Synergistic Effect of Combined Hyperthermia and a Nitrosourea in Treatment of a Murine Ependymoblastoma

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ABSTRACT
The effectiveness of heat therapy in combination with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea on a murine ependymoblastoma was investigated. Based on survival time and the number of survivors, whole-body hyperthermia (40°) increased the therapeutic effectiveness of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea. Heat alone did not modify the course of the tumor. Microscopic evidence of accelerated tumor destruction in hyperthermic mice was apparent within 24 hr of drug administration. A temporary drop in animal weight was observed with hyperthermia at the higher dose levels of drug. Mechanisms which may be involved in this synergism are discussed.

INTRODUCTION
The therapeutic efficacy of HPT combined with chemotherapy has been observed in animal and clinical studies (14, 22, 24). Both whole-body and localized HPT have been reported to enhance the action of several antineoplastic compounds. The effects of total-body HPT on chemotherapy have received less attention than that given local heating. Kolosov and Krupkin (13) observed that administration of pyrogens enhanced the effect of cyclophosphamide on a sarcoma 37 transplant and an Ehrlich carcinoma. Pettigrew and Lugate (24) noted that systemic HPT and chemotherapy in terminal melanoma patients with disseminated cancer were more effective than was drug alone. Suzuki (29) reported regression of Yoshida sarcomas in mice with a combination of nitrogen mustard and local heat therapy although, when used alone, neither was protective. Adriamycin combined with local HPT enhanced the destruction of a solid mouse mammary carcinoma when compared with either drug or heat treatment alone (22). Recently, Twentyman et al. studied the effects of local HPT and 1,3-bis(2-chloroethyl)-1-nitrosourea on a mouse mammary tumor both in vivo and in vitro. They observed that 1,3-bis(2-chloroethyl)-1-nitrosourea was more effective at 43° than at 37°. The interaction of HPT and several nitrosoureas was further demonstrated by Hahn’s (10) findings of suppressed colony formation in cultures of CHO.

Because controlled, localized heating of the central nervous system is difficult and because the prognosis of most cranial tumors is relatively poor, we have investigated whether concurrent whole-body HPT enhanced the effectiveness of a nitrosourea in the treatment of a murine ependymoblastoma.

MATERIALS AND METHODS
Tumor System. Barrier-maintained, female C57BL/6 mice weighing 18 to 22 g were obtained from the Primary Genetic Center, Leo Goodwin Institute for Cancer Research. The mutant strain, derived by Ausman et al. (1) from a methylcholanthrene-induced mouse ependymoblastoma described by Zimmerman and Arnold (33), was carried s.c. in passage for 4 years in our laboratory. Fragments were implanted intracerebrally by trocar as described by Geran et al. (7). In the usual experimental group, rats of 8 mice received no treatment, HPT alone, drug alone, or drug plus HPT. Animals were observed for 74 days. The evaluation period was 60 days as recommended for this murine tumor model (8). This is sufficient to determine treatment effect and the percentage of successful implants in the control mice.

Drug Treatment. CCNU was selected because of its effectiveness in this experimental system (7). It was supplied by Dr. R. Geran (Drug Evaluation Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.) and prepared fresh daily by triturating in hydroxypropyl cellulose (0.3%) followed by dilution with 0.85% NaCl solution. In therapeutic studies, a single dose of 2, 4, 8, or 16 mg/kg was administered i.p. in a volume of 0.25 ml on the day following tumor implantation (Day 1). In experiments where tissues were taken for histological examination, a single dose of 8 mg/kg was administered on Day 8 followed by heat therapy on Days 8 to 11 in the appropriate groups. (See below.)

Heat Treatment. Nonanesthetized mice were treated with HPT immediately following drug injection and for 3 additional days (Days 1 through 4 or Days 8 through 11). Animals were individually placed in circular plastic wells set into a transparent chamber through which heated water was circulated using a Haake thermostatic pump. The wells were fitted with a side entrance for rectal temperature probes. Rectal temperatures were gradually raised from 37.1 ± 0.2° to 40.0 ± 0.3° over a 20-min period and maintained there for 2 hr with oscillations between 39.6° and 40.6°. At the end of each treatment, the chamber was allowed to adjust to room temperature during a 30-min period before removal of the rodents to permit gradual acclimatization.

Histology. Brains of 40 tumor-bearing animals which were treated on the eighth day were removed on Days 9, 11, and 13. Evaluation of tumor destruction was made by examination of formalin-fixed tissues stained with hematoxylin and eosin.

Statistical Analysis. Individual survival times of animals from each experimental group were analyzed using a nested analysis of variance (27). This statistical approach distinguishes between variances of replicate experiments and those resulting from the effect of treatment. To determine the source of significance, control groups were compared with groups receiving only drug which, in turn, were compared with groups receiving...
both drug and HPT. The question of nonadditivity was approached by constructing an additive model to yield expected survival times when individual treatment results were combined. These were analyzed against observed results of combined therapy using the $\chi^2$ test.

The significance of survivors in each treatment group was determined by means of the distribution of arc sine transformation (27). Body weights of animals recorded during the 4 days of therapy in control and treatment groups were compared using a 2-way analysis of variance. The criterion of weight change was used as an indication of possible drug toxicity.

RESULTS

Effect of HPT Alone on Tumor Progression. It was found that normal C57BL/6 mice tolerated temperatures of 39.5 to 40.3° for 2 hr, whereas 40.6 to 41.2° resulted in a mortality of 33% or more. The MST and percentage of survivors of all experimental groups are listed in Table 1 for a comparison of the effects of HPT, CCNU, and the combination. There were no deaths during the 2-week period following final evaluation Day 60.

In 6 experiments comparing 39 control and 31 HPT-treated, tumor-bearing mice, the MST's of each group were 17.0 and 14.5, respectively. The range for the control was 14 to 24 days and for HPT was 13.5 to 21.0 days. However, statistical analysis of the data was based on individual survival times (Table 2) as explained earlier. (See above.) The effect of treatment was within the experimental variance as indicated by lack of significance ($p > 0.1$).

Effect of Drug Alone. When tumor-bearing mice received single doses of CCNU at 4, 8, or 16 mg/kg, the prolongation of survival time was significant. The number of survivors on Day 60 was highest at the maximum drug level, increasing from 4% at 4 mg/kg to 73% at 16 mg/kg. At 16 mg/kg, the MST was $>$ 60 days. During the 4 days of treatment, there was no significant change in animal weight of the drug-treated groups when compared with that of the control groups ($p > 0.05$) (Table 2).

Effect of Combined Drug and HPT. The therapeutic efficacy of CCNU at single doses of 8 or 16 mg/kg was significantly enhanced by HPT as indicated by prolonged survival time and/ or number of survivors at Day 60. The results of statistical analysis presented in Table 2 show that at 8 mg/kg, HPT significantly enhanced the effect of CCNU ($p < 0.001$) beyond its effect when used alone ($p < 0.001$). The percentage of survivors was significantly increased at both 8 and 16 mg/kg ($p < 0.001$). However, the effectiveness of the latter dose level (CCNU, 73%; CCNU plus HPT, 94% survivors) masked any effect of HPT on the MST. The lack of additivity was demonstrated by the significant increase in survival times of animals receiving combined therapy as compared with the expected survival times when the results of drug treatment were added to those of HPT ($p < 0.005$ [$\chi^2$ test]).

At 8 and 16 mg/kg, the combination treatment resulted in a significant decrease in animal weight ($p < 0.001$) which returned to normal after therapy was terminated (Table 2). There were no deaths due to drug toxicity.

Histological Evaluation of Tumor Destruction. Graphic confirmation of the accelerated tumor necrosis resulting from a combination of HPT with drug therapy was obtained by examination of tumor sections following treatment. For this purpose, a group of mice bearing an 8-day tumor was given a single treatment with HPT alone, CCNU alone, or the combination. The animals were sacrificed 24 hr later, and the morphological appearance of the tumors was compared.

The untreated tumors 9 days after implantation consisted of a canalized, dense mass of actively dividing, undifferentiated, polygonal cells with frequent mitoses. Giant forms were numerous (Fig. 1A). A single treatment with HPT did not produce any significant morphological change in the tumors (Fig. 1B). Tumors of mice treated with CCNU alone (8 mg/kg) showed a few small localized areas of necrosis, nucleolar retraction, and granulated chromatin (Fig. 1C). In marked contrast, concomitant administration of HPT and CCNU resulted in widespread necrosis within 24 hr (Fig. 1D).

Tissue sections taken on Days 11 and 13 showed progressive necrosis with both CCNU and CCNU plus HPT therapy; however, tumor destruction was always more advanced in the latter group. This observation is consistent with the therapeutic results which we have obtained.

DISCUSSION

Based on the number of Day 60 survivors as well as prolongation of survival time, the therapeutic efficacy of a single dose

<table>
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<th>Treatment</th>
<th>HPT</th>
<th>CCNU (mg/kg)</th>
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<th>MST</th>
<th>Day 60 survivors (%)</th>
</tr>
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<tr>
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<td></td>
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<tr>
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<td>14.7</td>
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<td>16</td>
<td>18</td>
<td>&gt;60</td>
<td>94*</td>
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* Significant difference from drug alone ($p < 0.001$).
of CCNU in treating a mouse ependymoblastoma was improved by whole-body hyperthermia of short duration. The effect was not additive as HPT alone was not therapeutic. In a limited number of experiments, combined HPT-CCNU treatment was therapeutically more effective than was CCNU alone when administered as late as Day 8. Histological examination of tumors treated on Day 8 confirmed these therapeutic results.

The augmented response of combined therapy was obtained only at critical levels of drug (8 and 16 mg/kg), an observation which is not unique. For example, Hahn (10) determined the cytotoxic effect of increasing concentrations of several nitrosoureas on CHO cells at 37, 38, 41, and 43°. At the lowest drug concentrations, a plateau occurred at all temperatures except 43°. Consequently, little change in cytotoxicity was apparent with increasing temperature. A similar plateau in mouse mammary tumor cell survival at lower doses of Adriamycin was observed by Marmor (18). She suggested that the concentrations of Adriamycin necessary for synergistic activity with HPT would be difficult to maintain in vivo for sufficient periods of time because of the short serum half-life of the drug. In contrast, the plasma half-life of CCNU, 74 hr for the alkylating moiety (20), could be expected to extend through 3 days of HPT which we administered after the inoculation of drug.

The dose level of 8 mg/kg is approximately double the clinical dose of CCNU (130 mg/sq m). No toxic deaths resulted in either of the groups given drug alone or drug plus HPT at any of the dose levels used. The temporary drop in weight with HPT at 8 and 16 mg/kg suggests an increase in drug-related stress. However, the weight recovery was immediate upon cessation of HPT. In four subsequent experiments, tumor-free animals were exposed to single and multiple doses of CCNU as high as 64 mg/kg with and without HPT. There were no deaths with HPT at single doses of 32 mg/kg or less and at multiple doses (4 times) of 16 mg/kg or less. As above, loss of weight which occurred with combination treatment was reversible.

The rapidity of HPT-CCNU augmentation, demonstrable histologically within 24 hr, suggests an acceleration of drug cytotoxicity. An alternative hypothesis is that CCNU increased the vulnerability of the tumor cell to HPT. Among the possible mechanisms for this response are a common cell cycle specificity; changes in membrane permeability, particularly of the blood-brain barrier, by elevated temperatures; and enhancement of CCNU activity on repair enzymes.

Several investigators have reported that cultures of L1210, CHO, P388, and HeLa cells are heat sensitive during the late S or early G2 cell cycle phases (2, 23, 32). Tobey and Crissman (30) demonstrated that CCNU prolonged these same phases in CHO cells. This suggests that extension of the heat-sensitive phases by CCNU could increase the lethality of HPT.

In vitro dye studies and uptake of labeled drugs have demonstrated a temperature-associated increase in tumor cell membrane permeability (6, 28). This factor might also increase the drug available to the tumor by acting on cells of the blood-brain barrier. This is consistent with observations of Moricca et al. (19) that patients succumbing to HPT exhibited brain edema. This suggests that HPT may alter autoregulation and produce edema by increasing capillary permeability. Since the magnitude of lipid-water partition coefficients of drugs also determines their rate of penetration into the central nervous system (25), a change in lipid viscosity resulting from HPT could increase transport of a hydrophobic compound such as CCNU.

Hahn (9) has suggested that HPT interferes with cellular repair processes. Alternatively, heat may increase the availability of the carbamylating moiety of CCNU which is proposed to play a role in inhibiting repair enzymes (11). This molecule binds 40 to 60% to plasma proteins (20), blocking its entry into the cerebrospinal fluid. An increase in temperature, which has been found to decrease binding constants for a number of drugs (12, 16, 21, 26), would provide a greater fraction of unbound drug available to the central nervous system.

Several reports suggest that a minimum core temperature of 42° maintained for 6 to 20 hr is necessary for effective cancer therapy (5, 24). Several investigators have been able to control and maintain total-body temperatures around 42° with only a few unmanageable side effects using appropriate life support measures (4, 15, 17). However, a level of 43° for 12 hr is suggested as a maximum safe level of body temperature in humans, leaving a very narrow margin between effective and dangerous heating (3). It is therefore encouraging that while hyperthermia of 40° applied for only 2 hr daily in our experiments was not therapeutically effective, it did enhance CCNU effectiveness at a physiologically tolerable temperature.

REFERENCES

19. Moricca, G., Cavaia, R., Caputo, A., Biggioni, A., and Colistro, F. Hyper-

Fig. 1. Murine ependymoblastoma 9 days after intracerebral implantation in female C57BL/6 mice. A. untreated tumor tissue; B. 24 hr after 2-hr heat therapy (40°) with no significant morphological changes; C. 24 hr after one treatment with CCNU (8 mg/kg) with early signs of necrosis; D. 24 hr after combined treatment of HPT plus CCNU with marked necrosis. H & E. × 940.
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