[CANCER RESEARCH 56, 3986–3992, September 1, 1996]

Intrathecal Chemotherapy with 1,3-Bis(2-chloroethyl)-1-nitrosourea Encapsulated into Hybrid Liposomes for Meningeal Gliomatosis: An Experimental Study[1]

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ABSTRACT

1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), one of the chloroethyl nitrosoureas, is effective against malignant glioma. To develop its use in intrathecal chemotherapy, we encapsulated BCNU in hybrid liposomes composed of dimyristoylphosphatidylcholine and micellar surfactants (Tween 20) and dissolved it in artificial cerebrospinal fluid (lipo-BCNU). We then studied the toxicity of hybrid liposomes and cellular proliferation inhibition of lipo-BCNU *in vitro*. We found that 3 mM hybrid liposomes did not affect the viability of human endothelial cells and that lipo-BCNU inhibited the proliferation of human glioma cell lines U-105MG, U-251MG, and U-373MG, and rat glioma cell lines C6 and 9L in a concentration-dependent fashion. Wistar rats that were administered lipo-BCNU intracisternally showed no weight loss, neurological symptoms, or histological changes of the brain and spinal cord. A Wistar rat model of meningeal gliomatosis was established by intracisternal inoculation of 0.1 ml cell suspension containing 1×10^6 or 5×10^6 viable C6 glioma cells. Two days after inoculation, lipo-BCNU (BCNU, 2.5 mg/kg) was administered intracisternally. When 1×10^6 glioma cells were inoculated (experiments 1 and 2), the median survival times were 24.5 and 26 days in the control groups and 32 and 45 days in the lipo-BCNU-treated groups, respectively. When 5×10^6 glioma cells were inoculated (experiments 3–6), the median survival times were 17–29.5 days in the control groups and 23–44 days in the treated groups, respectively. Significantly prolonged survival was obtained in three of six experimental groups. After the administration of 1 ml lipo-BCNU (BCNU, 4.67 mM) or 1 ml BCNU solubilized with 5% dextrose/water (BCNU, 4.67 mM) into the cisterna magna of dogs, the cisterna magna cerebrospinal fluid was sampled, and the BCNU concentrations were assayed by high-performance liquid chromatography. The half-life of the lipo-BCNU was longer than that of BCNU solubilized with 5% dextrose/water. These results suggest that the intrathecal administration of lipo-BCNU may be possible for the treatment of meningeal gliomatosis.

INTRODUCTION

Meningeal gliomatosis is an uncommon but serious complication of cerebral gliomas (1). Against meningeal gliomatosis, the effectiveness of radiation therapy and systemic chemotherapy appears to be limited. Although drugs such as methotrexate (2, 3), 1-β-D-arabinofuranosylcytosine (2–4), *N,N′,N″*- triethylenethiophosphoramide (2, 5, 6), and neocarzinostatin (7) have been administered intrathecally, they have had insufficient therapeutic effects. We investigated intrathecal chemotherapy using ACNU,[3] water-soluble nitrosourea, the drug of first choice in the treatment of malignant glioma in Japan. We found that in dogs, CSF distribution of ACNU was achieved by ventriculolumbar perfusion methods without serious toxicity (8), and we conducted clinical trials (9, 10). Among primary central nervous system tumors, medulloblastoma and primitive neuroectodermal tumors responded to the therapy; astrocytic tumors responded poorly. To improve these results, we investigated the intrathecal chemotherapy with BCNU, one of the nitrosoureas, the i.v. administration of which is effective for glioma (11, 12). However, BCNU is not suitable for intrathecal injection because of its lipophilicity and low aqueous solubility. To overcome these disadvantages, we encapsulated BCNU in hybrid liposomes composed of DMPC and Tween 20 for intrathecal administration. The hybrid liposomes we prepared have some characteristics that distinguish them from liposomes (13–16). The preparation is simple, without organic solvents that appear to be toxic for the central nervous system. In addition, these hybrid liposomes are uniform in size and stable for more than 2 weeks. Furthermore, we expected that the half-life of BCNU in the CSF would be prolonged by encapsulation into hybrid liposomes. In this study, we evaluated the toxicity of hybrid liposomes and cellular proliferation inhibition of lipo-BCNU *in vitro* and then used rats to evaluate the toxicity and antitumor activity and dogs to evaluate the pharmacokinetics of intrathecally injected lipo-BCNU.

MATERIALS AND METHODS

Hybrid Liposomes and Encapsulation of BCNU. BCNU was provided kindly by Bristol-Myers Squibb Co. (Evansville, IN). Artificial CSF was prepared as follows: 34.77 g NaCl, 1.08 g KCl, 0.920 g $CaCl_2 \cdot 2H_2O$, 0.545 g $MgCl_2 \cdot 6H_2O$, 0.880 g $MgSO_4 \cdot 7H_2O$, 0.385 g $NaH_2PO_4 \cdot 2H_2O$, and 3.0 g glucose were dissolved in 980 ml distilled water as a 5× stock solution. A vial of artificial CSF (108 ml) consisted of the 5× stock solution (21.6 ml), distilled water (85.1 ml), and 0.1 N HCl (1.3 ml). Immediately before use, 8.4% $NaHCO_3$ (2.53 ml) was added to a vial of the artificial CSF, which had a pH of 7.35. The components of artificial CSF were 142.02 meq/liter Na^+, 2.89 meq/liter K^+, 2.50 meq/liter Ca^{2+}, 2.49 meq/liter Mg^{2+}, 126.28 meq/liter Cl^-, 1.42 meq/liter SO_4^{2-}, 0.49 meq/liter $H_2PO_4^{2-}$, and 22.89 meq/liter HCO_3^-. Hybrid liposomes were prepared by dissolving both phospholipids and micellar surfactants (PEG) in the buffer solution with sonication (13–16). In this experiment, the hybrid liposomes were made of 3 mM DMPC as phospholipids and 0.33 mM Tween 20 as PEG surfactants sonicated in artificial CSF in an ultrasonicator (Branson model B2200; Yamato Scientific, Inc., Tokyo, Japan) for 5 min at 45°C in an atmosphere of nitrogen and filtered in a sterile manner through a 0.45 μm Millipore filter. The clear stock solution of hybrid liposomes encapsulating BCNU (lipo-BCNU) was prepared by dissolving DMPC, Tween 20, and BCNU in artificial CSF with sonication in various concentrations (Fig. 1). The average hydrodynamic diameter of lipo-BCNU thus prepared was about 80 nm by dynamic light-scattering measurement (14). These hybrid liposomes are uniform in size and stable for more than 2 weeks (Fig. 2).

Glioma Cells. Human glioma cell lines U-105MG, U-251MG, U-373MG (17–19); rat glioma cell line C6 (20); and rat gliosarcoma cell line 9L (21), cultured in our laboratory, were used. The glioma cells were cultured in tissue culture flasks (Corning Glass, Corning, NY) in RPMI 1640 medium containing penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM glutamine, and 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every 3 days, and the cells were subcultured before they became confluent.

MTT Assay. We modified methods described by Mosmann (22) and Nikkhah *et al.* (23). MTT (Sigma Chemical Co., St. Louis, MO) was dissolved in PBS at a concentration of 2 mg/ml, and 50 μl were added to each microculture well. After 4 h incubation at 37°C, the culture plates were

Received 3/22/96; accepted 7/2/96.

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[1] This work is supported in part by a Grant-in-Aid (05671172, 07808101) for Science Research from the Ministry of Education and the Science Research Promotion Fund from the Japan Private School Promotion Foundation.

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[3] The abbreviations used are: ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; lipo-BCNU, BCNU encapsulated in hybrid liposomes; DMPC, dimyristoylphosphatidylcholine; CSF, cerebrospinal fluid; MTT, 3-(4, 5-dimethyl-2-thiazolyl)-2,5 diphenyltetrazolium bromide; PEG, polyoxyethyleneglycol; FBS, fetal bovine serum.

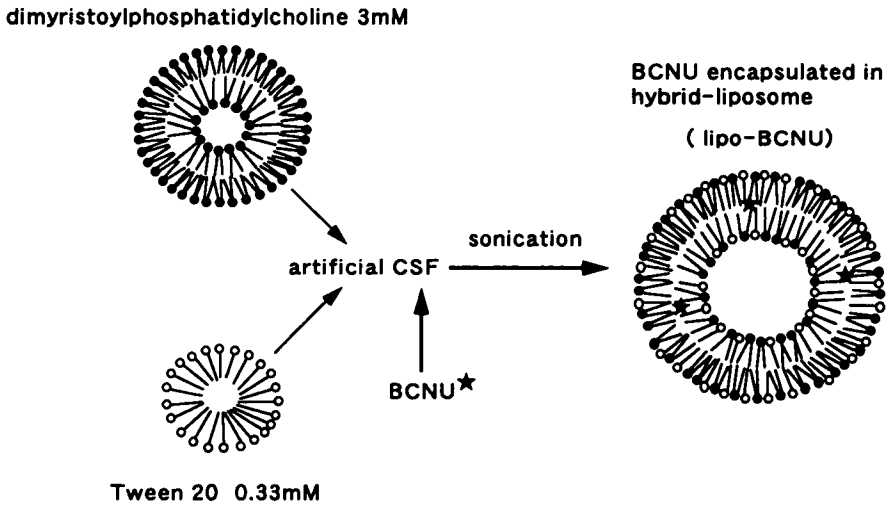

Fig. 1. Hybrid liposomes were prepared by dissolving both phospholipids and micellar surfactants (PEG) in the buffer solution with sonication. The clear stock solution of hybrid liposomes encapsulating BCNU (lipo-BCNU) was prepared by dissolving 3 mM DMPC, 0.33 mM Tween 20, and BCNU in various concentrations in artificial CSF with sonication for 5 min at 45°C in an atmosphere of nitrogen.

centrifuged at 1000 rpm for 10 min, and the medium supernatant was removed. Acid-isopropanol (100 μl; 0.04 N HCl in isopropanol) was added to each well, and plates were shaken for dissolving. After sufficient solubilization of the MTT-formazan product, absorbance at 570 nm was measured with a Bio-Rad model 2550 EIA reader (Hercules, CA). The percentage of control was calculated as a percentage of absorbance at 570 nm of the treated cells compared to absorbance at 570 nm of the control (untreated) cells. The *P* value was determined by the unpaired *t* test.

Assessment of Toxicity of Hybrid Liposomes *in Vitro*. Endothelial cells from human umbilical vein and culture medium E-GM were obtained from Kurabo, Inc. (Osaka, Japan). After harvesting, the cells were sorted by the trypan blue exclusion method, and 1×10^4 viable cells in 100 μl of the culture medium were incubated in 96-well microtiter plates. After 24 h, 3 mM hybrid liposomes not encapsulating BCNU, 3 mM phosphatidylcholine, and 0.33 mM Tween 20 in 10 μl artificial CSF were added to each well, and the plates were incubated again. After 48 h, the number of viable cells was checked using the MTT assay.

Assessment of Cytotoxicity of Lipo-BCNU *in Vitro*. Human glioma cell lines U-105MG, U-251MG, U-373MG; rat glioma cell line C6; and rat gliosarcoma cell line 9L were incubated in the medium that contained lipo-BCNU for 48 h. BCNU concentrations in the medium were 93.4, 233.5, 467.1, and 934.3 μM. Cell viability was tested using the MTT assay.

Assessment of *in Vivo* Toxicity of Intrathecal Hybrid Liposomes and Lipo-BCNU. Male Wistar rats, weighing approximately 180 g and obtained from Japan SLC (Shizuoka, Japan), were used in the *in vivo* experiments. The rats were maintained in a specific pathogen-free environment in the Laboratory Animal Research Center of Kumamoto University Medical School and fed sterile laboratory pellets and water. The rats were anesthetized by i.p. injection of sodium pentobarbital at a dose of 50 mg/kg. Hybrid liposomes not encapsulating BCNU or lipo-BCNU (0.1 ml) at BCNU concentrations of 5.25, 10.51, and 21.02 mM (the doses of BCNU were 0.625, 1.25, and 2.5 mg/kg body weight, respectively) were injected percutaneously into the cisterna magna. The rats were checked daily for changes in body weight and for neurological symptoms. At 23 days after the injection, the rats were killed by i.p. injection of sodium pentobarbital at a fatal dose, and their brains and spinal cords were removed, fixed in 10% formalin, and embedded in paraffin. Representative sections were stained with H&E and the Klüver-Barrera stain and inspected under a light microscope.

Meningeal Gliomatosis Model. Cultured C6 glioma cells were harvested, and the cell suspension was centrifuged at 800 rpm for 5 min; the supernatant

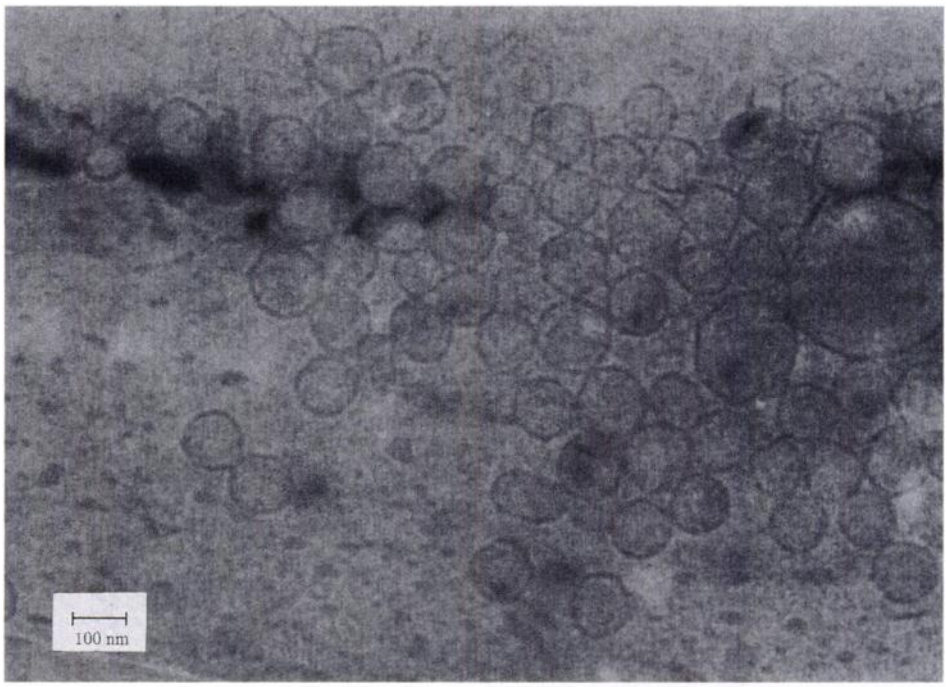

Fig. 2. Electron micrograph of hybrid liposomes composed of DMPC and PEG surfactants obtained by means of cryotransfer technique (JEM-2000, Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan). They are uniform in size, and the diameter is about 80 nm.

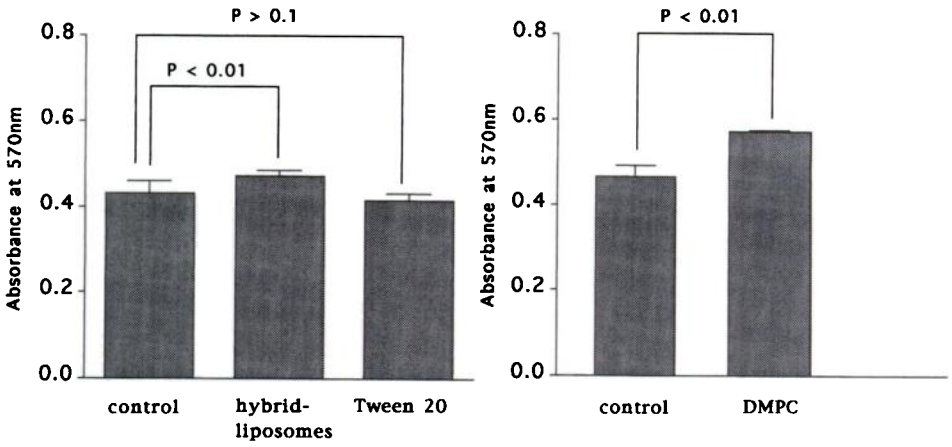

Fig. 3. Absorbance at 570 nm of human endothelial cells by MTT assay. The proliferation of human endothelial cells exposed for 48 h to 3 mM hybrid liposomes not encapsulating BCNU, 0.33 mM Tween 20, or 3 mM DMPC in the artificial CSF was not inhibited. The controls consisted of untreated endothelial cells. The *P* value was determined by unpaired *t* test. Data presented are means; *bars*, SDs.

fraction was aspirated off, and the cells were washed twice with RPMI 1640 containing 10% FBS. The cells were sorted by trypan blue exclusion, and 1×10^7 or 5×10^7 viable cells/ml in RPMI 1640 containing 10% FBS were kept on ice until the suspension was drawn into 1-ml syringes for intrathecal injection. For intrathecal inoculation, 0.1 ml of cell suspension containing 1×10^6 or 5×10^6 viable cells was inoculated percutaneously into the cisterna magna of rats using 27-gauge needles (24).

Assessment of *in Vivo* Antitumor Activity of Intrathecal Lipo-BCNU. Two days after tumor inoculation, the rats were divided randomly into two groups (control group and treated group). Lipo-BCNU (0.1 ml) at a BCNU concentration of 21.02 mM (the dose of BCNU was 2.5 mg/kg body weight) was injected percutaneously into the cisterna magna of rats in the treated group. All rats were checked daily for 60 days, and changes in body weight and neurological symptoms were recorded. The life span of treated rats was compared to that of the controls and the median life span as percentage of control was determined. The *P* value was determined by the generalized Wilcoxon test. If there were long-term survivors (more than 60 days) without microscopic evidence of tumor in the control group, they were excluded from the experiment, and the same number of long-term survivors in the treated group was also excluded.

Intrathecal Pharmacokinetics of Lipo-BCNU in Dog. Two adult mongrel dogs weighing 11.8 and 12.1 kg were anesthetized with ketamine and pentobarbital and placed in the sphinx position. Anesthesia was maintained with a mixture of nitrous oxide, oxygen, and halothane. One dog received a bolus injection of 1 ml lipo-BCNU (BCNU concentration, 4.67 mM; BCNU dose, 1 mg/body) into the cisterna magna, and CSF samples were withdrawn from the cisterna magna at 5, 10, 30, and 45 min postinjection. BCNU solubilized with 5% dextrose/water as described by Levin et al. (25) and diluted in artificial CSF at BCNU concentration of 4.67 mM was administered by bolus injection into the cisterna magna of the other dog (BCNU dose; 1 mg/body), and the CSF was sampled in the same way. The CSF samples were frozen immediately. The BCNU concentrations were assayed by reverse-phase high-performance liquid chromatography using a LC10A system (Shimazu Co., Tokyo, Japan) equipped with an octadecylsilica column (Cosmosil $5C_{18}$-AR 5 μm, 4.6 mm inside diameter × 150 mm; Nacalai Tesque, Inc., Kyoto, Japan) with a solvent system of acetonitrile and 0.1% acetic acid in

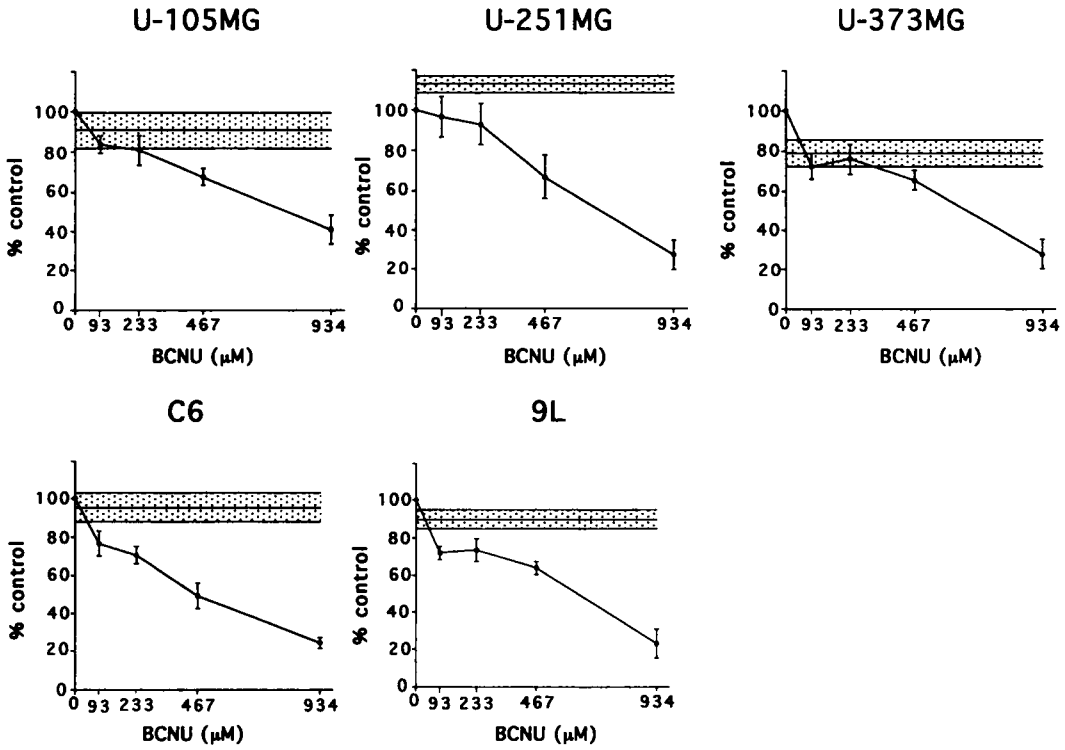

Fig. 4. Growth rate of glioma cell lines exposed to lipo-BCNU for 48 h. BCNU concentrations in the medium were 93.4, 233.5, 467.1, and 934.3 μM. The proliferation of human glioma cell lines U-105MG, U-251MG, and U-373MG, and of rat glioma cell lines C6 and 9L, was inhibited in a concentration-dependent fashion. *Plotted areas*, growth rate of glioma cell lines exposed to 3 mM hybrid liposomes not encapsulating BCNU for 48 h. Data presented are means; *bars*, SDs.

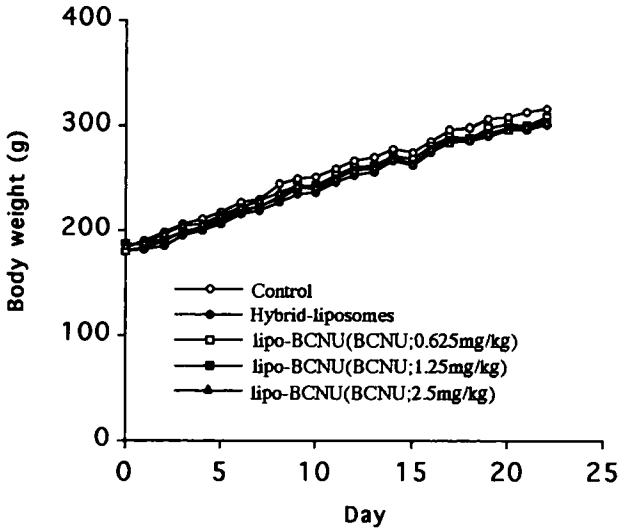

Fig. 5. Changes in average body weight of rats after intracisternal administration of 3 mM hybrid liposomes not encapsulating BCNU and lipo-BCNU (BCNU, 0.625 mg/kg, 1.25 mg/kg, and 2.5 mg/kg). No weight loss was observed.

distilled water (40:60, v/v) at a mobile phase. The flow rate was 1.0 ml/min. The column temperature was maintained at 40°C. Sample absorbance was measured at 230 nm. Samples of 10 μl were injected directly onto the high-performance liquid chromatography column. The BCNU concentrations were calculated with reference to standard curves. The detection limit of BCNU was 0.46 μM. To calculate the half-life, an exponential function was used to fit the data by using the least-squares method.

RESULTS

Toxicity of Hybrid Liposomes. Neither 3 mM hybrid liposomes not encapsulating BCNU, 3 mM DMPC, or 0.33 mM Tween 20 in artificial CSF damaged the viability of human endothelial cells. The absorbances at 570 nm of human endothelial cells exposed to 3 mM hybrid liposomes not encapsulating BCNU and 0.33 mM Tween 20 for 48 h were 0.471 ± 0.014 and 0.416 ± 0.014, respectively (for the control it was 0.432 ± 0.027). For endothelial cells exposed to 3 mM phosphatidylcholine for 48 h, the absorbance was 0.576 ± 0.004 (for the control it was 0.465 ± 0.027; Fig. 3). Hybrid liposomes did not affect the growth of human endothelial cells.

Cytotoxicity of Lipo-BCNU *in Vitro*. The percentages of control of C6 glioma cells exposed to lipo-BCNU at BCNU concentrations of 93.4, 233.5, 467.1, and 934.3 μM in the medium for 48 h were decreased to 76, 70, 49, and 24%, respectively. The half-life of BCNU in the liposomal preparations when in cell culture medium was 35.7 min (date was not shown). Similarly, lipo-BCNU inhibited the proliferation of U-105MG, U-251MG, U-373MG, and 9L cells in a concentration-dependent fashion (Fig. 4).

Toxicity of Hybrid Liposomes and Lipo-BCNU *in Vivo*. Neither weight loss nor neurological changes were observed in the rats that had received injections of hybrid liposomes or lipo-BCNU at BCNU doses of 0.625, 1.25, and 2.5 mg/kg into the cisterna magna (Fig. 5). Microscopically, no ependymitis, periventriculitis, meningitis, vasculitis, or demyelination was observed.

Antitumor Activity of Lipo-BCNU *in Vivo*. In this study, six separate experiments were performed. In experiments 1 and 2, 1×10^6 viable C6 cells were inoculated; in experiments 3–6, 5×10^6 cells were inoculated. In the terminal stage, almost all rats showed weight loss. Microscopically, the tumor appeared as a few layers or as a continuous multicellular mantle on the surface of the brain and spinal cord (Fig. 6). As shown in Table 1, in experiments 1 and 2, the median survival rates were 24.5 and 26 days in the control groups and 32 and 44.5 days in the treated groups, respectively. The median life spans (% control) were 130 and 171%, respectively. Similarly, in experiments 3–6, the median survival of the treated rats was longer than of the controls. There was a significant difference between the control and treated groups in experiments 2 ($P < 0.01$), 5 ($P < 0.05$), and 6 ($P < 0.05$). The survival curves for rats in experiments 2 and 6 are shown in Fig. 7.

Intrathecal Pharmacokinetics of Lipo-BCNU in Dog. When 1 ml lipo-BCNU (BCNU concentration, 4.67 mM; BCNU dose, 1 mg/body) was administered into the cisterna magna by bolus injection, the BCNU concentrations of cisterna magna CSF were 217.6, 56.5, and

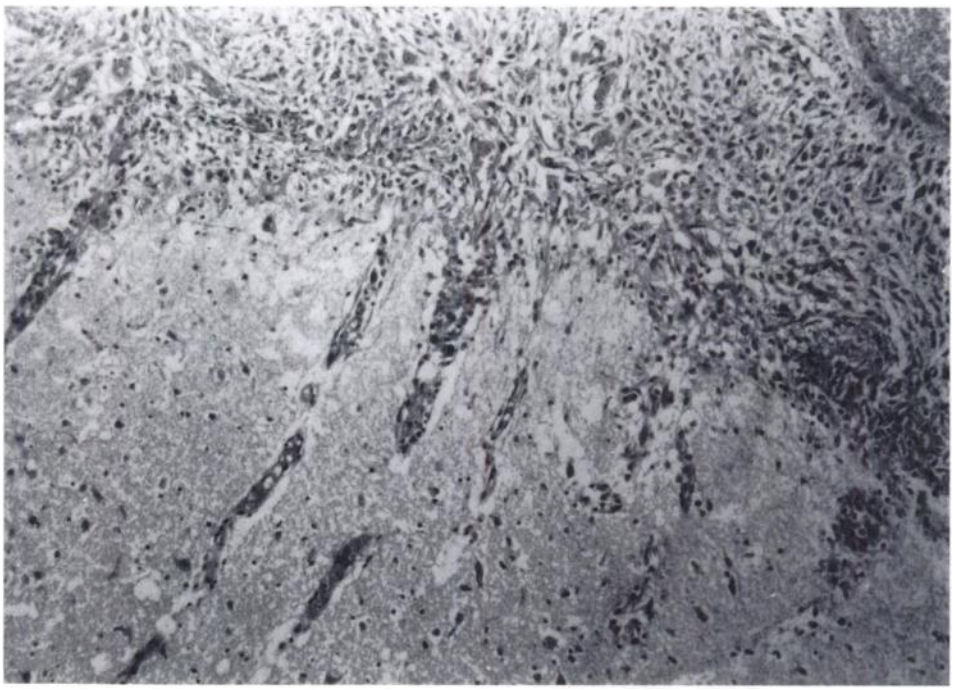

Fig. 6. Microphotograph showing parenchymal invasion extending from multiple layers of tumor cells on the surface of the brain 33 days after the inoculation of 1×10^6 viable C6 glioma cells. H&E; original magnification, ×50

Table 1 *Effect of intrathecal administration of lipo-BCNU against meningeal gliomatosis*

Experiment	Inoculum (C6 cells)	Group	n^a	Median survival (days)	Median life span (% control)	P value (Wilcoxon test)
1	1×10^6	Control	6	24.5	130	NS[b]
		Treated	7	32.0		
2	1×10^6	Control	8	26.0	171	<0.01
		Treated	8	44.5		
3	5×10^6	Control	11	29.5	108	NS
		Treated	10	32.0		
4	5×10^6	Control	8	17.0	135	NS
		Treated	11	23.0		
5	5×10^6	Control	5	26.0	169	<0.05
		Treated	5	44.0		
6	5×10^6	Control	15	27.0	116	<0.05
		Treated	16	31.5		

[a] n, number of rats.
[b] NS, not significant.

6.5 μM at 5, 10, and 30 min after the injection, respectively. Forty-five min postinjection, no BCNU was detected in the CSF. The BCNU concentration decreased exponentially; the half-life was 12.1 min (r^2 = 0.96). When BCNU solubilized with 5% dextrose/water and diluted with artificial CSF (BCNU concentration, 4.67 mM; BCNU dose, 1 mg/body) was administered into the cisterna magna by bolus injection, the BCNU concentrations of the cisterna magna CSF were 162.1, 14.4, and 1.4 μM at 7.5, 13.6, and 20 min postinjection respectively. BCNU was not detected at 30 and 45 min after the injection. The BCNU concentration decreased exponentially; the half-life was 4.2 min (r^2 = 1.00; Fig. 8).

DISCUSSION

In patients with meningeal gliomatosis, radiotherapy is effective for the treatment of bulky metastases of the spinal cord, but it cannot control widespread disease. Most types of systemic chemotherapy do not provide an effective drug concentration in the CSF and, although some drugs have been administered intrathecally, they failed to produce sufficient therapeutic effects (2–7, 9, 10).

To overcome these problems, we investigated the possibility of intrathecal chemotherapy with BCNU. BCNU has the following pharmacological characteristics: (*a*) it is a highly lipophilic drug with a log P (octanol/water partition coefficient) of 1.5; (*b*) its capillary transfer constant is high, and it crosses the blood-brain barrier easily; and (*c*) its time course of action is short. Levin and Levin (25) reported that BCNU can be solubilized in 5% dextrose/water at 60°C, thus making it available for intrathecal chemotherapy. Ueoka *et al.* (14) have recently produced specific hybrid liposomes composed of vesicular and micellar molecules, the physical properties of which (sizes, membrane fluidity, phase transition, and hydrophobicity) could be controlled by changing their composition. We encapsulated BCNU in these hybrid liposomes and dissolved it in artificial CSF to make intrathecal administration possible and to improve the CSF pharmacokinetics.

As a drug-delivery system in cancer therapy, liposomes and other lipid-based microspheres have been investigated with the aim of increasing efficacy, decreasing toxicity, or increasing the ease of administration (26). Many antitumor agents have been investigated, and BCNU and other nitrosoureas were found to exhibit increased antitumor activity in certain situations. Ritter and Rutman (27) reported that the effects of a low dose of BCNU on L1210 leukemia can be potentiated by the simultaneous administration of dipalmitoylphosphatidylcholine multilamellar liposome vesicles. Inaba *et al.* (28) reported that the inhibitory effect of 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea liposomes on the lung metastasis, induced by i.v.- or i.m.-planted Lewis lung carcinoma, was greater than that of the free drug. Takenaga *et al.* (29) found that lipid microspheres consisting of egg yolk lecithin and soybean oil and containing BCNU exhibited not only enhanced activity against L1210 leukemia in mice but also reduced toxicity. These findings show that it is reasonable to encapsulate lipophilic drugs such as BCNU in liposomes.

The intra-CSF administration of liposome-encapsulated drugs has been reported (30–32), and clinical trials are under way (33). Kim *et al.* (31, 32) showed that in rats and monkeys, liposomes containing

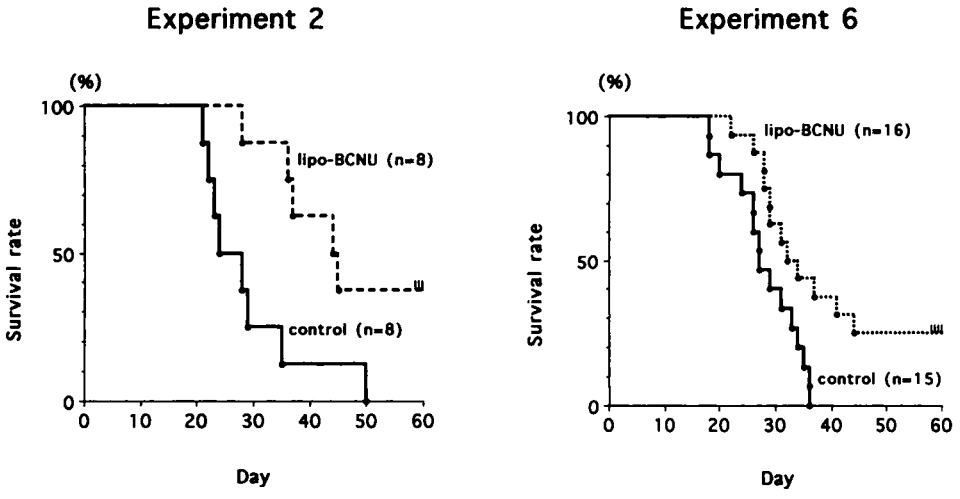

Fig. 7. Survival curves of rats treated with or without lipo-BCNU (BCNU, 2.5 mg/kg) 2 days after the inoculation of C6 glioma cells. In experiment 2, 1×10^6 viable cells, and in experiment 6, 5×10^6 viable cells were inoculated intracisternally.

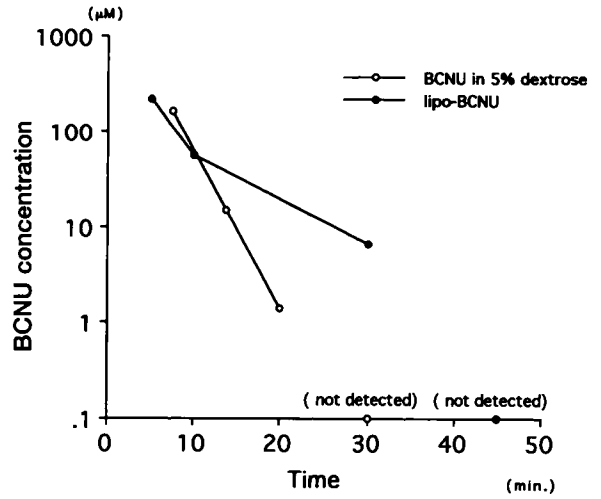

Fig. 8. The BCNU concentration in the CSF was tested by puncture of the cisterna magna of dogs that had received a bolus injection into the cisterna magna of 1 ml lipo-BCNU (BCNU, 4.67 mM) or BCNU solubilized with 5% dextrose/water and diluted with artificial CSF (BCNU, 4.67 mM). The detection limit of BCNU was 0.46 μM.

1-β-D-arabinofuranosylcytosine was capable of maintaining a cytotoxic concentration of free drug in the CSF for weeks. Their clinical Phase 1 study showed that seven of nine patients with neoplastic meningitis attained a complete cytological response upon intraventricular or intralumbar administration.

Our hybrid liposomes were not toxic to human endothelial cells *in vitro,* and in rats, intracisternally administered lipo-BCNU did not cause any neurological symptoms or histological changes in the brain and spinal cord. Levin *et al.* (34) observed no pathological changes in a dog that had received 8 weeks of 2 mg/week BCNU by intraventricular administration and another dog treated with 5 mg/week for 8 weeks. However, a dog that received 5 mg/week BCNU for 4 weeks exhibited partial obliteration of the aqueduct and hydrocephalus due to ventriculitis. Nagatani *et al.* (35) found that rats given ACNU intracisternally at more than 3.0 mg/kg lost body weight progressively; at more than 6.0 mg/kg, ACNU was fatal in 80% of the rats. In our experiments, the administration of lipo-BCNU at a BCNU dose of 2.5 mg/kg did not result in weight loss; nor were there any neurological symptoms or pathological changes in the brains and spinal cords of rats. Because it was not possible to encapsulate more than 21.02 mM (2.5 mg/kg) BCNU in the hybrid liposomes because of capacity limitations, we decided that the optimal dose of BCNU was 2.5 mg/kg.

With regard to cytotoxicity *in vitro,* lipo-BCNU inhibited the proliferation of glioma cells in a concentration-dependent fashion. We used the MTT assay to test antitumor activity *in vitro*. In a study that used a number of rat and human glioma cell lines (36, 37), there was a close correlation in chemosensitivity between the clonogenic assay and the MTT assay. In three of our six experiments, the intrathecal injection of lipo-BCNU (BCNU, 2.5 mg/kg) significantly prolonged the median survival of rats with meningeal gliomatosis. There is a possibility that some immune effect enhanced antitumor activity, because C6 glioma cells were suspended in medium containing 10% FBS. However, considering the chemoresistance of C6 glioma (38), our findings suggest that lipo-BCNU is effective in the treatment of meningeal gliomatosis.

The intrathecal half-life of lipo-BCNU was longer than that of BCNU solubilized with 5% dextrose/water. This is another advantage of intrathecally injected lipo-BCNU.

In summary, we demonstrated that in rats, intrathecal chemotherapy with lipo-BCNU is feasible to guard against subarachnoid dissemination of malignant glioma. Additional studies are under way to examine and improve the therapeutic effect.

ACKNOWLEDGMENTS

We thank Drs. Ryoko Matsushita and Yusuke Moriyama (Department of Pharmaceutics, Kumamoto University Medical School) for providing artificial CSF, Ikuyo Ishimatsu for preparing the histopathological specimens, and Takatoshi Ito for technical assistance.

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