoma</td>
<td>(3)</td>
</tr>
<tr>
<td>F. Anaplastic ependymoma</td>
<td>(2)</td>
</tr>
<tr>
<td>G. Gliosarcoma</td>
<td>(3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Tumors of the Peripheral Nervous System</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Neurinoma</td>
<td>(3)</td>
</tr>
<tr>
<td>B. Anaplastic neurinoma</td>
<td>(4)</td>
</tr>
</tbody>
</table>

One of the extra-neural tumors was an incidental finding at the time of necropsy. It consisted of a poorly differentiated sarcoma involving one-third of the left kidney. The other extra-neural tumor was a large squamous cell carcinoma of the salivary duct which developed over a period of 6 weeks.
I. Tumors of the Central Nervous System.

A definite predilection for tumor development in periventricular regions of the lateral ventricles and subcortical white matter was recognized. Most grossly detectable tumors extended to the brain's surface (Fig. 1) and many caused displacement of midline structures. Tumors were usually distinguishable from the adjacent neuropil which often had mild to moderate edema. Neoplasms were soft and usually cystic and hemorrhagic. In addition to grossly visible neoplasms (macrotumors), microtumors and areas of focal glial proliferation were present in brains of treated rats. Since proliferative foci and neoplasms occurred at the same predilection sites and the proliferating cells were not reactive or inflammatory, they were considered early neoplastic lesions. There was a gradual transition from focal glial proliferation to microtumors. Microtumors had fully developed characteristics of neoplasms, such as invasion, displacement, loss of differentiation, etc. Foci of hyperplastic oligodendrocytes represented the most common form of glial proliferation (Fig. 2), although astrocytes were also involved.

A. Oligodendrogliomas

Seven of the eight tumors classified as oligodendrogliomas were microtumors. Histologically they were composed of aggregates of isomorphic oligodendroglia which had proliferated from their intrafascicular or satellite position. Microtumors had little vascular proliferation and caused minimal compression. The isomorphism at the cellular and tissue level, lack of necrosis, and minimal peritumoral edema did not allow tissue selection for ultrastructural investigation of the
Figure 1. Glioblastoma in the left cerebral hemisphere of a male CD rat with a survival time of 257 days. (Scale in mm.).
Figure 2. Early neoplastic proliferation of periventricular glial cells. Mitosis (arrow), lateral ventricle (V). Hematoxylin and eosin. (580 X).
oligodendroglioma microtumors.

The remaining oligodendroglioma was a large, cystic, gray, subependymal tumor. It consisted of neoplastic cells resembling isomorphic oligodendrocytes with scattered astrocytes and was associated with prominent vascular proliferation (Fig. 3). Few mitoses were present. Ultrastructurally the tumor consisted of small to medium cells with electron-lucent cytoplasm, short processes and rounded nuclei (Fig. 4). Most of the neoplastic cells contained little endoplasmic reticulum, a poorly developed Golgi apparatus, moderate numbers of mitochondria and scattered ribosomes. Glial filaments and microtubules were rare.

B. Mixed gliomas.

The majority of mixed gliomas were microtumors. They consisted of nearly equal numbers of tumor cells resembling well-differentiated astrocytes and oligodendrocytes. In larger mixed gliomas, neoplastic oligodendrocytes dominated peripheral portions of the tumor, while the larger astrocytes were more common in the center. In mixed gliomas, minimal necrosis was evident and vascular proliferation was mild to moderate. Numerous organelles, including microtubules, large Golgi apparatuses, many vesicles and ribosomes, and variable amounts of glial filaments were demonstrated in most tumor cells. They often had long processes and eccentric, oval nuclei with single nucleoli.

C. Anaplastic gliomas

Small, dark, hyperchromatic cells interpreted as polymorphous oligodendrocytes predominated in peripheral portions of these tumors, while larger more pleomorphic cells populated more central regions of
Figure 3. Periphery of an oligodendroglioma with prominent vascular proliferation and scattered astrocytes (arrows). Hematoxylin and eosin. (560 X).
Figure 4. Neoplastic oligodendrocytes with round nuclei, patchy chromatin and few cytoplasmic organelles. (11,200 X).
the tumors. Necrosis, mitoses and vascular proliferation were common. Foci of adventitial cell hyperplasia accompanied vascular proliferation in some tumors. Increased amounts of reticulin were present in these areas, but did not extend into the tumors.

The neoplastic cells resembling oligodendrocytes contained rounded nuclei with a thin rim of condensed chromatin. Their cytoplasm was moderately dense, contained many rosettes of ribosomes and moderate numbers of mitochondria, microtubules and profiles of rough endoplasmic reticulum. More pleomorphic cells were usually larger and contained an irregular or oval nucleus in an eccentric position. The cytoplasm contained numerous organelles including glial filaments and dense-core vesicles (Fig. 5). Most tumor cells had one large process, although smaller processes were present. Giant cells containing cytoplasm rich in organelles were also scattered through the tumors.

D. Anaplastic glioependymoma.

The descriptive term, glioependymoma, has been applied to three pleomorphic cerebral tumors with ependymomatous and gliomatous areas. All of these tumors were located near lateral ventricles and two contained prominent areas of necrosis. In ependymomatous areas, tumor cells were lined up in columns and rarely in rosettes (Fig. 6). Intercellular spaces contained homogeneous to slightly fibrillar material in hematoxylin and eosin-stained sections. Gliomatous portions were pleomorphic, containing astrocytic elements and peripheral cords of polymorphous oligodendrocytes. The mitotic index was high, while vascular proliferation was moderate.
Figure 5. Poorly differentiated astrocyte in an anaplastic glioma. The cytoplasm contains a few glial filaments (f), scattered microtubules (MT) and dense-core vesicles (arrows). (29,500 X).
Figure 6. Ependymomatous portion of a glioependymoma. Neoplastic cells form rosettes (R) and are in mitosis (arrows). Hematoxylin and eosin. (560 X).
Ultrastructurally, most cells of glioependymomas appeared poorly differentiated. They contained a variably shaped nucleus which had at least one nucleolus, many vesicles, ribosomes, microtubules and mitochondria, moderate endoplasmic reticulum and variable numbers of lysosomes. In addition, dense-core vesicles measuring 60 to 140 mm were present in many cells. A few small processes containing microtubules usually extended into the extracellular space. Several undifferentiated cells were undergoing mitosis (Fig. 7). More differentiated cells were occasionally identified. Astrocytic elements contained a larger nucleus and scattered glial filaments, while the ependymal elements were polygonal with rounded nuclei, had few organelles, but many ribosomes. Cilia and desmosomes were occasionally demonstrated.

E. Anaplastic Ependymoma.

Two of the anaplastic tumors obliterating the hippocampus and periventricular tissues of two rats appeared to arise from ependymal lining and were considered to be ependymomas with a high degree of malignancy. Areas of normal ependyma bordered hyperplastic regions which were adjacent to neoplastic zones containing giant ependymal cells with frequent mitoses.

Numerous giant cells, both single and multinucleated were present throughout the tumor (Fig. 8). The large, round, strongly eosinophilic tumor cells invaded along blood vessels and formed pseudorosettes. Moderate amounts of necrosis were present in the tumor and adjacent tissues were severely edematous. Abnormal mitoses were common. When examined with the electron microscope, the tumor
Figure 7. Poorly differentiated cell in mitosis in a glioependymoma. The centriole, spindle (S) and a desmosome (D) are visible in the mitotic cell. An astrocytic process (A) is in the adjacent tissue. (11,300 X).
Figure 8. Neoplastic ependyma (E) line the lateral ventricle (V) and form giant cells (arrows). Hematoxylin and eosin. (580 x).
cells were smooth in outline (Fig. 9), had moderately electron-dense cytoplasm, abundant organelles and occasionally were connected by desmosomes and formed microvilli (Fig. 10). No cilia or collagen were present. Moderately electron-dense extracellular edema surrounded most tumor cells. Many of the blood vessels within the tumor were devoid of astrocytic end-feet and had endothelial cell separation.

F. Gliosarcoma.

No sharp distinction could be made between anaplastic gliomas with adventitial cell hyperplasia around tumor vessels and early gliosarcomas with neoplastic adventitial cells. In the gliosarcoma, the adventitial cells were more proliferative and produced greater amounts of reticulin. Cells in the larger more diffuse gliosarcomas were individually surrounded by fine reticulin fibers (Fig. 11). Coarser fibers traversed greater areas of the tumor. The reticulin-producing cells were extremely pleomorphic, varying from round to spindle-shaped cells. Their cytoplasm stained lightly eosinophilic to slightly basophilic. Peripheral portions of these tumors were diverse in cytodifferentiation. One tumor appeared to have extensive microglial infiltration around vessels and neurons. Others were predominantly gliomatous, with sarcomatous elements invading perivascular spaces and the pia mater.

Electron microscopy did not reveal collagen in the gliosarcomas. Sarcomatous elements had electron-lucent to moderately dense cytoplasm containing moderate amounts of rough endoplasmic reticulum, lysosomes, lipid bodies, and mitochondria. Their nuclei were usually oval and contained a prominent nucleolus. Neoplastic cells invading the peri-
Figure 9. Poorly differentiated ependymal cell with a smooth cytoplasmic membrane and an eccentric nucleus (N). Moderately electron-dense material is present in the extracellular space (E). (12,500 X).
Fig. 9
Figure 10. Microvilli-like projections (MV) and terminal bars (T) in an anaplastic ependymoma. (44,000 X).
Figure 11. Neoplastic cells of a gliosarcoma are surrounded by reticulin fibers. Wilder's reticulin stain (590 X).
vascular space were more pleomorphic and had numerous fine processes (Fig. 12). Many of the capillaries had at least a partial covering of astrocytic foot processes. Gliomatous elements were similar to those of anaplastic gliomas.

II. Tumors of the Peripheral Nervous System.

In contradistinction to the tumors of the central nervous system, only single peripheral nerve tumors were present in MNU treated rats. Four of the animals having peripheral nerve tumors also had grossly visible tumors of the CNS. The three remaining animals had additional microtumors of the CNS. The locations of peripheral nerve tumors included one in a trigeminal nerve and one in a cervical spinal root, two in thoracic spinal roots, and three in lumbar spinal roots.

A. Neurinomas.

More differentiated tumors of peripheral nerves were usually well demarcated, firm and light gray or tan. Microscopically, the tumors consisted of slender to plump, spindle-shaped cells with oval nuclei. The slender cells had long interwoven processes (Fig. 13) and formed patterns resembling the type A pattern present in human neurinomas. Reticulin fibers were intermingled between most cells. The amount of collagen produced by these tumors varied from very little to abundant. Mitoses were infrequent and tissue invasion was minimal.

A basement membrane could be demonstrated around most of the neoplastic cells. The amount of collagen corresponded closely to that demonstrated by light microscopy. When present, collagen fibers were intimately associated with tumor cells having a complete basement
Figure 12. Sarcoma cells are within the perivascular space. Glial basement membrane (BM). (10,200 X).
Fig. 12
Figure 13. Elongate processes (P) of neoplastic cells of a neurinoma form long interdigitations. Toluidine blue. (2,340 X).
membrane (Fig. 1). These cells contained oval nuclei, which had an irregular nuclear membrane, pale cytoplasm containing many fine fibrils, an active Golgi apparatus, numerous vesicles, varying amounts of rough endoplasmic reticulum, and moderate numbers of mitochondria and lysosomes. Concentric whorls of endoplasmic reticulum were present in several cells. Non-myelinated axons were frequently found within more differentiated cells.

B. Anaplastic Neurinomas

The more malignant peripheral nerve tumors were usually soft and more invasive. Histologically, they formed a heterogeneous group of tumors. One was composed primarily of large, plump, spindle-shaped cells with a high mitotic index. These cells produced little collagen and moderate amounts of reticulin. Two of the tumors were composed of small cells with hyperchromatic oval nuclei and indistinct processes. These cells were surrounded by fine reticulin fibers. The last tumor was very invasive, composed of long spindle-shaped cells, and had areas resembling both type A and type B patterns recognized in human neurinomas. Its tumor cells produced prominent amounts of reticulin, but little collagen.

Heterogeneity was also evident when these tumors were studied by electron microscopy. Small cells were often devoid of basement membrane, while larger cells and more differentiated cells had partial to complete basement membranes. Little collagen was visualized. Tumor cell processes formed complex interdigitations similar to those characteristic for human Schwann cells (Fig. 15). The number of organelles in neoplastic cells varied from a few in spindle cells to
Figure 14. Neoplastic Schwann cell with a basement membrane (arrow) and cytoplasmic filaments (f). Collagen fibers (C) are closely associated with the tumor cell. (20,800 X).
Figure 15. Many cell processes (P) interdigitate with those of a neoplastic Schwann cell which is surrounded by a basement membrane (BM). (18,700 X).
many in larger cells.

**Enzyme Histochemistry**

The enzyme activity of four tumors (2 anaplastic ependymomas, a neurinoma and an anaplastic neurinoma) was studied histochemically. The results are shown in Table 4.

Of the hydrolytic enzymes examined, acid phosphatase activity was consistently increased in all tumors. Neoplastic cells contained moderate amounts of particulate as well as diffuse ACP activity, while necrobiotic cells and macrophages had strong particulate activity. Beta-glucuronidase activity was less than that of ACP, but was increased over that of control tissues.

Cytochrome oxidase activity was greatly decreased in all tumors, when compared with normal brain. Neoplastic cells and blood vessels had moderate ATPase activity whereas it was primarily confined to blood vessels in control sections.

Alkaline phosphatase activity was confined to blood vessels in tumors and control tissues. The overall activities of TPNH, DPNH, LDH and G6PDH was similar to control materials, however, in control sections the activity was uniformly distributed while in tumors it was concentrated in scattered cells. The small cells of both neurinomas had much less activity than did larger cells. Non-specific esterase had slightly higher activity in neoplasms.
Table 4. Enzyme histochemical activity of MNU-induced neurogenic tumors.

<table>
<thead>
<tr>
<th></th>
<th>BG</th>
<th>ACP</th>
<th>ALP</th>
<th>EST</th>
<th>CO</th>
<th>ATP</th>
<th>TPNH</th>
<th>DPNH</th>
<th>G6PDH</th>
<th>LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaplastic Ependymoma</td>
<td>±</td>
<td>+</td>
<td>+++</td>
<td>±</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Neurinoma</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td>Anaplastic Neurinoma</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
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<td>++</td>
<td>+++</td>
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</tr>
<tr>
<td>Normal Brain</td>
<td>±</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Normal Gasserian Ganglion</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>
Discussion

A high incidence of neural tumors (94%) was produced in adult rats by multiple intravenous injections of methylnitrosourea. Utilization of the intravenous route decreased the number of non-neural neoplasms and increased the number of brain tumors when compared to previous experiments using oral (25) or intraperitoneal (26) inoculations. Terminal neurologic signs allowed animal selection and prediction of tumor site so that a variety of techniques, including electron microscopy and enzyme histochemistry, could be used to characterize the neoplasms. The selection of specific-pathogen-free rats for experimental tumor production eliminated animal losses due to inherent disease, allowing optimal evaluation of the experiment.

In this experiment, 10 out of 11 Sprague-Dawley and all 7 male Fisher rats developed tumors of the nervous systems. A squamous cell carcinoma of the salivary duct resulted in an early death of the one rat without a neural neoplasm. Since the number of females in the present study was low, specific statements on sex incidence cannot be made. It is of interest, however, that in a second group of rats composed of 11 Fischer females, only one has developed a brain tumor after 310 days (21). The apparent predilection for males corresponds to a higher incidence of neuroectodermal tumors in human males than females. Previous investigators have not mentioned sex differences, however, definite strain differences have been noted in rats treated with MNU. Tumor incidence in hooded rats given 10 mg/kg every two weeks to a total dose of 180 mg/kg was 72%, while Wistar rats, treated identically, developed neural tumors in only 32.7% (15). Both of
the strains employed in this study appear to be highly susceptible to MNU-induced neoplasms.

Focal areas of early neoplastic proliferation were frequent in brains of rats treated with MNU. Their location in areas associated with high tumor incidence and the apparent transition from focal glial hyperplasia to microtumor suggests that these areas represent preneoplastic or early neoplastic stages of MNU-induced brain tumors. Similar interpretations were made by other investigators (15,22).

By combining light and electron microscopy, several problems in classification and cell origin have been partially resolved. When tumors classified as oligodendrogliomas and mixed gliomas by light microscopy were studied at the ultrastructural level, characteristic oligodendrocytes and protoplasmic astrocytes were found to comprise the majority of tumor cells. Minimal regressive changes were present in the tumors and normal relationships between astrocytes and blood vessels were maintained.

Although neoplastic cells comprising the anaplastic ependymomas were demonstrated arising from the normal ependymal lining, characteristic features of ependyma were difficult to demonstrate. Electron microscopy supported the diagnosis of ependymoma demonstrating characteristics of ependyma cells such as smooth cytoplasmic membranes, lack of glial filaments, and the occasional formation of desmosomes and microvilli.

Tumors classified histologically as glioependymomas contained ependymomatous and gliomatous regions. Characteristics of both glia and ependyma were demonstrated ultrastructurally in neoplastic cells.
The majority of cells, however, appeared poorly differentiated. The significance of the dense-core vesicles present in gliopendymomas and gliomas is unknown.

The morphology of the anaplastic gliomas was heterogeneous. Peripheral cells resembled oligodendroglia at both the light and electron microscopic level while the more centrally located cells resembled protoplasmic astrocytes of a low degree of differentiation. The latter cells had abundant cytoplasm, many organelles, few processes and minimal numbers of glial filaments.

Tumors classified as gliosarcomas contained abundant reticulin, as demonstrated by silver impregnation, but lacked collagen fibrils at the ultrastructural level. Sarcoma cells were poorly differentiated, contained variable numbers of organelles, no glial filaments and few microtubules. Gliomatous areas were similar to less differentiated gliomas.

Peripheral nerve tumors appeared to arise from Schwann cells. A high proportion of cells examined with the electron microscope had a basement membrane. Only the small anaplastic cells of the poorly differentiated tumors lacked a basement membrane but some of these were observed incorporating unmyelinated axons. Tumor cell processes frequently interdigitated with each other in a similar manner to that recognized in human neurinomas. In general, however, the MNU-induced neurinomas were more malignant than their human counterpart. Conversely, they were considerably better differentiated than neurinomas induced transplacentally by ethylnitrosourea (28).
Several tumor types described by others using the MNU model were not represented in our study (15) (22). These included medulloblastomas, gangliocytomas, meningiomas, and primary sarcomas of the brain. The latter comprised over one-third of the MNU-induced tumors reported by Jänisch and Schreiber (15), who utilized a variety of routes, doses, and intervals of administration. Tumors classified here as anaplastic gliomas include the anaplastic oligodendrogliomas described by Jänisch and Schreiber (15) and Stroobandt and Brucher (22). The anaplastic ependymomas containing giant cells shared characteristics with MNU-induced tumors previously described as glioblastoma multiform (15).

Enzyme histochemistry revealed a common distribution of enzyme activity in most tumors. Acid phosphatase activity was increased in all tumors and represented the most reliable indicator of increased activity of hydrolytic enzymes. Biochemical enzyme determinations of samples from the same tumors revealed greatly increased ACP and BG activities (1). Similar increases in hydrolytic enzyme activity have been demonstrated histochemically in CNS tumors in man (11) (19).

Stavrou (20) (21) studied the activity of several of the same enzymes histochemically in MNU-induced brain tumors in rabbits and found comparable activities to that demonstrated in this experiment. Cytochrome oxidase activity was markedly decreased in all neoplasms. The activity of the remaining enzymes was not significantly different between neoplastic and non-neoplastic neuroectodermal cells. The enzyme histochemical findings in MNU-induced neurogenic tumors correspond to those reported for naturally occurring tumors of the
nervous system in man.
Summary

Repeated intravenous administration of methyl nitrosourea (5 mg/kg/week to a total dose of 180 mg/kg) resulted in the production of 54 neoplasms in 18 rats. The survival time ranged from 235 to 374 days following the initial injection of the compound. It was significantly shorter in males of the Sprague-Dawley strain than in those of the Fisher strain.

All but two of the tumors occurred in the nervous system, with the majority arising in the neuropil adjacent to the lateral ventricles or in the subcortical white matter. Tumors were classified on the basis of light and electron microscopy into the following categories: oligodendrogliomas, mixed gliomas, anaplastic gliomas, glioependymomas and ependymomas, gliosarcomas, and differentiated and anaplastic neurinomas.

Enzyme histochemistry of selected neoplasms revealed similar profiles of activity for all tumors. When compared with non-neoplastic brain the activity of hydrolytic enzymes was markedly increased and the activity of oxidative enzymes was decreased. These findings correspond to those reported for naturally occurring tumors of the nervous system in man.
Chapter II

TRANSPLENTAL PRODUCTION OF NEOPLASMS OF THE NERVOUS SYSTEM IN RATS WITH ETHYLNITROSOUREA

Introduction

Of the newly synthesized N-nitroso compounds with neurooncogenic activity, ethylnitrosourea (ENU) has been shown to be specifically suited for the transplacental induction of neoplasms of the nervous system since small doses of the carcinogen cause a high incidence of neural tumors in offspring, yet produce no tumors in the mother (8). Ivankovic and Druckrey (12), using BD IX rats, demonstrated that transplacental tumor production was not possible before the 12th day of gestation and that the susceptibility for neurogenic tumor induction increased with advancing pregnancy. The increased susceptibility culminated toward the termination of the gestation period and decreased after birth.

Previous experiments have concentrated on tumor induction on the 15th day of gestation in BD IX rats (13) (19). The objectives of this study were (a) to test the neurooncogenic effect of 50 mg/kg of ENU on offspring of Sprague-Dawley rats intravenously inoculated on the 20th day of pregnancy, (b) to determine the incidence and distribution of these neoplasms, and (c) to characterize the tumors by light microscopy, electron microscopy and enzyme histochemistry. The resulting tumors were compared with tumors induced during less mature stages of development.
Materials and Methods

Three specific-pathogen-free, pregnant female CD (Sprague Dawley) rats were inoculated with 50 mg/kg of ENU via the lateral tail vein on the 20th day of gestation. The ENU was freshly prepared by dissolving 10 mg/ml in sterile saline and adjusting the pH to 4.5 with crystalline ascorbic acid. A total of thirteen male and 12 female offspring were whelped by the inoculated mothers. The offspring were weaned at 28 days of age and housed in individual cages. All animals were fed autoclaved Purina Lab Chow 5010C and water ad libitum. Rats were examined daily and weighed weekly throughout the experimental period. Progressive neurologic signs and weight loss were utilized in selecting animals for euthanasia.

Animals selected for electron microscopy were deeply anesthetized with Metophane (Pitman-Moore, Indianapolis, Ind.) and perfused via the ascending aorta with either 3% glutaraldehyde or cacodylate buffer (pH 7.4) followed by a 4% paraformaldehyde-2.5% glutaraldehyde solution for ten minutes at 150 mm Hg. Tissue specimens were diced into one mm. cubes and immersed in the same fixative for one hour. Rats selected for both histochemistry and electron microscopy were deeply anesthetized with Metophane while the tumor was excised. Tissues for electron microscopy were immediately diced in 3% glutaraldehyde or 1% osmic acid and fixed for one hour. Samples for enzyme histochemistry were immediately placed in liquid nitrogen and cold formol-calcium. All experimental animals were subjected to a complete necropsy. Brains and spinal cords were fixed in Cajal's brom-formol solution, while non-neural tissues were fixed in 10% buffered formalin.
Samples for electron microscopy were post-fixed for one hour in buffered 1% osmic acid, dehydrated and embedded in Maraglas. Sections 1 μ thick stained with toluidine blue were utilized in selecting areas for electron microscopy. Thin sections were cut with glass knives on a Porter-Blum ultramicrotome, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips EM-200 electron microscope.

Tissues for enzyme histochemistry that had been frozen in liquid nitrogen were stored in a -70°C freezer. Formol-calcium fixed tissues were transferred to gum sucrose medium at 4°C after 18-24 hours of fixation. Frozen 8 μ sections, mounted on warmed coverslips, were made from all unfixed and formalin-fixed tissues. Coverslips coated with gelatin were used for formalin-fixed tissues. Alternate serial sections were stained with hematoxylin and eosin for orientation.

The activities of the coenzyme-linked dehydrogenases, glucose-6-phosphate (G6PDH) and lactate (LDH) were determined on cold acetone-fixed tissue by a method modified by Hess et al. (11), using nitro BT as the tetrazolium salt. The respiratory inhibitor was omitted in the LDH reaction and 0.01 M sodium fluoride was used in G6PDH. The osmolarity of the solutions was maintained at 0.44 M by addition of sucrose to the incubating medium. Control tissue sections were incubated without the addition of substrate to the medium.

The diaphorases, TPNH and DPNH, were investigated by the method of Scarpelli et al. (14). Cytochrome oxidase (CO) activity was determined on cold acetone fixed tissue by the method of Burstone (6). Control sections were placed in a substrate mixture containing
potassium cyanide which blocked the respiratory pathway. The activity of adenosine triphosphatase (ATPase) was demonstrated by the method of Wachstein et al. (18). Control sections were incubated without substrate.

Alkaline phosphatase (ALP), acid phosphatase (ACP), beta-glucuronidase (BG) and free esterase (EST) were demonstrated in formalin-fixed tissues with simultaneous coupling azo-dye techniques. A modified method of Burstone (5) utilizing a ammediol buffer at pH 9.2 was used to determine ALP activity. Naphthol AS-B1 phosphate (disodium salt) was used as substrate, and fast blue RR as the azo-dye. The method of Barka and Anderson (4) was used to determine the activity of ACP. Naphthol AS-TR phosphoric acid (Na salt) was used as the substrate, and hexazonium pararosanilin as the azo-dye. The method of Hayashi et al. (10) was employed for measuring BG activity and the method of Burstone (7) was utilized for determining free esterase. Control sections were incubated under identical conditions without substrate.

Sections of rat liver or kidney mounted on the same coverslips were incubated in each substrate mixture as positive controls.

Sections were mounted on glass slides with glycerine-jelly and rimmed with clear nail lacquer except BG, ACP and H&E sections which were dehydrated, cleared and mounted in picolyte.

All lesions, serial blocks of brain, and six selected segments of spinal cord were embedded in paraffin, sectioned and stained with hematoxylin and eosin. Wilder's reticulin, Masson's trichrome, cresyl violet and phosphotungstic acid-hematoxylin were utilized as additional histochemical or staining procedures.
Results

Incidence, Survival Time and Distribution

All 25 offspring of the three Sprague-Dawley rats inoculated intravenously with 50 mg/kg of ENU on the 20th day of gestation developed tumors of the nervous system. The survival time and average number of tumors per rat are given in Table 5. Survival times ranged from 85-346 days, with a mean of 211 days. The number of tumors per animal varied from one to nine, but averaged 4.36 tumors/rat.

Table 5. Survival time and average number of tumors in offspring of CD rats treated with 50 mg/kg ENU on the 20th day of gestation.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Number of Animals</th>
<th>Survival Time in Days</th>
<th>Number of Tumors/Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>13</td>
<td>201</td>
<td>4.69</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>222</td>
<td>4.00</td>
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<tr>
<td>Total</td>
<td>25</td>
<td>211</td>
<td>4.36</td>
</tr>
</tbody>
</table>

The number, location and size of neoplasms are given in Table 6. A total of 102 neural tumors and 7 non-neural tumors were produced in the 25 animals exposed transplacentally to the carcinogen. More than half of the tumors occurred in the brain, although only 25% of these were grossly visible. Two-thirds of the spinal cord and peripheral nerve tumors were detected grossly.
Table 6. Number, location, and size of tumors in offspring of male (M) and female (F) CD rats treated with 50 mg/kg ENU on the 20th day of gestation.

<table>
<thead>
<tr>
<th>Location</th>
<th>Gross Tumors</th>
<th>Micro-Tumors</th>
<th>Early Neoplastic Proliferation</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>Total</td>
<td>M</td>
</tr>
<tr>
<td>Brain</td>
<td>6</td>
<td>8</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Spinal Cord</td>
<td>7</td>
<td>5</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Peripheral N.S.</td>
<td>14</td>
<td>5</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>Other</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Total Number</td>
<td>28</td>
<td>23</td>
<td>51</td>
<td>30</td>
</tr>
<tr>
<td>%</td>
<td>26</td>
<td>21</td>
<td>47</td>
<td>27</td>
</tr>
</tbody>
</table>
Classification

The classification of neural tumors induced by ENU is given in Table 7. Oligodendrogliomas were the most common tumor, followed by mixed gliomas, anaplastic neurinomas and ependymomas. Each of the remaining tumor types represented less than 5% of the neural tumors.

Table 7. Incidence and Classification of 102 ENU-induced Tumors of the Nervous System.

<table>
<thead>
<tr>
<th>I. Tumors of the Central Nervous System</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Early neoplastic proliferation</td>
<td>4</td>
</tr>
<tr>
<td>B. Astrocytoma</td>
<td>4</td>
</tr>
<tr>
<td>C. Oligodendroglioma</td>
<td>31</td>
</tr>
<tr>
<td>D. Mixed glioma</td>
<td>16</td>
</tr>
<tr>
<td>E. Anaplastic glioma</td>
<td>5</td>
</tr>
<tr>
<td>F. Anaplastic glioependymoma</td>
<td>4</td>
</tr>
<tr>
<td>G. Anaplastic ependymoma</td>
<td>10</td>
</tr>
<tr>
<td>H. Meningioma</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. Tumors of the Peripheral Nervous System</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Neurinoma</td>
<td>3</td>
</tr>
<tr>
<td>B. Anaplastic Neurinoma</td>
<td>24</td>
</tr>
</tbody>
</table>
Non-neural tumors comprised 6.4% of the total. The heterogeneous group included one each of the following: myelogenous leukemia, ductular adenocarcinoma of the mammary gland with metastasis to the lung, mesothelioma, renal fibrosarcoma, arrhenoblastoma, and a papilloma of the cardiac portion of the stomach.

1. Tumors of the Central Nervous System (CNS).

Rats with brain tumors were usually acutely depressed in the terminal stage of the disease. The affected animals became comatose over a period of hours, with death following shortly thereafter. Definite predilection sites for tumors were present in brains of rats treated with ENU. The hippocampus and adjoining periventricular tissues, subcortical white matter, basal ganglia, cerebral cortex, and rarely cerebellum and medulla were involved with decreasing frequency. Three-fourths of the brain tumors were microscopic in size, while the remaining tumors varied from 3 to 13 mm in diameter. Large tumors extended to the surface and caused displacement of midline structures. Multiple tumors were present in ENU-treated rats more often than single neoplasms.

Clinical signs of spinal cord involvement usually developed over a period of days to weeks. Most commonly, a unilateral posterior paresis was followed by bilateral paralysis with urine retention. Tumors occurred in most segments of the cord, but were located in the lower thoracic and lumbar regions most frequently.

**Oligodendroglioma**

Oligodendrogliomas were the most common tumors induced by ENU. The majority of these were microscopic and located periventricularly. There was a transition from focal proliferation of oligodendrocytes, in
Figure 16. Early neoplastic proliferation of oligodendrocytes in packets and in satellite position around neurons (S). Hematoxylin and eosin. (560 X).
satellite position and packets (Fig. 16), to large, grossly visible neoplasms. Neoplastic cells were small to medium in size, had infrequent mitoses, and had the highest degree of differentiation at the periphery of the tumor. Extension to the meninges was present in two cases. Larger tumors frequently contained small aggregates of proliferating astrocytes and had moderate degrees of vascular proliferation.

In the spinal cord, most gliomas were located in the white matter and consisted of well differentiated oligodendrogliomas. Morphologically they resembled their counterpart of the brain.

**Mixed Glioma**

Mixed gliomas consisting of neoplastic oligodendrocytes and astrocytes without a predominant cell type, were the second most common neoplasm of the CNS. They varied in size from microtumors to grossly detectable tumors, with microtumors being most common (Fig. 17). Cerebral cortex and hippocampus were most frequently affected.

**Astrocytoma**

Tumors consisting primarily of astrocytes occurred much less frequently than oligodendrogliomas and mixed gliomas. Isomorphic populations of medium sized cells with round to oval nuclei and moderately abundant cytoplasm comprised the predominant cell type. Mild to moderate degrees of vascular proliferation occurred in the neoplasms.

**Anaplastic Glioma**

Tumors with prominent necrosis and cyst formation, a high mitotic index and cellular pleomorphism varying from poorly differentiated astrocytes to polymorphous oligodendrocytes were classified as anaplastic gliomas. These neoplasms were usually associated with hemorrhage and
Figure 17. A mixed glioma microtumor is adjacent to the lateral ventricle (V). Hematoxylin and eosin. (225 X).
peritumoral edema. Diffuse meningeal invasion, from the subdural space of the forebrain to the cauda equina, occurred in one rat.

Using electron microscopy, the two ectodermal glial cell types, oligodendrocytes and astrocytes, were detected in varying proportions and varying degrees of differentiation. Neoplastic oligodendrocytes were characterized by a round nucleus with patchy chromatin distribution and a rim of cytoplasm with distinct demarcation. They were moderately equipped with cytoplasmic organelles and had occasional short processes (Fig. 18). In less differentiated cells the nucleus was proportionately larger, the cytoplasmic rim narrower and almost devoid of cytoplasmic organelles.

The neoplastic astrocyte resembled the normal astrocyte in its main features (large cell, pleomorphic nucleus, many cytoplasmic processes) except glial fibrils were not as common (Fig. 19).

**Anaplastic Glioependymoma**

Two tumors of the brain and two of the spinal cord contained definite areas of ependymoma and glioma. The ependymomatous areas were characterized by columns and small groups of cells which occasionally formed rosettes (Fig. 20). One tumor from each location contained areas of well-differentiated neoplastic oligodendrocytes, while the remaining tumors contained pleomorphic glial cells. The mitotic index was moderately high in all tumors, but regressive changes were only evident in the neoplasms with pleomorphic glial cells.

**Anaplastic Ependymoma**

Anaplastic ependymomas were the most common tumor of the spinal cord, but were rare in the brain. A large cerebellar tumor which
Figure 18. Neoplastic oligodendrocytes with moderate numbers of organelles and few processes. (19,500 X).
Figure 19. Many glial filaments (f) comprise a large portion of the cytoplasm of a neoplastic astrocyte. (10,300 X).
Figure 20. Cerebellar gliopendymoma consisting of oligodendroglial (ol) and ependymal (E) regions. The neoplastic ependyma form cords and rosettes (r). Mitosis (arrow). Hematoxylin and eosin. (580 X).
extended along the aqueduct was characterized by medium-sized hyperchromatic cells which formed columns, palisaded and were associated with extracellular ground substance. The tumor contained extensive necrosis and a moderate degree of vascular proliferation. In contradistinction to the ependymoma of the brain, no direct connection between tumors and the ependymal lining of the central canal was demonstrated in ependymomas of the spinal cord. Most ependymomas involved the white matter, with compression of adjacent grey matter (Fig. 21). Tumor cells were characterized by an oval nucleus, a rim of deeply eosinophilic cytoplasm and numerous fine processes. They formed small aggregates or columns and were surrounded by eosinophilic extracellular ground substance (Fig. 22). Large cysts containing similar ground substance were present in many of the tumors. Extensive vascular proliferation, forming glomerulus-like structures, surrounded most ependymomas. Neoplastic cells occasionally spread along intrathecal routes.

The ependymoma cells were characterized by a large excentrically located nucleus of moderate density and with evenly distributed chromatin when studied ultrastructurally (Fig. 23). Usually one prominent nucleolus was present. Only a small rim of cytoplasm was demonstrated at the nuclear pole of the cell. The cytoplasm was rich in rough endoplasmic reticulum, consisting mostly of short profiles lined by ribosomes. Ribosomal rosettes were densely distributed throughout the cytoplasm. A fair number of mitochondria and lipid droplets were also present. The Golgi apparatus was prominent. Occasionally centrioles, but no cilia were observed (Fig. 24). Cells were attached to each other forming short cell cords. At the site of attachment their plasma
Figure 21. Anaplastic ependymoma of the spinal cord contains cysts (c) and many blood vessels (BV). Hematoxylin and eosin. (44 X).
Figure 22. Cells of an anaplastic ependymoma form long columns and packets. Many fine cell processes extend into the extracellular space (ECS). Toluidine blue. (2,320 X).
Figure 23. Anaplastic ependymoma cells with large oval nuclei, a thin rim of cytoplasm and many fine processes (P). (12,500 X).
Figure 24. A centriole (Ce) is present in the cytoplasm of a neoplastic ependymal cell. (128,000 X).
membranes followed a straight course with only occasional uncomplicated interdigitations and intercellular cyst formation (Fig. 25). Desmosomes were only infrequently observed. The free segments of the cytoplasmic membrane formed numerous villous projections interdigitating with similar processes of adjacent cells.

**Meningioma**

A meningioma causing compression of the frontal cortex occurred in one rat. It was composed of two cell types, a slender fibroblastic component producing abundant whorls of collagen, and a small oval hyperchromatic cell with indistinct cytoplasm (Fig. 26). The neoplasm was not invasive and had a low mitotic index.

**II. Tumors of the Peripheral Nervous System**

The trigeminal nerve was the most common single site for tumors of the peripheral nervous system. Head tilt, exophthalmus, impairment of facial movements and depression were the main clinical signs. Tumors of the spinal roots occurred most commonly in those roots forming the lumbosacral plexus. Clinical signs were similar to those of spinal cord tumors.

**Neurinomas**

Differentiated neurinomas were not common in rats treated with ENU. When present, they were characterized by oval nuclei and interconnecting eosinophilic spindloid processes. Moderate amounts of collagen and reticulin were produced. Mitoses were rare.

**Anaplastic Neurinomas**

Most neurinomas of ENU treated rats were anaplastic. Even early stages of tumor development, consisting of hypercellular spinal or
Figure 25. Prominent desmosomes (D) connect several neoplastic ependymal cells forming microcysts (Cy). (16,100 X).
Figure 26. Meningioma composed of small spindle cells wrapped around each other forming whorls. The cytoplasmic border is indistinctly outlined. Hematoxylin and eosin. (595 X).
trigeminal nerves, contained poorly differentiated cells. Larger neurinomas were composed of small oval hyperchromatic cells with indistinct cytoplasm or thin spindle-shaped cells with dark elongated nuclei. Malignant neurinomas commonly invaded the skull and muscle. The brain and spinal cord were frequently compressed, but not invaded by neurinomas (Fig. 27). Cysts containing eosinophilic material were the most common regressive change, especially in tumors of the trigeminal nerve. The mitotic index of these tumors was moderately high.

The ultrastructure of the ENU-induced neurinoma was characterized by a pleomorphic cell population which had one or several of the following features. They contained a plump, roughly oval, indented nucleus with 1 to 2 nucleoli in a slightly eccentric position. The cytoplasm was moderately rich in organelles. Prominent processes were characteristically intertwined with processes of other cells (bundling) or they wound around axis cylinders without detectable myelination (Fig. 28). A complete basement membrane, a characteristic feature of Schwann's cells, was not observed but many cells had a partial basement membrane (Fig. 29). These features strongly suggest that the neurinoma cell is a poorly differentiated Schwann cell.

Enzyme Histochemistry

Two gliomas, two neurinomas and a meningioma were characterized by enzyme histochemistry. All tumors had similar profiles of activity. Acid phosphatase activity was considerably increased over that of normal brain. It was present both in diffuse and particulate location. Beta-glucuronidase and esterase activity was slightly greater than normal brain, while alkaline phosphatase was the same. An exception was noted
Figure 27. Neoplastic Schwann cells have invaded the trigeminal nerve up to its junction with the brain (arrows). CNS invasion is restricted to perivascular spaces. Hematoxylin and eosin. (440 X).
Figure 28. Poorly differentiated Schwann cell has incorporated a non-myelinated axon (A) and has a partial basement membrane (arrow). (24,200 X).
Figure 29. A short segment of basement membrane (arrow) is opposed to a neoplastic Schwann cell. (24,500 X).
In areas of adventitial cell hyperplasia in the anaplastic glioma where increased ALP activity was present.

A marked decrease in cytochrome oxidase activity was evident in all tumors. ATPase reactions were weak overall, but moderate near blood vessels. Reactive astrocytes had moderately strong reactions for LDH although the neoplasms had weak LDH, DPNH, G6PDH, and TPNH activities.
Discussion

The intravenous injection of a single dose of ethylnitrosourea (50 mg/kg) to pregnant Sprague-Dawley rats during the 20th day of gestation resulted in the induction of neuroectodermal tumors in 100% of the offspring. Multiple tumors, at various stages of development, were present in most animals. In this study, the average number of neurogenic tumors per rat was 4.1. When BD IX rats were treated with 30 mg/kg ENU on the 15th day of gestation, an average of 2.65 neural tumors/rat were produced in the offspring (13) (19). The increased number of neoplasms probably resulted from a combination of higher dose and increased susceptibility during the terminal stages of pregnancy, although strain differences cannot be excluded.

Apparent differences in tumor location exist in offspring of rats treated with ENU on the 15th (13) (19) and 20th day of gestation. The number of spinal cord tumors increased from 0.2 to 0.7 neoplasms/rat. Brain tumors also increased, from 1.7 to 2.3 tumors per rat, while peripheral nerve tumors remained the same. This increase may be due to the different stage of maturation, to the higher dose of ENU utilized on the 20th day, or to strain differences. Evidence has been presented by Ivankovic and Druckrey (12) that the proportion of CNS tumors located in the spinal cord increased when compared with those of the brain in offspring of BD IX rats given 60 mg/kg ENU on the 20th day of gestation versus those treated on the 15th day. It should be pointed out, that at the 15th day of gestation the spinal cord is incompletely developed, while at the 20th day it is architecturally developed, but not mature (20).
By examining multiple sections from each animal, various stages of tumor development, from each neoplastic proliferation to microtumors, to grossly detectable neoplasms, were evident. Most of these were located near the lateral ventricle, suggesting the subependymal region as the site of origin. Since it is known that carcinogenic nitroso compounds are metabolized rapidly and the induction phase is completed within hours after exposure to the compound, it was surprising to find tumors in varying stages of development after a single injection of ENU. This may be explained by variations in the onset of neoplastic proliferation or by different growth rates between tumors. Support for the latter mechanism includes the high mitotic index and cellular pleomorphism in the majority of macrotumors compared to the isomorphic character and low mitotic rate of microtumors.

Electron microscopy proved to be an important method for tracing the pleomorphic neoplasms to their cell of origin. The malignant neurinoma was shown to be composed of poorly differentiated Schwann cells characterized by the presence of a partial basement membrane, bundles of interdigitating elongated processes, and their capability of incorporating axons.

The malignant ependymoma, of brain and spinal cord, was composed of cells retaining few characteristics of mature ependymal cells. Most cells had a marginated nucleus, multiple processes and villous projections occasionally resembling microvilli, and formed cell cords with scattered desmosomes between adjacent cells. The presence of occasional centrioles and the lack of cilia correspond to similar findings by Wechsler, et al. (19).
Neoplastic astrocytes and oligodendrocytes in various stages of differentiation were demonstrated electron microscopically in ENU-induced gliomas. When compared with gliomas induced in adult rats with methylaminoluroea, the tumor cells of the transplacentally induced gliomas had a higher degree of differentiation (17). In contrast, the ENU-induced neurinomas were more anaplastic than neurinomas produced in adult rats with MNU.

Enzyme histochemistry demonstrated increased activity of the hydrolases, especially acid phosphatase. Material from the same tumors and others induced by MNU and ENU has been studied biochemically (1). Significant increases (p < 0.001) were present in total acid phosphatase, B-glucuronidase, and glucosaminidase of rat tumors of brain, spinal cord and peripheral nerves. Similar results have been presented for human brain tumors (1) (9) (15) (16). The decreased cytochrome oxidase activity has also been confirmed biochemically (2) and represents a common change in the enzyme profile of human gliomas (3).

This experiment has substantiated the findings of Ivankovic and Druckrey (17) that the fetus is highly susceptible to the oncogenic effects of ENU during the last week of gestation. When compared to adult rats, the fetus is more than 50 times more susceptible to tumor induction. This high sensitivity has been shown to hold true for the Sprague-Dawley strain of rats, as well as the BD IX rats. These facts imply that a single contact with a carcinogen may induce neoplastic transformation in cells of the nervous system in utero, with transformation being expressed at a later stage of life.
Summary

Three Sprague-Dawley rats were inoculated intravenously with a single dose of 50 mg/kg of ethylnitrosourea on the 20th day of gestation. All 25 offspring developed tumors of the nervous system. A total of 102 neural tumors and 7 non-neural tumors were produced. The survival time ranged from 85 to 346 days (with a mean of 211 days) following the injection of the carcinogen.

The tumors were classified on the basis of light and electron microscopy into the following: 32 oligodendrogliomas, 4 astrocytomas, 19 mixed gliomas, 5 anaplastic gliomas, 4 glioblastomas, 10 ependymomas, 1 meningioma, and 27 neurinomas (3 differentiated, 24 anaplastic).

Predilection sites for tumors of the central nervous system were the periventricular areas of the lateral ventricles and the white matter of the lower thoracic and lumbar cord. Tumors of peripheral nervous system were most frequently located in the trigeminal nerve and the spiral roots forming the lumbosacral plexus. The majority of gliomas were well differentiated, while the neurinomas and ependymomas were anaplastic.

Enzyme histochemistry of selected neoplasms revealed similar profiles of activity for all tumors. When compared with non-neoplastic brain the activity of hydrolytic enzymes was markedly increased and the activity of oxidative enzymes was decreased. These findings correspond to those reported for naturally occurring tumors of the nervous system in man. This experiment demonstrated that a single transplacental exposure to a neurooncogenic compound is capable of inducing neuroecto-
dermal tumors in all offspring.
BIBLIOGRAPHY

CHAPTER I


CHAPTER II


