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Reduction of Glutathione Levels in Livers of Mice Treated with *N,N'*-Bis (2-Chloroethyl)-*N*-Nitrosourea

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Summary. *N-methyl-N-nitrosourea (MNU), N-(2-chloroethyl)-N'-(trans-4-methylcyclohexyl)-N-nitrosourea (methylCCNU), and N,N'-bis(2-chloroethyl)-N-nitrosourea (BCNU) were examined for their effect on glutathione (GSH) levels of various tissues of normal and L1210-leukemic mice. BCNU produced significant decreases in the GSH levels of livers of both groups, but caused no change in the GSH content of the L1210 tumor or in the lungs. The GSH content of the kidneys of L1210 tumor-bearing mice, however, was significantly decreased by BCNU at early time points. A small increase in the liver content of oxidized glutathione could not account for the decreased content of GSH. Methyl CCNU and MNU were without effect on any of the tissues examined. These data are consistent with our previous observation that BCNU is a substrate for GSH S-transferase, and suggest that a GSH-dependent process is an important pathway for the metabolism of BCNU.*

Introduction

Conjugation of foreign compounds with glutathione (GSH), the major nonprotein thiol present in body tissues, is a process for the detoxification of many harmful substances. Compared to other tissues, the liver has high levels of GSH [3]; and the measurement of hepatic GSH levels following administration of an agent provides a useful index of possible conjugation.

N-methyl-*N*-nitrosourea (MNU), *N*-(2-chloroethyl)-*N'*-(trans-4-methylcyclohexyl)-*N*-nitrosourea (methylCCNU), and *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) are important chemotherapeutic agents, primarily due to their activity against intracerebral tumor cells [10]. A recent report from our laboratory [6] has shown that BCNU, but not MNU or methylCCNU, is a substrate for GSH *S*-transferase of mouse liver cytosol. This information implicates GSH in the in vivo metabolism of BCNU. In the present study, we examined the GSH levels of various tissues in normal mice following IV administration of BCNU, methylCCNU or MNU, and in L1210 tumor-bearing mice following administration of BCNU. In addition, oxidized glutathione (GSSG) levels were determined in the livers of normal mice following treatment with BCNU.

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Materials and Methods

The nitrosoureas were provided by the Division of Cancer Treatment, National Cancer Institute. GSH, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and glutathione reductase (Type III from yeast) were purchased from Sigma Chemical Co., St. Louis, Mo.

Male BDF_1 mice weighing 25–30 g and with livers weighing an average of 1.3 g were used in all experiments. Some mice were injected SC with 10^6 L1210 ascites cells 1 week before use. All animals were given food and water ad libitum and were kept on a 12-h photoperiod. For injection, BCNU and methylCCNU were dissolved in Emulphor-EL620, 95% ethanol, and 0.9% saline (1 : 1 : 8, v/v); MNU was dissolved in 0.9% saline. Controls received the vehicle only. All drugs were administered IV in the tail vein. Doses were 50 mg/kg for BCNU and methylCCNU and 25 mg/kg for MNU. The injections were timed so that the experiments were finished between 8 and 11 A.M. Experiments for each time period after treatment were performed on different days.

At various times after treatment, the animals were killed by exsanguination, and tissues were removed, blotted, and weighed. The tissues were immediately homogenized in 5% trichloracetic acid (TCA) (5 ml/g) in a Brinkmann Polytron homogenizer with a P-10 generating head (Brinkmann Instruments, Westbury, NY), and the preparations were centrifuged at 2000 *g* for 10 min. Portions of the tissue supernatants (50 µl for liver and 100 µl for the other tissues) were added to 0.4 *M* phosphate buffer, pH 8.0, and this was combined with 0.1 ml of 0.01 *M* DTNB in phosphate buffer [4] to give a final volume of 1 ml. The samples, in duplicate, were incubated at 25° for 30 min, and the absorbance was measured at 412 nm with a Beckman DU-2 spectrophotometer. GSH standards in a 5% TCA were assayed concurrently with the samples.

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For estimation of the levels of GSSG [7], liver homogenates were prepared with 0.4 *M* phosphate buffer, pH 7.4. The homogenates were divided into two equal portions and treated as follows: (a) homogenate incubated at 37° for 10 min in 5 m*M* NADPH and with glutathione reductase (25 units/g liver protein); and (b) homogenate with no NADPH or enzyme, kept at 0° for 10 min. Supernatants of the homogenates were prepared with 10% TCA. The difference between the values obtained for homogenate (a) and (b) was used to estimate the level of oxidized glutathione.

As a check on interfering substances in the TCA extracts that might affect sulfhydryl color production, known amounts of GSH were added to extracts of liver, kidney, lung, and tumor. In kidney and liver extracts, 100% of the added GSH was recovered. However, only 82% and 58% of the added GSH was recovered in lung and tumor extracts, respectively. For these tissues, the values presented have been corrected for recovery of GSH.

Results and Discussion

Following administration of BCNU to normal mice, the GSH content of liver was significantly lower than control levels (Table 1). A decrease of 1.7 μmol/g occurred within 15 min; the average decrease for all time periods was 1.8 μmol/g. In L1210 tumor-bearing mice, BCNU caused a decrease of 3.0 μmol/g in the liver content of GSH at 1 h after treatment. In contrast to the course in normal animals, the GSH content of the livers of these mice returned to the control level within 6 h. A decrease of 1.8 μmol/g liver or 2.3 μmol/liver could represent reaction of 34% of the administered BCNU with hepatic GSH in normal mice. Similarly, a decrease of 3.0 μmol/g (3.9 μmol/liver) could represent reaction of 58% of the BCNU given to L1210 tumor-bearing mice.

In spite of the precautions taken (see above), some day-to-day variation in the GSH content of the livers of control mice was observed (Table 1). Accordingly, all values have been compared with control values obtained in concurrent assays. The GSH content of livers beyond 18 h after BCNU treatment was variable (not shown), probably due to the onset of toxicity in some of the mice [9].

Especially at 12 h and 18 h, there were low levels of GSH in the livers of tumor-bearing mice treated or not treated with BCNU (Table 1). Since these experiments were not terminated until day 8 after tumor implantation, which corresponds to the median day of death for the mice (M.W. Trader, Southern Research Institute, personal communication), it is likely that stress associated with the tumorous condition is responsible for the decrease in baseline GSH levels.

Although several workers [1, 5] have reported that BCNU inhibits glutathione reductase of erythrocytes, yeast, and mouse liver, there was no appreciable increase in the levels of liver GSSG in BCNU-treated mice compared with controls. At 1 h and 18 h after administration of BCNU (results not shown), GSSG was increased by about 16% (0.075 μmol/g liver). This increase cannot account for the decrease in liver GSH by 1.8 μmol/g following injection of BCNU.

It is possible that the decrease in the liver content of GSH in BCNU-treated mice is due to inhibition of GSSG reductase, with conversion of GSH to a product other than GSSG. This enzyme, however, is strongly inhibited in the kidneys and lungs of mice [5], where we observed no corresponding decrease in GSH content. It seems unlikely that inhibition of this enzyme is directly involved in lowering of the GSH content of mouse liver by BCNU.

At 15 min and at 1 h, the GSH content of the kidneys of tumor-bearing mice was significantly depressed; but at 6 h the level returned to that for controls (Table 1). No corresponding depression was observed for normal mice; in contrast, an increase, the basis of which is unknown, was observed at 6 h and 12 h.

There was no significant decrease in the GSH content (1.6 ± 0.3 μmol/g) of the L1210 tumor or in the GSH content (1.5 ± 0.4 μmol/g) of the lungs of normal

Table 1. Levels of GSH[a] in tissues of mice following administration of BCNU

Hours	Normal mice				Tumor-bearing mice			
	Control		BCNU-treated		Control		BCNU-treated	
	Liver	Kidneys	Liver	Kidneys	Liver	Kidneys	Liver	Kidneys
0.25	8.3 ± 0.6	2.6 ± 0.1	6.6 ± 0.6[b]	2.6 ± 0.2	5.1 ± 0.9	2.1 ± 0.2	5.7 ± 0.8	1.5 ± 0.1[b]
1	7.1 ± 0.7	2.6 ± 0.2	5.7 ± 0.4[b]	2.2 ± 0.4	6.4 ± 0.5	2.4 ± 0.3	3.4 ± 0.5[b]	1.2 ± 0.1[b]
6	6.8 ± 0.8	3.0 ± 0.2	5.6 ± 0.2[b]	4.0 ± 0.2[c]	5.2 ± 0.6	2.5 ± 0.1	4.7 ± 0.5	2.7 ± 0.1
12	6.5 ± 0.8	3.6 ± 0.3	4.4 ± 0.5[b]	4.3 ± 0.2[c]	3.4 ± 0.5	2.0 ± 0.1	3.2 ± 0.2	2.0 ± 0.1
18	7.3 ± 0.2	3.0 ± 0.6	4.7 ± 0.2[b]	3.7 ± 0.3	3.2 ± 0.1	2.2 ± 0.5	3.7 ± 0.4	3.0 ± 0.8

[a] Mean levels ± SD (μmol/g tissue) for three or more mice
[b] Significantly lower than control ($P < 0.05$)
[c] Significantly higher than control ($P < 0.05$)

or tumor-bearing mice following treatment with BCNU (results not shown). Since the L1210 tumor responds well to BCNU [8], it is apparent that the GSH content of the tumor is not depleted as a prerequisite for toxicity, as is the case for some compounds causing hepatotoxicity [7]. Depletion of GSH in the lungs is apparently not involved in the toxicity of BCNU to this organ [2].

Treatment of normal mice with MNU or methyl CCNU did not significantly decrease the GSH content of any of the tissues examined (data not shown). These observations are consistent with our previous report [6] that BCNU, but not methylCCNU or MNU, is a substrate for GSH *S*-transferase, and suggest that a GSH-dependent process is an important pathway for the metabolism of BCNU.

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