MYELOTOXICITY OF NITROSOUREA DERIVATIVE, CNUA

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After the injection of CNUA, chlorozotocin (CLZ) or ACNU into CDF_1 mice at the optimal doses of a single ip injection for L 1210 leukemia, the effects with time on peripheral white blood cells (WBC), nucleated bone marrow cells (BMC), CFU-c and CFU-s were investigated. All three nitrosoureas produced decreases of WBC and BMC counts, and the lowest count was observed 3 days after administration. The maximum reductions of WBC and BMC were 50% and 80% for CNUA, 60% and 80% for CLZ and 75% and 93% for ACNU, respectively. However, depression caused by the administration of CNUA or CLZ recovered to the normal level from 7 to 14 days following administration. Severe depression of the CFU-s also appeared on day 3 after drug administration of all drugs. In the case of CFU-c, however, early depletion was observed and it tended to recover on day 3 when CNUA or CLZ was administered. The quantitative inhibition of DNA synthesis of L 1210 leukemia and bone marrow cells by CNUA or ACNU was investigated *in vitro*. The ratio of 50% inhibitory dose on DNA synthesis of L 1210 leukemic cells and bone marrow cells were 6.5 for CNUA and 2.9 for ACNU. CNUA showed greater inhibition of L 1210 DNA synthesis than that of murine bone marrow cells *in vitro*.

INTRODUCTION

Since the antitumor effect of 1-methyl-1-nitrosoguanidine was reported[1], many N-nitroso containing compounds have been synthesized. Among these compounds, hydrophilic nitrosoureas such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and methyl-CCNU showed antitumor activity on animal tumors and have been established clinically as a useful group of antitumor agents. However, these hydrophilic nitrosoureas have delayed and cumulative toxicity on the bone marrow, which reduces the usefulness of nitrosoureas in clinical trials[2,3]. Derivatives have been synthesized in a effort to reduce bone marrow toxicity and chlorozotocin (CLZ) with chloroethyl nitrosourea bound to the C-2 position of glucose was reported to show only moderate toxicity on the bone marrow at the effective dose for L 1210 leukemia[4,5]. This finding suggested that bone marrow-toxicity may by reduced by the binding of the nitrosourea to the sugar moiety.

Since then, many nitrosourea-sugar derivatives have been reported including GANU[6~8], a C-1 substituted glucose derivative ; MCNU[9], a C-6 substituted glucose derivative ; and TA-077[10], a maltose derivative. As shown in Fig. 1, CNUA is a C-3 substituted allose derivative which showed marked antitumor activity against various experimental murine tumors with less myelotoxicity[11]. In this paper, sensitivity and recovery of hematopoietic stem cells for CNUA in mice were investigated and sensitivity of bone marrow cells and L 1210 leukemic cells to CNUA was defined *in vitro*.

I. MATERIALS AND METHODS

Animals and drugs. Male CDF_1 (BALB/c× DBA/2) mice were obtained from the Shizuoka Agricultural Cooperative Association (Hamamatsu, Japan). The animals were maintained on laboratory diet and water *ad libitum*. CNUA and CLZ were synthesized in our laboratory. ACNU was commercially available. They were dissolved in sterile physiological saline just before use and administered at a volume of 0.1 ml/10 g body weight. ^{3}H-Thymidine (^{3}H-TdR), ^{3}H-Uridine (^{3}H-UR) and ^{3}H-Leucine (^{3}H-Leu) were purchased from New England Nuclear, Boston, Mass.

Antitumor activity. Lymphoid leukemia L 1210 cells were kindly supplied by Dr. TSUKAGOSHI, Cancer Chemotherapy Center (Tokyo). Antitumor activity was evaluated by the increase in life span (ILS) : $(T/C-1)\times100\%$, where "T" is the median survival days (MSD) of the treated group and "C" is the MSD of the control group. Surviving mice were scored 60 days after inoculation of tumors and mice remaining alive at this time were considered as cured.

CFU-s and CFU-c. CFU-s was assayed by the method of TILL & MCCULLOCH[12]. Donor mice, 8 weeks of age, were given intraperitoneal injection of drugs at the optimal dose for L 1210 leukemia. The mice were sacrificed by cervical dislocation at the indicated days after administration, femurs were removed and the bone marrow cells were flushed out with 1 ml of cold MEM from each femur. Bone marrow cells from each donor were stained with Türk solution and nucleated cells were counted with a hemocytometer. For each experiment, the cell suspension was diluted to $0.5\sim1\times10^5$ cells/ml. One half milliliter of this cell suspension was injected iv into 3～5 recipient mice, which were given 950 rad total body irradiation to reduce the number of their endogenous hematopoietic stem cells about 20 hr. before marrow transplantation. The recipients were sacrificed 8～10 days later and their spleens removed and fixed in Bouin solution for 24 hr. The number of surface colonies on the spleen was counted under a dissecting microscope. CFU-c was assayed as follows. One milliliter of nucleated bone marrow cell suspension (5×10^5 cells/ml), prepared as described above, was added to 2 ml of 2.2%-methyl cellulose (two times strength MEM), 1 ml of calf serum and 1 ml of conditionized medium (murine L cells were incubated in MEM supplemented with 10%-calf serum at 37°C in a 5%-CO_2 incubator for 7 days ; the medium was removed and passed through a millipore filter). One milliliter of this cell suspension was incubated in Petri dishes at 37°C in a 5%-CO_2 incubator for 7 days. The number of colonies per dish was counted under a microscope.

Peripheral WBC count. Five microliter of blood was withdrawn from the caudal vein of donor mice before sacrifice, and the peripheral white blood cells (WBC) were counted using a Microcell counter (TOA model CC-108).

Macromolecular synthesis. Bone marrow cells obtained from normal CDF_1 mice were suspended at a concentration of 2×10^6 cells/ml in RPMI-1640 medium containing 10%-fetal calf serum. For experiments, 30 mm Petri dishes were seeded with 2 ml of cell suspensions, 20 μl of drug was added and they were incubated for 24 hr. at 37°C in a 5%-CO_2 incubator. One hour before termination of the culture, radioisotope was added to final concentrations of 0.1 μCi/ml (^{3}H-TdR), 0.2 μCi/ml (^{3}H-UR) and 0.4 μCi/ml (^{3}H-Leu). The cells were then harvested on a glass fiber filter by aspiration, and the filter was washed with ice-cold 5%-trichloroacetic acid. The radioactivity of acid-precipitable material on the filter was determined by a liquid scintilation spectrometer (Packard Tri-Carb).

II. RESULTS

The effects of CNUA, CLZ and ACNU on L1210 leukemia are shown in Fig. 2. The optimal doses of a single ip injection of CNUA, CLZ and ACNU were decided as 40 mg/kg, 25 mg/kg and 35 mg/kg, respectively.

Spleen and thymus weight. The changes in spleen and thymus weight after the administration

Fig. 1 Structure of CNUA

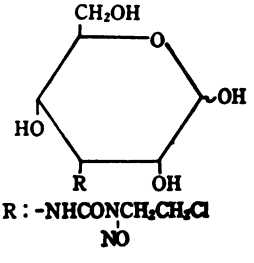


Fig. 2 Antitumor activity against L 1210 leukemia. L 1210 cells (1×10^5 cells/mouse) were inoculated ip into CDF_1 mice. Drugs were given as a single ip injection on Day 1. CNUA (●), CLZ (○), ACNU (▲).

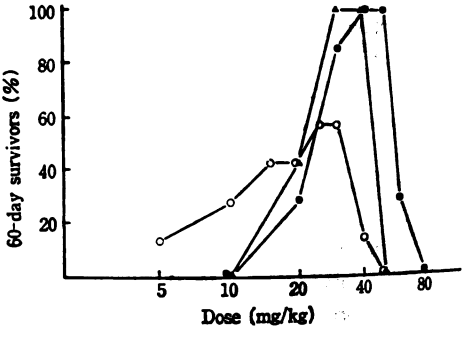
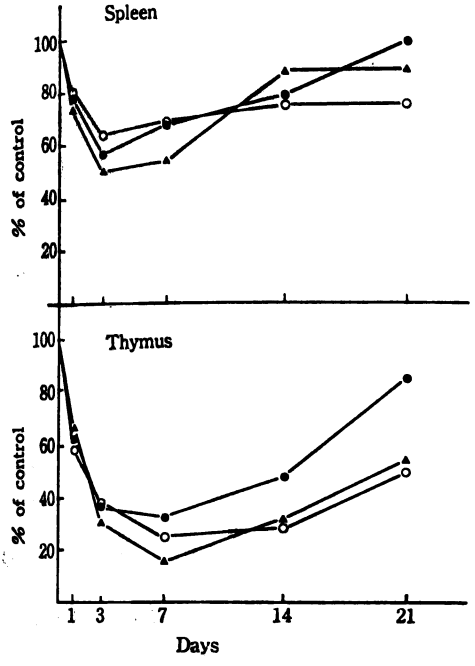


Fig. 3 Effect on spleen and thymus weight after a single ip injection of nitrosourea. CNUA 40 mg/kg (●), CLZ 25 mg/kg (○), ACNU 35 mg/kg (▲).



of these drugs were determined, and the lowest weight were observed 3 days or 7 days after administration. The recovery of thymus weight 21 days after administration was about 85% of control for CNUA and about 50% of control for CLZ and ACNU.

Effects on WBC count and bone marrow cellularity. All three nitrosoureas caused a decrease in WBC and BMC count and the minimum level of WBC and BMC occurred at 3 days after administration of the drugs (Fig. 4). However, WBC depression caused by the administration of CNUA or CLZ recovered to the normal level from 7 to 14 days following administration, while a 30% reduction in the WBC count was observed in mice treated with ACNU. The minimum reduction in the BMC was 80% for CNUA and CLZ, and 94% for ACNU. A relatively rapid recovery to pretreatment level was observed in the groups treated with CNUA or CLZ.

Effects on bone marrow CFU-s and CFU-c. The effects of nitrosoureas on CFU-s and CFU-c are shown in Fig. 5. Severe depression of CFU-s also appeared on day 3 after drug administration. When ACNU was administered, bone marrow CFU-s was reduced to a minimum of 2% of control. The recovery of bone marrow CFU-s showed a similar tendency to bone marrow cellularity. However, normal values were not restored 14 days after administration of drugs. The pattern of depletion and recovery of bone marrow CFU-c differed considerably from that of the CFU-s. Early depletion was observed, and it tended to recover on day 3. To determine the dose response, the survival of bone marrow CFU-s was measured 24

Fig. 4 Effect on peripheral WBC count and bone marrow cellularity after a single ip injection at the optimal dose for L 1210 leukemia. CNUA 40 mg/kg (●), CLZ 25 mg/kg (○), ACNU 35 mg/kg (▲).

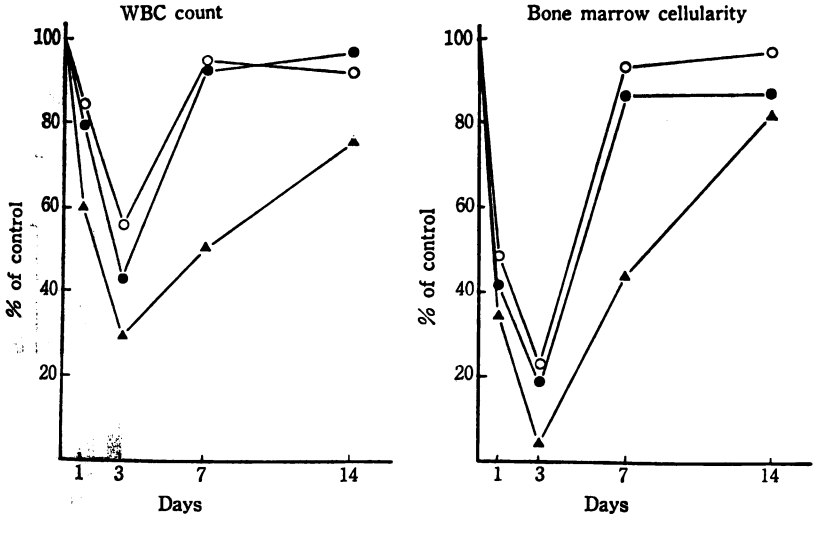
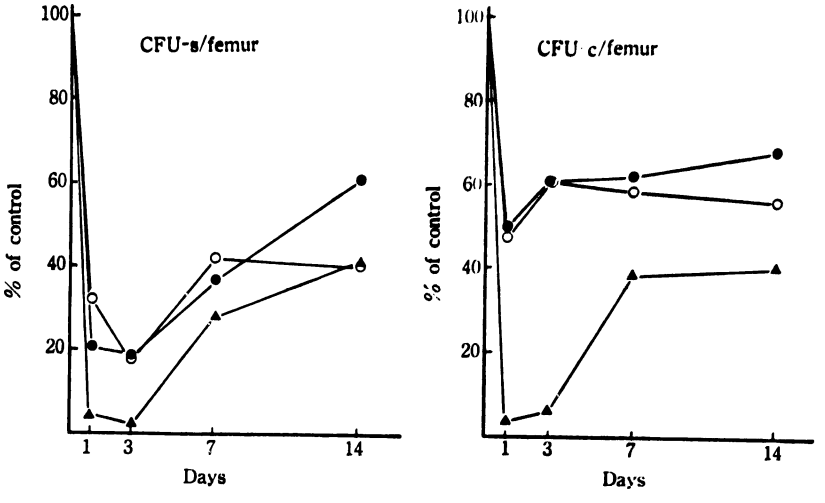
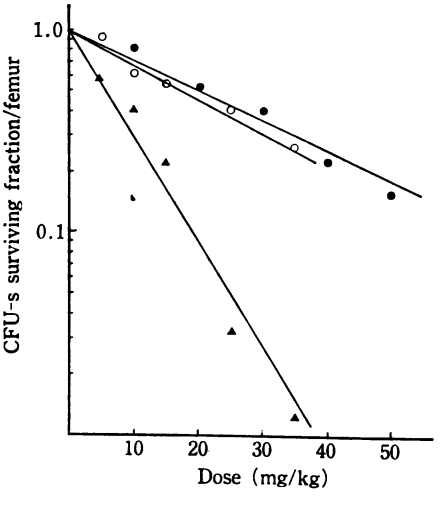


Fig. 5 Effect of nitrosoureas on bone marrow CFU-s and CFU-c in CDF_1 mice after a single ip injection at the optimal dose for L 1210 leukemia. CNUA 40 mg/kg (●), CLZ 25 mg/kg (○), ACNU 35 mg/kg (▲).



hr. after administration of nitrosoureas (Fig. 6). Survival of bone marrow CFU-s decreased in an exponential fashion with increasing dosages. The doses of CNUA, CLZ and ACNU required to reduce the cell population to 50% of control ($D_{1/2}$) were 20 mg/kg, 17 mg/kg and 6 mg/kg, respectively. The ratio of $D_{1/2}$: optimal dose for L 1210 was 0.50 for CNUA, 0.68 for CLZ and 0.17 for ACNU.

Fig. 6 Dose-survival curve of CFU-s to three nitrosoureas in mice. Cell survival was determined 24 hr. after ip injection of the drugs. CNUA (●), CLZ (○), ACNU (▲).



The ratio for CNUA and CLZ was 3 to 4 fold greater than that obtained for ACNU.

Macromolecular synthesis of L 1210 leukemia *in vitro*. Fig. 7 shows the inhibition of macromolecular synthesis of L 1210 leukemic cells exposed to various doses of CNUA for 24 hr. RNA and protein synthesis were inhibited to 26% and 15% of control at a concentration of 50 μg/ml of CNUA, while inhibition of DNA synthesis was observed at 85% of control. CNUA demonstrated preferential inhibition of DNA synthesis with comparable RNA or protein synthesis.

Fig. 7 Effect of CNUA on incorporation of radioactive precursors into macromolecules.

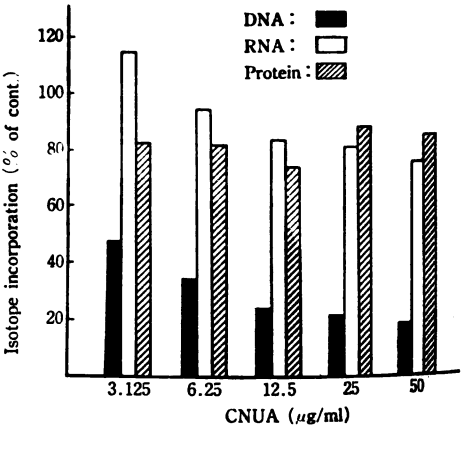
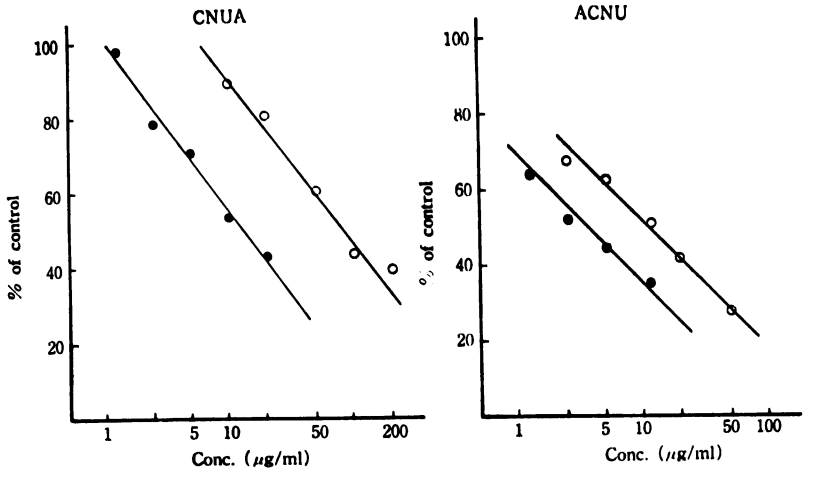


Fig. 8 Incorporation of ^{3}H-TdR into bone marrow cells (○) or L 1210 cells (●). The cells were exposed to the indicated concentration of drug for 24 hr.



DNA synthesis of L 1210 leukemia and bone marrow cells. The quantitative inhibition of DNA synthesis of L 1210 leukemia and murine bone marrow cells by CNUA or ACNU was investigated *in vitro*. The 50% inhibition doses for DNA synthesis in L 1210 leukemic cells were 13 μg/ml and 3.2 μg/ml of CNUA and ACNU, while the doses were 85 μg/ml and 9.2 μg/ml for bone marrow DNA synthesis. The 50% inhibitory dose ratio for DNA synthesis of L 1210 leukemic cells and bone marrow cells were 6.53 for CNUA and 2.87 for ACNU.

III. DISCUSSION

The present experiments demonstrated that nitrosoureas induced myelosuppression by decreasing the number of bone marrow cells, hematopoietic precursor cells and granulocyte-macrophage commited cells. CNUA and CLZ were less myelotoxic than ACNU. In these experiments, the minimum counts of peripheral white blood cells, nucleated bone marrow cells and CFU-s were observed 3 days after administration of the drugs. On the other hand, the minimum level of marrow CFU-c occurred 1 day after treatment, and recovery was faster than that of CFU-s which did not recovered to the pretreatment level 14 days after administration of drugs. HARVEY et al[13] reported that normal values of murine bone marrow CFU-s were not restored 42 days after administration of BCNU. However, return to the pretreatment pool size of CFU-c was regulary observed after 6~10 days[14,15].

In the sensitivity study of CFU-s, the surviving fraction was decreased exponentially with increasing doses, but sugar containing nitrosoureas, CNUA and CLZ, showed less toxicity on the bone marrow CFU-s with comparable antitumor activity. It has been reported[16,17] that the sensitivity of CFU-c to Cis-Pt varied with the animal species and murine cells were more sensitive than human cells. However, SCHEIN et al[18] reported that murine and human CFU-c showed similar sensitivity to nitrosoureas when treated *in vitro*. As demonstrated in these experiments, less toxicity on the bone marrow was observed by administration of CNUA than by that of ACNU. Whether this reduced toxicity was caused by the difference of direct sensitivity to the bone marrow cells and tumor cells was investigated *in vitro*. It appeared that the cytotoxic mechanism of nitrosoureas is alkylation of DNA and carbamoylation of protein, and alkylation of DNA is currently accepted as the mechanism responsible for antitumor activity of this class of compounds[19]. CNUA inhibited L 1210 DNA synthesis, and also a common property of nitrosoureas and inhibition of DNA synthesis was observed with a lag time after drug treatment. The direct cytotoxic activity of CNUA and ACNU was compared in bone marrow cells and L 1210 cells with respect to the ratio of DNA synthesis inhibition. CNUA showed greater inhibition of DNA synthesis of L 1210 cells than that of murine bone marrow cells *in vitro*. These results suggested that the reduced myelotoxicity on the bone marrow of CNUA when is administered *in vivo*

may be based on different cytotoxicity on the bone marrow cells and L 1210 cells. GREEN et al[20] reported that CLZ and GANU, glucose containing nitrosoureas, preferentially alkylated internucleosomal linker regions of bone marrow chromatin, while nucleosome core particles were the prefered targets of CCNU and ACNU. It has also been demonstrated[21,22] that DNA lesions whithin internucleosomal linker regions are more rapidly repaired than comparable lesions in the nucleosome core particles. Such differences may contribute to the differential cytotoxic properties of the allose containing nitrosoureas, CNUA.

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ニトロソウレア誘導体CNUAの骨髄毒性

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CNUAの骨髄に対する毒性をACNU, chlorozotocin (CLZ) と比較検討した。L 1210白血病に対する各薬剤の最適投与量を正常CDF_1マウスの腹腔内に投与し，その後，経時的に末梢白血球数，骨髄有核細胞数，骨髄幹細胞数 (CFU-s, CFU-c) の変化を調べた。末梢白血球数，骨髄細胞数は3薬剤共に投与3日後に最低値を示し，CNUAではそれぞれ対照の約50%，20%に，ACNUでは約25%，7%にまで減少した。しかしながらCNUA, CLZではその回復は比較的速やかで，投与7～14日後にはほぼ正常値にまで回復した。CFU-sも同様に3日後に最低値を示し，CNUAでは対照の約20%，ACNUでは約2%にまで減少した。一方，CFU-cは投与1日後に最低値を示し，3日後では回復する傾向が認められたが，いずれも投与14日後においても正常値には回復しなかった。また，*in vitro*の実験においてCNUAはRNA，蛋白合成よりもDNA合成をより強く阻害した。L 1210白血病細胞およびマウス骨髄細胞のDNA合成に及ぼす影響をACNUと比較検討したところ，それぞれの細胞に対するIC_{50}の比はCNUAで6.5 ACNUでは2.9でありCNUAはACNUと比較して骨髄細胞に対するDNA合成阻害は弱いものと思われた。