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Antitumor activity and toxicity of ACNU, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride, comparing two divided doses and a single dose

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Summary. *In order to determine whether it is possible to reduce the toxicity of the nitrosoureas, including the delayed type of hematologic toxicity, without diminishing antitumor activity by administering the drug according to a new treatment schedule other than the single high-dose treatment schedule, we examined the antitumor activity against murine tumors and toxicities to host mice of a nitrosourea derivative, 3-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-1-(2-chloroethyl)-1-nitrosourea hydrochloride (ACNU), which was administered according to two divided doses schedule. Experimental results indicated that the toxicity of ACNU with respect to lethality, as well as weight loss of host mice, was alleviated by administering ACNU (IV) at one-half of LD_{10} (20 mg/kg) on 2 successive days. However, no impairment of the antitumor activity of ACNU in various murine tumor systems was observed in comparison with that of ACNU administered according to the single high-dose schedule. The delayed type of hematologic toxicity (leukopenia) could not be alleviated by this treatment schedule.*

Introduction

Chloroethylnitrosourea derivatives are an important family of anticancer agents. The original lipophilic nitrosoureas, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (Me-CCNU), have achieved considerable antitumor efficacy in a variety of experimental tumors [18] and human malignancies [20, 22, 24, 25]. However, the delayed and cumulative hematologic toxicity [20, 22, 24, 25] has limited the clinical usefulness of these drugs.

To improve the nitrosourea class of anticancer agents, three strategies at least might be conceivable: (a) the development of new agents showing reduced hematologic toxicity while preserving antitumor activity, such as chlorozotocin [2, 13] and 1-(β-D-glucopyranosyl)-3-(2-chloroethyl)-3-nitrosourea (GANU) [3, 6, 14]; (b) combination treatment with non-anticancer agents which would reduce the hematologic toxicity of the nitrosoureas [23]; (c) the establishment of new nitrosourea treatment schedules to reduce the hematologic toxicity without diminishing the antitumor activity, if possible.

Previously [8, 16] we studied the relationship between the antitumor activity and hematologic toxicity of several nitrosourea derivatives, using mice bearing a 7-day-old Lewis lung carcinoma and non-tumored (normal) mice. By treatment with a single high dose of several nitrosoureas [i.e., 1-(2-chloroethyl)-3-(methyl-α-D-glucopyranos-6-yl)-1-nitrosourea (MCNU), Me-CCNU, and ACNU], tumor regressions below the predetermined weight (1,000 mg) were observed at maximum for 16 days [8], while leukopenia persisted for at least 21 days after administration of drugs [16], indicating that possibly chemotherapy failed to "cure" the tumor-bearing mice by repeating courses of a single high-dose schedule. Although the optimal treatment schedule for nitrosoureas has been considered to be a single high-dose treatment [18, 21], another treatment schedule for better therapeutic efficacy has been shown by many investigators [1, 8, 15]. The purpose of current studies is to determine whether it is possible to reduce toxicity of the nitrosoureas without concomitant loss of antitumor activity by administering the drugs on new treatment schedules other than the single high-dose treatment schedule. This report described the experimental results examining the antitumor activity against murine tumors and the toxicity to host mice of ACNU administered according to two divided doses schedule.

Materials and methods

Drug. ACNU was kindly supplied by Sankyo Co., Ltd., Tokyo, Japan. Solutions of ACNU, which were dissolved in physiological saline, were freshly prepared, diluted by a factor of 1.25, and administered IV or IP into either tumor-bearing or non-tumored (normal) mice in a volume of 0.01 ml/g body weight. Treatments were initiated at the indicated time and, in case of two doses, the second treatment with ACNU was given on 1, 3, 5, or 7 days after the first administration.

Animals and tumors. Adult male BDF_1 mice weighing 23–28 g and adult female BDF_1 mice weighing 21–24 g were used in these studies. L1210 murine leukemia was maintained by serial IP passage in female BDF_1 mice. Murine solid tumors, such as Lewis lung carcinoma and B16 melanoma, were maintained SC in syngeneic adult male C57BL/6 mice. All the mice and tumors were obtained from the Drug Research and Development (DR & D), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

Standardized protocols of the DR & D Program, NCI [11], with minor modifications as described previously [10], were followed for serial passage of and for implantation of tumors into BDF_1 or C57BL/6 mice. L1210 leukemia was implanted IP

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or IV at 10^5 cells/mouse on day 0. Lewis lung carcinoma was implanted SC at 5×10^5 viable cells/mouse and B16 melanoma was implanted SC or IP as 0.5 ml of a 1/9 (w/v) tumor brei on day 0.

Evaluation of antitumor activity. For the survival experiments, antitumor activity of ACNU against various tumors was assessed from two parameters: (a) the mean or median survival time of the drug-treated mice, excluding long-term survivors, versus saline-treated controls, expressed as percentage increase in life span (%ILS=T/C%-100); (b) the incidence of long-term (90-day) survivors. For the tumor-growth inhibition experiments, antitumor activity of ACNU against a 7-day-old Lewis lung carcinoma was assessed from three parameters [5]: (a) the tumor-growth delay expressed as treated minus control (T-C, days), (b) the tumor-growth inhibition expressed as (1-T/C), and (c) the complete tumor-regression rate. Tumor-free survivors were excluded from the calculations in parameters (a) and (b). Tumor sizes were measured three times a week, in general, and tumor weights were calculated according to the formula: length $\times$ $(\text{width})^2 \times 0.5$ [11].

The criteria of effective antitumor activity were the same as those employed for the "Division of Cancer Treatment Panel of Antitumor Screens" [12]. Statistical analysis was carried out according to Fisher's exact test for 90-day survival incidence; chemotherapy producing a significant number of survivors, compared with that (none) of control for each tumor, was evaluated as curative.

Evaluation of toxicity. Toxicity of ACNU against normal mice was assessed from three parameters: (a) lethality, (b) weight loss, and (c) hematologic toxicity.

A group of 10 or 6 BDF_1 mice were given a specified dose level of ACNU on various treatment schedules, and the mean body weight of mice in each group was measured, in general, daily for 30 days and the survival of mice was observed for 60 days after the initiation of treatments.

Hematologic toxicity was studied with respect to the bone marrow cellularity (as measured from the number of total nucleated cells per femur), as well as the white blood cell counts in the peripheral blood. At the indicated time after the last injection of ACNU on specified schedules into each group of 36 BDF_1 female mice, the blood was taken with a glass capillary from the supraorbital vein of mice (3 mice per assay, in general). Blood (0.02 ml) was diluted with 10 ml of chilled physiological saline, and total white blood cells were counted with a Coulter counter, model Z_B (lower threshold, 10) after hemolysis with 0.07% cetyltrimethylammonium chloride. In some experiments, total white blood cells were counted in a hemocytometer by staining the cells with Türk solution. Bone marrow cellularity was assayed by essentially the same method as described previously [7, 9]. Briefly, the mice used in the study of the white cell counts were killed by cervical dislocation, femurs (one femur/mouse) were removed, and the marrow was flushed out with 2 ml of chilled Hanks' balanced salt solution. The cell suspension was passed a few times through a 22-gauge needle to obtain a single isolated cell and was stained and diluted ($\times$ 10) with Türk solution in a melangeur. The nucleated cells were counted using a hemocytometer.

Statistical analysis. Experimental results were analyzed for significance by Fisher's exact test for 90-day survival incidence and by Student's *t*-test for prolongation of life span, tumor regression, and hematologic toxicity.

Results

Effects of dosage and time interval between two doses on toxicity of ACNU against normal BDF_1 mice

In order to determine the optimal condition of ACNU in two divided doses schedule, a interaction between treatment dosages and the time interval between two doses was examined, firstly, with respect to an incidence of toxic death of normal BDF_1 mice. Dosage employed were the LD_{10} (40 mg/kg), maximum tolerable dosage (32 mg/kg), and one-half of LD_{10} (20 mg/kg), respectively, when administered IV singly. As shown in Table 1, when ACNU at each dosage level was given twice at a 1-, 3-, 5-, or 7-day interval, almost all BDF_1 male mice receiving a dosage of 40 mg/kg per day died from toxicity regardless of the time interval. When the dosage of 32 mg/kg was given twice, a decrease (30% to 15%) in the lethality of mice of both sexes was observed according to increase in time interval. On the other hand, no deceased mice were observed at any time interval when the dosage of 20 mg/kg was given twice. Furthermore, no significant difference was observed in the patterns of weight loss and recovery of mice receiving 20 mg ACNU/kg twice at any time interval, and these patterns were much milder than that of mice receiving a single injection of 40 mg ACNU/kg (Fig. 1).

Effects of dosage and time interval between two doses on antitumor activity of ACNU

Secondly, a interaction between treatment dosages and the time interval between two doses was examined with respect to the antitumor activity against a 7-day-old Lewis lung carcinoma. As shown in Table 2, groups treated twice with ACNU at the dosage levels of 40 mg/kg or 32 mg/kg showed a larger tumor-growth delay (T-C value), a higher tumor-growth inhibition (1-T/C), and a higher incidence of completely tumor-regressed mice compared with those observed in the group treated twice with 20 mg ACNU/kg. Regression and regrowth patterns of the tumors receiving 32 mg ACNU/kg and 20 mg ACNU/kg are illustrated in Fig. 2. With respect to the prolongation of survival time of tumor-bearing mice, however,

Table 1. Effect of treatment schedule on lethal toxicity of ACNU against normal BDF_1 mice

Schedule	Dosage (mg/kg/day)					
	40		32		20	
	Exp. 1[a]	Exp. 2[b]	Exp. 1[a]	Exp. 2[b]	Exp. 1[a]	Exp. 2[a]
Day 0 only	1/6[c]	1/10	0/10	0/10	0/6	0/10
Days 0 and 1	6/6	NT[d]	2/10	4/10	0/6	0/10
Days 0 and 3	6/6	NT	1/10	3/10	0/6	0/10
Days 0 and 5	5/6	NT	2/10	2/10	0/6	0/10
Days 0 and 7	6/6	5/10	3/10	0/10	0/6	0/10

ACNU was administered IV
[a] Male
[b] Female
[c] No. of deceased mice/total
[d] NT = not tested

the highest ILS (74%) of all was observed in the group treated twice with 20 mg ACNU/kg on days 7 and 8. In addition, an obviously schedule-dependent antitumor activity was observed in the groups treated twice with 20 mg ACNU/kg, i.e., the shorter the interval between two doses, the higher the antitumor activity observed, resulting in the highest increased survival time, as well as the largest inhibition and delay of tumor growth by treatment with ACNU on daily two doses (Table 2 and Fig. 2B). Moreover, ACNU on this treatment schedule showed significantly greater inhibition of the tumor growth compared with that obtained by a single injection of 40 mg ACNU/kg (Fig. 2B). These results indicated that the schedule of two daily doses of 20 mg ACNU/kg appeared to be the most optimal of all treatment schedules studied with respect to both the toxicity and the antitumor activity and, therefore, further studies were carried out concerning this treatment schedule.

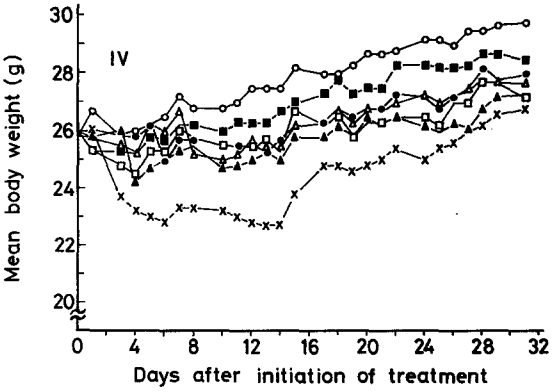

Fig. 1. Changes in mean body weight of mice after initiation of treatment with ACNU on various treatment schedules. ACNU (20 mg/kg/day) was administered IV to normal BDF_1 male mice (6 mice/group) on day 0 only (■), days 0 and 1 (□), days 0 and 3 (▲), days 0 and 5 (●), or days 0 and 7 (△), respectively. Other groups received either 40 mg ACNU/kg (×) or saline (○) on day 0. This experiment was carried out two times and similar results were obtained in both experiments

Antitumor activity of ACNU on the two-daily-dose schedule

Antitumor activity of ACNU on the schedule of daily two doses at a dosage level of one-half of LD_{10}, when ACNU was administered either IV (40 mg/kg) or IP (50 mg/kg) by a single injection, was examined and compared with that of ACNU at LD_{10} given by a single injection. As shown in Table 3, ACNU administered IV on either treatment schedule showed a curative antitumor activity against Lewis lung carcinoma as well as L1210 leukemia. ACNU administered IP on the two-daily-dose schedule also showed a curative effect on mice bearing IP L1210 leukemia, whereas some degree of toxicity was observed in the group received 50 mg ACNU/kg singly. As a whole, the antitumor activity in various murine tumor systems of ACNU administered on the two-daily-dose schedule was equal to or somewhat greater than that of ACNU administered by a single injection.

Hematologic toxicity of ACNU on the two-daily-dose schedule

Hematologic toxicity of ACNU (20 mg/kg per day) administered IV on the two-daily-dose schedule was studied with

Table 2. Effect of treatment schedule on antitumor activity of ACNU against advanced Lewis lung carcinoma

Dosage (mg/kg/day)	Schedule	ILS[a] (%)	Tumor growth		
			T-C value[b] (days)	1-T/C[c] (%)	Complete regression rate[d]
40	Day 7 only	55	12.0	96.2	0/6
	Days 7 and 8	11	23.0	98.3	2/5
	Days 7 and 10	49	26.5	99.3	4/5
	Days 7 and 12	28	26.5	99.2	4/5
	Days 7 and 14	32	27.0	98.9	5/6
32	Day 7 only	40	10.0	92.5	0/6
	Days 7 and 8	35	17.5	98.1	0/6
	Days 7 and 10	40	22.0	98.8	2/6
	Days 7 and 12	38	25.5	98.5	1/6
	Days 7 and 14	33	28.5	99.1	3/6
20	Day 7 only	−25	4.0	60.5	0/6
	Days 7 and 8	74	14.0	98.3	0/6
	Days 7 and 10	64	11.5	94.8	0/6
	Days 7 and 12	17	11.5	93.7	0/6
	Days 7 and 14	−11	6.5	76.4	0/6

[a] Trypan-blue-excluding Lewis lung carcinoma cells (5×10^5 cells/mouse) were implanted SC into axillary region of six BDF_1 male mice per group on day 0, and ACNU at specified dose levels was given IV on the indicated schedules. ILSs were calculated with the median survival time of each group. Median survival days of saline-treated controls were 26.5–27.5

[b] T-C value (days) = time required for the treatment-group tumors (the mean value) to reach 1,000 mg minus the time required for the control-group tumors to grow to the same size, as reported in [5]

[c] 1-T/C (%) = percent drgree of the tumor growth inhibition calculated by the mean tumor weight of the group treated, excluding the completely tumor-regressed mice, divided by the mean tumor weight of the control-group tumors (T/C). The maximum growth inhibition throughout the observation periods is shown

[d] Number of the completely tumor-regressed mice/total number of mice in the group. The maximum rate throughout the observation periods is shown. All these mice died with tumor within 90 days after the implantation of tumor

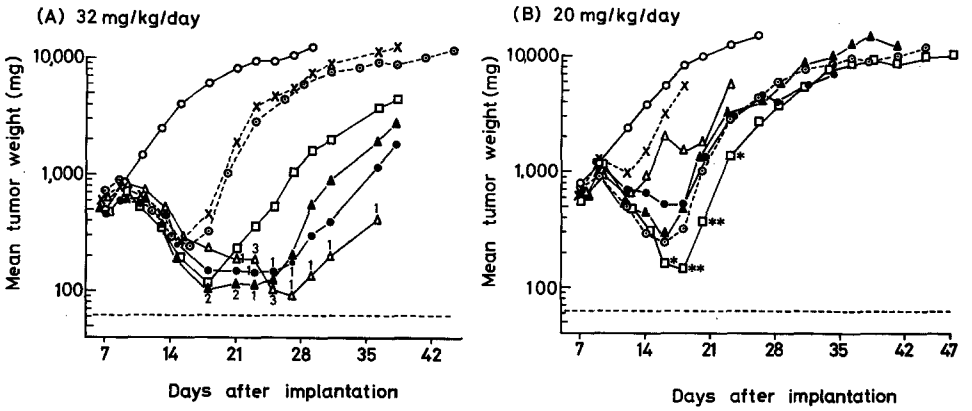

Fig. 2A and B. Tumor regression patterns of advanced Lewis lung carcinoma after initiation of treatments with ACNU on various treatment schedules. **A** ACNU at the dosage levels of 32 mg/kg/day or **B** 20 mg/kg/day was administered IV to tumor-bearing mice on day 7 only (×———×), days 7 and 8 (□———□), days 7 and 10 (▲———▲), days 7 and 12 (●———●), or days 7 and 14 (△———△), respectively. Other groups were treated with either 40 mg ACNU/kg (⊙———⊙) or saline (○———○) on day 7. The plots were terminated at the approximate median survival time of tumor-bearing mice in each group. ------ Limit of palpation of tumor (62.5 mg). Figures around symbols indicate the number of completely tumor-regressed mice. * $P < 0.05$, ** $P < 0.01$, compared with the group received 40 mg ACNU/kg

Table 3. Comparison of antitumor activity of ACNU administered singly or by two daily doses against various murine tumors

Tumor	System	Drug	Schedule	Dosage (mg/kg/day)	MST ± SD (days)	ILS (%)	90-day survivors
L1210 leukemia	IV-IV	Saline	Day 1		7.3 ± 0.5		0/8
		ACNU	Day 1	40	24.7 ± 0.6	238	5/8*
		ACNU	Days 1 and 2	20	33.0	352	6/8**
	IP-IP	Saline	Day 1		8.3 ± 0.7		0/8
		ACNU	Day 1	50	17.0 ± 12.6	105	1/8
		ACNU	Days 1 and 2	25	26.0	213	6/8**
Lewis lung carcinoma	SC-IV	Saline	Day 1		32.6 ± 4.6		0/8
		ACNU	Day 1	40	85.0	161	7/8***
		ACNU	Days 1 and 2	20	56.0	72	7/8***
B16 melanoma	SC-IV	Saline	Day 1		33.6 ± 7.2		0/8
		ACNU	Day 1	40	31.9 ± 15.5	−5	1/8
		ACNU	Days 1 and 2	20	42.7 ± 19.1	27	2/8
	IP-IP	Saline	Day 1		21.1 ± 3.4		0/8
		ACNU	Day 1	50	26.3 ± 1.5	25	1/8
		ACNU	Days 1 and 2	25	37.4 ± 17.9	77	1/8

L1210 cells (10^5 cells/mouse) were implanted IV or IP, trypan-blue-excluding Lewis lung carcinoma cells (5×10^5 cells/mouse) were implanted SC, B16 homogenate (tumor : Hanks' balanced salt solution = 1 : 9) was implanted SC or IP (0.5 ml/mouse), respectively, into eight BDF_1 male mice per group on day 0, and ACNU or saline was administered IV or IP on the indicated schedules. MST = mean survival time of tumor-bearing mice excluding 90-day survivors. SD = standard deviation. ILS = increase in mean life span of drug-treated groups excluding long-term survivors over saline-treated control

* $P < 0.02$, ** $P < 0.005$, *** $P < 0.001$, compared with the individual control group

respect to femoral bone marrow cellularity as well as peripheral leukocytes counts, and compared with that of ACNU (40 mg/kg) administered IV by a single injection. As shown in Fig. 3, decrease in the bone marrow cellularity in the group treated twice with 20 mg ACNU/kg was significantly milder than that elicited in the group receiving a single injection of 40 mg ACNU/kg. Recovery from the bone marrow suppression of the former group was identical to or one day faster than that of the latter group when compared in the patterns either after the initiation (panel B) or after the termination (panel A) of both treatments, respectively. Changes in the peripheral leukocyte counts in the group treated twice with 20 mg ACNU/kg also appeared to be milder than that elicited in the group receiving a single injection of 40 mg ACNU/kg during the first 5 days after termination (and after initiation) of both treatments, whereas the former group conversely encountered lower leukocyte counts than the latter group 7 and 14 days after the termination of both treatments (panel A). This delayed type of more severe suppression in the peripheral leukocyte counts of the group receiving two daily injections of 20 mg ACNU/kg was examined further in another study. As shown in Table 4, although the delayed type of leukopenia (significantly lower leukocyte counts than the controls) was equally observed in both groups 7 and 14 days after the last injection of drug, more severe leukopenia was observed on day 14 in the group receiving the two daily injections of 20 mg ACNU/kg.

It is interesting to known the hematologic toxicity of those groups receiving such a chemotherapy which induced far more intensive tumor-regressive activity against advanced Lewis

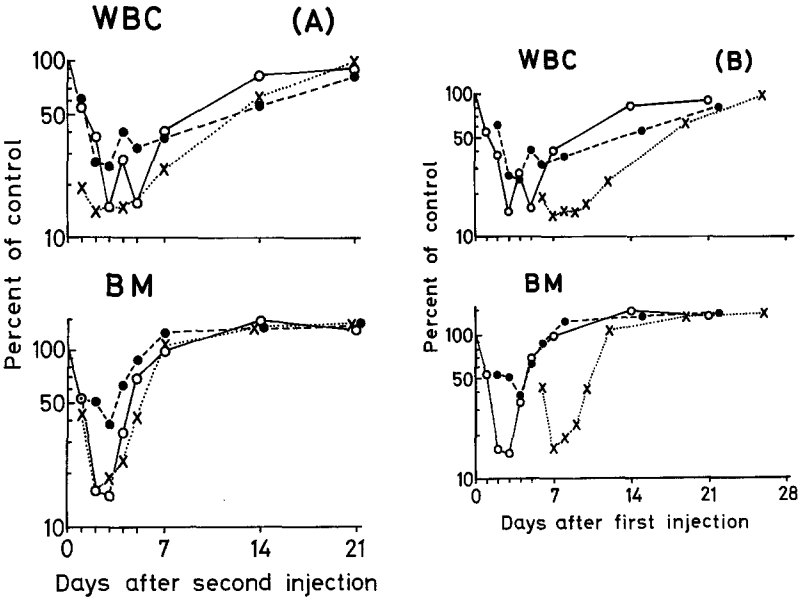

Fig. 3A, B. Sequential changes in peripheral white blood cell counts and femoral bone marrow cellularity of BDF_1 mice after treatment with ACNU on three different schedules. Thirty-six BDF_1 female mice per group were given IV a single injection of 40 mg ACNU/kg (○), daily two injections of 20 mg ACNU/kg (●), or two injections at 5-day interval of 32 mg ACNU/kg (×). Three mice from each group were examined, as described in Materials and methods, for both the number of white blood cell counts and the bone marrow cellularity on 1, 2, 3, 4, 5, 7, 14, and 21 days after the last injection of drug in each group. **A** Sequential changes after the second injection and **B** those after the first injection are shown, respectively. This experiment was carried out twice and similar results were obtained in both experiments. Control consisted of eight mice and the cell number (mean ± standard deviation) of the control (no treatment) was 6,602 ± 2,005/μl for white blood cell count and 106 ± 22 × 10^5/femur for bone marrow cellularity, respectively, in this experiment

Table 4. Comparison of peripheral white blood cell count at days 7 and 14 after treatment with ACNU given by single injection or by two daily injections

Group	Day 7	Day 14
Control	6,780 ± 2,719[a]	4,140 ± 1,171
ACNU	3,480 ± 1,547**	2,560 ± 796**, *
40 mg/kg (day 0)		
20 mg/kg/day (days −1 and 0)	2,660 ± 792***	1,790 ± 761***, *

Sixty BDF_1 female mice were distributed into six groups (10 mice per group). Two of six groups received a single IV injection of 40 mg ACNU/kg on day 0 and another two groups received two daily IV injections of 20 mg ACNU/kg on days −1 and 0. The remaining two groups served as controls (no treatment). Peripheral blood was taken from the supraorbital vein of mice 7 and 14 days after the last injection of drug (day 0). White blood cells were stained and diluted (× 20) with Türk solution and counted using a hemocytometer

[a] Mean ± standard deviation

* $P < 0.05$ by Student's t-test, ** $P < 0.01$, *** $P < 0.001$, compared with control (by t-test)

lung carcinoma than did daily two injections of 20 mg ACNU/kg (Table 2). Among them the hematologic toxicity of the group receiving two injections of 32 mg ACNU/kg at a 5-day interval was examined. As shown in Fig. 3, a clear second suppression of the bone marrow cellularity was observed after the second injection of 32 mg ACNU/kg (panel B) since mice which received a single injection of 32 mg ACNU/kg showed both a decrease and recovery pattern of the bone marrow cellularity (data not shown) that were essentially similar to those observed in the group receiving a single injection of 40 mg ACNU/kg (Fig. 3). However, after the second injection of drug in the group receiving two injections of 32 mg ACNU/kg at a 5-day interval, the decrease and recovery patterns were similar to those of the group receiving a single injection of 40 mg ACNU/kg (panel A). Also, a second suppression (which was not as clear as that observed in the bone marrow cellularity) in the peripheral leukocyte counts was observed after the second injection of 32 mg ACNU/kg, resulting in a longer period of leukopenia than that of the other two groups (panel B).

Discussion

The nitrosourea class of anticancer agents were relegated to those classes of cell-cycle nonspecific drug on the basis of cell-cycle specificity [21] and concentration-dependent drug with respect to rate of cell killing [21], indicating that a single high-dose therapy appears to be the most optimal for this class of drugs [18, 21]. However, as described previously, a single high-dose therapy with some nitrosourea derivatives failed to cure the mice bearing a 7-day-old Lewis lung carcinoma in relation to the delayed type of hematologic toxicity [8, 16].

In order to determine whether it is possible to reduce toxicity to host animals, including the delayed type of hematologic toxicity of nitrosoureas, without concomitant loss of antitumor activity, we examined in the present study the effects of a new treatment schedule (a schedule of two divided doses) on the antitumor activity against murine tumors and toxicity to host mice. The experimental results indicate that the toxicity of ACNU can be successfully alleviated with respect to lethality as well as weight loss of host mice by administering the drug at one-half of LD_{10} on a two-daily-dose schedule (Table 1 and Fig. 1). In addition, no impairment of antitumor activity was observed in various murine tumor systems (Tables 2, 3, and Fig. 2). However, the delayed type of hematologic toxicity could not be alleviated by this treatment schedule (Fig. 3 and Table 4), although a minor improvement could be observed during the early stage of hematologic toxicity (Fig. 3).

As indicated by Schabel [17], to approach a "curative" drug treatment of advanced (clinically recognized) tumors of experimental animals or of man, the first drug which should be used for induction therapy leading to complete regression of the tumor appears to be the cell-cycle nonspecific drug because of the presumably low growth fraction of tumor cells in the advanced diseases. The nitrosourea class of anticancer agents were relegated to this class of drugs [21] and, moreover, it was suggested that some nitrosoureas are as effective against resting tumor cells as proliferating tumor cells [4, 19]. Thus, the nitrosourea class of anticancer agents appears to be one of the most appropriate candidates for employment in the induction therapy of advanced tumors because of its considerable antitumor efficacy in a variety of experimental tumors

[18] and human malignancies [20, 22, 24, 25]. In addition, by scheduling the administration of nitrosoureas, more intensive tumor-regressive activity was observed in the present study (Fig. 2A) and in other studies [8, 15]. Therefore, further studies are clearly necessary and now in progress in order to establish, if possible, a true optimal treatment schedule for the individual nitrosourea derivatives, leading to successful induction therapy with this class of anticancer agents.

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