Increased in Vitro Toxicity to Mouse Bone Marrow with 1,3-Bis(2-chloroethyl)-1-nitrosourea and Hyperthermia

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ABSTRACT

Hyperthermia has been shown to increase the cytotoxic effect of certain cancer chemotherapeutic agents in vitro and in vivo. It is not known whether the combination of hyperthermia plus these chemotherapeutic drugs will also increase the toxicity of the drugs. This study was undertaken to evaluate the toxic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea combined with hyperthermia on normal mouse bone marrow in vitro. An increased effect of the combination was seen when the marrow was heated at 42° in the presence of the drug for 15 min (p < 0.025) and for 1 hr (p < 0.005). Incubations for 2 hr produced a significant decrease of control growth, such that no increased effect of the combination of drug plus hyperthermia could be seen. In order for the increased effects to be seen, heating had to be done simultaneously in the presence of the drug. Clinicians using the combination of whole-body hyperthermia plus 1,3-bis(2-chloroethyl)-1-nitrosourea should be aware of possible increased marrow toxicity.

INTRODUCTION

Hyperthermia has been demonstrated to have cytotoxic effects against neoplastic tissues in vitro (4, 7, 8, 13) and in vivo (10). Heat has been demonstrated to potentiate the effects of radiotherapy (6, 21) and certain cancer chemotherapeutic agents (9, 11, 22). There is evidence that tumor cells may be more sensitive to heat than is normal tissue (4). Further, the tumor cells that are theoretically most sensitive to heat are those now believed to be most resistant to chemotherapy and radiotherapy, namely, cells in poorly vascularized or poorly oxygenated states (8). The combination of heat plus these other antineoplastic modalities may therefore be very beneficial in enhancing the effectiveness of current therapy.

Little is known about the toxicity of whole-body hyperthermia in conjunction with chemotherapeutic agents, but this combination will be useful only if the therapeutic ratio is favorable. Heat may conceivably increase the toxic effects of chemotherapeutic agents and make any potential therapeutic benefit that may be obtained at a higher cost to the patient. The primary and often limiting toxicity of many cancer chemotherapeutic agents is hematological, and the studies of whole-body or local hyperthermia combined with chemotherapy have not addressed the potential problem of increased bone marrow toxicity (16, 20).

Marmor et al. (11) and Twentyman et al. (22) have demonstrated that the combination of nitrosoureas plus heat is more effective than either modality alone in treating mouse tumors. This study was undertaken to investigate the toxic effects of hyperthermia together with nitrosoureas on the bone marrow. The assay for in vitro CFU-c has been utilized to study these effects. The CFU-c assay has been used extensively to study the relative toxicity of chemotherapeutic agents on normal hematopoietic precursors (12, 14).

MATERIALS AND METHODS

The method of Brown and Carbone (1) was used for assaying CFU-c’s in the mouse. The effect of BCNU on these CFU-c’s was compared at 42° versus 37°, keeping other variables constant. Pairs at the 2 temperatures were also compared, varying the time of incubation (15 min, 30 min, 1 hr, 2 hr) to assess the time-drug-heat interaction.

Femoral marrow was obtained aseptically from C57BL mice (NIH, Bethesda, Md.) 12 to 18 weeks old. Marrow suspensions were obtained after removal of the femur by gently flushing the femoral marrow cavity with McCoy’s plus medium (Grand Island Biological Co., Grand Island, N. Y.). The marrow was then diluted to approximately 2 x 10⁶ nucleated cells/ml; 0.5 ml of this suspension (containing a total of 1 x 10⁸ cells) was then added to graded concentrations of freshly prepared BCNU (Division Cancer Treatment, National Cancer Institute, Bethesda, Md.), also diluted in 0.5 ml McCoy’s plus medium. Cells diluted with 0.5 ml of McCoy’s plus medium alone served as controls.

The marrow cells plus BCNU in medium or plus medium alone (controls) were then incubated in water baths at 37 or 42° (water baths accurate to 0.2°), and, after variable time periods (15 min, 30 min, 1 hr, 2 hr), paired specimens were assayed for CFU-c’s. Initially after incubation, the cells were washed and resuspended in 1 ml of medium, but this step did not produce a difference in the CFU-c’s seen at the various temperatures and was omitted after the initial experiments. Before assaying for CFU-c’s, the number of cells was again counted, and the CFU-c’s were expressed per 10⁵ cells actually plated.

One-half ml of the specimens incubated (approximately 5 x 10⁵ cells) was added to tubes containing 2.5 ml methylcellulose (2.2%) (Fisher Scientific Co., Fairlawn, N. J.), 0.2 ml bovine serum albumin (Armour Industrial Chem. Co., Chicago, Ill.), 0.5 ml fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), and 1.25 ml McCoy’s plus medium. A supernatant from mouse L-cells (0.5 ml) (Microbiological Associates, Inc., Bethesda, Md.) served as the source of colony-stimulating activity (2). The specimens were then mixed thoroughly, plated in four 10– x 30-mm Petri dishes, and incubated in a humidified incubator at 37° in a 7.5% CO₂ atmosphere. Colonies defined as collections of greater than 50 cells were counted after the...

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2 The abbreviations used are: CFU-c, colony-forming units committed to granulocyte-macrophage differentiation; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea.
7 days. Each experiment included controls at 37 and 42° and pairs incubated at 37 and 42° in the presence of BCNU at several concentrations for variable time periods.

Statistical analysis of the paired values was done using the Wilcoxon signed-rank test (19). The data are displayed, however, in numbers using the mean ± S.E. of all the points at a particular concentration and time of incubation.

RESULTS

A dose-response curve for BCNU at 37° revealed that the most effective concentrations were between 10⁻³ and 10⁻⁷ M. There was 100% decrease in colony formation at 10⁻³ M, 86% decrease at 10⁻⁴ M, 33% decrease at 10⁻⁵ M, and 8% decrease at 10⁻⁶ M, compared to control.

Chart 1 gives the data after a 15-min incubation (30-min incubation is similar). There is a decrease in colony formation at 42° at 5 x 10⁻⁵, 5 x 10⁻⁶, and 5 x 10⁻⁷ M BCNU compared to 37° with these same concentrations, which is statistically significant (p < 0.025; n = 16, where n equals the number of observations of matched pairs of 37 and 42° points.)

At 1 hr (Chart 2), the difference in colony growth was also significant at 42° versus 37° in the presence of BCNU (p < 0.005; n = 27). There was no significant difference in control growth between 37 and 42° at 15 min, (n = 8) or 1 hr (n = 10). The combined effect of heat plus BCNU is most marked at the highest concentrations of BCNU. The majority of experiments were done testing the effects between BCNU doses of 10⁻⁷ and 10⁻⁴ M. Only 2 experiments were performed at the lower dose levels (5 x 10⁻⁸ and 5 x 10⁻⁷) in order to complete the dose-response curve. Thus in Chart 2, no S.E.'s of the mean are calculated at these latter points.

At 2 hr (Chart 3), there was a significant decrease in colony formation at 42° versus 37° (n = 16) without BCNU, such that no increased effect of the combination could be seen over that seen with heat alone.

The potentiating effects of the combination were seen only when the heating was done in the presence of BCNU. When the cells were heated first for 1 hr and BCNU was added after heating, no increase in effect of the BCNU was seen. The response was equivalent to adding BCNU to unheated cells. When BCNU was heated for 1 hr and then added to the cells, the results were comparable to control colony growth. The BCNU was without cytotoxic effects against the CFU-c's after heating.

BCNU requires a polar solvent to be solubilized. In these experiments, alcohol was used. The alcohol alone in the concentrations used had no effect on colony growth at 37 or 42°.

DISCUSSION

Nitrosoureas have been shown to have a wide range of clinical activity and are among the most active agents against some tumors very refractory to chemotherapy, such as gastrointestinal cancers, melanoma, brain tumors, and squamous cell carcinoma of the lung (5, 23). In animal models, heat has been shown to increase the effectiveness of BCNU (11, 22).

The clinical usefulness of the chloroethylnitrosoureas is often limited by prolonged dose-dependent and cumulative toxicity (5, 15, 23). The hematological effects are delayed in comparison with the antitumor effect. The mechanism for this is unknown but may be due to damage to marrow stem cells (17).

If heat can potentiate the antineoplastic effects of these useful agents, it is also important to assess whether the toxic effects of these agents are magnified when used in conjunction with heat.
with heat. These results suggest that heat potentiates the toxic effects of BCNU against the marrow.

Previous data have shown that the effects of BCNU on mouse CFU-c's are similar to the effects on human CFU-c's (12–14); furthermore, effects seen in vitro have correlated with marrow toxicity seen in vivo (12, 14, 18). However, the in vitro system is not analogous to the in vivo situation. Such factors as tissue perfusion and the pharmacokinetics of the drug in vivo make the situation far less static than in vitro. The levels of BCNU that have been used in our incubations are achieved in vivo after usual doses of BCNU (18), but in vivo, the concentrations rapidly change. BCNU concentrations stay above \( 10^{-5} \) m for less than 10 min. However, even a period of heating as short as 15 min is associated with an increased toxicity of BCNU on in vitro bone marrow growth. This is most marked at the higher drug concentrations. Increased toxicity of the combination was seen only when the drug was present simultaneously with the heating. This observation may have important clinical application for the timing of hyperthermia plus chemotherapy. Maximum toxic and therapeutic effects may be seen with simultaneous administration, but perhaps differential effects could be seen with changes in timing of the combination.

Another aspect of the in vitro system that differs from the in vivo situation is the lack of potential for drug metabolism by other organs. Metabolites of BCNU may be responsible for the toxic effects (12, 14, 18), and it is unknown whether heat will alter this metabolism and the effects of the metabolic products.

At 2 hr in these experiments, there was a significant decrease in control growth of colonies at 42\(^\circ\). A marrow toxic effect has not been seen in patients treated with whole-body hyperthermia for periods greater than 2 hr (3, 16), and this decrease in our system most likely represents changes due to the in vitro conditions.

For these reasons, the in vitro data may not be applicable to the in vivo situation. However, these data do suggest that an increase in hematological toxicity may be seen in vivo with whole-body hyperthermia plus BCNU. The crucial parameter that remains to be measured is the differential therapeutic and toxic effects seen with the addition of heat to nitrosourea therapy. Clinicians using the combination of heat combined with BCNU should be aware of the possible increased toxicity and begin therapy with low doses of the chemotherapeutic agent.

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JULY 1979

2549
Increased *in Vitro* Toxicity to Mouse Bone Marrow with 1,3-Bis(2-chloroethyl)-1-nitrosourea and Hyperthermia
