Enhanced Acute Lung Damage in Mice following Administration of 1,3-Bis(2-chloroethyl)-1-nitrosourea

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ABSTRACT

1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), also known as carmustine, is a lipid soluble anticancer drug which produces pulmonary fibrosis in up to 30% of the patients who receive this drug. The major risk factor for this disorder is preexisting lung damage. Animal models of this interaction have not been reported previously. A diffuse alveolar lesion was produced in male BALB/c mice by the administration of butylated hydroxytoluene (BHT). Total lung hydroxyproline levels, an index of fibrosis, were not increased in mice 21 days after single doses of BHT or BCNU. Total lung DNA synthesis, an index of pulmonary damage, was slightly increased after 15 and 18 days in rats and mice treated with BCNU (15 and 35 mg/kg, respectively). This suggested that a single dose of BCNU had only a minimal toxic effect on lung tissue. Combined treatments in mice given BHT (350 or 400 mg/kg), followed on Day 1 by BCNU (35 mg/kg), resulted in the deposition of significantly more hydroxyproline than with either agent alone. This enhancement was not seen following lower doses of BHT and was diminished when the dose of BCNU was decreased. Delaying the administration of BCNU (35 mg/kg) until Day 3 or 5 eliminated increases in hydroxyproline content, but not histological evidence of enhanced lung damage. Additional histological analyses confirmed the presence of an increased fibrotic reaction, especially when high doses of BCNU were administered 1 day after BHT (400 mg/kg). Most of the lungs were totally consolidated with numerous hyperactive fibroblasts and a large number of giant type II cells with atypical nuclei. These effects may be related to the ability of BCNU to inhibit pulmonary glutathione reductase activity and the increased DNA synthesis normally seen after BHT. These data show that BCNU treatment can enhance BHT-induced lung damage resulting in a fibrotic lesion similar to that seen in some human patients. This effect is dependent on the extent of the initial BHT lesion as well as the time when BCNU is administered and may represent an animal model of the primary risk factor for the development of pulmonary fibrosis in human patients receiving this drug.

INTRODUCTION

BCNU, also known as carmustine, is a potent, lipid soluble alkylating agent. It is used for the treatment of numerous neoplasms and is particularly effective against gliomas (1). A major problem with the clinical use of BCNU is the occurrence of pulmonary toxicity (2). Lung damage and fibrosis have been estimated to occur in 20–30% of the patients receiving this drug (3). Early animal studies with BCNU failed, however, to reveal any direct lung toxicity (2). Recent work has described lung damage in tumor bearing mice after a single dose of BCNU (4). The administration of multiple, but not single, doses of BCNU is also able to induce marked lung damage in rats (5–7).

The mechanisms underlying BCNU induced pulmonary fibrosis are not known. The presence of preexisting lung damage is the major risk factor for the development of fibrosis in humans treated with BCNU (8). Thoracic irradiation, the administration of other lung toxic antineoplastic agents, and hyperoxic exposures have also been suggested as risk factors (3, 9). Studies in animal models of some of these risk factors showed that hyperoxia does not enhance the deposition of hydroxyproline in lung tissue from mice treated with single doses of BCNU (9) while fibrosis developed in irradiated rats given multiple doses of this drug (10). The effect of BCNU treatments on the development of pulmonary fibrosis in animals with preexisting lung damage has not been investigated previously.

BHT produces a diffuse alveolar epithelial cell lesion in mice which mimics many of the cell changes seen in some types of human lung damage (11). Data presented here indicate that single doses of BCNU alone elicit only minimal lung damage in either rats or mice and produce no increase in total lung hydroxyproline, an index of fibrosis. However, the administration of single doses of BCNU to mice with BHT induced lung damage enhanced the development of pulmonary fibrosis. Although the mechanism of this effect is not clear, it was dependent on the extent of the initial BHT induced lesion and the dose and time when BCNU was administered.

MATERIALS AND METHODS

Chemicals. Reduced and oxidized glutathione, glutathione reductase, NADPH, and BHT were obtained from Sigma Chemical Company (St. Louis, MO). [methyl-3H]Thymidine, 53.4 mCi/mmol, was obtained from New England Nuclear (Boston, MA) and 3,5-dihydroxy[2-14C]proline, 21 mCi/mmol, was from Amersham (Arlington Heights, IL). BCNU was purchased from Bristol Laboratories (Syracuse, NY). All other chemicals were reagent grade.

Animals and Treatments. Male Sprague-Dawley rats (150–220 g) and male BALB/c mice (23–28 g, 9–12 weeks of age) were used for these studies. All animals were bred and maintained in the Animal Resources Center at the University of Texas at Austin. BHT was dissolved in corn oil and administered to mice i.p. BCNU was administered i.p. as a solution in corn oil or s.c. as a solution in 10% ethanol in saline. Concentrations of BHT and BCNU were adjusted so that mice received 0.1 ml and rats received 0.05 ml/10 g body weight. Control animals received equal volumes of the appropriate vehicles. Food and water were available ad libitum. Mice were killed by cervical dislocation and...
rats were killed by decapitation.

Pulmonary DNA Synthesis. DNA synthesis was assessed by measuring the incorporation of thymidine into total lung DNA. Mice and rats were given i.p. injections of 0.5 and 1.0 ¿Ci [3H]thymidine, respectively. After 90 min animals were killed and the specific activity of pulmonary DNA was determined (12). Briefly each individual lung was removed and homogenized in 3 ml (mice) or 5 ml (rats) water. The entire mouse lung homogenate or 1 ml of the rat lung homogenate was then mixed with 2 ml or 0.7 ml 0.5 n HClO4, respectively. After centrifugation the pellets were washed twice with 5 ml cold 0.5 n HClO4. The pellets were then suspended in 4 ml 1.5 n HClO4 and digested at 70°C for 20 min. This mixture was centrifuged, and 2 ml of the resultant supernatant fraction were counted for radioactivity by liquid scintillation. A 0.2-ml aliquot of each supernatant fraction was assayed for total DNA using Richard’s modification of the diphenylamine reaction (13).

Hydroxyproline Analyses. Total lung collagen was estimated by measuring hydroxyproline, an amino acid found primarily in collagen (14). Lung tissue was excised and typholized intact. The entire lung (or right lobe for rats) was hydrolyzed for 18 h at 107°C in 4 ml of 6 n HCl. An internal standard of 0.01 ¿Ci [14C]hydroxyproline was added to the hydrolysate, which was assayed for hydroxyproline by the method of Prockop and Udenfriend (15) as described previously (12). Recoveries of hydroxyproline, generally 70-90%, were calculated by counting a 0.2-ml aliquot of the hydrolysate.

Enzymatic Assays. Glutathione reductase and glutathione peroxidase activities were measured in the 10,000 x g supernatant fractions of saline-perfused lung tissue homogenized in 0.1 n sodium phosphate buffer, pH 7.6, containing 0.5 mM EDTA. Glutathione reductase activity was measured by the method of Carberg and Mannervik (16) and glutathione peroxidase activity was measured by the method of Paglia and Valentine (17). Data were calculated as nmol NADPH oxidized per min per mg protein using 6.22 as the millimolar extinction coefficient for NADPH at 340 nm. Protein was determined by the microbiuret method (18).

Histopathology. Lung tissue was fixed without trapped air by immersion for 1 h in a solution containing 10 ml 8% glutaraldehyde, 8.2 ml 40% formaldehyde, 0.95 g NaH2PO4, 0.22 g NaOH and 72.6 ml H2O, pH 7.4. The tissue was then sliced into approximately 2-mm strips and fixed for an additional 3 h in the same solution. The fixative was then removed and replaced with 0.1 M cacodylate buffer, pH 7.4. Lung tissue was embedded in paraffin or Epon 812. Thick sections of plastic embedded tissues, as well as from paraffin blocks, were stained with hematoxylin and eosin or toluidine blue. For transmission electron microscopy the tissue was postfixed with 2% osmic acid in 0.1 M cacodylate buffer (pH 7.4), dehydrated in graded acetone solutions, and embedded in Epon 812. Ultrathin sections were cut, double stained with uranyl acetate and lead citrate, and examined.

Statistics. All data are expressed as means ± SE. Comparisons between two treatment groups were done with Student’s t test. Multiple group data were analyzed by one way analysis of variance and comparisons between groups were done with the Newman-Keuls test (19). A P value of less than 0.05 was considered significant.

RESULTS

A single 35-mg/kg dose of BCNU had no effect on mortality (not shown) or total lung DNA synthesis in mice until 18 days after treatment (Chart 1). Between 18 and 24 days after BCNU there was a small but significant increase in pulmonary thymidine incorporation. BCNU (35 mg/kg) did not increase total lung hydroxyproline levels at 3 weeks compared to vehicle treated controls (Table 1). Similar results were seen in rats given a single 15-mg/kg dose of BCNU. Total lung DNA synthesis was significantly increased between Days 15 and 27 in this species and there was no measurable increase in total lung hydroxyproline at Day 30 (Chart 1; Table 1).

BHT alone, up to 400 mg/kg, had no significant effects on mortality (not shown) or total lung hydroxyproline content in mice (Chart 2) compared to vehicle treated controls (Table 1) in this series of experiments. Treatment of mice with BCNU (35 mg/kg) 1 day after BHT (350 or 400 mg/kg) produced significant increases in total lung hydroxyproline content compared to mice treated with vehicle, BCNU, or BHT alone (Chart 2). BCNU treatment also increased mortality from zero to 50 and 44% after BHT (350 and 400 mg/kg, respectively).

The enhanced deposition of hydroxyproline in BHT-damaged mouse lung tissue was highly dependent on the doses of BHT and BCNU administered and on the time when BCNU was given. The lung damage induced by BHT (250 or 300 mg/kg), which is less extensive but qualitatively similar to that seen at higher doses (20), was not enhanced following treatment with BCNU (35 mg/kg) (Chart 2). BCNU did, however, increase mortality to 10 and 24% after 250- and 300-mg/kg doses of BHT, respectively. In addition, although decreasing the dose of BCNU to 10 and 24% after 250- and 300-mg/kg doses of BHT, respectively. In addition, although decreasing the dose of BCNU to 10
or 15 mg/kg resulted in pulmonary hydroxyproline levels that were significantly greater than those in vehicle or BCNU treated controls, these lower doses, or a delay in treatment with BCNU until Day 3 or later, did not produce any increases in total lung hydroxyproline content compared to mice given BHT alone (400 mg/kg) (Table 1).

Histologically the enhanced lung damage seen in mice treated with both BHT and BCNU showed an increased number of macrophages, fibroblasts, and lymphocytes in the alveolar walls (Fig. 1A) with areas of total consolidation of the parenchyma (Fig. 1B). This lesion was evident 21 days after the initial 400-mg/kg BHT treatment followed on Days 3 or 5 by BCNU (35 mg/kg). A similar but more severe lesion was evident when relatively low doses of BCNU (10 and 15 mg/kg) were given 24 h after the initial BHT treatment.

When the dose of BCNU administered 24 h after BHT was increased to 35 mg/kg, the lung changes seen 21 days later were markedly more severe. Most of the lungs were totally consolidated with areas of intense fibrosis in which fibroblasts and collagen fibers predominated (Fig. 1C). In addition to numerous hyperactive fibroblasts (large cells with basophilic cytoplasm and a large nucleus with prominent nucleoli), a large number of atypical, sometimes giant cells with bizarre large nuclei and nucleoli, could be seen (Fig. 1D). Changes in the airways were minimal, however, with the bronchial epithelium appearing entirely normal and the bronchiolar epithelium looking normal to slightly hyperplastic.

Electron microscopy showed the presence of a large number of type II pneumocytes and an almost total absence of type I pneumocytes (Fig. 2A). In addition, the presence of numerous fibroblasts and collagen fibers could be confirmed (Fig. 2B) and the large cells seen by light microscopy could be identified as type II pneumocytes. Most of these cells appeared several times larger than normal type II pneumocytes and contained large irregular or lobulated nuclei with numerous nuclear inclusions of a granular or fibrous nature (Fig. 2, C and D). Some nuclear pseudoinclusions of a cytoplasmic nature, as well as nuclear segregation, could also be observed. None of these changes were seen in mice given equivalent doses of BHT or BCNU alone or in vehicle treated controls.
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with BCNU (2, 3). Although the mechanism of this toxicity is not known, patients with existing lung damage resulting from some underlying disease or previous radio- or chemotherapy are known to be at a greater risk for the development of fibrosis (8). The direct toxicity of BCNU to the lung appears to be quite low and animal models of this disorder have been difficult to develop. The induction of lung damage with BCNU in animals has required multiple dosing regimens in rats (5–7) or the treatment of irradiated rats (10) or tumor bearing mice (4).

The data presented here have shown that a single dose of BCNU only slightly increased rat or mouse pulmonary DNA synthesis, a parameter which can be used as an index of the amount of cellular repair which is occurring and thus indirectly to quantify any damage (21). Furthermore BCNU did not increase lung hydroxyproline content of these animals when measured up to 4 weeks after treatment. Higher, lower, (9) and multiple doses* of BCNU were also without effect on mouse lung hydroxyproline levels indicating a greater resistance of this species, than rats, to BCNU induced lung damage.

Based on the thymidine data, the minimal lung damage caused in mice and rats by a single dose of BCNU appears to be delayed. Busulfan and cyclophosphamide, two other lung toxic anticancer drugs, produce a similar delayed cell proliferation (22). Although 3 weeks is sufficient time for fibrosis to develop following some types of mouse lung damage (23, 24), it is possible that pulmonary fibrosis might become detectable if hydroxyproline analyses were performed at later times after treatment with BCNU.

Preexisting lung damage may explain the discrepancy between the significant clinical pulmonary toxicity of this drug and its minimal toxicity to normal lung tissue. It is possible that BCNU has few direct effects on the lung but that it is able to enhance even subclinical lung lesions making it appear that BCNU itself is toxic to this tissue. The administration of BCNU to mice with BHT induced lung damage may provide an animal model of this human risk factor. Combined treatments with these drugs resulted in the development of a severe fibrotic lesion. Biochemically this was reflected by significant increases in total lung hydroxyproline content. Although these increases did not occur at low levels of BHT induced lung damage or when BCNU treatment was delayed beyond 1 day, histopathological analyses did reveal pulmonary abnormalities.

It is interesting to note that, from the histopathological point of view, the combined treatment of mice with BHT and BCNU produces a fibrotic lung with a visible and marked increase of collagen fibers that can be easily detected with both light and electron microscopy. This is in contrast with fibrosis induced with BHT alone or with BHT plus oxygen, which is morphologically characterized by a preponderance of a cellular reaction over fibrogenesis (25–27).

Murine lung fibrosis produced by BHT and BCNU is markedly similar to the pulmonary fibrotic changes induced by BCNU in astrocytoma patients (28–30). These authors described the presence of large atypical cells with bizarre nuclei in the fibrotic lungs and identified them as hyperplastic type II pneumocytes. Electron microscopy showed that the large atypical cells present in our experimental model were also type II pneumocytes. We also found that these cells contained large nuclear inclusions of several types characteristic of hyperactive cells (31). Although these cells are able to continue growing, the presence of large lobulated nuclei in giant cells is indicative of an alteration in the ability of these cells to divide properly (32). This effect could be a consequence of the cytotoxicity of BCNU, and similar changes have been described in epithelia subjected to either ionizing radiation or other anticancer drugs (31, 33–35).

The mechanisms by which BCNU might enhance preexisting lung damage are not known. Alveolar damage is normally required by a series of steps including epithelial cell division followed by endothelial and interstitial cell proliferation (36, 37). Interference with epithelial cell repair has been hypothesized to lead to a loss of control over interstitial fibroblasts by the epithelium resulting in the development of pulmonary fibrosis (25). Several studies have supported this hypothesis (23, 24, 38), although it is probably not the only factor involved in the development of fibrosis (21). Nevertheless, the inhibition of BHT induced increases in pulmonary thymidine incorporation by BCNU indicates that this drug may enhance existing lung damage by a similar mechanism.

BCNU has, however, other biochemical effects that could also play a role in its pulmonary toxicity. Mice treated with BCNU exhibit an increased susceptibility to oxygen toxicity (9) and this factor may contribute to actions of this drug on damaged lung tissue. Various studies have shown that fibrotic changes can develop in an injured lung if oxygen is administered at a concentration that would be considered nontoxic to a healthy lung (39). The increased susceptibility to oxygen toxicity following BCNU treatment may lead to enhanced toxicity in damaged lung tissue even at the oxygen concentrations present in room air.

The mechanisms underlying the increased toxicity of oxygen in BCNU treated mice are not known. One possibility is related to the ability of BCNU to inhibit glutathione reductase activity in RBC (40) and several other tissues (41, 42). Glutathione reductase is responsible for the reduction of glutathione disulfide to glutathione and is believed to be a major component of the glutathione detoxication system (43). Several studies have supported the critical importance of glutathione reductase in protecting cells from oxidative damage (44, 45) and this inhibitory effect, which we also found in BHT damaged lung tissue, could contribute to the enhancement of existing lung damage by BCNU. In addition, the inhibition of glutathione reductase activity occurs at lower doses of BCNU in rats and this species is more susceptible to the lung toxic effects of this drug (7).

These data have shown that BCNU can enhance BHT induced lung damage in mice. Since BHT does not damage rat lung tissue (46), it is not yet known whether this effect is chemical or species specific. However, the mouse model described in this paper appears to mimic human lung damage induced by BCNU and to reflect the primary risk factor of existing lung damage for the development of a fibrotic lesion in patients treated with this anticancer drug.

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REFERENCES


* J. P. Kehrer, unpublished data.
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Fig. 1. A, marked cellular infiltration and thickening of the alveolar walls 21 days after treatment of mice with BHT (400 mg/kg) followed on Day 1 by BCNU (10 mg/kg). Epon section, toluidine blue stain, × 140. B, total parenchymal consolidation with effacement of the alveolar architecture 21 days after treatment of mice with BHT (400 mg/kg) followed on Day 1 by BCNU (35 mg/kg). Epon section, toluidine blue stain, × 140. C, marked consolidation and fibrosis of the lung parenchyma following the same treatment as in B. Paraffin section, H & E, × 80. D, higher magnification of C showing large atypical cells in an area of fibrosis. Paraffin section, H & E, × 270.
Fig. 2. In A, type II pneumocytes constitute the main cell type lining the alveolar spaces 21 days after treatment of mice with BHT (400 mg/kg) followed on Day 1 by BCNU (35 mg/kg). × 3200. In B, increased collagen is seen in the interstitium. Same treatment as in A. × 4000. C, giant type II pneumocyte exhibiting large nuclear inclusions. Same treatment as in A. × 4100. D, giant type II pneumocyte with multilobulated nucleus. Same treatment as in A. × 3500.
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