Comparative Study of Chemotherapy and/or Immunotherapy of Established Experimental Brain Tumors

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Abstract

Intracerebral injection of 5×10^5 nitrosourea-induced glioma cells (T_9 tissue culture line) consistently produced brain tumors in syngenic Cesarean Derived Fisher rats. The tumors were 1 mm in diameter within 7 days and caused death within 23 days (mean results). The effects of therapy with ACNU and/or *Corynebacterium parvum* (*C. parvum*) were evaluated. *C. parvum* alone was apparently ineffective. ACNU (10 mg/kg body weight) given on days 7 and 14 prolonged the mean survival time to 33.4 days. Combined treatment with ACNU and intradermal immunization with T_9 cells and *C. parvum* on days 7 and 14, or on days 0 and 7, prolonged the mean survival time to 35.3 and 39.6 days, respectively. Eleven rats that survived following this treatment showed specific immunity against T_9 cells, as demonstrated by subsequent challenge with T_9 cells. The Winn's test procedure showed that spleen lymphocytes from immune rats were effective against T_9 cells. The results suggest that immunochemotherapy may be an effective treatment for rats with established brain tumors.

Key words: brain neoplasms, chemotherapy, immunotherapy, *Corynebacterium parvum*

Introduction

Despite recent achievements in operative methods, radiation therapy, and chemotherapy, either alone or combined, results of treatment of human malignant brain tumors are still poor.^{34,35)} The use of anticancer nitrosourea compounds, BCNU, CCNU, Me-CCNU, PCNU, chlorozotocin, and streptozotocin in the treatment of brain tumors has been only moderately effective.³⁴⁻³⁷⁾ This is also true for ACNU, a water-soluble nitrosourea compound³⁾ that is similarly effective in the treatment of both experimental^{17,24)} and human malignant brain tumors.^{29,30)} Other means of treating induced brain tumors, such as immunologic modalities, have also shown only minor and inconsistent success.^{25,26,42)} A combination of chemotherapy and immunotherapy may be synergistic, since chemotherapy may retard tumor growth and thus allow more time for im-

munologic mechanisms to destroy tumor tissue. This approach has been tried clinically,^{2,22,31,41)} with conflicting results. Further experimentation with both treatment modalities is therefore necessary.

A formalin-killed suspension of *Corynebacterium parvum* (*C. parvum*) was used because it appears to be a nonspecific immunostimulant^{12,37)} as well as a specific immunomodulator^{19,32,33)} and has also been shown to be somewhat effective in the treatment of experimental non-neural tumors.^{12,39)} In our experiments, we sought to determine the effects on neurogenic tumor growth of treatment with *C. parvum* alone, ACNU alone, ACNU plus *C. parvum*, tumor immunization alone, and ACNU plus tumor immunization. We also studied the immunologic competence of rats whose mean survival, following combined modality treatment (chemoimmunotherapy), was more than twice as long as that of controls.

Received May 28, 1984; Accepted November 12, 1984

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Materials and Methods

I. Tumor cell line

Cell line T₉ was obtained in culture form, through the courtesy of Dr. R. Denlinger. This line originated from a young male inbred Cesarean Derived Fisher (CDF) rat injected weekly, for 36 weeks, with 5 mg/kg body weight of methylnitrosourea (MNU).⁵⁾ The brain tumor from which the T₉ cell line was derived was histologically designated as astrocytoma, grade III. We have previously reported the *in vitro* sensitivity to ACNU of this tumor cell line.²⁰⁾ We used another rat glioma cell line, EB679, to investigate specificity of immunity. This line was originally a cerebral glioma induced with ethylnitrosourea transplacentally by Dr. W. Wechsler of West Germany. A clone (RG-2) of this cultured rat glioma (*in vitro* passage 5) was kindly given to us by Dr. D. Bigner of Duke University in North Carolina.

II. Animals

CDF inbred male rats were purchased from Charles River Co. and were used throughout these experiments. Their mean weight was approximately 150 g.

III. Development of brain tumor model

Adult male rats were anesthetized with inhalation of ether and intraperitoneal injection of ketamine and placed in a small-animal stereotaxic head holder. The scalp was incised and the skull drilled with a dental drill 1 mm anterior to the bregma and 2 mm to the right of the mid-sagittal line. A 20 μ l tumor cell suspension containing 5×10^5 T₉ cells was injected intracerebrally (IC) through this opening, *via* a 27 G Hamilton microsyringe, to a depth of 5 mm (coincident with the right caudate nucleus). The skull was then sealed with bone wax and the scalp was sutured. The rats were observed daily for their general condition and development of neurological signs. When they became preterminal, they were sacrificed. The brains were removed and fixed in 10% formaldehyde, cut into 1–2 mm coronal slices, and embedded in paraffin. Sections were stained with hematoxylin-eosin.

IV. Treatment of the brain tumor model

ACNU: 25 mg of ACNU in powder form was dissolved in 5 ml of Dulbecco's phosphate buffer saline and injected into the animals intravenously (IV).

C. parvum: A formalin-killed suspension of *C. parvum* supplied at a concentration of 7 mg dry weight bacteria/ml was administered intraperitoneal-

Table 1 Therapeutic regimens used in experimental brain tumors in rats

Group	Treatment regimens
1	Control (no treatment)
2	<i>C. parvum</i> ID on days 7 and 14
3	<i>C. parvum</i> IP on days 7 and 14
4	T ₉ 10 ⁶ + <i>C. parvum</i> ID on days 0 and 7
5	T ₉ 10 ⁶ + <i>C. parvum</i> ID on days 7 and 14
6	ACNU alone IV on days 7 and 14
7	ACNU IV on days 7 and 14 + <i>C. parvum</i> IP on days 8 and 15
8	ACNU IV on days 7 and 14 + <i>C. parvum</i> IV on days 8 and 15
9	ACNU IV on days 7 and 14 + (T ₉ 10 ⁶ + <i>C. parvum</i>) ID on days 8 and 15
10	ACNU IV on days 7 and 14 + (T ₉ 10 ⁶ + <i>C. parvum</i>) ID on days 0 and 7

C. parvum: *Corynebacterium parvum*, ID: intradermal administration, IP: intraperitoneal administration, IV: intravenous administration.

ly (IP), intradermally (ID), or IV.

Ten different methods of treatment were employed in our experiment, as shown in Table 1. *C. parvum* was administered as 2×10^8 formalin-killed bacterial particles (0.14 mg) in each injection at days 7 and 14. ACNU was injected at 10 mg/kg body weight at days 7 and 14. *C. parvum*, either alone or mixed with T₉ tumor cells, was used for active immunization. When *C. parvum* was combined with ACNU, it was injected at days 8 and 15. In groups 4 and 10, this tumor immunization was given at days 0 and 7. Previous experiments¹⁹⁾ have shown that after ID immunization with 2×10^8 *C. parvum* mixed with 1×10^6 T₉ cells, the ID tumor challenges are rejected, whereas IC challenges are not fully suppressed.

V. Evaluation of the immunological status

Rechallenge with T₉ cells: Rats that survived for more than 50 days were considered long survivors. They were challenged ID with 5×10^5 T₉ cells, and then with 10⁶ cells ID if the first challenge was rejected. Subsequently, the rats were challenged with 10⁶ T₉ cells IC in the left frontal lobe.

Challenge with EB679 cells: Some of the long survivors were injected ID with 5×10^5 EB679 gliosarcoma cells to study the specificity of the immunity. These ID tumors were measured with calipers weekly and compared with those of the controls.

Winn's assay: After ID and IC challenges, as described above, the rats were sacrificed and their spleens were used for Winn's test.³⁸⁾ Briefly, the Winn's test was performed as follows. Spleen cells

were harvested aseptically from treated and from normal, untreated rats. Mixtures of spleen cells as a ratio of 25:1 (7.5×10^6 spleen cells mixed with 3×10^5 T_9 cells) were injected ID into the backs of six normal rats. Tumor growth was measured weekly for up to 28 days after injection.

VI. Statistical analysis

The data obtained in our experiments were studied with the combined Student's T test for comparison of tumor size and survival time.

Results

I. Effects of treatment

Injection of 5×10^5 T_9 cells consistently produced tumors in syngenic CDF rats. All IC injected rats had established tumors, 1 mm in diameter, within 7 days (Fig. 1), and all died from the tumor (within an

Fig. 1 Intracerebral tumor of control rat on 7th day after inoculation. The tumor is 1 mm in diameter. No necrotic foci or significant lymphocytic infiltration are evident. HE stain, $\times 40$.

Table 2 Results of treatment of established T_9 brain tumor with ACNU and/or *C. parvum*

Group	Total No.	Mean survival (days)	Increased lifespan (%)	Long survivors (> 50 days)
1	14	23.0 ± 1.5 SE	—	0
2	8	21.6 ± 0.7	-6.1	0
3	6	18.3 ± 0.2	-20.0	0
4	8	25.0 ± 2.4	8.7	0
5	8	23.1 ± 1.2	0.4	0
6	8	33.4 ± 3.2 ($p < 0.001$)*	45.2	1 (12.5%)
7	8	31.4 ± 2.1 ($p < 0.005$)*	36.5	0
8	8	28.3 ± 4.8	23.0	1 (12.5%)
9	14	35.3 ± 2.3 ($p < 0.001$)*	53.5	3 (21.4%)
10	15	39.6 ± 2.9 ($p < 0.001$)* ($p < 0.05$)**	71.7	6 (40.0%)

Statistically significant when compared with *controls, and with **ACNU alone.

average of 23 days). The results of the various treatments are shown in Table 2. When the rats were treated with IP or ID injections of 0.14 mg of *C. parvum* alone at days 7 and 14, the survival time of treated rats was not prolonged. IP injection of *C. parvum* actually decreased the survival time of treated rats, compared with that of controls. When rats were immunized with injections of 10^6 live T_9 cells mixed with 0.14 mg of *C. parvum* at days 0 and 7 or days 7 and 14, the survival time was not significantly prolonged (mean survival: 25.0 and 23.1 days, respectively).

ACNU (10 mg/kg body weight) given on days 7 and 14 significantly prolonged the mean survival time to 33.4 days (range, 22 to 43 days); one rat survived more than 50 days. Combination treatment with ACNU and IP or IV *C. parvum* also extended the survival time, but by no more than treatment with ACNU alone. ACNU plus tumor immunization at days 8 and 15 lengthened survival time more than did ACNU alone, but the difference was not significant. However, when tumor immunization was started at day 0 and followed by ACNU administration, the effects were more obvious and significant; the mean survival time increased to 39.6 days, and all such treated rats survived more than 30 days. Six of 15 rats treated in this manner survived more than 50 days.

II. Immunologic status of long survivors

Eleven rats survived more than 50 days. This

Table 3 Immunologic status of "long survivors"

	Rat No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Nos. 7-11
Treatment	ACNU alone	ACNU+ (T ₉ +C.p.) ID (8 & 15 d)	ACNU+ (T ₉ +C.p.) ID (8 & 15 d)	ACNU+ (T ₉ +C.p.) ID (8 & 15 d)	ACNU+ C.p. IV (8 & 15 d)	ACNU+ (T ₉ +C.p.) ID (0 & 7 d)	ACNU+ (T ₉ +C.p.) ID (0 & 7 d)
Rechallenge with 10 ⁶ T ₉ ID	rejection	≈ control	≈ control	rejection	rejection	rejection	rejection*
Rechallenge with 10 ⁶ T ₉ IC	—	—	—	—	rejection	rejection	—
Challenge with 5×10 ⁵ EB679 ID	≈ control	—	—	—	≈ control	—	—
Winn's test	negative	—	—	—	positive**	positive**	—
Brain histology	no tumor	no tumor	large IC tumor	no tumor	no tumor	no tumor	no tumor

*See Table 4, **see Fig. 2. IC: intracerebral.

represents 13.3% of all treated rats and 20.8% of rats treated with either ACNU alone or ACNU combined with *C. parvum*. None of the rats treated without ACNU survived more than 50 days. A summary of the immunologic status of the long survivors is shown in Table 3.

Rechallenge with T₉ cells: ID tumor challenge with 10⁶ T₉ cells (injected on day 50) was rejected by nine of 11 long-surviving rats (Table 3). Subsequent IC challenge of 10⁶ T₉ cells to two rats who rejected the ID challenge was also rejected. Comparison of ID tumor growth in the five long survivors with that in normal rats is shown in Table 4; there were marked differences in tumor "take" (tumorigenesis) and size.

Challenge with EB679 cells: ID injection of 5×10⁵ EB679 gliosarcoma cells in two of the long survivors induced tumors in both rats as in controls (Table 3).

Winn's assay: Winn's assay was performed in three long survivors. When tested, two rats had an ID EB679 tumor and the other one was totally free of the tumor (Table 3). Although one of them showed no significant effects, the other two demonstrated

a remarkable suppression of tumor growth when compared with controls, as shown in Fig. 2.

Pathology of the brain: With one exception, autopsies revealed no tumor growth in the brains of any of the long survivors. The brain injection sites were filled with fibrous tissue or cystic lesions surrounded by lymphocytic infiltration.

Discussion

The tumor we used was originally induced with MNU and has been perpetuated as T₉ or 9L gliosarcoma by serial subcultures and animal passages.⁵⁾ Other investigators^{4,10,21)} have also used the T₉ brain tumor model to determine the efficacy of various therapeutic regimens. Levin *et al.*²¹⁾ compared the therapeutic effects of seven nitrosourea compounds in this model, and observed an increase in survival time with PCNU treatment followed by BCNU, CCNU, and Me-CCNU administration. Barker *et al.*⁴⁾ showed that a dose of BCNU, at approximately 40% of the 10% lethal dose, increased the lifespan of 38% of the animals when given 9 and 16 days after tumor inoculation. In our investigation with induced T₉ brain tumors, weekly injection of 10 mg/kg of ACNU increased the mean survival time by 45.2%. Of interest is that ACNU treatment, either alone or combined with other treatment modalities, "cured" 20.8% of rats with brain tumors. These results corroborate those of our *in vitro* studies of T₉ cell sensitivity to ACNU.²⁰⁾

It has been reported^{6,12,27,33)} that both specific and nonspecific immune mechanisms are involved in the antitumor action of *C. parvum*. However, in several published studies, the systemic injection of *C. parvum* disturbed normal immune reactions⁶⁾ and facilitated tumor growth, depressed *in vitro*

Table 4 Results of ID challenges in "long survivors" (Nos. 7-11) with T₉ tumor cells

	Tumor "take" and size (mean, mm)			
	1 week	2 weeks	3 weeks	4 weeks
Long survivors (n=5)	2.0 (3/5)	2.3* (2/5)	3.0* (1/5)	3.6* (1/5)
Control (n=3)	2.0 (3/3)	9.8 (3/3)	14.3 (3/3)	29.6 (3/3)

*Statistically significant (p<0.05). n: number of rats.

Fig. 2 Winn's test with spleen cells from two "long survivors," No. 5 rat (left) and No. 6 rat (right). $\circ-\circ$: injected ID with a mixture of 3×10^5 T_9 tumor cells and 7.5×10^6 spleen cells of long survivors (1:25 ratio), $\blacksquare-\blacksquare$: injected ID with a mixture of 3×10^5 T_9 tumor cells and 7.5×10^6 normal spleen cells, $\square-\square$: injected with 3×10^5 T_9 tumor cells only (control). Each mark represents the average tumor size in six rats.

cell-mediated cytotoxicity,^{16,23)} and increased the myelotoxicity of concomitant chemotherapy.^{13,14)} Moreover, only a small amount of *C. parvum* is distributed to the central nervous system after IV injection^{11,28)} and it may raise the intracranial pressure.³¹⁾ Therefore, it appears inappropriate or premature to use *C. parvum* systemically for the treatment of brain tumors. In our experiments, IV and IP injections of *C. parvum* alone could not suppress brain tumor growth, and actually shortened the lifespan of rats with brain tumors. When ACNU was combined with systemic administration of *C. parvum*, the antitumor effects of ACNU apparently decreased and its myelotoxicity may have been enhanced. On the other hand, some investigators have reported that *C. parvum*, injected as a form of admixture with tumor cells, consistently suppressed tumor growth and induced systemic tumor immunity, especially in highly immunogenic tumors^{6,19,23)} and sometimes even in poorly immunogenic tumors.⁹⁾ These findings applied to the T_9 brain tumor model. In fact, immunization with *C. parvum* mixed with T_9 tumor cells suppressed subsequent ID and IC tumor challenges and induced specific antitumor immunity, which was partially effective in brain tumors¹⁹⁾ and readily transferable to normal rats.³²⁾

In studies of extracranial tumor systems, the effects of chemotherapy have been increased by preimmunization or adoptive immune transfer with antibody or cytotoxic cells. However, in the treatment of established brain tumors this is not the case. In the chemoimmunotherapy of extracranial tumors, possible roles for chemotherapeutic drugs are: (1) reduction of tumor cells to the extent that the immune system can eliminate them,⁸⁾ (2) retardation of tumor growth until effective tumor immunity can be developed by the host,⁸⁾ (3) modification of tumor cells to increase their susceptibility to immune mechanisms,⁷⁾ (4) alteration of tumor cells to increase tumor immunity,¹⁵⁾ and (5) removal of suppressor T cells or suppressor macrophages that may inhibit the host's antitumor immunity.¹⁸⁾

Immunochemotherapeutic trials have been conducted with experimental brain tumors. Albright *et al.*¹⁾ reported the results of chemoimmunotherapy with CCNU and mitomycin-treated glioma cells and/or BCG that was started 3 or 4 days after tumor inoculation. Compared with CCNU administration alone, the combination treatment did not increase the survival time of animals with the brain tumors. In our experiments, immunization with live tumor cells mixed with *C. parvum* apparently enhanced the

chemotherapeutic effect of ACNU, though the combined effects were not great enough to eradicate the brain tumors completely. However, when the active immunization was applied earlier, the results appeared to be better.

The specific antitumor immunity demonstrated by long survivors is notable. Long survivors rejected or retarded IC inoculated tumor cells as well as subsequent IC and ID T₉ cell challenges. Therefore, it is conceivable that antitumor immunity was developed during treatment and contributed to the "cure" of brain tumors. More importantly, the induction of specific immunity might be indispensable for a complete "cure" following chemotherapy. It has been reported that following inoculation of brain tumors in mice, a specific killer T cell is generated in their spleens, and that spleen T cells from these mice are cytotoxic to the inoculated tumor cells.⁴⁰⁾ The Winn's test we performed tends to confirm this work. Whether or not humoral immunity was also involved in the eradication of brain tumors is still unclear. In our next study, we will attempt to identify a humoral tumor antibody in the serum of long survivors.

Acknowledgment

We are grateful to Drs. Gerald M. Hochwald and Jeannette G. Thorbecke for their advice and criticism of our manuscript.

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