[CANCER RESEARCH 59, 2107–2114, May 1, 1999]

Paracrine Immunotherapy with Interleukin-2 and Local Chemotherapy Is Synergistic in the Treatment of Experimental Brain Tumors[1]

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ABSTRACT

Potent immune responses against malignant brain tumors can be elicited by paracrine intracranial (IC) immunotherapy with interleukin (IL)-2. Additionally, IC delivery of carmustine via biodegradable polymers has been shown to significantly prolong survival in both animal models and clinical trials. In this study, we show that the combination of paracrine immunotherapy, with nonreplicating genetically engineered tumor cells that produce IL-2, and local delivery of chemotherapy by biodegradable polymers prolongs survival in a synergistic manner in mice challenged intracranially with a lethal murine brain tumor. Animals receiving IL-2-transduced cells and polymers containing 10% 1,3-bis(2-chloroethyl)-1-nitrosourea had significantly improved survival compared with animals receiving IL-2-transduced cells or 10% 1,3-bis(2-chloroethyl)-1-nitrosourea alone. Median survival for the control group was 19 days. Survival in animals receiving IL-2-transduced cells and 1% carboplatin-containing polymers was also significantly improved compared with either therapy alone. Histopathological examination on day 14 of animals receiving combination treatment showed rare degenerating tumor cells. In addition to tissue necrosis surrounding the polymer, a marked inflammatory reaction was observed. In long-term survivors (all animals receiving combination treatment), no tumor was observed and the inflammatory reaction was completely resolved. The brains of animals receiving combination therapy showed both tissue necrosis due to local chemotherapy and strong inflammation due to paracrine immunotherapy. The demonstration of synergy between paracrine IL-2 and local IC delivery of antineoplastic drugs is novel and may provide a combined treatment strategy for use against both primary and metastatic IC tumors.

INTRODUCTION

Despite significant advances in neuroimaging, microsurgery, radiation therapy, and conventional chemotherapy, the prognosis for most patients with malignant brain tumors remains dismal (1–3). The current standard of care for medium- to large-sized accessible tumors is surgical debulking, followed by external beam radiation therapy. Although these treatment modalities improve quality of life and prolong survival, the median survival after diagnosis is still <1 year (1). As a result, the focus has shifted to developing innovative and more effective treatments. In recent years, efforts at improving survival for patients harboring malignant brain tumors have been directed at controlling local disease. This is based on clinical and experimental observations that most of these tumors recur locally, within 2 cm of the original resection field (4).

Identification and cloning of genes encoding cytokines has led to the development of a promising strategy for treatment of brain tumors that involves stimulation of a local immune response against tumor cells (5–7). Cytokines, such as IL[3]-2, rarely have a direct cytotoxic effect on neoplastic cells, but rather exert their immunomodulatory activity in a paracrine fashion. Under these circumstances, tumor cells genetically engineered to release cytokine adjacent to tumor antigens, can produce a strong local inflammatory response specific to the particular cytokine (8). For IL-2, use of this paracrine physiology with autologous cells has been shown to exhibit potent antitumor responses in experimental brain tumors (9). IL-2 production by tumor cells is believed to bypass T-helper function in the generation of an antitumor response (10), thereby modulating host immune responses to the neoplasm. IL-2 is also required for CTL growth and enhances natural killer cell and LAK activity (8, 11). Recently, intratumoral administration of allogeneic cells genetically modified to secrete IL-2 has also been shown to significantly prolong survival in nonimmunized mice (12, 13).

Implantable polymers that release chemotherapeutic agents directly into the CNS are another local anticancer therapy that provides a novel approach to the treatment of malignant brain tumors (14, 15). This technology makes it possible to achieve very high local concentrations of anticancer agents while minimizing systemic toxicity and circumvents the need for a drug to cross the blood-brain barrier. This strategy, using BCNU-loaded biodegradable polymers, was first shown to be effective against experimental IC tumors (16) and has now demonstrated statistically significant improvement in survival in clinical trials in patients with malignant gliomas, both at recurrence (17, 18) and at initial presentation (19, 20).

In an effort to improve further on these two antitumor strategies, we hypothesized that the combination of paracrine immunotherapy, with nonreplicating genetically engineered tumor cells that produce IL-2, and local delivery of chemotherapy by biodegradable polymers may act synergistically against a murine brain tumor model. The rationale for this study is based on theoretical and experimental evidence of increased antigenicity of tumors after exposure to cytotoxic drugs (21, 22). As a neoplasm grows, the immune system has either failed to recognize antigens or failed to respond appropriately. Therefore, enhancing the intrinsic immunogenicity of tumors may be an important modality to increase the effectiveness of immunotherapy (23). Furthermore, chemotherapeutic drugs may abrogate tumor-derived T-cell suppressor factors that inhibit antigen-induced T-cell function (24, 25) and also cause cytoreduction of the tumor, resulting in a tumor of a size that can be targeted and handled by the immune system (21). In this study, we analyze the therapeutic efficacy of IL-2 delivered locally via implantation of IL-2-transduced cells used in combination with either of two local antineoplastic chemotherapeutic agents, BCNU and carboplatin, in a murine IC B16-F10 melanoma model.

Received 9/9/98; accepted 3/2/99.

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[1] The research reviewed in this manuscript was partially funded by the National Cooperative Drug Discovery Group (Grant UO1-CA52857) of the National Cancer Institutes of Health (Bethesda, MD) and by Guilford Pharmaceutical Corporation (Baltimore, MD). Generous support for this research was received from the Norton family in memory of Robin Norton. P. S. is recipient of the NIH National Research Service Award CA-09574. J. H. is the 1997–1998 Bonnie Blair/American Brain Tumor Association postdoctoral fellow. H. B. is consultant to Guilford Pharmaceutical, Inc. and to Rhone-Poulenc Rorer, and Guilford Pharmaceutical, Inc. has provided a gift for research in the laboratory of H. B. The Johns Hopkins University and H. B. own Guilford Pharmaceutical, Inc. stock, the sale of which is subject to certain restrictions under University policy. The terms of this arrangement are being managed by the University in accordance with its conflict of interest policies.

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[3] The abbreviations used are: IL, interleukin; LAK, lymphokine-activated killer; CNS, central nervous system; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; IC, intracranial; LTS, long-term survivor; pCPP:SA, poly[1,3-bis(carboxyphenoxy)propane-*co*-sebacic acid] anhydride.

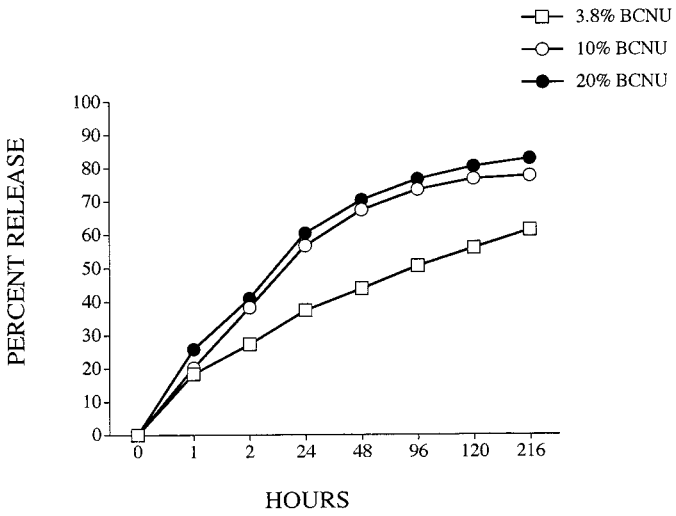

Fig. 1. Drug-release kinetics from pCPP: SA polymer at 3.8% (□), 10% (○), and 20% (●) loading by weight

Because B16-F10 is an aggressive, poorly immunogenic tumor, it provides a rigorous model for testing the ability of immnotherapy to alter endogenous antitumor immune responses (9).

MATERIALS AND METHODS

Study Design. Using a murine model for metastatic melanoma to the brain by stereotactically implanting B16-F10 melanoma cells into the left parietal lobes of C57BL/6 female mice (6–12 weeks of age), we tested the efficacy of local paracrine IC immunotherapy, using nonreplicating genetically engineered tumor cells that produce IL-2 with and without subsequent locally delivered carmustine (BCNU) or carboplatin. Long-term animal survivors (> 70 days) were examined histopathologically, as were a separate set of animals that were similarly implanted and sacrificed 14 days later.

Tumor Line. The B16-F10 melanoma cell line was obtained from the National Cancer Institute–Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD). The cells were maintained in DMEM containing 10% FCS and penicillin/streptomycin in humidified incubators gassed with 5% carbon dioxide. B16-F10 melanoma cells were transduced with the murine *IL-2* gene by using the replication-defective MFG retroviral vector, as previously described (26). The amount of IL-2 produced by the transformed tumor cells was quantified routinely by a standard ELISA (Endogen, Cambridge, MA). Cultured tumor monolayers were harvested with 0.025% trypsin, counted, and resuspended in DMEM before IC implantation. Transduced tumor cells were exposed to 5000 rad from a 137Cs source (Gammacell model no. 62 irradiator; Nordin International, Inc., Kanata, Ontario, Canada) discharging 1378 rad/min, immediately before injection to render them replication-incompetent.

Animals. C57BL/6 female mice, 6–12 weeks of age, were obtained from Harlan Sprague Dawley (Indianapolis, IN). Animals were allowed free access to water and rodent chow. They were housed and treated in accordance with the policies and principles of laboratory care of the Johns Hopkins University School of Medicine Animal Care and Use Committee.

Chemotherapeutic Agents. Carmustine (BCNU) and *cis*-diammine-1,1-cyclobutane-dicarboxylate platinum (II) (carboplatin) were obtained from Bristol-Myers-Squibb (Princeton, NJ) and were stored at 4°C and -20°C, respectively.

Polymer Preparation. pCPP:SA, with a 20:80 molar ratio, was supplied by Guilford Pharmaceuticals Corp. (Baltimore, MD). CPP:SA polymers containing BCNU at 3.8%, 10%, and 20% loading by weight or carboplatin at 1% loading by weight were prepared as described previously (27, 28). The polymers for implantation were pressed into disc shapes weighing 5 mg each (1.5 mm in diameter, 0.5 mm in height).

Drug-release Kinetics. *In vitro* BCNU and carboplatin release from pCPP:SA were determined by reversed-phase C18 high-pressure liquid chromatography, as described previously (27, 28). Briefly, polymer discs of 20:80 pCPP:SA with 3.8%, 10%, 20% BCNU or 1% carboplatin were placed in 1.5-ml vials containing 1.0 ml of normal saline. The vials were capped to prevent evaporation and placed in an incubator at 37°C. The medium was periodically removed and replaced, and the recovered solution was assayed for the presence of drug by high-pressure liquid chromatography. These analyses were performed with a Beckman System Gold (consisting of Autosampler 507, Programmable Solvent Module 126AA, and Programmable Detector Module 166; Beckman Instruments, San Ramon, CA), controlled and integrated by a personal computer and equipped with a 3.9 × 300-mm μBondpack C18 column (Waters Associates, Milford, MA).

Tumor Inoculation. Mice were anesthetized with an i.p. injection of 0.1 ml of a stock solution containing 25 mg/ml ketamine hydrochloride, 2.5 mg/ml xylazine, and 14.25% ethanol, all diluted 1:3 in 0.9% NaCl solution. The surgical site was shaved and prepared with 70% ethanol and iodine-containing solution. After a midline incision, a 2-mm burr-hole was made 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture. The animals were then placed in a stereotactic frame, and the tumor cells were delivered over 3 min by a 26-gauge needle inserted to a depth of 3 mm. The site of injection was the center of the burr-hole, allowing subsequent local therapy with polymer implanted through the burr-hole to be centered on the tumor bed. The needle was then removed, and the site was irrigated with 0.9% NaCl solution and closed with 4.0 Vicryl sutures.

Polymer Implantation. The surgical incision used for inoculating the tumor was reopened 5 days later, and a single polymer was inserted in the cortex entirely below the level of the inner table of the parietal bone. After hemostasis was obtained, the placement site was irrigated and closed with 4.0 Vicryl sutures.

Polymer Dose-escalation Studies. Groups of C57BL/6 mice received IC injections of 10^2 B16-F10 tumor cells, followed by implantation of 3.8%, 10%, or 20% BCNU polymer 5 days later to determine the optimal and maximally tolerated polymer loading dose for future studies. The cells were injected stereotactically into the left parietal lobe, as described above. Animals were closely monitored for signs of toxicity, including early failure to thrive and neurological deficits. Survival was assessed, and autopsies were performed whenever possible. Survival curves were prepared for each loading dose of polymer, and median survivals were noted for subsequent efficacy trials. The maximally tolerated carboplatin loading dose (1%) had been previously determined (29).

Efficacy Studies. Each polymer loading dose was tested in an independent experiment in the IC B16-F10 model. For each experiment, four groups of 10 animals initially underwent tumor implantation with 10^2 B16-F10 melanoma cells. Groups 1 and 2 received coinjection of 75,000 nonreplicating, wild-type B16-F10 melanoma cells, followed by implantation of blank polymer on the 5th postoperative day (group 1, control) or implantation of BCNU polymer at 3.8%, 10%, or 20% loading or carboplatin at 1% loading on the 5th postoperative day (group 2, chemotherapy only). Groups 3 and 4 received coinjection of 75,000 nonreplicating, IL-2-producing B16-F10 melanoma cells (which produce 80 ng/10^6 cells/24 h, as determined by ELISA), followed by implantation of blank polymer on the 5th postoperative day (group 3, immunotherapy only) or BCNU polymer at 3.8%, 10%, or 20% loading or carboplatin at 1% loading on the 5th postoperative day (group 4, combination therapy).

Table 1 *BCNU polymer dose-escalation study*

BCNU loading	Median survival (days)	Range (days)	LTSs	*P versus* control[a]
Control (n = 10)	18	17–19		
3.8% BCNU (n = 10)	27.6	19–80	10%	0.124
10% BCNU (n = 10)	33.6	25–80	10%	0.0084
20% BCNU (n = 11)	47.5	13–80	17%	0.0034

[a] Using nonparametric (Kruskal-Wallis) statistical analyses.

Table 2 *Efficacy of BCNU-loading polymers and carboplatin-loaded polymer with or without local IL-2 immunotherapy*

Experiment[a]	Median survival (days)	Range (days)	LTSs	P^b
1				
Group 1 (control, $n = 10$)	25.7	19–54	0%	
Group 2 (3.8% BCNU, $n = 10$)	19	18–20	0%	0.06 *vs.* control
Group 3 (IL-2 alone, $n = 10$)	34.5	22–70	0%	0.18 *vs.* control
Group 4 (combination, $n = 10$)	41.9	27–90	10%	0.05 *vs.* control
				0.14 *vs.* IL-2
				0.02 *vs.* BCNU
2				
Group 1 (control, $n = 10$)	20.6	19–27	0%	
Group 2 (20% BCNU, $n = 10$)	125.4	14–160	30%	0.0023 *vs.* control
Group 3 (IL-2 alone, $n = 10$)	39.4	14–160	20%	0.034 *vs.* control
Group 4 (combination, $n = 10$)	118.7	42–160	30%	0.0018 *vs.* control
				0.55 *vs.* IL-2
				0.86 *vs.* BCNU
3				
Group 1 (control, $n = 9$)	15.8	13–18	0%	
Group 2, (10% BCNU, $n = 10$)	26.4	22–39	0%	<0.001 *vs.* control
Group 3 (IL-2 alone, $n = 9$)	33.8	13–70	11%	0.077 *vs.* control
Group 4 (combination, $n = 10$)	Not reached	18–70	70%	0.0023 *vs.* control
				0.042 *vs.* IL-2
				0.0033 *vs.* BCNU polymer
4				
Group 1 (control, $n = 10$)	20.6	19–27	0%	
Group 3 (1% Carboplatin, $n = 10$)	23.3	13–40	0%	0.26 *vs.* control
Group 3 (IL-2 alone, $n = 10$)	38.4	14–160	30%	0.035 *vs.* control
Group 4 (combination, $n = 10$)	Not reached	22–160	80%	0.0001 *vs.* control
				0.017 *vs.* IL-2
				0.002 *vs.* carboplatin

[a] In each experiment, combination therapy included drug-polymer with IL-2 treatment. Experiment 1, 3.8% BCNU-loaded polymer; experiment 2, 20% BCNU-loaded polymer; experiment 3, 10% BCNU-loaded polymer; experiment 4, 1% carboplatin-loaded polymer.

[b] Using nonparametric (Kruskal-Wallis) statistical analyses.

Histological Evaluation. An additional twelve C57BL/6 mice (three per group) received IC injections of 10^2 B16-F10 tumor cells, with either 75,000 nonreplicating, wild-type B16-F10 melanoma cells or 75,000 nonreplicating, IL-2-producing B16-F10 melanoma cells, followed by implantation of 10% polymer or empty polymer 5 days later for histological evaluation. On day 14 after tumor implantation, animals from each group were sacrificed, the brains were removed, and brain tissue was fixed in 10% formalin, blocked in paraffin, sectioned in a coronal plane in 10-μm sections, and stained with H&E.

On day 70 after tumor implantation, 7 of 10 animals receiving combination treatment in the efficacy studies were still alive. These surviving animals were sacrificed, and the brains were removed and were similarly sectioned and stained.

Outcome and Statistical Analysis. For all efficacy studies, survival was the primary end point. All animals were monitored for any signs of neurotoxicity and autopsied, when possible, to confirm that death was due to IC tumor. In previous studies in our laboratory with these models, animals that were moribund or paralyzed uniformly died within 24 h. The distribution of the intervals until death was determined by the method of Kaplan and Meier (30). Two nonparametric statistical analyses, the Mann-Whitney *U* test and Kruskal-Wallis test, were used to compare survival between groups. Statview Version 4.51 software (Abacus Concepts, Inc.) was used for statistical analyses. "Synergism" for combination experiments was defined as statistically significant improvement in survival over either therapy alone.

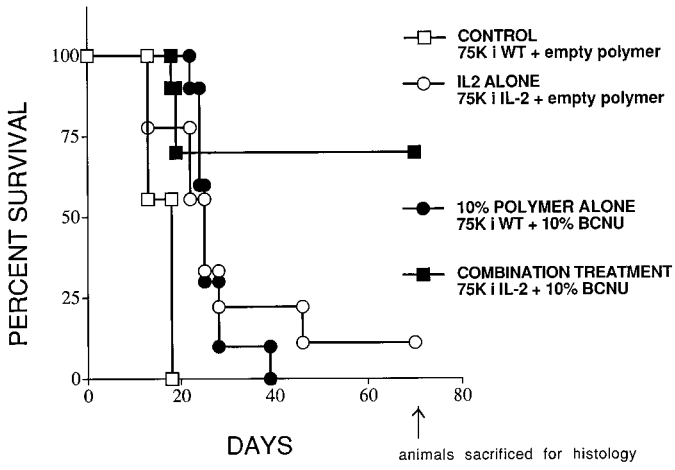

Fig. 2. Kaplan-Meier survival curve showing survival for animals after IC B16/F10 melanoma challenge treated with empty polymer (□), IL-2-transduced cells alone (○), 10% BCNU polymer alone (●), or combination of 10% BCNU polymer and IL-2-transduced cells (■).

RESULTS

***In Vitro* Polymer Release of Drug.** Studies of the BCNU-release kinetics from polymer wafers loaded with 3.8%, 10%, and 20% BCNU by weight showed a dose-dependent increase in the amount of drug released (Fig. 1). In all cases, sustained BCNU release was observed over 96 h.

BCNU Polymer Dose-escalation Studies. BCNU polymer, at 10% and 20% loading by weight, significantly prolonged survival in the B16-F10 IC melanoma model (Table 1).

Efficacy of 3.8% BCNU-loaded Polymer with or without Local IL-2 Immunotherapy. Combination IC immunotherapy with IL-2 and 3.8% BCNU-loaded polymer improved survival over either antitumor therapy alone (Table 2).

Efficacy of 20% BCNU-loaded Polymer with or without Local IL-2 Immunotherapy. No survival advantage was observed when 20% BCNU-loaded polymer was used in combination with IL-2 immunotherapy (Table 2).

Efficacy of 10% BCNU-loaded Polymer with or without Local IL-2 Immunotherapy. In contrast to polymers loaded with 20% BCNU, polymers with 10% BCNU and IL-2 immunotherapy showed synergism in the treatment of IC B16-F10 melanoma (Fig. 2 and Table 2).

Efficacy of 1% Carboplatin-loaded Polymer with or without Local IL-2 Immunotherapy. Polymer with 1% carboplatin and IL-2 immunotherapy also showed synergism in the treatment of IC B16-F10 melanoma (Fig. 3 and Table 2).

Histological Evaluation of Animals Receiving 10% BCNU-loaded Polymer with or without Local IL-2 Immunotherapy. In a separate experiment, 12 animals were implanted for planned sacrifice

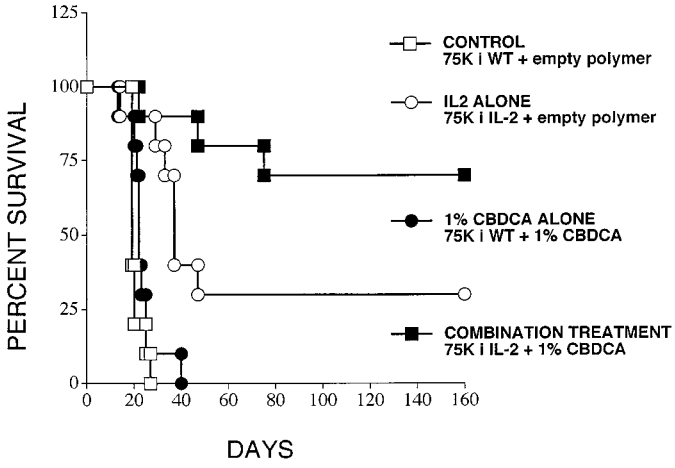

Fig. 3. Kaplan-Meier survival curve showing survival for animals after IC B16/F10 melanoma challenge treated with empty polymer (□), IL-2-transduced cells alone (○), 1% carboplatin polymer alone (●), or combination of 1% carboplatin polymer and IL-2-transduced cells (■).

on day 14 for histopathological analysis. Control animals ($n = 3$, group 1) had large viable, solid IC tumor masses with minimal reactive changes, including scattered lymphocytes and macrophages as well as cavitation, underlying the polymer implantation site (Fig. 4, *A* and *B*). Animals receiving 10% BCNU-loaded polymer alone ($n = 3$, group 2) showed scattered tumor cells and a ring of tissue necrosis surrounding the polymer with minimal reactive changes in the surrounding brain parenchyma (Fig. 5, *A-C*). Animals receiving B16/IL-2 alone ($n = 3$, group 3) had aggregates of tumor with a surrounding striking mixed inflammatory reaction containing both acute and chronic inflammatory components. Polymorphonucleocytes, macrophages, and lymphocytes were all seen. In addition, no ring of necrosis was present (Fig. 6, *A-C*). In animals receiving combination treatment ($n = 3$, group 4), rare degenerating tumor cells were identified. In addition to tissue necrosis surrounding the polymer, a marked mixed inflammatory reaction was observed (Fig. 7, *A-D*).

On day 70, in LTSs (all animals receiving combination treatment of B16/IL-2 and 10% BCNU), no tumor was observed, and the inflammatory reaction was completely resolved (Fig. 8, *A-C*).

DISCUSSION

We have demonstrated that combination IC immunotherapy with IL-2 and local delivery of chemotherapy via biodegradable polymers improves survival in experimental brain tumors over either antitumor therapy alone. More importantly, at 10% BCNU and 1% carboplatin polymer loading, synergy was observed with respect to survival in animals challenged with an otherwise lethal dose of IC tumor. Histological examination suggests that immunotherapy acts by an immune-mediated phenomenon with recruitment of a large number of inflammatory cells. Unique to the brains of animals receiving combination therapy, both tissue necrosis, due to local chemotherapy, and strong inflammation, due to paracrine immunotherapy, were evident 14 days after tumor challenge. In animals surviving long-term, no tumor cells were present, and the inflammatory cell infiltrate had completely resolved.

Given the poor prognosis for most patients with malignant brain tumors, efforts at improving survival have centered on controlling local disease. Improving treatment for malignant brain tumors has been hindered by the unique environment of the CNS. Certain chemotherapeutic drugs, immune response modifiers (*e.g.*, cytokines), antiangiogenesis agents, and other anticancer therapies, are unable to cross the blood-brain barrier and are associated with significant systemic toxicity. Therefore, strategies at improving local delivery of therapeutic agents have come to the forefront. The introduction of implantable polymers that release chemotherapeutic agents directly into the CNS is one such local therapy that makes it possible to achieve very high local concentrations of anticancer agents, while minimizing systemic toxicity, and circumvents the need for a drug to cross the blood-brain barrier (14, 17, 18). Nitrosoureas, including carmustine (BCNU), are a class of chemotherapeutic agents that have been incorporated into biodegradable polymers and, on IC implantation, have demonstrated efficacy in clinical trials in patients with both recurrent (17, 18) and newly diagnosed (19, 20) malignant brain tumors. Importantly, in contrast to systemic administration of nitrosoureas, this treatment has not been associated with any systemic side effects (17–20).

Adoptive immunotherapy is another "locally" active antitumor strategy (31, 32). The treatment of malignant brain tumors may be improved by activating the defenses of the body (9, 12, 13, 33, 34). For this approach, elements of the immune system of the host are isolated, genetically modified in the laboratory, and then readministered to the host with the goal of selectively seeking out and destroy-

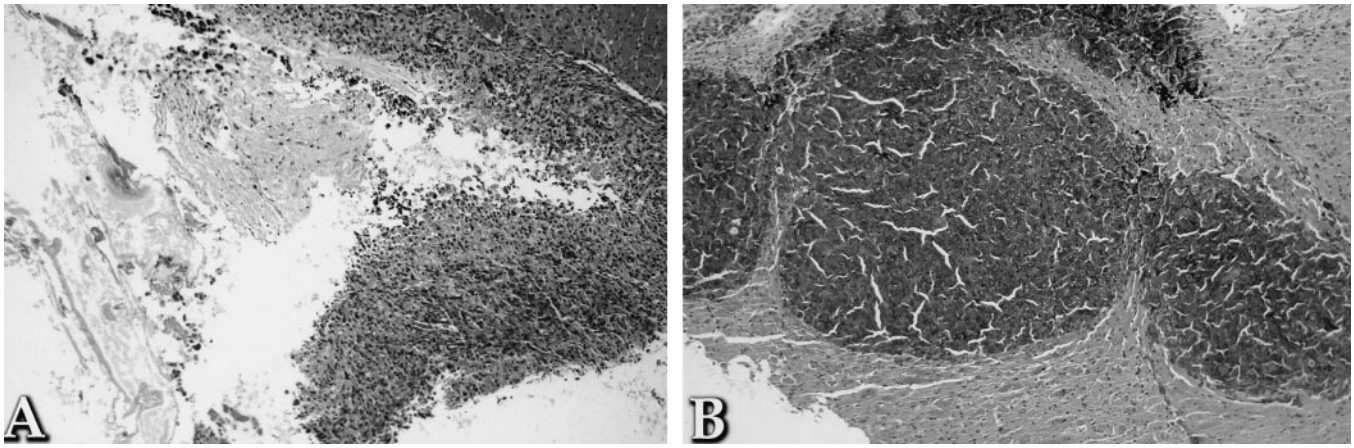

Fig. 4. Photomicrographs of H&E-stained coronal section of mouse brain obtained on day 14 after IC injection of 10^2 B16-F10 tumor cells and 75,000 nonreplicating wild-type B16-F10, followed by interstitial implantation of empty polymer on day 5. *A*, note the cavitation underlying the polymer implantation site with residual polymer surrounded by viable tumor cells and minimal reactive changes including scattered lymphocytes and macrophages. *B*, same coronal section showing the presence of large islands of tumor cells.

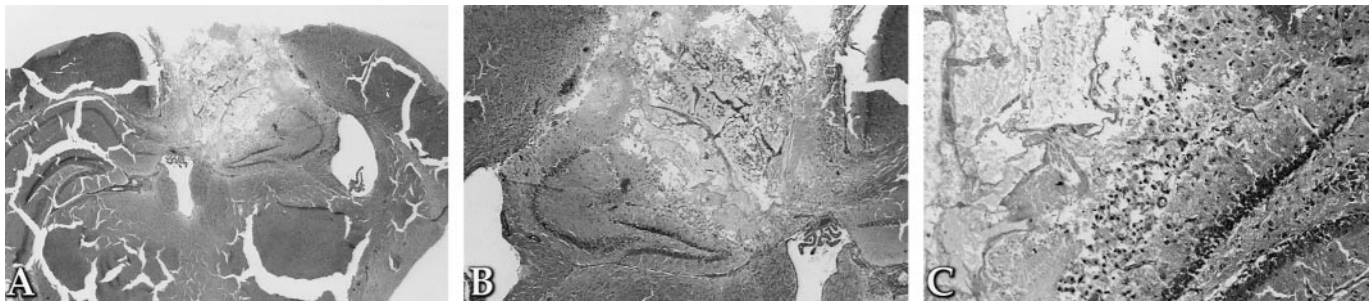

Fig. 5. Photomicrographs of H&E-stained coronal section of mouse brain obtained on day 14 after IC injection of 10^2 B16-F10 tumor cells and 75,000 nonreplicating wild-type B16-F10, followed by interstitial implantation of 10% BCNU loaded polymer on day 5. *A* and *B*, note the extensive tissue necrosis surrounding the polymer with only minimal reactive and inflammatory changes. *C*, higher magnification of the brain parenchyma underlying the polymer cavity showing rare macrophages and scattered degenerating tumor cells.

ing tumor cells. Unfortunately, various systemic immunotherapeutic approaches have, up to this time, met with limited success (35–38). This is due to a combination of factors, including problems posed by the blood-brain barrier, which tightly restricts immune effector cells across brain epithelium (39), poor target recognition in the CNS, immune resistance, and other protective mechanisms intrinsic to brain tumors (35, 40), and limited major histocompatibility antigen expression by tumor cells in the brain (41).

Identification and cloning of genes encoding specific cytokines has provided an important advance in activating immunological effector responses in the brain. Because cytokines exert their immunomodulatory effect in a paracrine fashion, local to the site of antigen, cells genetically engineered to secrete a specific cytokine can be placed directly into the CNS to bypass limitations imposed by systemic administration of cytokines (8). Furthermore, this paracrine biology more closely resembles the natural biology of cytokines and allows for very high concentrations directly at the site of tumor antigens. Using this strategy, we have previously shown that paracrine IC IL-2 with transduced autologous tumor cells is highly effective in treating experimental brain tumors (9). For many years, the CNS was thought to be an immunologically "privileged" site; however, recent evidence suggests the brain may have antigen presentation mechanisms distinct from the periphery (42). Therefore, interest has now shifted to enhancing the intrinsic immunogenicity of IC tumors to increase immunological effector responses.

A novel, emerging strategy against malignant neoplasms is the use of chemotherapeutic agents in combination with immunotherapy (21, 22, 43). The rationale for this cotreatment is based on the finding that chemotherapeutic agents may potentiate immune responses local to the tumor (21, 22). Cell death from cytotoxic agents may increase the number of tumor peptide antigens restricted by class I molecules of the MHC. This "release" of intracellular antigens may provide a powerful set of reagents to promote an inflammatory antitumor response. Moreover, chemotherapeutic agents have been shown to enhance delayed-type hypersensitivity responses at the site of the tumor (44). This mechanism has been attributed to the immune response observed against large tumors eradicated by treatment with cyclophosphamide and IL-12 (22). In addition, immunomodulatory properties of antineoplastic drugs administered in conjunction with granulocyte macrophage colony-stimulating factor-secreting cancer cell vaccines have been reported (21). Chemotherapeutic drugs may also abrogate tumor-derived T-cell suppressor factors that inhibit antigen-induced T-cell function (24, 25), cytotoxic T-cell proliferation, the production of tumor-infiltrating lymphocytes, and the generation of LAK cells (40). This may lead to tumor "unmasking," allowing susceptibility to host immunological effectors.

Perhaps more importantly, chemotherapy may act in concert with immunotherapy by reducing tumor burden. In previous studies with systemic tumors, the curative potential of cytokine-secreting tumor cells seemed to be limited to animals with relatively small tumors (26, 45, 46). It is possible that for malignant CNS neoplasms, surgical resection, followed by local chemotherapy, can result in sufficient cytoreduction, that the tumor will be small enough for it to be targeted and handled by the immune system. Moreover, chemotherapy alone may not be able to eradicate all residual tumor cells after surgical debulking, especially with pleomorphic heterotropic tumors, such as gliomas. Therefore, a combination treatment may be required to overcome potential tumor cell resistance. Moreover, use of local, rather than systemic, chemotherapy may prove helpful in preserving the systemic source of immune effector cells.

Use of local chemotherapy and paracrine IC immunotherapy in combination against IC malignant brain tumors has not been previously examined. In this study, we use an IC B16-F10 tumor model, an aggressive, poorly immunogenic tumor that is uniformly fatal if untreated. The maximal tolerated IC IL-2 dose was previously shown to be 75,000 nonreplicating, IL-2-producing B16-F10-transduced

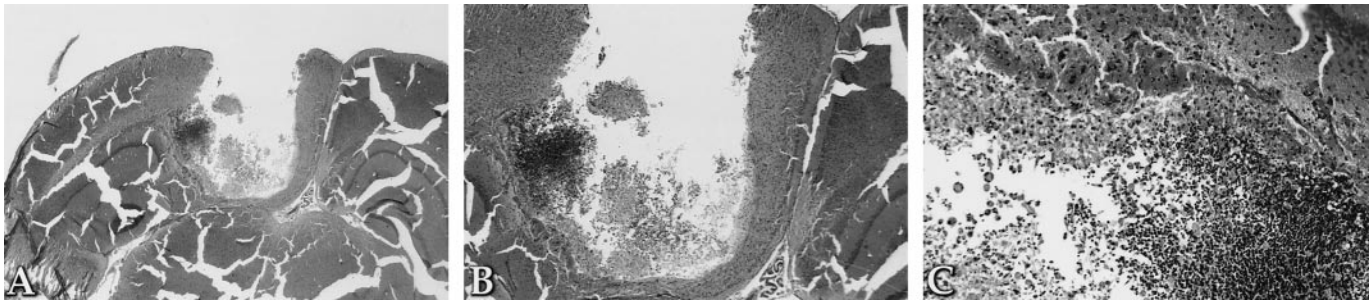

Fig. 6. Photomicrographs of H&E-stained coronal section of mouse brain obtained on day 14 after IC injection of 10^2 B16-F10 tumor cells and 75,000 IL-2-producing, nonreplicating B16-F10, followed by interstitial implantation of empty polymer on day 5. *A* and *B*, note the presence of a striking inflammatory infiltrate surrounding the polymer implantation site. *C*, higher magnification of the inflammatory infiltrate showing both acute and chronic inflammatory components and small clusters of viable tumor cells. Brain surrounding the cavity shows reactive changes, but no evidence of tissue necrosis.

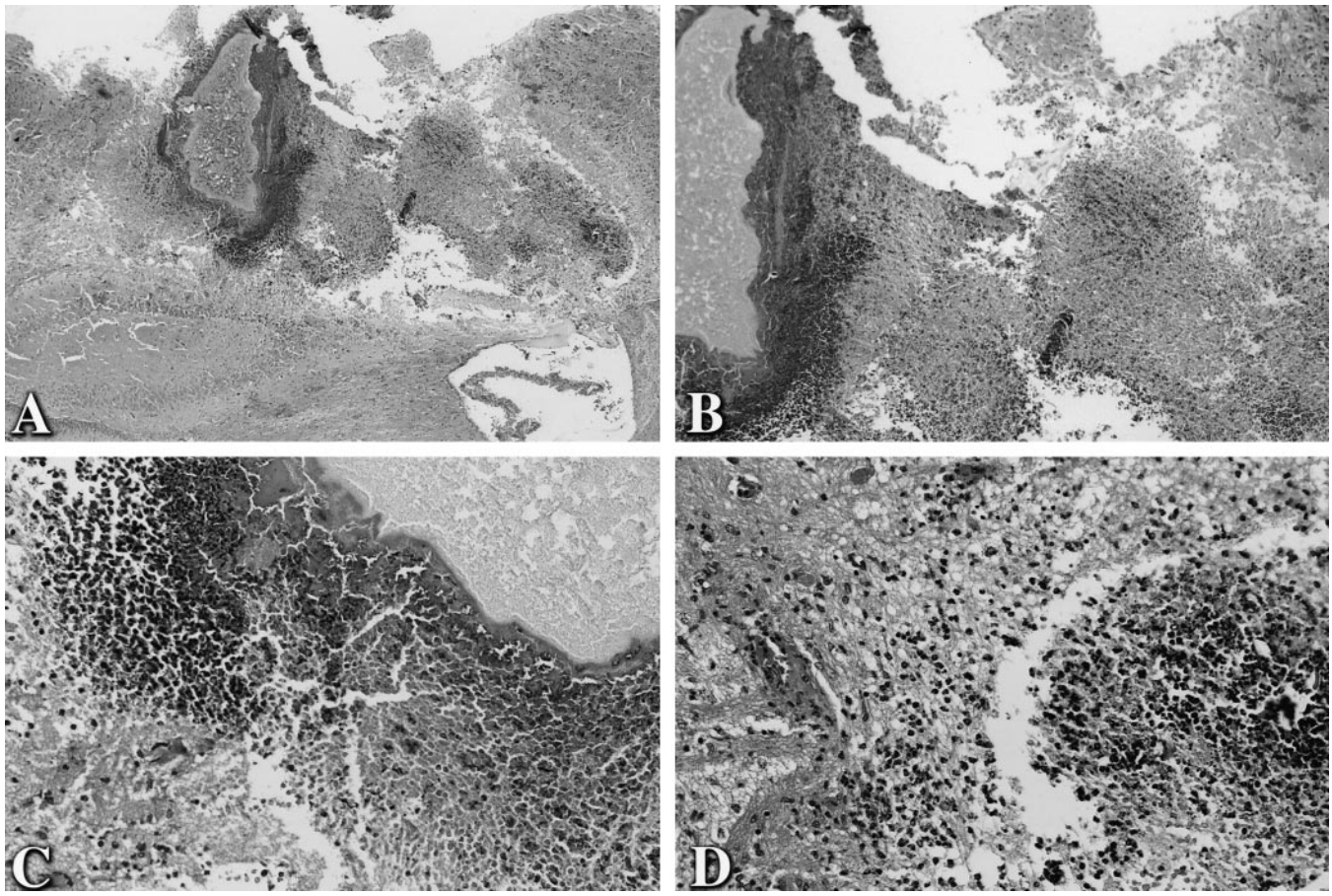

Fig. 7. Photomicrographs of H&E-stained coronal section of mouse brain obtained on day 14 after IC injection of 10^2 B16-F10 tumor cells and 75,000 IL-2-producing, nonreplicating B16-F10, followed by interstitial implantation of 10% BCNU-loaded polymer on day 5. *A* and *B*, note the presence of tissue necrosis and marked inflammatory reaction in the tissue adjacent to the polymer. *C* and *D*, infiltrate of acute and chronic inflammatory cells and high density of degenerating cells, most likely representing dying melanoma cells.

melanoma cells (9). To control for antigen load in the CNS, animals not receiving paracrine IL-2 treatment (groups 1 and 2) received IC injection of irradiated, nonreplicating, non-IL-2-producing B16-F10 (wild type) tumor cells. In the present study, two chemotherapeutic agents were selected, each with unique properties that make it ideal for local polymer delivery. BCNU, an alkylating agent, was chosen because it has a large number of binding sites (47) and has been shown to be especially effective at the high doses achieved by local delivery in rats after IC 9 L glioma challenge (27) and in murine IC tumor models (28, 29). Carboplatin is a water-soluble alkylating agent that is less neurotoxic than cisplatin, but systemic use is still associated with severe marrow toxicity (28, 48). The three loading doses (3.8%, 10%, and 20%) of BCNU were chosen based on previous experimental experience (27) and also on the current use of 3.8% BCNU-impregnated polymers (marketed as Gliadel) in patients with recurrent and newly diagnosed gliomas (49). In addition, a Phase I clinical trial is currently recruiting patients with malignant gliomas to assess whether higher loading doses of BCNU polymer can be tolerated (49). Preliminary data suggest loading doses as high as 20% can be tolerated with acceptable toxicity.

Because a dose-dependent increase in efficacy with BCNU polymer was observed (Table 1) and was well tolerated, efficacy studies of chemotherapy and paracrine IL-2 were performed for each polymer-loading dose. Combination IC immunotherapy with IL-2 and 3.8% BCNU-loaded polymer improved survival over either antitumor therapy alone (Table 2, experiment 1), but synergy was not observed. This

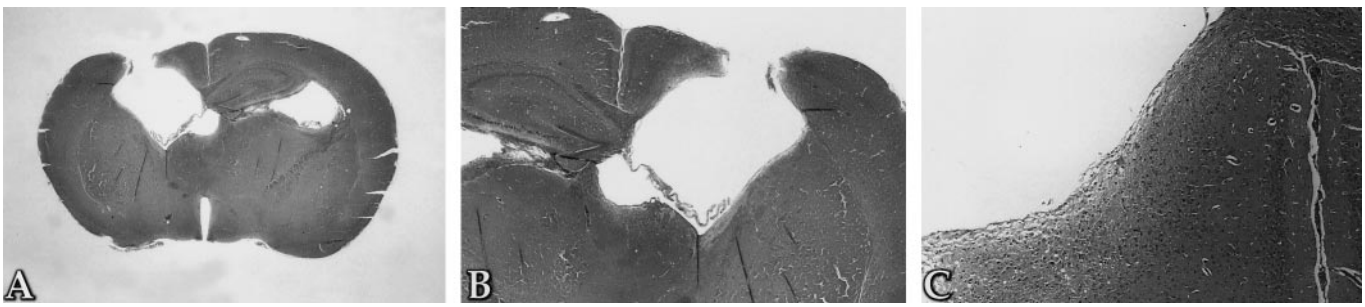

Fig. 8. *A*, *B*, and *C*, photomicrographs of H&E-stained coronal section of mouse brain sacrificed on day 70 after IC injection of 10^2 B16-F10 tumor cells and 75,000 IL-2-producing, nonreplicating B16-F10, followed by interstitial implantation of 10% BCNU-loaded polymer on day 5. Surrounding the cavity, only mild reactive changes were seen. No significant inflammation or tissue necrosis was present. No tumor was present.

may be due to the finding that 3.8% BCNU alone (group 2) did not show any survival advantage over control animals in the B16/F10 IC model. Therefore, potential enhancement of tumor immunogenicity or decrease in tumor burden due to chemotherapy-induced cell death may not have occurred to an adequate degree. No survival advantage was observed when 20% BCNU-loaded polymer was used in combination with IL-2 immunotherapy (Table 2, experiment 2). In this case, 20% BCNU proved to be so effective in prolonging survival (30% LTSs), that it may be difficult to demonstrate an additive or synergistic effect with immunotherapy. This suggests there may be a critical "window of opportunity" where chemotherapy can potentiate immune response modifiers. If the cytotoxicity of the chemotherapeutic agent is too strong, the immune system may be unable to mount an effective response, even in the presence of immune modulating cytokines.

When IL-2 immunotherapy was used in combination with either 10% BCNU-loaded polymer or 1% carboplatin-loaded polymer, statistically significant improvement of survival over either antitumor therapy alone was observed. There may be several reasons for this improvement. Chemotherapy may increase the intrinsic immunogenicity of B16-F10 tumor cells through its cytotoxic effects, thereby enhancing the effect of paracrine immunotherapy. Indeed, mice receiving combination treatment showed a marked mixed inflammatory reaction in addition to tissue necrosis surrounding the polymer. Because IL-2 production by tumor cells is believed to bypass T-helper function (10) and directly promote CTL growth and enhance natural killer/LAK activity (8, 11), the synergistic efficacy seen in these animals may be due to direct stimulation of the effector arm of the immune system in the presence of increased tumor antigens as a result of cell lysis from chemotherapy. Interestingly, in long-term surviving animals receiving combination treatment, no tumor was observed, and the inflammatory reaction was completely resolved. This suggests that continued antigen exposure may be required to maintain an active immune response or that continuous local IL-2 may be necessary to keep effector immune cells in the vicinity. Recent studies have demonstrated that immunological memory is conferred on long-term surviving animals rechallenged with wild-type tumor, raising the specter of "primed" surveillance cells in the CNS.[4]

Another possible explanation for this synergistic effect may be the result of marked cytoreduction caused by local chemotherapy, thereby reducing overall tumor burden. Histological examination of animals receiving chemotherapy polymer alone (group 2) showed scattered tumor cells in the brain parenchyma with a ring of tissue necrosis surrounding the polymer with minimal inflammation (Fig. 4*B*). In contrast, control animals (group 1) showed a large foci of tumor. This finding suggests that in order for anticancer immune strategies to realize their full clinical potential, they may need to be combined with other cancer treatment strategies, such as chemotherapy, to reduce host tumor burden. It is possible that immunotherapy may be ideally suited to target residual tumor cells that escape or are resistant to standard anticancer drugs.

A final plausible reason for synergy between immunotherapy and chemotherapy may be abrogation of tumor-derived T-cell suppressor factors that inhibit the immune responses of the host. This may be important in using cytokine-based therapy against malignant gliomas, where suppression of T-cell function is believed to occur (24, 25).

This study provides the basis for the application of IC paracrine cytokine delivery in combination with chemotherapy-impregnated polymers to brain cancer therapy. The potential of enhancing the intrinsic immunogenicity of malignant brain tumors or reducing tumor burden with cytotoxic agents may provide an avenue for effective use of immunotherapy in CNS neoplasms and may prove to be a means of eradicating residual disease after surgical resection. The demonstration of synergy between paracrine IL-2 and local IC delivery of antineoplastic drugs is novel. This treatment strategy may represent an adjunct to controlling local disease and add to the armamentarium available for the treatment of malignant brain tumors.

[4] M. Ewend, R. C. Thompson, E. Jaffee, R. Anderson, K. Stavely-O'Carroll, B. M. Tyler, K. Babel, A. K. Sills, M. Thomas, H. Levitsky, D. Pardoll, and H. Brem. Intracranial paracrine interleukin-2 therapy stimulates prolonged antitumor immunity and antigen presentation by intrinsic CNS cells, submitted for publication.

ACKNOWLEDGMENTS

We thank Drs. Pamela Talalay, Hy Levitsky, and Ritu Goel for assistance in the preparation of this manuscript. In addition, we gratefully acknowledge the generous support of the Hunterian Laboratory by Laurence Lomax.

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