The effect of amphotericin B on the survival of brain-tumor-bearing mice treated with CCNU

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Although the nitrosoureas appear to be the best available chemotherapeutic agents for the treatment of malignant brain tumors, their efficacy must be improved if long-term survival is to be achieved in patients suffering from malignant gliomas. The nitrosoureas have been shown to be highly effective in prolonging survival in a number of transplantable brain-tumor models and in an autochthonous model, and are superior to other chemotherapeutic agents thus far tested. A number of Phase II clinical trials have revealed some therapeutic effectiveness of nitrosoureas against malignant brain tumors. However, Reagan, et al., showed, in their Phase III study, that the addition of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) to radiotherapy was of no value in increasing survival. Weir, et al., in their cross-over study, had a similar experience. In the cooperative Phase III trial...
TABLE 1
Intraperitoneal amphotericin B (AMP) and intraperitoneal CCNU in the treatment of intracerebral mouse ependymoblastoma*

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<thead>
<tr>
<th>Experiment No.</th>
<th>No. of Mice</th>
<th>Dose of AMB (mg/kg)</th>
<th>Dose of CCNU (mg/kg)</th>
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<th>Change in Life Span (%)</th>
<th>No. of Long-Term Survivors (&gt; 100 days)</th>
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*All mice in each experiment received the same tumor-cell suspension, administered on Day 1. All drugs were delivered by the intraperitoneal route. The AMB was given on Day 4, and CCNU on Day 5. NS = not significant. Tox indicates a toxic level.

†Significance of treated groups as compared to control.

‡Significance of groups treated with AMB compared to group receiving no AMB but an identical dose of CCNU.

Conducted by Walker and Gehan, it was demonstrated that 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) increased median survival in patients with malignant astrocytomas, but the increase was small when compared to the results of surgery plus radiotherapy without BCNU. Thus, the effects of the nitrosoureas must be improved if long-term survival is to be attained in the treatment of malignant brain tumors.

The efficacy of the nitrosoureas in the treatment of cerebral tumors is related to their lipid solubility and ability to cross the blood-brain barrier. There are, however, limits to this drug delivery system. Both BCNU and CCNU have been shown to be more effective against intraperitoneal than intracerebral implants of the same tumor. Furthermore, it has been suggested that, although they easily penetrate the blood-brain barrier, the nitrosoureas have impaired effectiveness against intracerebral tumors because of their failure to reach some parts of the tumors in adequate concentration. Potentiation of the effects of various chemotherapeutic agents by the membrane-active polyene, amphotericin B (AMB), has been demonstrated in several tissue-culture systems. The ability of AMB to potentiate the effects of the nitrosoureas has been demonstrated in vivo. Medoff, et al., were able to dramatically increase the survival of AKR leukemic mice by the administration of AMB 24 hours prior to a dose of BCNU which by itself was of marginal value. Laurent, et al., have extended this observation to solid tumors by showing an increased rate of complete regression and cure of a subcutaneous mouse ependymoblastoma when AMB was given 10 hours before CCNU.

To our knowledge, there have been no previous studies of chemotherapeutic potentiation in an intracerebral brain-tumor model. We have examined the effect on survival of AMB administered intraperitoneally or intracerebrally, alone or in conjunction with CCNU, in a transplantable intracerebral murine ependymoblastoma.
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Materials and Methods

Animals and Tumor

This study was performed using C57B1/6J female mice* that weighed 16 to 18 gm. A mouse ependymoblastoma has been maintained in our laboratory by serial subcutaneous transplantation every 2 weeks since 1963. The tumor was originally induced by Zimmerman and Arnold in mouse brain with intracerebrally implanted methylcholanthrene.

Tumor-Cell Suspension and Intracerebral Implantation

Approximately 2 gm of 14-day-old subcutaneous ependymoblastoma tumor tissue was harvested and trypsinized in phosphate-buffered saline to produce a tumor-cell suspension containing 10^5 cells/ul as previously described. Penicillin (50 units/ml) and streptomycin (50 mg/ml) were added to the tumor-cell suspension. With a No. 30 needle attached to a micrometer syringe assembly, 3 to 6 µl of tumor-cell suspension were injected into the right hemisphere of recipient mice by means of a stereotaxic frame.

Drugs

The amphotericin B (Fungazone) was purchased in vials each containing 50 mg AMB, 41 mg sodium desoxycholate, and 25.7 mg of sodium phosphate buffer. The AMB solution was prepared immediately before injection by dissolving a vial of Fungazone in an appropriate volume of sterile water.

The CCNU suspension was prepared by grinding an appropriate weight of CCNU† in 2.0 ml of 0.4% methylcellulose in a Potter-Elvehjem homogenizer‡ for 10 minutes in an ice bath. After a uniform suspension of CCNU was achieved, 0.4% methylcellulose was added to the initial suspension to bring it to the desired concentration. The final suspension was kept uniform by frequent mechanical mixing, and was left in an ice bath until use. The time from drug preparation to completion of administration to the mice did not exceed 90 minutes.

Experimental Protocol

Tables 1 and 2 show the experiments conducted. All mice in each individual experiment received the same suspension of tumor cells. Each experiment had its own simultaneous control. Control animals received 0.4% methylcellulose in place of CCNU and sterile water in place of Fungazone by the appropriate route through the same gauge needle as the treatment groups. Intracerebral injection volume was 4.5 µl and intraperitoneal injection volume was 100 µl. The day of intracerebral tumor implantation was designated as Day 1; AMB was administered on Day 4 and CCNU on Day 5 in all experiments.

Statistical Analysis

The median day of death was determined graphically by plotting the cumulative percentage of deaths against survival days after tumor implantation (Figs. 1–4). Comparison was then made between the median day of death of treated and control groups, and the percentage increase or decrease in life-span was thus determined. Long-term survivors were defined as animals living more than 100 days from the time of tumor implantation. Significant prolongation (therapeutic) or shortening (toxic) of survival was assessed from the graphs by the Kolmogorov-Smirnov test as adapted by Tate and Clelland. To test the significance of differences between proportions as in the evaluation of the number of long-term survivors, a table prepared by Csima and Reid, based on Fisher's exact procedure was used.

Results

Table 1 contains the results of three survival studies using intraperitoneal AMB and CCNU, alone or in combination. At a dose of 50 mg/kg, intraperitoneal AMB was highly toxic (Experiment 1), whereas 25 mg/kg intraperitoneal AMB (Experiments 2 and 3) was not toxic but did not significantly prolong survival (Figs. 1 and 2). Administration of
CCNU alone was ineffective at 5 mg/kg, moderately effective at 10 mg/kg, and highly effective at 20 to 30 mg/kg (Figs. 1 and 2). Administration of 25 mg/kg intraperitoneal AMB 24 hours before intraperitoneal doses of CCNU from 5 to 30 mg/kg did not significantly increase the life-span, nor the number of long-term survivors (Table 1).

Table 2 contains the results of three survival studies using intracerebrally administered AMB and intraperitoneal CCNU, alone or in combination. At a dose of 1.5 mg/kg intracerebral AMB (Experiment 4), sufficient early toxicity occurred to render the survival distributions bimodal and thus prevented a significant improvement in survival from becoming manifest in the combined treatment groups. In Experiment 5, 1.0 mg/kg intracerebral AMB produced early toxicity without significantly altering the median day of death. Intracerebral AMB, 1.0 mg/kg, 24 hours prior to CCNU, 20 mg/kg, resulted in the early death of 20% of the mice, although no further mortality occurred for 80 days. This administration of AMB, 1.0 mg/kg, before CCNU significantly (p < 0.05) prolonged survival when compared with the group receiving CCNU alone. At a dose of 0.2 mg/kg, intracerebral AMB was nontoxic, and significantly (p < 0.01) prolonged survival when compared to the group receiving 20 mg/kg CCNU alone.
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**Fig. 2.** Graph showing the percentage of cumulative death plotted against days after intracerebral tumor implantation. The AMB was administered intraperitoneally on Day 4 and CCNU intraperitoneally on Day 5. See Table 1, Experiment 2, for details.

Furthermore, the number of mice surviving more than 100 days was significantly (p < 0.01) increased in the combined AMB-CCNU groups compared to the group receiving CCNU alone.

In Experiment 6, 0.5 mg/kg of intracerebral AMB alone did not alter survival (Fig. 4). The survival with intraperitoneal CCNU alone at 20 mg/kg was near optimal with 85% survival at 60 days, 60% survival at 100 days, and 50% survival at 140 days. Nevertheless, the addition of 0.5 mg/kg of intracerebral AMB 24 hours before the CCNU resulted in 90% survival during the entire period of observation (144 days) (p < 0.01). Administration of 10 mg/kg CCNU alone did not significantly increase survival (17% change in life-span) over control, but the administration of 0.5 mg/kg intracerebral AMB before this dose of CCNU did significantly (p < 0.05) increase survival (61% change) over control. At a dose of 5 mg/kg CCNU there was no therapeutic effect whether or not it was combined with intracerebral AMB.

**Discussion**

The first barrier that a chemotherapeutic agent must penetrate in order to affect the tumor cells in brain-tumor-bearing subjects is the blood-brain barrier. This barrier is disrupted in the central portions of glial tumors...
but intact in the peripheral regions where glial tumor cells infiltrate brain and where tumor growth is believed to be maximal. The last barrier is the membrane of the tumor cell itself, and if the permeability of this barrier could be increased then the effects of anti-neoplastic agents might be enhanced once they had passed beyond the cerebral capillary.

The enhancement of the effects of various agents on cells in tissue culture is thought to be the result of increased uptake of the "second agent." Autoradiographic studies have demonstrated a sixfold increase in the uptake of labeled actinomycin D in actinomycin-resistant HeLa cells when these cells were incubated in AMB before the actinomycin treatment. Furthermore, it is the effect of the "second agent" that is enhanced rather than simply the addition of the cytotoxic activity of AMB.

In vivo studies have tended to confirm the potentiation property of AMB but not the mechanism of action. In the AKR leukemia model, Medoff, et al., demonstrated a dramatic increase in the survival of leukemic mice when AMB preceded the administration of BCNU. When leukemic colony-forming units were measured, there was not only an initial fivefold decrease compared to BCNU treatment alone but also a delayed decrease in the survivors. Although the initial fall in
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colony-forming units can be explained by increased uptake of CCNU by the leukemic cells, the delayed decrease requires another explanation. The authors postulated enhancement of immune mechanisms by AMB. Immune modification has been demonstrated for AMB\textsuperscript{16,36} and vitamin A\textsuperscript{10}, both of which are polyene compounds. In a subcutaneous mouse ependymoblastoma, Laurent, et al.\textsuperscript{12} have shown an increased rate of regression and cure when AMB was administered 10 hours before CCNU treatment. However, they were unable to demonstrate an increased uptake of C\textsuperscript{14}-labeled CCNU by the tumor.

In our transplantable intracerebral murine ependymoblastoma we have been unable to demonstrate any increase in survival with AMB administered intraperitoneally. Moreover, there was no potentiation of the effects of CCNU when intraperitoneal AMB was given 24 hours before the CCNU. This failure of response to intraperitoneal AMB was likely due to a failure in drug delivery since AMB crosses the blood-brain barrier quite poorly.\textsuperscript{7} This defect in drug delivery was circumvented by direct intracerebral injection of AMB into the tumor-bearing hemisphere. With this direct route, potentiation of CCNU was demonstrated. Administration of 1.0, 0.5, and 0.2 mg/kg intracerebral AMB 24 hours before 20 mg/kg intraperitoneal CCNU significantly increased survival over

Fig. 4. Graph showing the percentage of cumulative death plotted against days after intracerebral tumor implantation. The AMB was administered intracerebrally on Day 4 and CCNU intraperitoneally on Day 5. See Table 2, Experiment 6, for details.
TABLE 2
Intracerebral amphotericin B (AMB) and intraperitoneal CCNU in the treatment of intracerebral mouse ependymoblastoma*

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<th>Dose of CCNU (mg/kg)</th>
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*All mice in each experiment received the same tumor-cell suspension, administered on Day 1. The AMB was administered by the intracerebral route on Day 4 and CCNU by the intraperitoneal route on Day 5. NS = not significant.

†Significance of the treated groups compared to control.

‡Significance of groups treated with intracerebral AMB compared to group receiving no AMB but an identical dose of CCNU.

The group treated with CCNU alone (Table 2). Intracerebral AMB, 0.5 mg/kg, 24 hours before 10 mg/kg intraperitoneal CCNU significantly increased survival over control when CCNU alone did not (Table 2).

These experiments demonstrate the potentiating property of AMB on CCNU in a brain-tumor model. It is clear that the enhancement of CCNU's tumoricidal activity occurred only after the AMB was delivered directly to the tumor-bearing hemisphere. This suggests that AMB exerts its potentiating effects primarily on the tumor cell. Furthermore, the CCNU must be effective alone before it is enhanced by AMB pretreatment. For example, 5 mg/kg CCNU was ineffective and not improved by intracerebral AMB pretreatment, whereas 10 mg/kg CCNU was minimally effective and minimally potentiated by intracerebral AMB pretreatment. Administration of 20 mg/kg CCNU was markedly effective in prolonging survival and greatly potentiated by intracerebral AMB.

The optimum application of AMB's potentiating property is not yet defined. The time interval between AMB and CCNU administration may be of critical importance. For example, Medoff, et al., 20 found the optimum interval to be 24 hours in AKR leukemic mice. Laurent, et al., 12 employed a 10-hour interval in their subcutaneous mouse ependymoblastoma. Since CCNU is so rapidly degraded in vivo, 28 it seems clear that the AMB must precede the nitrosourea treatment.

The potentiating of chemotherapeutic agents by membrane-active polyenes, such as AMB, is a recent advance in the therapy of experimental tumors. Our studies demonstrate the possibility of enhancing or potentiating the effect of a nitrosourea in an intracerebral brain-tumor model. The use of membrane-active polyenes, such as AMB, in the treatment of human brain tumors must await further experimental confirmation of their value, as well as adequate toxicology trials.
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Acknowledgments

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References


26. Rosso R, Donelli MG, Innocenti IRD, et al:

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