Morphologic and Biochemical Characteristics of Transplantable Neurogenic Tumors Induced by N-Ethyl-N-nitrosourea in Inbred BD IX Rats 1, 2

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ABSTRACT—Brain and nerve tumors were induced transplacentally in inbred BD IX rats by systemic application of N-ethyl-N-nitrosourea. Because primary gliomas and neurinomas produced in this way are composed of heterogeneous cell populations, changes in tumor morphology were expected to occur during serial transplantation in syngeneic hosts. In this study such changes in morphology were correlated with the expression of two biochemical nervous system markers, S-100 protein and 2',3'- cyclic nucleotide 3'-phosphohydrolase. Several changes were observed during serial transplantation, including increased growth rate (even after one passage), preferential growth of anaplastic versus differentiated glial and Schwann's cells, varying degrees of fibroascomatous changes after prolonged serial transplantation, and reduced levels of S-100 protein. In contrast, tumors derived from biologically differentiated clonal cell lines retained their morphologic and biochemical characteristics to a much greater extent, even after prolonged periods of sc transplantation.—J Natl Cancer Inst 62: 811-817, 1979.

The transplacental induction of tumors of the nervous system in rats with the alkylating agent ENU was discovered by Druckrey et al. in 1966 (1). The pathology of such tumors of both the CNS and PNS has been described and reviewed (2-6). The importance of this experimental tumor model for cancer research and human neuro-oncology is well recognized. To develop the system further, transplantable tumors and tumor cell lines have been established that are used to study transplantation behavior, immunologic aspects of tumor cells, host-cell interaction, and therapeutic approaches.

Attempts to transplant primary neurogenic tumors have been successful in several rat strains (7-12). In addition, permanent cell culture lines and clones have been developed from both primary and transplantable tumors experimentally induced in the rat nervous system ([13-24]; reviewed in (25); Pfeiffer SE, Wechsler W, Sunderraj N: Manuscript in preparation; Wechsler W, Pfeiffer SE, Sunderraj N: Manuscript in preparation). In most instances, transplantable tumors and propagated tumor cell lines in culture show varying degrees of cellular heterogeneity, the extent being dependent on the type of primary tumor and the number of passages in animals or of subcultures. The presence of nervous system-specific biochemical markers in these tumors suggested the usefulness of such markers for investigations in neuro-oncology. For example, S-100 protein unique to the nervous system (26, 27) has been found in gliomas and neurinomas (28, 33-33) and experimental animals (34, 35). A membrane-bound biochemical marker, CpNase, was present at relatively high activity in both normal nervous tissue and brain tumors, as well as in neoplastic clones of glial and Schwann's cells ([19, 33, 36, 37]; Pfeiffer SE, Wechsler W, Sunderraj N: Manuscript in preparation). In this study, we compare morphologic and biochemical parameters of 3 experimentally produced rat tumors, 1 glioma and 2 neurinomas, during long-term serial transplantation experiments.

MATERIALS AND METHODS

Primary tumors.—As described by Druckrey and coworkers (7), the tumors used in this study were induced transplacentally in pregnant BD IX rats by a single iv dose (tail vein) of 80 mg ENU/kg administered on the 15th day of gestation. BD IX is a well-characterized inbred rat strain (38, 39). BD IX rats are not prone to spontaneous tumor proliferation, particularly not to tumors of the CNS and PNS. A brain tumor (animal No. A140) was taken from the left hemisphere of an offspring 215 days after birth. The 2 PNS tumors, a cervical nerve root lesion and a lumbar spinal nerve root tumor (animal No. 1637), have been previously described (11).

Transplantation.—Minced tissue removed from the primary tumors under sterile conditions was inoculated sc into adult syngeneic BD IX rats of both sexes. Several transplantation tumor lines were established from brain tumor A140, but only one line was maintained over 20 passages. Three permanent lines (A11, A12, and A13) were developed from the cervical nerve root tumor, and one line (A14) from the

ABBREVIATIONS USED: CNS = central nervous system; CpNase = 2',3'- cyclic nucleotide 3'-phosphohydrolase; ENU = N-ethyl-N-nitrosourea; H & E = hematoxylin and eosin; PNS = peripheral nervous system.

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Table 1.—Transplantation behavior of ENU-induced CNS and PNS tumors in syngeneic BD IX rats

<table>
<thead>
<tr>
<th>Growth of transplanted tumors, days*</th>
<th>11-15</th>
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<th>21-25</th>
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<td>Growth of primary tumor, days</td>
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RESULTS

Cerebral Glioma

Primary tumor.—The tumor was a glioma of the left hemisphere that had invaded the basal ganglia. The prominent cell type was the astrocyte, but anaplastic small glial cells and a few neoplastic oligodendrocytes were also present (fig. 1A). No obvious sarcomatous proliferation was detectable; however, blood vessels were prominent. The number of mitotic figures was moderate. This tumor was diagnosed as a mixed astrocytic glioma with a moderate degree of anaplasia.

Transplantation tumors.—Tissue of the primary glioma was used for sc transplantation in 6 syngeneic animals. After an observation time of more than 1 year, 2 recipients demonstrated no tumor take and 4 rats developed tumors [2 fibroma-like neoplasms (A270, A274) and 2 glioma-like tumors (A272, A273)]. The first passage of tumor A273 was used for long-term serial transplantation studies. This tumor was a rather differentiated astrocytic glioma with few neoplastic oligodendrocytes and anaplastic cells. Small and large gliomatous areas were separated by thick fibromatous portions (fig. 1B). A connective tissue capsule surrounded the tumor periphery. Although this morphology remained constant through the second passage, anaplastic changes characteristic of a mixed tumor, a so-called gliosarcoma with predominance of anaplastic glial tumor cells and fibrosarcomatous portions producing modest amounts of reticulin fibers, became apparent in the third passage and remained fairly constant until termination of the tumor line at passage 22 (figs. 1C, 1D). A long latent period and slow growth rate characterized the first passage. Thereafter, the lumbar nerve root tumor. In addition, cell cultures from neurinoma clones RN-1, RN-2, and RN-3 (19, 25), isolated from the tumor line AI2, were used for serial propagation as subcutaneous clonal tumors.

Histology.—The tissues were fixed in 4% neutral aqueous formaldehyde and stained with H & E or Masson trichrome stain. Silver impregnation for reticulin fibers was performed by the method of Tibor-Pap.

Biochemical investigation.—Tumor tissue that had been rapidly frozen and stored at -70°C was thawed, suspended in Tris-buffered saline, disrupted in a Dounce homogenizer, and centrifuged at 3,000 × g for 20 minutes. Supernatant fractions were analyzed for S-100 protein by microcomplement fixation as described previously (19, 35, 40). S-100 protein is expressed as the percentage of the 600,000 × g-min supernatant protein.

The sensitivity of the assay is 0.005 μg S-100 protein/ml solution. The total protein concentration of the extracts was determined by the method of Lowry et al. (41), with bovine serum albumin as a standard. CpNase was assayed by the method of Olafson et al. (42), as described by Glastris and Pfeiffer (43). Specific activities are given in units of micromoles of substrate converted per minute per milligram of protein. The sensitivity of the assay is 7.5 nmoles/minute.
latent period declined sharply and the growth rate increased markedly (table 1).

Biochemical results.—The primary glioma contained a relatively high level of S-100 protein (1.0%), which exceeded that found in normal brains of BD IX rats. This S-100 protein level decreased sharply after the first sc transplantation (0.02%) and remained constant in subsequent passages with only slight fluctuations (table 2). In contrast, the CpNase activity remained high, irrespective of the morphology and the passage number of the transplanted tumors (table 2).

Cervical Neurinoma

Primary tumor.—The tumor originated from a dorsal root and its sensory ganglia at the level of the brachial plexus. It was a relatively small cell-rich neoplasm with slight microcystic alterations and some hemorrhagic changes, was composed predominantly of spindle and stellate cells, and had a low mitotic index (fig. 2A). A moderate amount of fine delicate reticulin fibers, but no collagen, was present. The tumor was diagnosed as a malignant neurinoma with a relatively high degree of differentiation.

Transplantation tumors.—Three subcutaneous transplantation tumors (A1, A12, and A13) were developed. Details concerning growth behavior are summarized in table 1, and corresponding biochemical data are given in table 2.

The histology of the first passage of tumor A1 was typical of a differentiated microcystic neurinoma with increased numbers of mitotic figures. Slight anaplastic changes occurred after the fifth passage, including partial loss of cyst formation and a more pronounced tendency for central necrosis. Reticulin fibers, in contrast to collagen, were produced in each transplantation tumor. The tumor line had a fairly stable morphology until it was terminated at the 12th passage.

At the first passage, tumor A12 was a rapidly proliferating typical fibrillary neurinoma composed of stellate and spindle cells (fig. 2B). Cyst formation was a constant feature during early serial propagation. After the fifth passage slight anaplastic changes occurred (fig. 2C), becoming more prominent after the 10th passage (fig. 2D). These changes persisted, and the number of plump spindle cells that produce large amounts of fine reticulin fibers increased. Only slight fibromatous morphologic changes occurred thereafter, and the line was terminated after the 32d passage. RN-1, RN-2, RN-3, and RN-4 neurinoma clones were derived from the second transplantation and characterized as sarcoma and neoplastic Schwann's cell clones (19).

At the first passage, the multicystic tumor A13 was composed of spindle and stellate cells typical for Antoni A and B neurinoma patterns. The moderate number of fine delicate reticulin fibers was compatible with the diagnosis of neurinoma. However, scattered afibrillar areas were also detected. Although this morphology remained fairly constant in early passages, cellular anaplasia with predominance of large plump spindle cells and loss of cyst formation tended to increase between passages 5 and 10. An increased amount of medium and thick reticulin fibers and a few collagen fibers were also noted. The line was terminated at the 14th passage, when a fibrosarcoma-like morphology was prominent.

Biochemical results.—The S-100 protein values of the primary tumor and the three transplantable tumor lines are shown in table 2. In the primary cervical

<table>
<thead>
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<th>No. of passages</th>
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<th>Cervical neurinoma</th>
<th>Lumbar neurinoma*</th>
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<td>CpNasee</td>
<td>S-100c</td>
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* Tumor line, A273.
† Tumor line, A14.
‡ Percent of 700,000×g-min supernatant protein.
§ μmoles adenosine-2'-monophosphate released/min/mg cellular protein.
∥ Tumor line, A11.
© Tumor line, A12.
' Tumor line, A13.
neurinoma, 0.3% of the total soluble protein was S-100 protein. During transplantation, S-100 protein was detectable in all tumors examined. The content of S-100 protein was relatively high in all three sublines after their first passage, but dropped significantly at the second passage. All transplantable tumors examined between the 2d and 22d passages had CpNase activity, with values ranging from 0.3 to 0.6 μmoles/minute/mg.

**Lumbar Neurinoma**

**Primary tumor.**—The tumor originated from a dorsal nerve root and its ganglion and was of medium cell density. Spindle and small irregular cells were prominent, and mitoses were rare. The production of fine reticulin fibers was moderate, but collagen was absent. The tumor tissue showed rare microcystic changes. The lesion was diagnosed as a malignant neurinoma with a fibrillary differentiation and minor cellular anaplasia.

**Transplantation tumors.**—Transplantation line A12 was established from the primary tumor. Tumors from passages 1-6 presented the morphology of a fibrillary cystic neurinoma with many mitoses. Reticulin fibers were extensive, but collagen was absent. Beginning with passage 9, a solid and more pleomorphic spindle cell tumor containing many irregular reticulin fibers was observed. The number of anaplastic plump spindle cells increased until the 14th passage; this finding indicated a slight fibrosarcomatous change.

**Biochemical results.**—The primary lumbar neurinoma contained only 0.06% S-100 protein (table 2). This value remained fairly constant until the 22d passage. CpNase activity ranged from 0.4 to 1.1 μmoles/minute/mg.

**Clonal Neurinoma Tumor Lines**

Four tumor clones designated RN-1, RN-2, RN-3, and RN-4 have been isolated from the second passage of the transplantable BD IX rat neurinoma line A12 (19). On the basis of morphologic and biochemical criteria, clone RN-2 was identified as a neoplastic Schwann's cell clone. The other three clones were S-100 negative and were considered to be sarcomatous. Cell suspensions from the Schwann's cell clone RN-2 and from two sarcoma clones (RN-1 and RN-3) were injected sc into adult BD IX rats to produce clonal tumors.

**Neoplastic Schwann's cell clone RN-2.**—Cell suspensions of clone RN-2 produced a cell-rich subcutaneous tumor composed of a homogeneous population of medium-sized spindle cells and small plump stellate cells (fig. 3A). No cyst formation was noted. The amount of reticulin fibers was moderate. Most fibers were fine; few were of medium and thick types. Collagen fibers were absent. Tumor tissue from the first animal passage was cultivated in vitro, and only after several subcultures was it reintroduced sc into animals. These cells then formed a solid spindle-cell tumor that remained unchanged over subsequent passages and was similar in morphology to the tumor from which RN-2 tumor cell clones were originally derived. The S-100 protein levels were approximately 0.1% for both the original RN-2 monolayers and cultures derived subsequently from serial sc transplanted tumors. Transplantable clonal tumors consistently had about 0.05-0.07% S-100 protein, and CpNase activity was between 0.3 and 0.9 μmoles/minute/mg. Two years later, S-100 protein values for cells in monolayer cultures were still approximately 0.07%, and those for subcutaneous clonal tumors approximately 0.05%.

**Transplantable RN-1 and RN-3 clonal tumors.**—Several sc passages were studied histologically. All tumors presented a similar fibrosarcomatous pattern (fig. 3B). The spindle-cell tumors produced coarse, medium, and thick reticulin fibers abundantly. Scattered mature collagen fibers were present in scant to moderate numbers. S-100 protein was negative, and CpNase activity was low, i.e., between 0.01 and 0.05 μmoles/minute/mg. Recently, Thy-1 differentiation antigen has been detected in line RN-1 (44).

**DISCUSSION**

Tumors of the nervous system produced transplacentally with ENU in BD IX rats are characterized by a heterogeneous cell population (3-5, 45-49). If these tumors are used to develop permanent tumor lines by syngeneic transplantation, subsequent alterations of the tumor morphology are to be expected.

Table 1 illustrates that the growth potential of transplanted tumors increased after the first three passages, which indicates that selection occurred for cell types with more rapid proliferation under the conditions of sc syngeneic transplantation. For example, 4 tumors that developed from transplantation of a primary mixed glioma of the brain demonstrated at the first passage either a fibroma- or glioma-like pattern. By the second and third passages, an overgrowth with fibrosarcomatous cells was apparent. We assume that a few sarcomatous cells were already present in the primary glioma and that these overgrew the glial tumor cells during transplantation or culture passages. However, exceptions to this pattern are illustrated by tumor line A273 that retained over many passages the morphologic features of an anaplastic glioma type with varying degrees of a fibrosarcomatous involvement.

Whether a sarcomatous participation and overgrowth takes place in malignant neurinomas induced transplacentally with ENU is more difficult to ascertain by histologic techniques because neoplastic Schwann's cells and fibroblasts have morphologically more in common than do neoplastic glial cells and fibroblasts. One cerebral mixed glioma and 2 neurinomas established as subcutaneous transplantation tumors retained characteristic biochemical functions of the nervous system, i.e., synthesis of S-100 protein and CpNase. The presence of S-100, although at lower values, over prolonged serial transplantations (for 20 or more passages) indicates the persistence of glial and Schwann's cells capable of performing this specific nervous system function.
function. Changes in both the morphology and the biochemical parameters occurred more rapidly for the cerebral glioma than for the neurinomas.

In contrast to the transplantable neurinomas A11, A12, A13, and A14, the transplantable Schwann’s cell clonal tumor RN-2 maintained its morphologic stability after serial propagation in culture and sc transplantation. Similar observations were made for the neurosarcomatous clonal tumors RN-1 and RN-3. In contrast to the easy transplantability of these clones in syngeneic BD IX rats, RN-2 clonal neoplastic Schwann’s cells were repeatedly rejected from several other strains of rats with different major histocompatibility antigens (45). This suggests that the normal major histocompatibility antigens of the clonal RN-2 tumor cell lines have remained normal.

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FIGURE 2.—Spinal nerve root neurinoma after serial syngeneic transplantation as tumor line A12: A) 1st passage; B) 2d passage; C) 6th passage with slight anaplasia; and D) 17th passage with moderate partial fibrosarcomatous changes. H & E. × 175

FIGURE 3.—Histology of subcutaneous clonal tumors from A) neoplastic Schwann’s cell clone RN-2; and B) the sarcomatous neurinoma clone RN-1. H & E. × 175