Sensitivity of Human and Murine Hematopoietic Precursor Cells to 2-[3-(2-Chloroethyl)-3-nitrosoureido]-D-glucopyranose and 1,3-Bis(2-chloroethyl)-1-nitrosourea

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ABSTRACT

The sensitivity of mouse and human bone marrow hematopoietic precursor cells [colony-forming units committed to granulocyte-macrophage differentiation (CFU-C)] was determined after in vitro incubation with chlorozotocin (2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose), or 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), with the use of drug concentrations achieved during clinical administration. Chlorozotocin, at a concentration of 1 x 10^{-4} M, did not decrease mouse CFU-C below the control of 44 colonies/10^6 nucleated cells; 5 x 10^{-4} M produced a 70% reduction in CFU-C, and 1 x 10^{-3} M chlorozotocin eliminated all colony formation. In contrast, BCNU at 1 x 10^{-4} M resulted in a 55% reduction in CFU-C, and at 5 x 10^{-4} M it eliminated all colony formation. For human marrow the threshold concentration for chlorozotocin toxicity was 1 x 10^{-4} M, which resulted in a 25% reduction in CFU-C as compared to the control of 32 colonies/10^6 nucleated cells. In contrast, BCNU at 5 x 10^{-3} M decreased human CFU-C to 47% of control, and at 1 x 10^{-4} M it eliminated all colony formation. Twenty-four hr after in vitro exposure to 1 x 10^{-4} M chlorozotocin, there was no reduction in human bone marrow DNA synthesis in contrast to a 42% reduction with 1 x 10^{-4} M BCNU. The plasma concentration of drugs following a therapeutic dose in patients was measured and was found to correspond to the concentration range used in the in vitro studies of marrow toxicity.

INTRODUCTION

The chloroethylnitrosoureas have been established as an important class of antitumor agents. However, these drugs frequently produce treatment-limiting delayed and cumulative bone marrow toxicity associated with extended periods of depressed WBC and platelet counts (17). Prior structure-activity studies had suggested that the attachment of a nitrosourea cytotoxic group onto C-2 of glucose would reduce this hematological toxicity (13). For a test of this hypothesis, a new compound, 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose (chlorozotocin), was synthesized (7). Chlorozotocin has curative antitumor activity against the mouse L1210 leukemia that is comparable to that of the clinically active agent, BCNU at doses that produce little or no bone marrow depression (1). As a direct correlate of this reduced myelosuppressive activity, chlorozotocin did not significantly reduce the in vivo incorporation of tritium-labeled thymidine into mouse bone marrow DNA. In contrast, BCNU and CCNU produced a significant reduction in bone marrow DNA synthesis with a maximal depression at 24 hr after administration (1, 14).

In this study, we extend our initial observations to normal human bone marrow and compare the toxicity of chlorozotocin and BCNU on in vitro bone marrow colony formation using drug concentrations achieved during clinical use with the 2 agents.

MATERIALS AND METHODS

Hematopoietic Cell (CFU-C) Assay. Human bone marrow was aspirated from the posterior iliac crest of 15 normal volunteers from 21 to 35 years old. A differential cell count was made on each specimen to verify that there was no morphological abnormality. The washed buffy coat was suspended in McCoy's 5A medium (NIH media, NIH, Bethesda, Md.) at a final concentration of 2.5 to 3.0 x 10^6 nucleated cells/ml (3). Murine bone marrow was obtained from the femurs of 12- to 18-week-old C57BL mice. The bone was aseptically isolated, and the marrow was gently flushed by repeated injection of 1.0 ml McCoy's 5A medium through a No. 23 needle and syringe and suspended in media at a final concentration of 0.8 to 1.0 x 10^6 nucleated cells/ml (3). Human and mouse bone marrow cells were incubated with chlorozotocin or BCNU (Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.) at graded drug concentrations ranging from 1 x 10^{-3} to 1 x 10^{-4} M. Following a 2-hr incubation at 37°C in a 10% CO_2 incubator, the cells were washed twice with McCoy's medium and added to the methyl cellulose semisolid culture medium. Untreated cells prepared in the identical manner served as controls (3).

Human CFU-C were assayed by the method of Carbone et al. (3) with the use of a supernatant prepared from human embryonic kidney cells (Flow Laboratories, Rockville, Md.) as a granulocyte colony-stimulating factor. For mouse marrow, a supernatant from mouse L-cell cultures was used as a stimulant (19). Mouse colonies were counted...
after 7 days, and human colonies were counted after 10 days of incubation if they contained more than 50 cells. The number of colonies per plate was determined, and the mean and 2 S.E. of 4 plates were computed.

**Bone Marrow DNA Synthesis.** Human bone marrow cells were prepared in an identical manner, as described for the CFU-C assay. Following a 2-hr incubation with graded molar concentrations of chlorozotocin, BCNU, or control media, the bone marrow cells were washed twice with McCoy's medium. They were then resuspended in the original volume of McCoy's 5A medium containing 10% fetal calf serum at 37°C in a 10% CO₂ incubator. It was not technically feasible to perform repeated bone marrow aspirations in order to measure DNA synthesis over a range of time points. A 24-hr period was chosen for study for 2 reasons. It allowed for greater than 90% viability of control and treated marrow cells, and this time point correlated with the maximum depression of DNA synthesis after chlorozotocin and BCNU treatment in our previous studies with murine bone marrow (1). One hr prior to study, 23 hr after initial exposure to nitrosourea, 100 μCi of [³H]thymidine (1.9 Ci/m mole; New England Nuclear, Boston, Mass.) were added to each tube. DNA was extracted by the method of Schneider (16). A portion of the final supernatant was dissolved in Aquasol (New England Nuclear) for measurement of ³H radioactivity in DNA, and a second portion was used for determination of the concentration of DNA by the method of Burton (2). Results were expressed as dpm/μg DNA.

**Clinical Pharmacology and Hematopoietic Toxicity.** The effect of chlorozotocin on circulating WBC and platelet count in humans following a single i.v. dose was measured during the clinical Phase 1 trial. Seven patients who had had less than 6 months of prior chemotherapy and/or radiation limited to the cervicothoracic region were treated with a single i.v. dose of 120 mg/sq m every 6 weeks; serial WBC and platelet counts were obtained weekly.

Plasma concentration of N-nitroso intact drug following i.v. administration in 9 patients receiving chlorozotocin and in 3 patients receiving BCNU was determined with the use of a spectrophotometric method that is a modification of the procedure of Forist (5) for streptozotocin and the method of Loo and Dion (8) for BCNU. Data obtained from 1 patient of each drug group are presented; each of these is representative of the data of the entire group receiving the respective drug.

Following rapid i.v. administration of chlorozotocin given over 1 min and BCNU given over 2 to 3 min due to pain at the site of injection, venous blood was drawn into heparinized syringes at 1-min intervals for the first 15 min and then at 5-min intervals for an additional 45 min. Immediately upon withdrawal, each 2-ml sample was spun at 8,000 x g for 1 min. A 0.8-ml sample of plasma was removed and added to 0.2 ml of 3 N perchloric acid, shaken vigorously for 15 sec, and centrifuged for 1 min. This results in cleavage of the intact N-nitroso group to yield nitrous acid in the resulting supernatant. A 0.5-ml aliquot of this supernatant was added to 2.5 ml of color reagent [0.5% sulfanilic acid and 0.5% N-1-(naphthyl) ethylenediamine dihydrochloride in 30% acetic acid] and 0.5 ml of 6 N HCl. The reaction mixture was then incubated at 60°C for 45 min. In this reaction the nitrous acid diazotizes sulfanilic acid, which then couples to the naphthyl ethylenediamine to form a colored product with a maximum absorbance at 550 nm. A similarly prepared pretreatment blood sample served as a control.

The plasma concentration of drug was calculated with the use of a standard curve that followed Beer's law through a concentration of $5 \times 10^{-6}$ to $2.5 \times 10^{-4}$ M.

**RESULTS**

**Bone Marrow CFU-C.** The sensitivity of mouse CFU-C to chlorozotocin and BCNU is presented in Chart 1 and is representative of 8 studies conducted in this species. The mean control mouse CFU-C was $44 \pm 6$ colonies/10⁶ nucleated cells plated. Chlorozotocin at concentrations of $5 \times 10^{-5}$ and $1 \times 10^{-4}$ M did not decrease the number of mouse marrow CFU-C below control, whereas $5 \times 10^{-4}$ M concentrations caused a 70% reduction in colony formation. In contrast BCNU produced a concentration-dependent decrease in colony formation; BCNU at concentrations of $5 \times 10^{-5}$ and $1 \times 10^{-4}$ M resulted in 22 and 55% reductions in CFU-C, which were significantly less than those for chlorozotocin at both drug levels ($p < 0.005$). BCNU, $5 \times 10^{-4}$ M, eliminated colony formation.

The sensitivity of human marrow CFU-C is presented in Chart 2 and is representative of 5 studies conducted in this species. The mean control human CFU-C was $32 \pm 3$ colonies/10⁶ nucleated cells plated. Chlorozotocin, 1 and $5 \times 10^{-3}$ M, did not cause a decrease in human bone marrow CFU-C, whereas the $1 \times 10^{-4}$ M concentration of this drug resulted in a 25% reduction in colony formation. When compared to chlorozotocin, BCNU produced a concentration-dependent decrease in number of surviving colonies at all concentrations ($p < 0.005$), with elimination of all colony formation at $1 \times 10^{-4}$ M (Chart 2).

**Bone Marrow DNA Synthesis.** No significant decrease in human bone marrow DNA synthesis was demonstrated at 24 hr after exposure to chlorozotocin at concentrations of $1 \times 10^{-4}$ to $5 \times 10^{-4}$ M. At $1 \times 10^{-3}$ M, chlorozotocin
produced a 24% reduction of [3H]thymidine incorporation into DNA when compared to control (Table 1). BCNU was studied with the use of identical concentrations and demonstrated a concentration-dependent decrease in bone marrow DNA synthesis. At $1 \times 10^{-4}$ M the incorporation of [3H]thymidine into bone marrow DNA was 58% of control. At $1 \times 10^{-3}$ M, DNA synthesis was virtually eliminated by the 24-hr study period (Table 1).

**Clinical Pharmacology and Toxicity.** Following rapid i.v. administration, the peak plasma concentration of both drugs was approximately $1 \times 10^{-4}$ M. After an initial distribution phase, the half-life of the prolonged phase of N-nitroso intact Chlorozotocin was 7 min compared to 9 min for BCNU. Both drugs remained at a concentration in excess of $1 \times 10^{-5}$ M for a minimum of 10 min (Chart 3).

After a single dose of chlorozotocin, 120 mg/sq m, the mean nadir WBC in the 7 patients was 6,800/cu mm (range of 5,800 to 9,800), which was not significantly different from pretreatment values. Similarly, there was no reduction in mean platelet count, 275,000/cu mm (range, 147,000 to 357,000).

The 120-mg/sq m dose of chlorozotocin was demonstrated to be therapeutically active during the Phase 1 clinical trial. Partial responses (a 50% or greater decrease in tumor mass as determined by the product of the longest and widest diameter) were demonstrated in 1 patient with Stage IV nodular, poorly differentiated lymphocytic lymphoma and in 2 cases of melanoma.

The in vitro CFU-C assay measures cells that are committed to granulocyte-macrophage differentiation (9) and has been extensively used to study the relative toxicity of chemotherapeutic agents on normal hematopoietic precursor cells. Species differences have been encountered when the relative myelosuppressive activities of some classes of cancer chemotherapeutic agents have been compared. However, in previous studies of BCNU in this system, the toxicity to mouse and human marrow CFU-C has been essentially identical (9, 11). In this study, Chlorozotocin, when compared to BCNU, quantitatively spared the mouse and human marrow CFU-C from toxicity over a range of drug concentrations achieved with clinical use. These results could be correlated with the failure of chlorozotocin to produce a significant reduction in human bone marrow DNA synthesis over the same range of drug concentrations. This was contrasted with the concentration-dependent decrease in DNA synthesis demonstrated 24 hr after exposure to BCNU, consistent with our prior in vivo studies in mice (1, 14).

The 2 principal chemical activities of the nitrosourea antitumor agents are alkylation, mediated through the formation of carbonium ions (4, 10), and carbamoylation of the lysine residues of protein after the formation of an isocyanate (4, 15). Structure-activity analyses have suggested that nitrosourea antitumor activity correlates with the relative alkylating activity of each compound (18). It has been demonstrated that chlorozotocin possesses a 2- to 3-fold increased chemical alkylating activity when compared to BCNU or CCNU (6, 12). In the in vivo murine leukemia L1210 system, chlorozotocin produces curative antitumor activity comparable to that of BCNU or CCNU, but at one-half to one-third the molar dose of the latter 2 analogs (6, 12).

After in vivo administration or in tissue culture, chlorozotocin has the potential of exposing normal bone marrow cells to twice the alkylating activity of an equimolar concentration of BCNU. Nevertheless, these studies of murine and human bone marrow colony formation and the Phase 1
clinical trial conducted with comparable plasma concentrations have demonstrated that chlorozotocin is significantly less myelosuppressive. Chlorozotocin represents a unique instance when an anticancer agent was prospectively designed to have reduced myelotoxicity.

The full therapeutic activity of chlorozotocin for human cancer remains to be determined in the recently initiated Phase 2 clinical trials. Should this agent be demonstrated to have efficacy comparable to BCNU, it can be anticipated that chlorozotocin will be readily incorporated into future combination chemotherapy regimens.

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