Development of an Animal Brain Tumor Model and Its Response to Therapy with 1,3-Bis(2-chloroethyl)-1-nitrosourea

Marvin Barker, Takao Hoshino, Ozdemir Gurd, Charles B. Wilson, Surl L. Nielsen, Robert Downie, and Joseph Eliason

Howard C. Naffziger Laboratories for Neurosurgical Research, Department of Neurological Surgery, Cancer Research Institute [M. B. T. H., O. G., C. B. W., R. D., J. E.], and Department of Pathology [S. L. N.], University of California, San Francisco, California 94122

SUMMARY

A chemically induced rat glioma, carried in cell culture, was transplanted to the brain of CDF rats. The tumor thrived in cell culture, producing a typical malignant astrocytoma when implanted in rat brain. Survival of tumor-bearing rats varied in different experimental groups, but the range of survival within each experimental group was within acceptable limits.

The rat glioma in cell culture grew progressively through three phases, namely, exponential, stationary, and a variable phase, limited by space and medium. The cell-cycle time of the glioma in vivo was 20 hr, the growth fraction was 0.35 to 0.46, the observed doubling time was 72 hr, and the cell-loss factor was 0.42. These data, along with the chronology of the cell-cycle phases, enhance the usefulness of this system as a model for brain tumor chemotherapy.

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) significantly increased the survival time of rats bearing intracerebral gliomas. A dose of BCNU approximating 40% of the 10% lethal dose, when given 9 and 16 days after tumor implantation, produced an increased life-span of 38%. A dose of BCNU equivalent to 80% of the 10% lethal dose given on Days 9 and 16 produced an increased life-span of 84%. When BCNU was withheld until the 16th and 23rd days, a significant increase in survival time was still evident.

The advantages of this animal model for brain tumor chemotherapy are that (a) the transplanted tumor is of glial origin, (b) the tumor take of the model has been 100%, (c) the mean survival of animals is approximately 30 days, permitting the start of a treatment schedule after the tumor has grown to significant size, and (d) the rat is suitable for various routes of therapy.

INTRODUCTION

There have been numerous approaches to establishing an animal model for the testing of chemotherapeutic agents against brain tumors. The most extensive studies have used the following methods: (a) heterotransplantation of human brain tumors to the brain, anterior chamber, and flank of animals; (b) heterotransplantation of nonglial tumors to animal brain; (c) viral induction of brain tumors in animals; and (d) chemical induction of brain tumors in animals.

Wilson and Bates (36) recently reviewed all of these methods for producing transplantable brain tumors, and several have been utilized as models for testing chemotherapeutic agents (30). A nonglial tumor transplanted to the brains of animals has been used by Schabel et al. (26), Norrell and Wilson (22), Wilson et al. (38), Chirigos et al. (5), Kotsilimbas et al. (17, 18), Donelli et al. (8), and Rosso et al. (25). A glial tumor transplanted to the flanks of animals has been used by Wright et al. (39), Donelli et al. (8), and Rosso et al. (25). To date, the most widely used model has been the chemically induced glioma transplantable to the brains of mice [recent studies with this model having been reported in studies by Edelman et al. (9), Ausman et al. (1, 2), Levin et al. (20), and Shapiro and Ausman (28-30)].

Knowledge of cell generation times of primary brain tumors would enhance any approach to their treatment, but presently available information is fragmentary and probably misleading. The few reported in vivo studies of human glioblastomas were based on calculations made with assumed DNA synthesis times (10, 15, 33). Although the recent advances in the treatment of leukemia resulted directly from studies in an animal model of great predictive value and the majority of all data on tumor cell kinetics have been obtained with this and similar animal models (40), such data are not applicable to the study of intracerebral tumor kinetics.

The alkylating agent BCNU has been found to be highly effective against extracerebral tumors such as leukemias, carcinomas, and sarcomas (7, 14, 21, 24, 26). It crosses the blood-brain barrier, and has thus been investigated in the chemotherapy of brain tumors. Wilson et al. (37) reported an objective response in 7 of 18 glioblastoma patients, 4 of 6...
astrocytoma patients, and 4 of 8 patients with miscellaneous tumors.

Two animal models have shown that BCNU is effective against intracerebral tumors. Schabel et al. (26) and Chirigos et al. (5) reported increases in survival time and number of long-term survivors when mice in which leukemia L1210 had been implanted intracerebrally were treated with BCNU. Ausman et al. (1, 2) and Shapiro and Ausman (29) reported that BCNU significantly prolonged the life-span of mice with glioma.

This report describes the development of an animal model for brain tumors, including its cell proliferation kinetics and its response to therapy with BCNU. This model was used previously to study the antitumor effect of methylprednisolone acetate (12, 13).

MATERIALS AND METHODS

Development of Animal Model

The tumor used in this study was provided by William H. Sweet, Paul T. Kornblith, Janette R. Messer, and Beverly O. Whitman of the Massachusetts General Hospital, Boston, Mass. The tumor was induced in CD Fischer rats by weekly i.v. injections of N-nitrosomethylurea. Tissue culture and preservation methods have been described by Benda et al. (4).

Upon receipt of frozen cells from the above source, we placed them in Eagle’s basal medium supplemented with 10% fetal calf serum, L-glutamine (398 μg/ml), and antibiotics (penicillin, 80.5 units/ml; streptomycin, 80.5 μg/ml), and grew them by the monolayer method for continuous cell culture described previously (3, 35). The morphology of the tumor cell line was checked by observation of Giemsa-stained coverslip cultures.

Transplantation of the cultured cells to the brain of rats was done as follows. Forty-eight-hr cultures were harvested by trypsinization (0.25% for 15 min) and adjusted to a concentration of 4 × 10⁶ cells/ml in Gey’s balanced salt solution. Adult, male CDF rats (150 g body weight) were anesthetized with pentobarbital and placed in a small-animal stereotaxic head holder (Trent Wells Mechanical Co., South Gate, Calif.). The scalp was opened electrosurgically, and a 0.07-inch hole was drilled at a point 2.5 mm to the left of the midline and 4.0 mm anterior to the frontal zero plane, according to the method of König and Klippel (16). The hole was plugged with a stainless steel, 0.40- x 0.0625-inch screw with a 0.0292-inch center-drilled hole.

The cell suspension (0.01 ml) was injected into the central white matter through a blunt 22-gauge needle attached to a 0.1-ml chromatography syringe (the needle extending 3.0 mm beyond the screw). The central screw hole was immediately sealed with bone wax to prevent reflux, and the scalp incision was closed with a skin clip. Less than 1% of the animals died from overdoses of anesthetic.

Some rats were killed on successive days following tumor implantation, and others were allowed to die from the effects of their tumor so that symptoms of tumor growth and length of survival could be recorded. In several experiments, the weights of the rats were recorded daily during the last 10 days before death. Brains were removed from all rats and were studied grossly and microscopically.

Cell Kinetics

In Vitro. Thirty-one 3-oz prescription bottles, each containing a glass coverslip, were seeded with 40,000 rat glioma cells contained in 10 ml of Eagle’s basal medium. The cultures were incubated at 37°.

Starting on Day 1 after passage, and each day thereafter, the coverslip was removed from 1 bottle and incubated in TdR-3H (1 μCi/ml) for 15 min. After fixation in methyl alcohol, the coverslip was prepared for autoradiography. The labeling and mitotic indexes were determined microscopically. The remaining cells in each bottle were trypsinized and counted with the use of a standard hemocytometer. These cell counts were plotted to permit determination of the doubling time in vitro.

On Day 5, the remaining 26 bottles were divided into 2 groups of 13 bottles. In 1 group of cultures, one-half of the medium was replaced each day (5 ml of old medium were extracted and 5 ml of fresh medium were added). Cultures in the other group received no fresh medium during the entire experiment.

The study was continued for the next 21 days, and the culture from 1 bottle from each group was incubated with TdR-3H each day, or every 2 or 3 days, depending on the results at that time.

In Vivo. Adult male CDF rats received tumor implants by the method described previously. On the 26th day after implantation, the rats received an i.v. injection of 200 μCi TdR-3H. Three rats were killed every 1.5 hr thereafter, beginning with the 3rd hr and ending at the 30th hr after the administration of TdR-3H. The brains were removed and fixed in formalin. Autoradiograms of the tumor and adjacent brain sections were prepared. The labeling index and the percentage of labeled mitoses were determined for each tumor. From these data, the generation time, growth fraction, and cell-loss factor of the tumor were calculated.

The tumor-mass doubling time was determined in 3 separate experiments. In each, 2 to 4 tumor-bearing rats were killed on successive days (from Day 14 through Day 28), and their tumors were removed, freed of adherent brain, and weighed.

Chemotherapy Trials

The animal model was prepared as previously described. BCNU was obtained in vials, each of which contained 100 mg of BCNU and 400 mg of mannitol. For reconstitution, 3 ml of absolute ethanol and then 27 ml of sterile water were added to the contents of the vial. The drug was administered immediately upon reconstitution. Five separate experiments were done to determine the effectiveness of BCNU in prolonging the survival of tumor-bearing rats.

Experiment 1. Twenty-four tumor-bearing rats were randomly divided into 3 groups. Nine days after tumor implantation, 8 rats received 5.2 mg of BCNU per kg [40% of the LD₁₀ (19)] i.p. The same dose was repeated on Day 16. The 2nd group of 8 rats received 10.4 mg of BCNU per kg
RESULTS

Development of Animal Model

The tumor grew well in cell culture, providing sufficient cells for 2 transplantation studies (25 rats each) per week. The morphology of the tumor in cell culture was quite similar to that of cultured human astrocytomas (34) (Fig. 1).

The median survival of the animals allowed to die from their tumors changed slightly from experiment to experiment (Table 1). We attribute variation in the survival of untreated animals in different experiments to changes occurring in the stock cultures during continuous cultivation over a period of 1.5 years. Death in tumor-bearing animals was preceded by a definite symptomatic period of 5 to 6 days. Signs consistently observed were (a) weight loss of 5 to 10 g/day; (b) increased tear secretion and red pigmentation around the eyes; (c) paresis (progressing to paralysis), starting in the right side and becoming bilateral, first involving the hindlegs and not always appearing in the forelegs before death; and (d) generalized convulsions.

Autopsy revealed that death was caused by caudal displacement of the brain stem and cerebellum through the foramen magnum. Intracerebral tumor was present in all 500 animals. In a few, s.d. and s.c. spread of tumor had occurred from leakage of the cell suspension during injection. The tumor was grayish, well-circumscribed, and firmer than, and easily dissected from, the surrounding brain. It was visible microscopically 7 to 10 days after implantation and macroscopically by the 14th day. Lethal tumors were 5 to 7 mm in diameter and weighed 200 to 250 mg.

The original induced tumor was densely cellular and had a finely fibrillar background (Fig. 2). The individual nuclei varied in shape from a polygon to an oval to a spindle, and an occasional multinucleated cell was present. Generally, the nuclear chromatin was evenly dispersed and nucleoli were infrequent. Mitoses were easily identified. The cell cytoplasm had poorly defined limits, often showing tapering cytoplasmic processes that blended into the fibrillar background. The amount of cytoplasm varied considerably in individual cells. A mild degree of endothelial proliferation was evident, but necrosis was not a conspicuous feature. The desmoplastic response was not significant. The histology of the original tumor conforms to that of an astrocytoma with anaplastic features (27).

After approximately 6 months in cell culture, the implanted tumor exhibited morphological changes. This change was gradual until, finally, after continuous culture for 2.5 years, the tumor produced after intracerebral transplantation was well circumscribed but not encapsulated. Although it was similar to the original tumor, it had more bizarre nuclear forms and more zones of necrosis surrounded by palisading tumor cells (Fig. 3). Staining with phosphotungstic acid-hematoxylin demonstrated the glial nature of this portion of the tumor, although relatively few fibers stained positively. In addition to this glial component, there were, coursing through the tumor, interdigitating bundles of spindle cells that had vesicular, elongated nuclei with more prominent nucleoli. These cells manifested somewhat less pleomorphism than did those of the distinctly glial component and were associated with abundant reticulin and considerable collagen production. Mitoses could be demonstrated in this component, although less frequently than in the glial component. In some areas, the 2 cell types intermingled closely. The profound desmoplastic response exceeded the limits of a purely reactive process and, on the basis of histological features, was best interpreted as neoplastic. The presence of the 2 distinct but closely intermingled cell types satisfied our criteria for classifying a tumor as a mixed glioblastoma multiforme and sarcoma, or a gliosarcoma.

Cell Proliferation Kinetics

In Vitro. Chart 1 shows the in vitro growth curves for the induced rat glioma. The dark solid line represents the cell population that received one-half new medium starting on Day 5; the light solid line represents the unfed group. The lower broken lines on the chart indicate the pulse labeling of cells from each culture.
Animal Model for Brain Tumor Chemotherapy

Cells in unfed cultures grew exponentially to 1.5 million cells per 3-oz. prescription bottle. The population remained at this point from Day 7 to Day 22, when it began to fall steadily. Cultures given one-half new medium each day (starting on Day 5) grew exponentially to a population of 15 million cells per bottle. After remaining at a plateau for 4 days, the cell population decreased to 1.5 million cells per bottle on Day 18. Shortly thereafter, a gradual increase was noted, until the cell population again reached 15 million cells per bottle.

Chart 1 indicates that the labeling index (lower lines) was high during exponential growth and was moderate thereafter in cultures that received new medium and gradually declined thereafter in unfed cultures.

Although a grain index was not determined on these cultures, it was evident that the average number of radioactive grains per cell in the unfed cultures was less than the average grain count in cells of fed cultures. Also, the number of mitotic figures was high during exponential growth, moderate thereafter in fed groups, and low thereafter in starved cultures.

From Chart 1, doubling time of the cell population during the exponential phase can be estimated at 18 hr.

In Vivo. We scored labeled mitoses in tumors receiving TdR-3H on Day 26 after implantation to determine the basic kinetic parameters of the tumor in vivo. Chart 2 shows the labeled mitoses curve constructed from this study. The cell-cycle time (T_c) was calculated as the period between the 0.5 values of the ascending arms of the 1st and 2nd waves of the labeled mitoses curve. The period for the S phase (T_s) was taken as the time between the 0.5 intercepts of the ascending and descending arms of the 1st wave. The time interval between injection of TdR-3H and 0.5 value of the ascending arms of the 1st wave was equal to \( T_{G_1} + \frac{1}{2} T_M \) (the length of the G1 phase + one-half the time of mitosis).

\[ T_M \] is estimated, which does not introduce serious error (14). We calculated the length of G1 phase (\( T_{G_1} \)), using the relationship of \( T_{G_1} = T_C - (T_S + T_{G_2} + T_M) \). From these calculations, the following kinetic parameters were obtained:

\[ T_C = 20 \text{ hr}, \quad T_S = 10 \text{ hr}, \quad T_M = 1 \text{ to } 2 \text{ hr}, \quad T_{G_1} = 6 \text{ to } 7 \text{ hr}, \quad T_{G_2} = 2 \text{ to } 5 \text{ hr}. \]

The growth fraction of the tumor was calculated by the use of Cleaver's formula (6). In this formula, the growth fraction (GF) equals the ratio of observed labeling index (LI) to theoretical labeling index (TLI). The observed labeling index is between 15 and 20%, and the theoretical labeling index is equal to \( (T_S/T_C) \log 2 + \frac{1}{2} [(T_S/T_C) \times \log 2]^2 + [(T_S \times T_{G_2})/T_C^2] \) (log 2)^2. The following calculations show how the growth fraction was determined:

\[ GF = \frac{LI}{TLI} = 0.35 \text{ to } 0.46. \]

Chart 3 is a plot of the weights of tumors removed on successive days, from Day 14 through Day 28. From these weights, it is evident that the observed doubling time (\( T_d \)) of the tumor in vivo is approximately 72 hr.

The last kinetic parameter to be determined was the cell-loss factor (CLF), calculated by the method presented by Steel in 1968 (32):

\[ CLF = 1 - \frac{T}{T_d} \]

\[ T_d = 72 \text{ hr} \]

To derive \( T \) (potential doubling time):

\[ T = k \frac{T_S}{LI} \]

\[ LI = 17.5\% \]

\[ T_S = 10 \text{ hr} \]

\[ k = 0.77 \] [method of Steel (32)]

\[ T = 42 \text{ hr} \]

\[ CLF = 1 - \frac{42}{72} = 0.42 \]

Chemotherapy Trials

Experiment 1. All untreated control rats died during the 4th week, giving a median survival time of 25 days with a survival range of 8 days.

Rats receiving BCNU equivalent to 40% of the LD10 had a median survival time of 34.5 days with a survival range of 20 days and an ILS of 38%.
DISCUSSION

The ideal experimental model of human brain tumors should satisfy the following criteria. (a) The tumor should be derived from cells existing in normal brain. (b) The tumor should be serially transplantable to the animal brain. (c) The tumor take should be predictable and reproducible. (d) The survival span should be long enough to permit therapy well after the implantation procedure. (e) The host animal should be suitable for all clinical routes of therapy.

In this study, the original induced tumor was a typical malignant astrocytoma. The tumors resulting from implantation of the cultured cells remained typical astrocytomas until sarcomatous elements were noted after 6 months of continuous culture. More recently, we avoided this morphological transformation by freezing large quantities of tumor cells from early cultures and by discarding tumor cultures after 6 months of continuous cultivation.

The ease with which the tumor may be transplanted to the brain satisfies one of the major criteria for such an animal model.

The tumor take (100%) of this model is surprisingly good, since there is an incidence of “no take” even in the best of systems. The median range of survival among different groups of untreated tumor-bearing rats varied, in part because of technical errors (e.g., cell count) and the proportion of viable cells implanted. We believe that the major factor contributing to the changing survival was a biological change in the cell cultures over a period of 18 months. The increasing participation of sarcomatous cells supports this view.

The survival time of more than 20 days allows the administration of drugs well after the immediate postimplantation period. Treatment during the first few days after implantation is generally believed to be of limited value in predicting the chemotherapeutic response of solid tumors, although it may have value in the screening of unknown agents. When effective drugs are being analyzed for the most suitable routes and scheduling, such analysis is best delayed until a growing, well-vascularized tumor can be attacked. A treatment schedule ending near the onset of neurological signs in the control animals would most closely resemble the clinical situation.

In most of the chemotherapy studies on animal models, treatment has closely followed implantation (5, 20, 25, 26, 28, 31). Although survival has been prolonged in most of these studies, it does not necessarily follow that this was the direct result of oncolytic activity against growing tumor cells. In our model, the tumor cell mass was not microscopically evident before the 7th day after implantation and was not grossly visible before the 12th day. Treatment in the 1st week after transplantation may interfere with tumor take rather than inhibit tumor cell growth. In the 1st 2 weeks after transplantation, all of the viable tumor cell population almost certainly exhibits log phase growth, and the effect of drug on this population of cells differs substantially from its effect on older (and larger) tumors that contain noncycling cells. Beyond the 14th day, the tumor assumes the kinetic characteristics of brain tumors in the clinical situation.

Another advantage of this particular model is that the
Table 2
Statistical analysis of effect of BCNU on survival of rats bearing brain tumors
All BCNU-treated animals received 80% of an LD10, unless otherwise indicated.

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of animals</th>
<th>Survival time (days)</th>
<th>Median survival (days)</th>
<th>Range (days)</th>
<th>ILS (%)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>8</td>
<td>24.5 ± 3.16c</td>
<td>25</td>
<td>8</td>
<td>38</td>
<td>0.0001</td>
</tr>
<tr>
<td>BCNU</td>
<td>8</td>
<td>37 ± 11.3</td>
<td>34.5</td>
<td>20</td>
<td>84</td>
<td>0.0001</td>
</tr>
<tr>
<td>2. Control</td>
<td>10</td>
<td>29.1 ± 4.72</td>
<td>28</td>
<td>14</td>
<td>41</td>
<td>0.0001</td>
</tr>
<tr>
<td>BCNU</td>
<td>12</td>
<td>40 ± 6.8</td>
<td>40.5</td>
<td>23</td>
<td>41</td>
<td>0.0001</td>
</tr>
<tr>
<td>3. Control</td>
<td>12</td>
<td>29.75 ± 6.56</td>
<td>28</td>
<td>25</td>
<td>41</td>
<td>0.0001</td>
</tr>
<tr>
<td>BCNU</td>
<td>12</td>
<td>39.75 ± 7.4</td>
<td>39.5</td>
<td>25</td>
<td>41</td>
<td>0.0001</td>
</tr>
<tr>
<td>4. Control</td>
<td>10</td>
<td>35.8 ± 7.64</td>
<td>36.5</td>
<td>25</td>
<td>75</td>
<td>0.0001</td>
</tr>
<tr>
<td>BCNU</td>
<td>8</td>
<td>66 ± 19</td>
<td>64</td>
<td>56</td>
<td>47</td>
<td>0.0001</td>
</tr>
<tr>
<td>5. Control</td>
<td>10</td>
<td>37.4 ± 4.76</td>
<td>36</td>
<td>15</td>
<td>47</td>
<td>0.0001</td>
</tr>
<tr>
<td>BCNU</td>
<td>10</td>
<td>55.3 ± 10.7</td>
<td>53</td>
<td>47</td>
<td>47</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* Determined by Wilcoxon rank sum test.
© Received 40% of an LD10.
* Mean ± S.D.

animal is large enough to permit alternative routes of drug administration, e.g., intrathecal (38) and arterial injection or infusion (22).

This animal model has been used to test the antitumor effect of several drugs. Gurcay et al. (12, 13) have shown that methylprednisolone acetate given on Days 10, 13, and 19 postimplantation reduced the weight of tumors removed on Day 21 from an average of 157.5 mg in the controls to an average of 36.25 mg in the drug-treated animals. In another experiment, they found the average weight of control tumors to be 113 mg, while the average weight of methylprednisolone-treated tumors was 34.1 mg. Control animals in this experiment survived an average of 28.7 days; treated animals survived an average of 37.4 days.

The proliferation characteristics of these rat glioma cells in culture varied according to the quantity of nutrients in the medium and the availability of a surface on which to grow. The tumor cells grew exponentially to a population of 1.5 million cells per 3-oz prescription bottle (available growth area, 30 sq cm). Then, if the medium was not changed, the population became stationary. Replacing one-half of the medium each day permitted proliferation to a population of 15 million cells per 3-oz bottle. At this population, the growth space became exhausted, and a stationary phase resulted for a brief period. A sudden decrease in cell population to 1.5 million cells per bottle was then noted. If one-half of the medium was replaced each day, the population gradually increased again to 15 million cells. Thus, the growth pattern of these cultures could be classified in 3 categories, namely, cells in exponential growth, cells in a stationary phase due to starvation, and cells with variable proliferation due to overcrowding.

Although the number of mitoses was not counted, the frequency of mitosis appeared to be very high during exponential growth, moderate thereafter in the cultures fed daily, and quite low thereafter in the unfed cultures. Surprisingly, the unfed cultures continued to exhibit a considerable number of labeled cells, although the labeling index was considerably decreased. However, the grain index was considerably lower in the unfed cultures, an indication that DNA synthesis had slowed.

The kinetic parameters of the rat glioma in the rat brain could not be determined during the early stages of tumor growth, since tumor cells could only rarely be identified before the 12th day postimplantation. Therefore, kinetic characteristics were obtained on a tumor in the late stages of proliferation and just before the onset of signs in the animal host. The long doubling time and the high cell loss of this tumor indicate its similarity to the human brain tumor.

Information concerning cell population kinetics should provide a more rational basis for the design of drug schedules in brain tumor chemotherapy. This is true not only for phase-dependent drugs but also for drug combinations, e.g., a cell-cycle-nonspecific drug such as BCNU and a cell-cycle-specific drug such as vincristine sulfate (23). In this example, vincristine sulfate could be scheduled to arrest the proliferation of cycling cells in the intervals between pulses of BCNU. Combination chemotherapy can be applied to the rat model and, with additional knowledge of the kinetic parameters of human gliomas, schedules successful in the laboratory can be translated into clinical chemotherapy.

It is evident from these experiments that BCNU, when given in tolerated doses i.p., not only increased the survival of rats bearing intracerebral gliomas if given on Days 9 and 16, but also produced a significant ILS if given as late as the 16th and 23rd days. This was true even in rats that already had neurological signs when first treated. These results not only confirm the findings of Schabel et al. (26), Chirigos et al. (5), Ausman et al. (1, 2), and Shapiro and Ausman (29), but indicate as well that BCNU is just as effective against an older, larger tumor that contains many nonproliferating cells.

Of major interest are the 4 BCNU-treated animals that were
References


Fig. 1. Rat glioma in early cell culture. Giemsa, x 280.
Fig. 2. Original, induced rat glioma. H & E, x 140.
Fig. 3. Tumor produced in the brain of a rat, with cells cultured for more than 6 months. H & E, X 140.
Fig. 4. Morphological appearance of a glioma from an untreated animal. H & E, X 140.
Fig. 5. Morphological appearance of a glioma from a BCNU-treated animal. H & E, X 140.
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