INDUCTION OF TUMORS IN THE BRAIN, KIDNEY, AND OTHER EXTRA-MAMMARY GLAND ORGANS BY A CONTINUOUS ORAL ADMINISTRATION OF N-NITROSOBUTYLUREA IN WISTAR/FURTH RATS

(Plates CII-CIII)

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Synopsis

A continuous oral administration of N-nitrosobutylurea (NBU) for 6 months induced a variety of tumors besides mammary tumors in variously conditioned Wistar/Furth (W/Fu) rats of both sexes. The animals were castrated before the NBU treatment or castrated and supplemented with biweekly injections of 0.4 mg of progesterone and 0.01 mg of estradiol benzoate or with an ovarian graft under the kidney capsule.

Leukemias of myeloid and undifferentiated-cell types developed in 7 rats with a mean latent period of 237±37 days, kidney tumors mostly of the so-called nephroblastoma and clear-cell carcinoma in 8 rats with a latent period of 289±34 days, and brain tumors in 14 rats, mostly oligodendrogliomas except one case of ependymoma, with a latent period of 320±21 days, out of 64 rats. In a single or a few rats, tumors of the thyroid, liver, ear duct, duodenum, and peripheral nerve were found.

This study clearly demonstrated the multicarcinogenicity of NBU and strain specificity of the experimental animals in regard to the spectrum of the target organs by NBU; low incidence of leukemias and gastrointestinal tumors compared to the prevalence of those neoplasms in other strains of rats, and subsequent occurrence of tumors in the kidney and brain at a relatively high rate among the long survivors of W/Fu rats.

There have been no evidences indicating the hormonal implications in the genesis and progression of the afore-mentioned tumors other than mammary tumors.

INTRODUCTION

Among the 65 N-nitroso derivatives tested by Druckrey and his associates,1) acylnitrosamides such as methyl-, dimethyl-, trimethyl-, and ethyl-nitrosourea have been found to induce neurogenic tumors selectively in both central and peripheral nervous tissues at a high rate by oral or intravenous administration. However, N-nitrosobutylurea (NBU) has not been tested for organotropic carcinogenesis by them. More recently, NBU has been found to be selectively leukemogenic or mammary tumorigenic by oral administration in certain strains of mice and rats.3, 8, 11, 14, 19, 20, 22)

In a series of our study for rat mammary tumorigenesis by NBU, several types of tumors were found among the variously conditioned W/Fu rats. The present data will be compared with those by others studying the tumorigenicity of NBU, with special

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reference to the difference in tumor site, tumor type, and incidence among the different species and strains of the test animals.

Materials and Methods

Details on the animals, experimental groups, and NBU administration were already described in the previous paper. Only the procedures for animal autopsy will be outlined herein, since the purpose of this paper is to report the induction of tumors in organs other than the mammary gland by oral administration of NBU.

When the animals were found moribund or sick, numbers of white and red blood cells from the tail vein were counted and the blood film was stained with May-Grünwald-Giemsa solution. In the case of leukemia or suspected, imprints from the liver, spleen, and occasionally from the lymph nodes and bone marrow were taken and stained with May-Grünwald-Giemsa, periodic acid-Schiff (PAS), special staining for peroxidase (McJunkin), and alkaline phosphatase (Naphthol AS-MX).

At autopsy, the stomach was opened along the greater curvature, or injected with 10% neutral Formalin after ligating both cardiac and pyloric ends. The liver, spleen, and kidney were weighed. Other organs such as the adrenals, ovaries, uteri, lungs, mediastinal lymph nodes, thymus, and cervical organs, were also carefully examined. Finally, the cranial cavity was opened routinely, but the spinal cord was examined only occasionally. The brain was weighed and fixed in 10% neutral Formalin for 1 to 2 days. After fixation, the brain was sliced on the frontal or horizontal plane.

All the tissue pieces were embedded in paraffin and cut to 4-μ thickness for regular tissues and 6 to 8 μ for the brain, and stained with Hematoxylin and Eosin, Azan Mallory, or Van Gieson stainings.

Results

Brain Tumors Table I is a list of brain tumors developed in rats treated with NBU. Total number of animals with brain tumors, either grossly or microscopically, was 14 out of 25 rats which were subjected to autopsy later than 273 days after the beginning of NBU administration. The brain tumors developed either in the castrated female rats with or without supplement of ovarian hormones or in normal females, with a relatively long latency ranging from 273 to 342 days after the beginning of NBU administration.

Although they occurred in any lobe of the cerebral hemisphere, frontal lobe or hippocampal region might be regarded as the predilection site for the NBU-induced brain tumors. Only 6 of 18 tumors were macroscopically recognizable, measuring 1 to 15 mm in diameter. In 4 cases (W1322, 1326, 1332, and 1350) two or more lesions of brain tumor were found. Histological studies on Hematoxylin-Eosin stained preparations revealed that they were gliomas, mostly originating in oligodendroglia cells except in one case of ependymoma (W1326) (Photos 1~4).

Most of the animals bearing brain tumors also had tumors in the tissues other than the brain; mammary tumors in 6 rats, kidney tumors in 4 rats, duodenal tumors in 2 rats, and tumors of the thyroid, liver, peripheral nerve, and ear duct in one rat each.
BRAIN TUMOR INDUCTION BY NITROSOBUTYLUREA

Table I. Brain Tumors Induced in W/Fu Rats by Oral Administration of NBU

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sex</th>
<th>Treatment</th>
<th>Day sacrificed[a]</th>
<th>Brain tumor Site</th>
<th>Brain tumor Size (cm)</th>
<th>Histology[b]</th>
<th>Tumors in other tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1268</td>
<td>♂</td>
<td>—</td>
<td>273</td>
<td>left frontal</td>
<td>0.2</td>
<td>ol</td>
<td>mammary gland kidney</td>
</tr>
<tr>
<td>W1276</td>
<td>♂</td>
<td>Ovariectomy</td>
<td>278</td>
<td>left frontal</td>
<td>0.1 - 0.2</td>
<td>ne</td>
<td>peripheral nerve</td>
</tr>
<tr>
<td>W1319</td>
<td>♂</td>
<td>Ovariectomy</td>
<td>311</td>
<td>right frontal</td>
<td>1.5 x 1.0</td>
<td>ol</td>
<td>—</td>
</tr>
<tr>
<td>W1322</td>
<td>♂</td>
<td>Ovariectomy</td>
<td>313</td>
<td>left and right hippocampal</td>
<td>0.5</td>
<td>ol</td>
<td>kidney</td>
</tr>
<tr>
<td>W1326</td>
<td>♂</td>
<td>—</td>
<td>316</td>
<td>microscopic</td>
<td>gl</td>
<td>ep</td>
<td>mammary gland thyroid</td>
</tr>
<tr>
<td>W1331</td>
<td>♀</td>
<td>Orchidectomy</td>
<td>320</td>
<td>left hippocampal</td>
<td>1.0 x 0.5</td>
<td>gl</td>
<td>—</td>
</tr>
<tr>
<td>W1332</td>
<td>♀</td>
<td>Orchidectomy + hormone[c]</td>
<td>320</td>
<td>right frontal and left temporal</td>
<td>0.4</td>
<td>ol</td>
<td>mammary gland kidney liver</td>
</tr>
<tr>
<td>W1343</td>
<td>♂</td>
<td>Ovariectomy + hormone[c]</td>
<td>328</td>
<td>temporal</td>
<td>microscopic</td>
<td>gl</td>
<td>mammary gland ear duct</td>
</tr>
<tr>
<td>W1344</td>
<td>♂</td>
<td>—</td>
<td>328</td>
<td>right temporal</td>
<td>0.3</td>
<td>ol</td>
<td>mammary gland duodenum</td>
</tr>
<tr>
<td>W1348</td>
<td>♂</td>
<td>Ovariectomy</td>
<td>331</td>
<td>frontal</td>
<td>microscopic</td>
<td>ol</td>
<td>—</td>
</tr>
<tr>
<td>W1350</td>
<td>♂</td>
<td>—</td>
<td>332</td>
<td>frontal</td>
<td>microscopic</td>
<td>ol</td>
<td>mammary gland</td>
</tr>
<tr>
<td>W1358</td>
<td>♂</td>
<td>Orchidectomy</td>
<td>342</td>
<td>frontal</td>
<td>microscopic</td>
<td>ol</td>
<td>—</td>
</tr>
<tr>
<td>W1359</td>
<td>♀</td>
<td>Orchidectomy</td>
<td>342</td>
<td>frontal</td>
<td>microscopic</td>
<td>ol</td>
<td>duodenum</td>
</tr>
<tr>
<td>W1360</td>
<td>♀</td>
<td>Orchidectomy</td>
<td>342</td>
<td>frontal</td>
<td>microscopic</td>
<td>ol</td>
<td>—</td>
</tr>
</tbody>
</table>

[a] Days after the beginning of NBU treatment.
[b] ol=oligodendroglioma, gl=glioma, not specified, ep=ependymoma, ne=not examined.
[c] Injected with 0.4 mg of progesterone and 0.01 mg of estradiol benzoate, twice weekly for 7 months.

Kidney Tumors  Table II is a list of kidney tumors developed in rats treated with NBU. Overall incidence of the kidney tumors was 8 out of 45 cases examined during the period of 236 to 321 days after the beginning of NBU treatment. Total number of tumors in these rats was 12 including 3 whose sections were not available.

The tumors were classified tentatively into nephroblastoma (3 cases), anaplastic tumor (1 case), and clear-cell carcinoma (5 cases) according to the histological type. The size of the tumors varied from miliar to 5 cm in diameter in no relation to the latency.

Histology of the nephroblastomas obtained in this experiment was varied: undifferentiated (Photo 5) or hyalinized type (Photo 7). Intermediate form was the one in which the tubules were surrounded by concentric mass of tumor cells (Photo 6). On the contrary, the clear-cell carcinoma was uniformly well-differentiated (Photo 8).
Leukemia

Seven cases of leukemia developed among 61 rats treated with NBU with a latent period of 195 to 273 days after the beginning of the treatment (Table III). They were 4 cases of undifferentiated cell type and 3 cases of myelogenous type. In the former cases, the leukemia cells consisted of small to medium-sized round cells with a scanty basophilic cytoplasm and a round nucleus with fine chromatin network. In the latter cases, the cells were mainly myeloblasts (W1171) or promyelocytes (W1265 and W1267). Peripheral blood cell counts ranged from 19,000 to 54,900/mm³ for white cells and from 129 to 585 × 10⁴/mm³ for red cells.

There was no case of erythroid leukemia in the present study. All the leukemic rats developed a moderate to marked hepatosplenomegaly due to the extensive leukemic infiltration. Five out of 7 leukemic rats had one or two small, sometimes microscopic, mammary tumors.

The leukemias induced by NBU thus far tested were invariably transplantable. Intraperitoneal injections of 10⁶ or 10⁷ cells from the leukemic liver and spleen into syngeneic rats caused leukemic death of the recipients within 2 months in case of undifferentiated cell type leukemia (W1180 and W1231) and within 4 or 5 months in case of myelogenous leukemia (W1265 and W1267).

Table II. Kidney Tumors Induced in W/Fu Rats by Oral Administration of NBU

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Sex</th>
<th>Treatment</th>
<th>Days sacrificed</th>
<th>Site</th>
<th>Kidney tumor size (cm)</th>
<th>Histology</th>
<th>Tumors in other tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1220</td>
<td>♀</td>
<td>Ovariectomy + hormone</td>
<td>236</td>
<td>left</td>
<td>5 × 5 × 4</td>
<td>Nephroblastoma</td>
<td>mammary gland</td>
</tr>
<tr>
<td>W1230</td>
<td>♂</td>
<td>Orchidectomy + ovarian graft</td>
<td>245</td>
<td>right</td>
<td>0.5 across</td>
<td>Nephroblastoma</td>
<td>mammary gland</td>
</tr>
<tr>
<td>W1268</td>
<td>♀</td>
<td>—</td>
<td>273</td>
<td>right</td>
<td>0.4 × 0.5</td>
<td>Nephroblastoma</td>
<td>mammary gland, brain, mammary gland</td>
</tr>
<tr>
<td>W1294</td>
<td>♂</td>
<td>Orchidectomy + hormone</td>
<td>291</td>
<td>right</td>
<td>0.8 across</td>
<td>Clear-cell carcinoma</td>
<td>mammary gland</td>
</tr>
<tr>
<td>W1321</td>
<td>♀</td>
<td>Ovariectomy</td>
<td>313</td>
<td>left right</td>
<td>1 across</td>
<td>not studied Anaplastic tumor</td>
<td>mammary gland</td>
</tr>
<tr>
<td>W1322</td>
<td>♀</td>
<td>Ovariectomy</td>
<td>313</td>
<td>left right</td>
<td>miliary miliary</td>
<td>not studied</td>
<td>brain</td>
</tr>
<tr>
<td>W1332</td>
<td>♂</td>
<td>Orchidectomy + hormone</td>
<td>320</td>
<td>left right</td>
<td>0.4 across</td>
<td>Clear-cell carcinoma</td>
<td>mammary gland, brain, liver</td>
</tr>
<tr>
<td>W1333</td>
<td>♂</td>
<td>Orchidectomy + hormone</td>
<td>321</td>
<td>left right</td>
<td>0.4 across</td>
<td>Clear-cell carcinoma</td>
<td>mammary gland, liver</td>
</tr>
</tbody>
</table>

a) Days after the beginning of NBU treatment.
b) Injected with 0.4 mg of progesterone and 0.01 mg of estradiol benzoate, twice weekly for 7 months.
c) Syngeneic ovary was grafted under the capsular space of the kidney 2 weeks before NBU treatment.
Miscellaneous Tumors  Reticulum cell sarcoma was found in an ovariectomized rat which died 236 days after the beginning of the treatment with NBU. The animal had a distended abdomen filled with ascites. There were many yellowish nodules of various sizes on the surface of the liver. Mesenteric lymph nodes were greatly enlarged and tumorous. The tumor cells were large and polygonal in shape. There were some giant cells.

Thyroid tumor was found in a female rat sacrificed 316 days after the initial NBU treatment. The right lobe of the thyroid was replaced by a tumorous tissue showing a follicular pattern. The animal also had two mammary tumors in the cervical and axillary pads, and a microscopic glioma in the brain.

In two rats, orchidectomized and supplemented with ovarian hormones, sacrificed on 320th and 321st day, hepatoma was induced together with a clear-cell carcinoma in both kidneys, and multiple mammary tumors and brain tumors.

In a rat sacrificed on 328th day, right ear duct tumor was found. Two cases of duodenal tumor were encountered in the rats autopsied on 328th and 342nd day after the beginning of NBU treatment.

A spayed rat which showed incontinentia urinae at the terminal stage had a huge retroperitoneal tumor (2 × 1.5 × 1 cm), probably derived from the lumbar radixus of the peripheral nerves. The animal had a tiny tumorous lesion in the brain and several hyperplastic nodules in the liver.

Among the control rats, there was no occurrence of such tumors as developed among the NBU-treated rats by the age of 1 year.18)

Tumor Distribution  Table IV summarizes the tumor distribution among the variously conditioned groups. As to the brain tumor, the prolonged life span of 305±47 days in group D has apparently favored the prevalence of the tumors. Nevertheless,
there was no significant difference among the experimental groups on the incidence of
the brain tumor, indicating no hormonal effect on the genesis of the neurogenic tumor.
There was no specific pattern on the tumor distribution among the various groups with
respect to kidney tumors, leukemia, and other types of tumor.

**DISCUSSION**

Present study clearly indicates that NBU is a multipotent carcinogen. Selective
induction of either leukemia in mic and rats\(^3,11,14,22\) or mammary tumors in rats\(^3,8,19,20,22\) was repeatedly shown in a number of experiments with various strains of
animals, in which the multicarcinogenicity of NBU was also suggested.

Major discrepancies between our present data and others lie in the following: (1)
Incidence of leukemia and forestomach tumor. Odashima was successful in inducing
100% of leukemia in female Donryu rats with a short latency by a continuous oral
administration of NBU with a daily dose of 6 mg per rat.\(^11\) He also found a frequent
occurrence of tumors in the forestomach and esophagus by a smaller dose of NBU in
accord with reduction in the incidence of leukemia.\(^11\) In the experiments using Sprague-
Dawley rats\(^9\) and ACI rats,\(^22\) nearly 100% of leukemia was obtained by pulse or
continuous administration of NBU. On the other hand, induction rate of leukemia was
only 11% in the present study with W/Fu rats. By increasing the daily dose of NBU
to 10 mg, the maximum dose tolerated by the animals, the incidence rose to 30% in this
strain of rats.\(^20\) (2) Induction of brain tumors at a high rate in the present study. To
the best of our knowledge, there has been no report concerning the development of
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brain tumors in NBU-treated rats. Prolongation of the observation period and marked reduction in early mortality by castration have presumably enabled us to obtain such late-occurring tumors.

It is rather unusual that a brain tumor has been induced by systemic administration of the carcinogen. In the literature, only four cases of intracranial tumors were induced by the oral administration of 2-acetamidofluorene. Later, Druckrey and his associates reported the selective induction of neurogenic tumors in the brain, spinal cord, and peripheral nerves by various forms of nitrosourea. NBU was the one left untested by them.

Induction of brain tumor has been attempted by implanting the carcinogen or injecting the oncogenic viruses or tumor cells harboring the viruses into the brain tissue. The results of such experiments have contributed to the understanding of the tumor cytology and its classification. In this connection, the discovery of the agents capable of inducing neurogenic tumors by systemic administration seems beneficial.

Transplacental induction of various types of tumors in the nervous system has been reported by Ivanovic and Druckrey using ethylnitrosourea and by Spatz and Laqueur using crude cycad material. These facts clearly indicate that the growing nervous tissues are apt to undergo malignant changes by such agents. The brain tumors induced by NBU in the present study are gliomas and mostly oligodendrogliia in origin. Further studies on the histogenesis of these tumors are being planned.

Kidney tumors have been efficiently induced in rats by various nitrosamines and nitrosamides, and less efficiently by total body irradiation with ionizing radiations. Thomas and Schmähl postulated that these chemical carcinogens should be given in a high single dose, only for a short time.

In our present study, continuous oral administration of NBU resulted in 8 cases of kidney tumors; 3 cases of nephroblastoma at an early stage, 1 case of anaplastic tumor, 3 cases of clear-cell carcinoma, and 1 case not examined at a later stage. According to Fukunishi et al., 7 cases of nephroblastoma were obtained from both sexes of 67 Sprague-Dawley rats given oral administration of a large dose of NBU four times at an interval of 2 weeks, starting at the age of 21 days.

Magee and Barnes stated that renal tumors induced by dimethylnitrosamine (DMN) fell into two types; well differentiated (renal cell type) and anaplastic (nephroblastoma). They also reported that when DMN was given to newborn rats all the kidney tumors were of the nephroblastoma type. Recently, Ito suggested, after analysing a large number of experimental kidney tumors, that tumor of the renal cell type is derived from the proximal convoluted tubules or uriniferous tubules, while nephroblastoma from undifferentiated cell or its precursor “metanephrogenic tissue.” However, Hard and Bulter claim, in their recent report, that hitherto called “nephroblastoma” induced by DMN is vascular nature of mesenchymal origin. In any case, the histological classification of renal tumors in rats seems to be unsettled and apparently needs further studies.

The incidence of leukemia was low in the present study with W/Fu rats as previously described. Ovariectomy before NBU administration prevented early death from uterine hemorrhage and mammary tumors, and consequently prolonged the life span of the
animals. However, such surgical intervention was not particularly effective in augmenting the induction rate of leukemia.

These findings remind the observation that by treatment with 7,12-dimethylbenz[a]anthracene, there were a high yield of mammary tumors and a low yield of leukemia in Sprague-Dawley rats, while the relation was reversed in Long-Evans rats treated with the same carcinogen. The difference in susceptibility of these strains of rats to carcinogen-induced mammary tumor or leukemia might be a reflection of the genetically determined variation in their endocrine or hematopoietic constitution.

It is noteworthy that the chemically-induced rat leukemias are mostly of undifferentiated cell type and myelogenous type, in sharp contrast to the dominant occurrence of thymic lymphoma in mice. In certain strains of rats, the development of erythroblastic leukemia is also common after the systemic administration of chemical carcinogens.

Possible participation of a leukemogenic virus in NBU-leukemogenesis in mice has been strongly indicated by the recent observations in this laboratory that leukemic cells reacted to the Gross virus-specific cell-surface antigen, and the leukemia could be transmitted to the susceptible recipients by a cell-free inoculation (unpublished data). On the other hand, there has been no evidence of viral implication in NBU-leukemogenesis in rats. An apparent difference in leukemogenic mechanism in the two species deserves further analysis.

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EXPLANATION OF PLATES CII~CIII

Photo 1. A general view of the brain (W1331). Note asymmetric enlargement of the left hemisphere.

Photo 2. Horizontal cross-section of the above. Left temporal lobe was occupied by the tumorous tissue.


Photo 5. Nephroblastoma, undifferentiated type, measuring 5×5×4 cm, found in an ovariectomized and hormone-injected rat (W1220) which was sacrificed 236 days after the beginning of NBU treatment. H–E. ×100.

Photo 6. Another type of nephroblastoma, in which the tubules were surrounded by a concentric mass of tumor cells (W1268). H–E. ×100.

Photo 7. Marked fibrosis and hyalinization in a case (W1230) of nephroblastoma. Van Gieson. ×100.

Photo 8. Well-differentiated clear-cell carcinoma, measuring 0.2 cm across, in both kidneys of a rat (W1332) autopsied 320 days after the beginning of NBU treatment. H–E. ×100.

H–E=Hematoxylin-Eosin stain