SUMMARY: A simple and reproducible injection technique has been developed for inducing an in vivo experimental astrocytoma model in rats. Newborn rats were injected with a suspension of astrocytoma C-6 cells in doses ranging from 5 to $1 \times 10^4$ cells. The optimum dose of tumor cells was found to be between $10^4$ and $10^5$ cells, with a consistent 100% take rate seen above $1 \times 10^4$ injected cells. The injection of less than $10^4$ cells resulted in a decreased ability to induce tumors, and a prolonged survival.

The pathology was consistent with that of a glioblastoma multiforme. The tumor showed a diffuse infiltrating border, necrosis, and pseudopalisading. Light microscopy revealed undifferentiated tumor cells while electron microscopy demonstrated rough endoplasmic reticulum, Golgi complexes, mitochondria, and the occasional cilium and centriole. The nature of the astrocytoma C-6 cell line, and the advantages, disadvantages and possible uses of the model are discussed.

INTRODUCTION

Radiation, viruses and chemical carcinogenesis are the three methods of experimental cerebral tumorgenesis commonly employed (Janisch and Schreiber, 1977). These methods do not easily offer the opportunity to create large numbers of animals bearing identical tumors in similar intracerebral locations. Carcinogen implantation, although resulting in the generation of experimental astrocytomas requires a craniotomy with its attendant morbidity and mortality and results in a host of tumors with unpredictable biological activity only after prolonged and variable latent periods (Janisch and Schreiber, 1977). Serial syngeneic tumor transplantation is also cumbersome requiring repeated craniotomies and at times living tumor-bearing animals to carry the tumor line. These factors hamper the production of large numbers of tumor-bearing animals.

Astrocytoma C-6 is a well established in vitro cell line initially induced in rats by N-nitrosomethylurea (Benda et al, 1968) and thereafter extensively characterized (Bissell et al, 1974, Parker et al, 1980). It was the goal of this study to examine the ability of these cells to induce a simple and reproducible in vivo glioma model.

MATERIALS AND METHODS

1. Induction of Intra-Cerebral Tumor:

One hundred and eight Wistar rats of both sexes from thirteen litters were used. Inoculation with astrocytoma C-6 cell suspensions was performed in the first 48 hours of life in all litters. The C-6 cells were stored at -70°C in Minimal Essential Medium (Eagle, 1955: MEM, Flow Labs Inc., Mississauga, Ont.) supplemented with 10% fetal bovine serum and 10% Dimethylsulfoxide (DMSO). Culture was re-established after thawing by incuba-
tion in MEM, Penicillin G (100 Units ml⁻¹) and Streptomycin (100 μg ml⁻¹). This medium was supplemented with 5% fetal bovine serum which was complement-deactivated at 56°C. At confluence, cells were harvested after trypsinization with 0.25% trypsin (GIBCO Canada, Burlington, Ont.) centrifuged for one minute at 650 g., and resuspended in MEM. The number of resuspended cells, and the volume of diluant MEM could each be adjusted to give the desired inoculum of cells in the desired volume. Cell counts were verified using a haemocytometer.

A 10 microliter syringe (Hamilton Co., Reno, Nevada) was used to perform intracerebral inoculation into the right parietal lobe of newborn immunologically incompetent rats. The thin skull at this age permitted direct trans-calverial inoculation via the needle, obviating the need for craniotomy. If the needle shaft was cleansed with an alcohol swab between injections, and volumes of 2μl or less were used, extra-cerebral growth fell to zero.

2. Light Microscopy:
In initial experiments, rats were sacrificed by chloroform overdose. The brains were removed, and fixed in 20% formalin. Eight micron paraffin sections were stained with hematoxylin and eosin, phosphotungstic acid hematoxylin, and cresyl violet.

3. Electron Microscopy:
Animals were first anesthetized with twice the dose of sodium pentobarbital (Abbot Laboratories, Montreal) and Chloral Hydrate (Matheson, Coleman and Bell, Norwood, Ohio) necessary for surgical anesthesia (Valenstein, 1961). A midline thoracotomy was quickly performed, and the aorta was cannulated via the left ventricle.

The following solution was perfused via gravity from a height of 100 cm. for 20 minutes; 1% glutaraldehyde, 1% paraformaldehyde, 5% sucrose and 0.1 M sodium cacodylate buffer at pH 7.4. This was followed by perfusion of an identical solution except for concentrations of 2.5% glutaraldehyde and 2.0% paraformaldehyde. The brains were removed, and sectioned coronally at 2 mm thickness. Tumor and peritumoral areas were sampled and post-fixed for 90 min. in a solution containing 1% osmium tetroxide, 0.1 M sodium phosphate at pH 7.3, and 5% sucrose. Tissues were then treated overnight in 2% uranyl acetate (Fisher Scientific, Fair Lawn, New Jersey), embedded in Spurr (Taab Laboratories, Reading England) and examined with a Philips E.M.-201 electron microscope.

<table>
<thead>
<tr>
<th>LITTER</th>
<th>NO. OF CELLS INJECTED</th>
<th>MEDIAN SURVIVAL</th>
<th>TUMOR TAKE RATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 X 10⁵</td>
<td>16</td>
<td>6/6 100%</td>
</tr>
<tr>
<td>2</td>
<td>1 X 10⁵</td>
<td>18</td>
<td>9/9 100%</td>
</tr>
<tr>
<td>3</td>
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<td>10/10 100%</td>
</tr>
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<td>4</td>
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<td>9/9 100%</td>
</tr>
<tr>
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<td>1 X 10⁴</td>
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<tr>
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</tr>
<tr>
<td>8</td>
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</tr>
<tr>
<td>12</td>
<td>1 X 10</td>
<td>—</td>
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</tr>
<tr>
<td>13</td>
<td>5</td>
<td>—</td>
<td>0/3 0%</td>
</tr>
</tbody>
</table>

Figure 1—Tumor take rate in percent (ordinate) vs. number of cells injected in the inoculum (abscissa) yields a sigmoid shaped curve. Although doses near 10³ cells occasionally give rise to tumors, doses of 10⁴ cells and higher produce consistently high take rates.
RESULTS

A definite symptomatic period of 3 to 4 days preceded death in tumor-bearing animals. Clinical signs consistently observed included rough fur, lethargy, generalized convulsions and various degrees of hemiparesis. Since the study was intended to examine factors influencing tumor production and subsequent survival, rats were allowed to succumb from their tumor or were sacrificed when clearly moribund.

1. Volume of Inoculate:

In initial experiments litters were inoculated with 20 to 40 ul volumes of cells. This large volume produced reflux around the syringe with concomitant loss of inoculum, growth of tumor in the scalp, and marked deviation from the pattern of survival of subsequent injections. The inoculation of 1 or 2 µl volumes were used subsequently, which resulted in no reflux, and solely intracerebral tumor growth.

2. Age at Time of Injection:

All litters in this study were injected within 48 hours of birth. However in other experiments one litter was injected with only 300 cells at age five days: three of the litter of eight succumbed to tumor which became manifest after long latencies of 48, 48, and 54 days respectively (µ = 50 days).

Tumor did not grow in adult rats when does of 10^5 cells were injected intracerebrally via craniotomy, into the interscapular connective tissue, or into the peritoneum.

3. Dose of Cells:

Figure 1 shows the number of cells injected vs. the tumor take rate, and yields a sigmoid shaped curve. Survival times are seen in Figure 2. Doses higher than 2 x 10^4 cells were redundant, but shortened survival in litters 1, 2 and 3 (Fig. 2). No dose of 100 cells or less yielded tumor induction at any age studied (Table 1).

Figure 2 — Number of cells injected in inoculum (ordinate) vs. survival time (abscissa) yields a negatively sloping set of points.

Figure 3 — Gross appearance of tumor, demonstrating necrotic and hemorrhagic intracerebral growth, with massive right to left brain herniation. There is mild hydrocephalus of the left lateral ventricle.
4. Gross Neuropathologic Findings: The tumor was yellow-grey, well-circumscribed and gelatinous with occasional hemorrhage. The tumor grew as an intracerebral mass lesion resulting in significant herniation (Fig. 3) and occasionally causing marked hydrocephalus. Subarachnoid and intraventricular tumor was occasionally observed.

5. Light microscopy: The tumor grew as a highly malignant astrocytoma invading brain, with necrosis and pseudopalisading (Fig. 4) akin to that seen in human glioblastoma multiforme. There was brain shift and herniation due to massive intracerebral growth (Fig. 5) and in addition, subarachnoid and intra-ventricular spread was seen in some cases. Proteinaceous eosinophilic edema fluid (Fig. 6) was seen, but more commonly the associated edema fluid was clear. Vascular hyperplasia and hypertrophy was present in the tumor. Cytologically, the tumor cells were undifferentiated, with no PTAH positive fibrils. Mitotic figures were numerous (Fig. 6). There was a pronounced tendency for tumor cells to grow around blood vessels, seen more easily at the tumor interface with brain. (Fig. 7).

6. Electron Microscopy: The extracellular space was greatly expanded in the tumor and immediate peri-tumoral region (Fig. 8). Tumor cells showed the following cytoplasmic organelles; ribosomal rosettes, rough endoplasmic reticulum, Golgi apparatus, centrioles, microfilaments, mitochondria and the occasional solitary cilium with basal body (Fig. 9). Tight junctions in the tumor-induced vasculature were incompletely formed (Fig. 10) but were intact in the peri-tumoral tissue.

DISCUSSION
Barker et al (1973) used a nitrosomethylurea-induced rat glioma to demonstrate the chemotherapeutic effectiveness of 1,3-Bis (2-chloroethyl)-1-nitrosourea (BCNU). Serial transplantation via craniotomy was used to propagate the tumors. Waldbaur et al (1978) produced a glioblastoma multiforme in rats after 271 days using weekly intravenous injections of methyl nitrosourea, and used serial transplantation for the purpose of chemotherapeutic trials with CCNU. Druckrey et al (1965) found that the carcinogenic effect of the nitrosoureas...
was selective for neur ectodermally derived tumors, and that this selective effect persisted transplacentally (Druckrey et al, 1966, Ivankovic and Druckrey, 1968).

Astrocytoma C-6 is a tumor cloned for its high content of the brain protein S-100 (Moore et al, 1968, Cicero et al 1970, Haglid et al, 1971), from a tumor originally induced by N-nitrosothiapropylurea (Benda et al, 1968). It has been extensively characterized biochemically, and most of its features are those of an astrocytoma. Transdifferentiation in serial cultures of C-6 cells from oligodendrocytic to astrocytic enzyme markers was observed by Parker et al (1980). Serial passages revealed an increase in the astrocytic marker enzyme glutamine synthetase, and a decrease in the oligodendrocytic marker enzyme cyclic nucleotide phosphohydrolase (Zanetta et al, 1972). Mixed gliomas with differentiating potential for more than one glial lineage; astrocytic, oligodendrocytic and ependymal, have long been recognized in human tumor biology (Bailey and Cushing, 1926, Hart et al, 1974). The multi-potential nature of transplantable experimental gliomas, and their dependence on environment for expression of varying potential lines of differentiation has been stressed by Zimmerman (1955). Netsky (1963) categorically stated that “pure gliomas do not exist”, in his study of experimental gliomas.

C-6 cells have been found to be a rich source of the acidic brain protein S-100 (Moore et al, 1968), which is specific for astrocytes (Cicero et al, 1970, Haglid et al 1971). C-6 cells have produced glial fibrillary acidic protein in organ culture systems (Bissell et al, 1974). Astrocytic differentiation has also been observed in the form of increased foot processes, microtubules, and Golgi apparatus under the influence of the DNA and RNA synthesis inhibitor amphoteronin B, and accumulation of glycogen under the influence of the DNA-complexing agent Actinomycin D (Reich, 1963, Silbert and Goldstein, 1972). Synthesis of acid mucopolysaccharides (Dorfman and Ho, 1970) has also been observed. The presence of ribosome-studded rough endoplasmic reticulum and Golgi complexes is morphologic evidence of continued synthetic and secretory capability of the C-6 line in our in vivo model. It is common to find ciliary profiles in normal astrocytes, usually only one to a cell (Peters et al, 1976), and hence this finding in our tumor cells is compatible with astrocytic morphology, and does not necessarily imply ependymal differentiation.
Other gliomas have been induced with nitrosomethylurea, and have been propagated in culture (Benda et al, 1971). These tumors have also had glial characteristics, including astrocytic morphologic features, and the synthesis of S-100 protein. Stereotactic injection of a variety of nitrosourea-induced neurectodermal tumors was used by Kobayashi et al (1980) to produce spheroidal tumors in the head of the caudate nucleus. They used 1% agar in a 10 μl injection volume to minimize extracerebral growth, whereas in the present study this was achieved by reducing the injection volume to 1 or 2 μl, and by careful cleansing of the outside of the syringe needle with alcohol between injections. Kobayashi et al (1980) found a similar optimum tumor induction dose of between $10^4$ and $10^5$ cells while Mandybur (1981) needed $10^5$ cells to induce brain metastases via intra-arterial injection.

The disadvantages of the tumor model included its extreme malignancy and the variable position of the tumor using freehand injection. The latter can be controlled using a stereotaxic approach (Pellegrino et al, 1979, Kobayashi et al, 1980).

Advantages of the described tumor model are that it is simple to induce, and frozen cells may be stored indefinitely until needed without constant passage in animals. The trans-calvarial injection method is reliable and permits the cheap and rapid production of large numbers of tumor bearing animals. The astrocytoma C-6 cell line has been very extensively studied and many of its in vitro characteristics are known.

In summary this model may be useful to study intrinsic properties of brain gliomas and the effectiveness of various treatment modalities.

ACKNOWLEDGEMENTS

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Figure 10 — Tumor-induced capillaries, with lumen at top (L). Intereendothelial tight junctions (zonulae occcludentes) are variably incomplete (arrows). Intact tight junction on left. The extracellular space (E) is expanded. Uranyl acetate, X 200,000. Bar = 50 nm.

REFERENCES


