Chemotherapy of Experimental Metastatic Brain Tumors in Female Wistar Rats

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ABSTRACT

"Metastatic" brain tumors were produced in female Wistar rats by intracarotid inoculation of Walker 256 carcinoma cells and subsequent treatment with small doses of cyclophosphamide (CTX) which eradicated extracranial tumors, giving time for intracranial tumors to grow. Seven drugs currently in use for brain tumor chemotherapy were tested using this model. After toxicity for each drug was determined, symptomatic animals were treated with each drug 28 to 39 days after tumor inoculation, and their survival was compared to untreated or vehicle-treated controls. 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea and a new water-soluble nitrosourea, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea, along with CTX were very effective and increased median survival time 135 to 213% of control. Methotrexate was minimally effective if given in a single i.v. dose, and procarbazine was ineffective. Dexamethasone was also ineffective at prolonging survival. The value of i.v. methotrexate and also of CTX in the therapy of this disease suggests that once metastatic brain tumors have developed, blood-brain barrier phenomena do not impede the entry of such agents into the tumor. Nevertheless, despite therapy, all animals eventually died of massive multiple intracranial metastatic tumors. Similar results were obtained in rats directly inoculated i.c. with tumor and in rats inoculated s.c. The facts that the metastatic brain tumor-bearing animals were not cured and that systemic therapy alone could not prevent the development of the metastatic tumor may mean that the blood-brain barrier is important along the edge of growing tumors and in the intact brain where metastases develop.

INTRODUCTION

Malignant brain tumors in adults are either primary, arising within the brain substance, or metastatic, spread from distant sites via the blood stream. Therapy of metastatic brain tumors is rarely curative. Therapy includes steroids, surgical excision, irradiation, and chemotherapy for the primary tumors (9) and usually steroids and irradiation (rarely surgery) for the metastatic tumors (8). Attempts to improve the therapy of primary tumors have included increasingly complex chemotherapy (11), but there have been few attempts to fashion new therapeutic approaches to metastatic brain tumors. Furthermore, it is difficult to evaluate the therapy of cerebral metastases in patients because they often succumb to systemic disease even if the brain tumor is successfully treated.

While numerous animal brain tumor models have been developed to assist in choosing new chemotherapeutic agents for primary brain tumors, none can be used directly to test for hematogenous metastatic disease because they depend on direct tumor (or viral) implantation into the brain which necessarily traumatizes brain tissue. In fact, this mechanical disruption of the blood-brain barrier calls into question the significance of all chemotherapy results obtained in such models. In order to: (a) circumvent the problem of mechanical disruption of brain tissue so as to examine blood-brain barrier function in brain tumors; and (b) develop a system to test agents specifically for metastatic brain tumors, we developed an animal model of metastatic brain tumor that does not require direct implantation into the brain (15). The model consists of the intracarotid inoculation of the Walker 256 carcinoma into Wistar rats, followed by treatment in 14 days with low-dose cyclophosphamide. The chemotherapy reduces the incidence of extracerebral disease, permitting the development of brain tumors. The massive and multiple intracranial metastatic lesions which develop kill animals shortly after the appearance of symptoms.

Having established this model of metastatic brain tumor, we undertook to test several chemotherapeutic regimens. We included therapy of s.c.-implanted tumor to circumvent entirely the question of blood-barrier interference and to compare those results with those of treatment of the intracerebral tumor. We also treated i.c. implanted tumor to compare 2 different animal models in response to chemotherapeutic agents and to circumvent the possibility that we may produce cyclophosphamide-resistant tumor by pretreating with low-dose cyclophosphamide. The rationale for the choice of drugs was as follows. Corticosteroid hormones (dexamethasone) are given to all patients with metastatic brain tumor and have been thought by some to be chemotherapeutic in themselves (13). Lipid-soluble nitrosoureas (e.g., CCNU and methyl-CCNU) have been used successfully in the treatment of primary brain tumors. A new water-soluble nitrosourea (ACNU) has been recently found to be effective against a primary experimental brain tumor model (4). We also chose a drug to which the tumor is highly sensitive (cyclophosphamide) and one to which it is relatively resistant but water soluble (MTX). Finally, we also evaluated a drug which crosses the blood-brain barrier and which has been used in the treatment of primary brain tumors (procarbazine) (17).

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MATERIALS AND METHODS

Tumor Models

The method for the production of the metastatic brain tumor model has been described in detail elsewhere (15). Briefly, female Wistar rats weighing approximately 150 g are anesthetized with sodium pentobarbital (40 mg/kg i.p.), and the right common carotid artery is exposed under a dissecting microscope. Using a 32-gauge needle, the right common carotid artery is inoculated with 0.01 to 0.02 ml of a tumor cell suspension of Walker 256 carcinoma cells containing 0.5 to 1 x 10⁶ viable cells. On the 14th day following inoculation, the animals are treated with a single injection of cyclophosphamide (15 mg/kg i.v.). Animals are observed daily and graded according to their neurological condition: 0, normal, running actively; 1, lethargic but runs with stimulation; 2, lethargic, does not run but can move about; 3, still upright but may have paresis; 4, moribund, unable to stand. They are weighed every other day. Most of the animals regain weight after treatment with cyclophosphamide but begin to lose weight about Day 28 after initial inoculation. In the experiments reported here, at the time the rats were losing weight and when they had reached Grades 1 to 3, they were divided into control and treatment groups of similar weight and grade. The treated animals received the drugs as described below; the controls received 0.9% NaCl solution or vehicle.

In the s.c. studies, the tumor was implanted into the right axilla of Wistar rats weighing 150 g. The rate of tumor take was 77%. For chemotherapy trials, groups of rats were divided into control and treatment groups and treated on Day 8 after s.c. inoculation. The tumor size was followed by measuring the long (A) and short (B) axis of the tumor weekly, and the tumor volume was calculated as follows:

\[ Volume = \frac{4}{3} \pi \left( \frac{B^2}{2} \right) \times \left( \frac{A}{2} \right) \]

In the i.c. tumor studies, a 1-cu mm fragment of tumor was inoculated p.c. with a modified 19-gauge spinal needle into the right cerebral hemisphere of Wistar rats. The rats began losing weight about Day 14 after tumor inoculation and clinically became Grades 1 to 3 by Day 16. On Day 16, sick animals were randomly divided into 5 groups of 5 rats each. One group, the controls, were treated with 0.9% NaCl solution; the other 4 were treated with ACNU, CCNU, MTX, and cyclophosphamide.

Drugs

The drugs, methods of administration, and dose schedules were as follows.

Cyclophosphamide. Cyclophosphamide was supplied by Mead, Johnson & Co. (Evansville, Ind.) in vials containing 200 mg of the drug plus 90 mg of sodium chloride and was made up by dissolving it in 20 ml of sterile water. It was injected i.v. via the tail vein. For the metastatic tumor model, it was given at a dose of 15 mg/kg and was subsequently used in therapy studies at doses of 60 and 90 mg/kg. Toxicity of cyclophosphamide was determined in normal animals using 5 rats/dose in 7 dosages between 50 and 200 mg/kg and was calculated by the method of Litchfield and Wilcoxon (7). The LD₅₀ was 185 mg/kg (154 to 222 mg/kg; 95% confidence limits), and the LD₁₀ was 132 mg/kg (110 to 158 mg/kg).

Nitrosoureas. CCNU and methyl-CCNU were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). The drugs were prepared according to the method of Davignon et al. (2) for i.v. use. CCNU was kept on ice and immediately before its use was dissolved in 24% Emulphor El 620 (GAF Corp., New York, N. Y.)/ethanol in dextrose solution to make 6 mg of CCNU per ml of vehicle. Methyl-CCNU was kept on ice and immediately before use was dissolved in 12% Emulphor El 620/ethanol in dextrose solution to make 6 mg of methyl-CCNU per ml of vehicle. ACNU was supplied by Sanko Co. (Tokyo, Japan) and dissolved in sterile water. The toxicity of the nitrosoureas in normal animals was as follows: For CCNU, the LD₅₀ was 68 mg/kg (55 to 83 mg/kg), and the LD₁₀ was 48 mg/kg (39 to 58 mg/kg) (test range, 50 to 150 mg/kg in 5 dosages; 5 rats/dose). For methyl-CCNU, the LD₅₀ was 59 mg/kg (53 to 65 mg/kg), and the LD₁₀ was 50 mg/kg (45 to 55 mg/kg) (test range, 50 to 150 mg/kg, in 5 dosages; 5 rats/dose). The LD₅₀ for ACNU was 46 mg/kg (41 to 51 mg/kg), and the LD₁₀ was 38 mg/kg (34 to 42 mg/kg) (test range, 40 to 150 mg/kg, in 5 dosages; 5 rats/dose). The nitrosoureas were administered according to the doses as described in Tables 1, 2, and 3 for the various experiments.

MTX. MTX was purchased from Lederle Laboratories (Pearl River, N. Y.) and dissolved in sterile water for administration. The toxicity for MTX was difficult to determine because of wide variation of animal tolerance. The calculated LD₅₀ for MTX in normal animals was 195 mg/kg by single injection i.v. (165 to 230 mg/kg), and the LD₁₀ was 145 mg/kg (122 to 171 mg/kg) (test range, 5 to 500 mg/kg, in 12 dosages; 5 rats/dose).

Procarbazine. Procarbazine hydrochloride was purchased from Hoffman-LaRoche Inc. (Nutley, N. J.) and dissolved in sterile water for injection. The LD₅₀ was 740 mg/kg (666 to 821 mg/kg), and the LD₁₀ was 650 mg/kg (585 to 721 mg/kg) (test range, 100 to 1000 mg/kg, in 7 dosages; 5 rats/dose).

Dexamethasone. Dexamethasone sodium phosphate was purchased from Merck, Sharp & Dohme (West Point, Pa.) and administered in a dose of 10 mg/kg i.m. twice daily for 3 or 10 doses (see Table 3). The drug was not toxic at these doses.

Pathological Studies

Forty-nine animals were selected at random from both control and treated groups for histological study. The animals’ heads were fixed in 10% formalin and decalcified. Multiple sections were taken and examined following staining with hematoxylin and eosin.

RESULTS

Studies (s.c.). The effect of the various drugs on the growth of s.c. tumor is shown in Table 1 and Chart 1. The dose of the various drugs is listed in Table 1. The tumor volume for tumors which were palpable is listed as a percentage of control for each of the drugs on Day 22 following inoculation, i.e., 14 days after therapy. However, many tumors were not at all palpable, and the ratio of palpable tumors versus those inoculated is also shown. However, most of the tumors regrew in another 3 weeks. As demonstrated graphically, the most effective drugs were the 3 nitrosoureas and cyclophosphamide. MTX produced a definite but transient effect at Day 14; by Day 22, the
tumor had already begun to regrow. Procarbazine retarded tumor growth so that it was smaller than controls on Day 22, but there were palpable tumors in all 5 inoculated animals.

**Tumor Inoculated i.c.** The results of chemotherapy of i.c.-inoculated tumor are given in Table 2. Listed are the dose for each drug, the median survival time after treatment of the treated group (T) and the control group (C), as well as the median life span as a percentage of control (control = 100%). The results were statistically analyzed using Gehan's modification of the Wilcoxon analysis (3). ACNU and CCNU significantly increased median survival, and some animals lived more than 60 days after inoculation. Methyl-CCNU at 2 doses increased survival from 140 to 213%, and again at the higher dose, 3 animals survived more than 60 days after inoculation. Cyclophosphamide at 60 mg/kg increased survival by 206%, but median survival at 90 mg/kg was not significantly better than at the lower dose, and 2 animals died of obvious drug toxicity. MTX increased survival 50 and 100 mg/kg, but there were no long-term survivors. Procarbazine did not increase survival significantly, nor did twice-a-day steroids. In none of the animals were there cures; almost all tumored animals died within 62 days following therapy.

**Pathological Studies.** The distribution of tumor found at
postmortem examination of 49 animals is shown in Table 4. Although we excluded rats with detectable extracranial tumor from those that eventually were treated, 67% of animals studied pathologically had extracranial tumors at postmortem examination. Most were located near the base of the skull, involving the ethmoid and sphenoid bones. One hundred % of the animals had massive multiple intracranial tumors that appeared to be the cause of death (Fig. 1). The incidence of cerebellar tumor was higher than expected considering the unilateral nature of the inoculation, but the rat circulation around the circle of Willis is such that it produces considerable anterior-to-posterior cross-circulation. Meningeal tumor spread occurred frequently, although the extent of meningeal involvement was considerably less than in models we have developed and have reported elsewhere (14, 16). Under these circumstances, tumor is injected directly into the cisterna magna, and meningeal carcinomatosis is complete within 7 to 15 days. In the current study, hydrocephalus occurred in about one-third of the animals.

A comparison was made between the pathological changes in the treated animals and those in the controls. There was no difference in the size of the CNS parenchymal tumors between treated and control groups. Cytotoxic tumor changes (necrosis, fibrous proliferation, cell transformation, and bizarre giant cells) were observed in the treated animals, but both treated rats and controls showed recurrent tumor cell proliferation. Parenchymal CNS tumors had infiltrating tumor cells in the periphery of the tumors. Similarly, jaw tumors showed invasion of surrounding tissues by actively proliferating cells. Thus, while cytotoxic changes were more evident in the treated animals in both CNS and jaw tumors, both groups of rats showed recurrent tumor infiltration in both sites.

**DISCUSSION**

Animal models of human cancer must deal with 2 problems, the pathological nature of the tumor and its location in the body. If the model is designed to help define and choose effective therapy, it must be able to predict with reasonable accuracy human tumor response to the therapy under consideration. While the problem of the pathological similarity of the animal tumor to that of humans must be solved for all cancers, the problem of the location of the tumor is especially important when dealing with brain tumors. The question may be asked whether an animal model using s.c. implanted brain tumor predicts for human brain tumor response as well as a model using brain tumor implanted directly into the brain. To approach this problem, W. R. Shapiro helped to develop an i.c. model of brain tumors using chemically induced ependymoblastomas in mice (1). Such a model examined brain tumors in situ, and the results of chemotherapy proved more predictable for human brain tumors than did those from s.c. implanted tumors. For example, mithramycin had demonstrated effectiveness against a murine glioma implanted s.c. (5) but was ineffective against an i.c. murine glioma (12). It subsequently proved ineffective when used in patients with primary brain tumors (19). These results implied that the i.c. implantation technique could more readily distinguish agents that were effective against brain tumors because they crossed the blood-brain barrier than could models utilizing s.c. implanted tumors. Nevertheless, a specific problem of any such modeling is that the brain is partially destroyed by the process of implanting the tumor into its substance. Since such trauma breaks down the barrier, it may be argued that even the direct i.c. implanted tumors would not be good models to study blood-brain barrier phenomenon in brain tumors since the implantation process already broke the barrier. To answer this criticism, we developed the metastatic brain tumor model (15). In this model, the cells were introduced via the carotid artery and produced a brain tumor which was at least comparable to a metastatic brain tumor in humans if not to a primary malignant glioma. The blood-brain barrier could now be studied without the danger of direct damage from implantation, since this model avoided direct trauma to the brain. The simple technique of introducing cells via the carotid artery was in itself not sufficient to create the model since such cells tended to grow in extracerebral sites and, if otherwise unchecked, would kill the animal by tumor growth in the jaw. The expedient of giving small doses of a highly effective agent, i.e., cyclophosphamide, early after intracarotid inoculation reduced the incidence of extracerebral tumor and permitted the brain tumors to take and grow. As presented in this report, such tumors could then be treated with a variety of chemotherapeutic agents and, just as in i.c. implanted models, survival could be used as an endpoint to define effective and ineffective agents.

As noted in the report, the drugs we found effective included the nitrosoureas and cyclophosphamide, drugs which have been found to be effective in i.c. tumor models reported elsewhere (12). Furthermore, these drugs were also effective against the Walker 256 tumor implanted directly i.c. (Table 2). The results cannot be quantitatively compared because the 2 models are not comparable, the metastatic tumor received prior therapy with small doses of cyclophosphamide, and the direct i.c. inoculated tumor did not receive such treatment. The results qualitatively were similar, however. A more important comparison is the question of whether or not the breakdown of the blood-brain barrier induced by the i.c. tumor inoculation specifically permitted better therapy than was possible with the intracarotid-administered tumor without experimental traumatic blood-brain barrier rupture. Since the results we obtained were

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**Table 4**

**Pathological study of metastatic brain tumor in rats**

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<th>Location of the tumors</th>
<th>Complications</th>
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<td>Meningeal spread</td>
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<td>Control</td>
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<td>Treated</td>
<td>29 (100)</td>
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<td>Total</td>
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* N, number of rats examined.
* Numbers in parentheses, percentage of tumors.
* Not significantly different from control group (p).

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similar in the 2 circumstances, it is likely that blood-brain barrier function in the 2 circumstances was similarly impaired. If such impairment of the blood-brain barrier is important in the therapy of such tumors, it would appear that the tumor itself is responsible for the barrier breakdown. In fact, there is growing evidence that a significant barrier does not exist in fully grown brain tumors (18). It is probable that neovascularization of growing tumors produces relatively leaky blood vessels that lack significant barrier phenomena. This can easily be seen visually in experiments with our model in which Evans blue is injected i.v. prior to sacrifice of the animal (Fig. 2); the stained tumor stands out sharply against the unstained surrounding brain. Since Evans blue forms a complex with albumin, it is apparent that the albumin/dye complex readily leaks into the tumor. Thus, even the metastatic tumor model, once having grown and developed neovascularization, permitted drugs which normally do not cross the blood-brain barrier to reach the tumor cells and prove effective.

Also in this report, we attempted to define the effectiveness of the drugs against a s.c. implanted tumor. Here there is no question about blood-brain barrier function, and here again similar results were obtained in the s.c. study as were obtained both with the direct i.c. implantation and with the metastatic model. All the nitrosoureas were effective, procarbazine was essentially ineffective, and even MTX was transiently effective in all 3 models, although perhaps best so in the s.c. tumor and least well in the i.c. tumor model.

Since the results in the s.c. tumor were similar to those of the i.c. and metastatic brain tumor, the question remains as to why not just use s.c. tumor models to identify drugs which might be effective against brain tumors. Treating s.c. implanted tumors would indeed permit direct measurement of drug effect (10). If one wished to substitute animal survival for tumor volume as an endpoint, tumors could be implanted i.p. to determine specific tumor sensitivity.

We believe, however, that both i.c. and metastatic tumor models are still necessary in the study of brain tumor therapy for several reasons: (a) such models measure survival within narrow limits, i.e., survival based on the minimal increase in growth required to produce cerebral death versus the larger change required to produce animal death in tumors implanted i.p. or s.c.; (b) as noted above, previous studies (12, 19) suggested that drug effect as measured by survival of i.c. implanted tumors more closely predicts the results in patients similarly treated where survival is also the endpoint. (c) i.c. tumor models still represent the only way that one can study blood-brain barrier function in brain tumors, and there is reason to believe that this problem is still important. Although the blood-brain barrier appears to be broken down in fully grown tumors, there is still the problem that it is almost impossible to cure animals harboring fully grown brain tumors. In this case, failure to cure animals may mean that the drugs simply cannot get to the growing edge of the tumor where blood-brain barrier function may well be preserved (8). Furthermore, the metastatic brain tumor is developed in animals by giving the tumor by intracarotid inoculation and adding systemic chemotherapy. In this case, it appears that small clusters of hematogenously disseminated cells implant in the intact brain and grow despite systemic chemotherapy because the blood vessels near where the tumors begin their growth still maintain an intact blood-brain barrier, thus preventing the drugs from reaching the cells. It is exactly the kind of model described in this report, i.e., a metastatic tumor model which compares early versus late therapy, that permits inferences about blood-brain barrier function.

Thus, all 3 tumor models appear to be necessary if one is to utilize animal experiments to assist in determining effective therapy of human brain tumors. Tumor models (s.c.) can define minimum effective drug dosage, direct i.c. implanted tumors can define drug effectiveness in an animal harboring a brain tumor, and the metastatic brain tumor model can define blood-brain barrier phenomena not disturbed by experimental manipulation.

Finally, what do these experiments tell us about the problems of metastatic brain tumors in humans? It would seem that systemic chemotherapy so far does not prevent CNS metastases and that this is true for both humans and animals; new drugs are needed that prevent such metastases. Our model appears to be able to determine the value of such drugs in preventing subsequent tumor take. Preliminary studies in our laboratory have shown that nitrosoureas given 2 weeks after intracarotid Walker 256 cell inoculation similarly do not prevent the occurrence of subsequent brain tumors. Furthermore, while Walker 256 carcinoma was selected because of its sensitivity to cyclophosphamide, the drug did not completely eliminate extracranial tumors, as noted in the pathological study. These extracranial tumors were undetectable at the time of subsequent treatment and appeared not to be direct causes of death. If other animal tumors can be found which similarly can be treated with effective systemic agents and therefore permit the development of a metastatic brain tumor model, such tumors could be used to test several primary systemic tumor systems. One could then have a breast tumor, a lung tumor, a tropho-blastic tumor, and a melanoma, all metastatic to brain. The requirement appears to be an effective systemic therapy. Since that is the prerequisite in the patient as well, one could use such animal modeling to test agents that prevent metastasis to brain or treat it once such metastases occur. The tumors one chooses should resemble their human counterpart. This was not possible with Walker 256 carcinoma, which was chosen to develop the prototype model but should be possible as new agents become available. This then would appear to be the appropriate goal for this kind of modeling experiment.

ACKNOWLEDGMENT

We thank Doris Fok for her technical assistance.

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7. Litchfield, J. T., and Wilcoxon, F. A simplified method of evaluating dose-
Fig. 1. Metastatic brain tumor in the rat. a, coronal section through the midbrain and occipital lobes of a rat dying 41 days after tumor inoculation. This animal served as a control in the chemotherapy experiments. Two tumor masses are seen in the left occipital lobe, and there is meningeal tumor spread surrounding the midbrain; b, coronal section through the pons and cerebellum of a rat dying 41 days after tumor inoculation. This animal received CCNU as part of a chemotherapy experiment. There is a large cerebellar metastatic brain tumor.

Fig. 2. Metastatic brain tumor in the rat. Left, gross photograph of brain of rat 48 days after tumor inoculation; right, another rat 43 days after tumor inoculation. Evans blue (2%) was injected i.v. 3 hr before the animals were killed. The tumors stand out as dark areas against the light background of the unstained normal brain. Left, one large tumor is seen in the brain; right, 2 tumors in the brain, one frontal and one midhemisphere. The dark color means that the blood-brain barrier was disrupted by the tumor, permitting the albumin-dye complex to penetrate into the tumor.
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