Effects of Aclacinomycin-A on Murine Leukemia

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Effects of aclacinomycin-A (ACM) on leukemic cells and normal hematopoietic stem cells were studied for the purpose of evaluating the usefulness of this agent as an antileukemic agent. ACM suppressed not only leukemic colony-forming unit (LCFU) in the radiation-induced murine erythroleukemia, but also normal hematopoietic stem cells (CFU-S, CFU-C) in mice. However, LCFU was the most sensitive to ACM. After the administration of ACM at a dose of 5 mg/kg, LCFU was suppressed to 0.02 of the control on day 3 and recovered within 5 days. Splenic CFU-S and CFU-C were suppressed to the nadirs on day 2 and day 1, respectively, and they recovered within 5 days. On the basis of the differences in the sensitivities to ACM and recovery patterns between LCFU and normal hematopoietic stem cells, ACM at a single dose of 5 mg/kg was administered every 3 days (total dose of 25 mg/kg) into leukemic mice, and the increase in mean lifespan obtained 113.9%. Effectiveness of ACM in the treatment of acute leukemia was discussed.

ACLACINOMYCIN-A (aclarubicin hydrochloride, ACM) isolated from the culture of Streptomyces galileus was developed in 1975. This agent belongs to the anthracycline antitumor antibiotics, which could be divided into two classes on the basis of their effects on DNA and RNA syntheses. Class I anthracyclines, such as adriamycin (ADM) and daunorubicin (DNR), inhibit DNA and RNA syntheses at approximately equivalent concentrations, and there is specific interference with the late S or G2 phase of the cell cycle. Class II anthracyclines, including ACM, inhibit whole cellular RNA synthesis at 6–7-fold lower concentrations than those required to inhibit DNA synthesis, and nucleolar RNA synthesis at 170–1250-fold lower concentrations than necessary to inhibit DNA synthesis. ACM interferes with not only the late S or G2 phase of the cell cycle but also with the mid-G1 phase. The presence of di- or trisaccharides linked to the anthracycline aglycone in class II anthracyclines is considered to be related with nucleolar RNA synthesis inhibitory specificity.

ACM showed the same degree of antitumor activity against leukemia L-1210 and P-388 as DNR and somewhat less than ADM. However, ACM was still effective against the ADM-resistant mutant subline of mouse lymphoblastoma L-5178Y cells. Cardiac toxicity, which is the serious side effect of anthracyclines, of ACM has been recently used clinically and revealed to be effective for human acute leukemia. Given as a single agent, ACM has produced 35% complete remission rate in patients with previously untreated acute myelocytic leukemia. This value is considerably comparable with the remission rate of 35%–50% obtained by DNR.

The present study was designed to examine the cytotoxic effects of ACM on leukemic cells and normal hematopoietic stem cells using animal models. Based on the differences in the sensitivities and recovery patterns between leukemic cells and normal hematopoietic stem cells, some regimens for the treatment of acute leukemia were discussed.

MATERIALS AND METHODS

Murine Leukemia

Acute leukemia was newly induced in a B6C3F1, mouse (C57B1/6J × C3H/He hybrid) 12 mo after a single whole-body x-irradiation of 900R followed by the syngeneic bone marrow transplantation. Leukemic cells were large round cells with large nucleus and scanty amount of basophilic cytoplasm, resembling proerythroblast. Histopathologically, leukemic cells infiltrated in red pulps and subcapsular regions in spleen, sinusoids in liver, and diffusely in bone marrow. By chromosomal analysis, all leukemic cells had marker chromosomes (2n = 40, XX, -12, + marker, abn. 2, 5q-, 16q+, 18q+, abn. X). These findings were compatible with those of murine erythroleukemia.

To passage the leukemia, a spleen obtained from a preterminal leukemic mouse was homogenized in normal saline and forced through a 23-gauge needle to disaggregate any clumps, and 107 cells were inoculated into syngeneic mice via tail vein. Thus, leukemia was maintained with the preservation of the characters of this leukemia, which had been confirmed by successive cytologic, histologic, and cytogenetic studies. Transplanted mice died within 19.8 ± 1.8 days (mean ± SD). Cell-free extracts of a leukemic spleen never induced leukemia.

All mice used in the present work were 8–12-wk-old female B6C3F1 mice bred in the animal colony of the National Institute of Radiological Sciences.

Aclacinomycin-A

ACM was kindly provided by Sanraku Ocean Co., Tokyo. This agent was dissolved in normal saline and was injected into mice via tail vein.
**Effects of ACM on Leukemic Cells**

Sixteen mice were inoculated with $10^7$ leukemic spleen cells obtained from a mouse with advanced disease. Four mice were sacrificed on each 4th, 7th, 10th, and 14th day thereafter, and peripheral blood counts, the weights of spleen and liver, and bone marrow nucleated cell counts were assessed. These studies were also performed in mice injected intravenously with ACM at a dose of 5 mg/kg on days 4 or 6 after the leukemic cell inoculation.

Next, $5 \times 10^4 \sim 10^5$ leukemic spleen cells were inoculated into syngeneic mice. Twelve days after the inoculation, mice were sacrificed and spleens were removed. Discrete white colonies were found on the splenic surface. The histologic sections of them revealed the clusters of leukemic cells. There was a statistically significant linear correlation between the number of inoculated colonies and the number of formed colonies (unpublished data), and these colonies were determined to be derived from leukemic colony-forming unit (LCFU). In the present work, LCFU per $5 \times 10^4$ leukemic spleen cells were scored macroscopically after the Bouin's fixation. Using this assay system, effects of ACM on LCFU in spleen were examined. Five mg/kg of ACM was administered to mice that had been inoculated with $10^7$ leukemic cells 6 days before. On days 1, 3, 5, 8, and 12 thereafter, mice were sacrificed and spleens were removed. From the pooled spleen cells of 3 mice in each group, $5 \times 10^4$ nucleated cells were injected to 8 assay mice, and LCFU were counted. Thus, changes in LCFU affected with ACM were studied. The dose–survival curve of LCFU was obtained from leukemic mice given the injection of ACM at various doses.

**Effects of ACM on Normal Hematopoietic Stem Cells**

Peripheral blood counts, bone marrow nucleated cell counts, splenic cell counts, pluripotent stem cells (CFU-S) in bone marrow and spleen, and granuloid committed progenitor cells (CFU-C) in bone marrow and spleen were assayed following the intravenous injection of $5 \text{ mg/kg}$ of ACM into normal B6C3F, mice. Bone marrow nucleated cells were obtained from femur by flushing out with 2 ml of McCoy's modified 5A medium (GIBCO, Grand Island, N.Y.). Spleen was carefully minced with scissors, homogenized in 5 ml of McCoy's modified 5A medium, passed through a sterile stainless mesh, and splenic cells were collected. CFU-S was assayed according to the spleen colony assay by Till and McCulloch. Briefly, 7 days after the intravenous injection of $10^5$ bone marrow cells or $2 \times 10^5$ spleen cells into syngeneic mice given 800R x-irradiation, discrete colonies formed on the splenic surface of the recipient were scored as CFU-S with the aid of the inverted microscope (Olympus Co., Tokyo, Japan). Triplicate cultures containing 1 ml in 35-mm diameter standard dishes (Lux Co., Newbury Park, Calif.) were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. After 7 days, colonies containing more than 50 cells were counted as CFU-C with an inverted microscope (Olympus Co., Tokyo, Japan).

The dose–survival curves of CFU-S and CFU-C were studied in mice given the injection of ACM at various doses.

**Effects of ACM on Survival of Leukemic Mice**

ACM was intravenously administered to leukemic mice in various schedules. Each group studied consisted of 6 mice, and 2 or 3 experiments for each group were repeated. Survival of the treated mice was measured as the percentage increase in mean survival time (MST) compared to untreated control mice from the start of therapy, and the increase in mean lifespan (%ILS) was calculated: [%ILS=(MST of treated group – MST of control group)/MST of control group × 100 (%)].

**RESULTS**

**Changes in Hematologic Parameters Following the Leukemic Cells Implantation**

Following the inoculation of $10^7$ leukemic cells, anemia progressed, peripheral blood nucleated cells increased in number, and the weights of spleen and liver increased after the latent period of about 4 days (Fig. 1). Most nucleated cells in the peripheral blood were leukemic cells. Leukemic cells infiltrated in bone marrow, spleen, and liver.

**Effects of ACM on the Leukemic Cell Proliferation in Spleen**

Leukemic cells proliferated rapidly in spleen. The volume doubling time of spleen in mice inoculated with $10^7$ leukemic cells was estimated to be about 1.5 days (Fig. 2). When ACM was administered intravenously at a dose of 5 mg/kg on day 4 (group II) or day 6 (group III) after the inoculation of leukemic cells, the proliferation of leukemic cells in spleen was definitely suppressed for about 4–5 days (Fig. 2). Thereafter, spleen weights began to increase, and the regrowth curves ran almost parallel with that of the sham-treated group (group I). By the back-extrapolation of the regrowth curves, the fractional spleen weight equivalency on the time of the injection of ACM was
Changes of leukemic spleen weights

Fig. 2. Regrowth curves of leukemic spleen weights after the injection of aclacinomycin-A. Group I: 10^7 leukemic cells were inoculated, and ACM was not administered. Group II: ACM at a dose of 5 mg/kg was administered 4 days after the inoculation of leukemic cells. Group III: ACM at the same dose was administered 6 days after the inoculation of leukemic cells. Mean ± SD of 4 mice are shown.

Changes in Normal Hematopoietic Stem Cells After the Administration of ACM

Figure 5 shows the changes in CFU-S after the injection of ACM at a dose of 5 mg/kg to normal B6C3F1 mice. CFU-S in bone marrow decreased to about 0.23 of the control on day 1 and recovered within 5 days. In contrast, CFU-S in spleen was suppressed more strongly up to 0.023 of the control on day 2 and also recovered within 5 days.

Figure 6 shows similar changes in CFU-C. Changes in CFU-C were similar to those in CFU-S except that bone marrow CFU-C recovered within 3 days.

Effects of ACM on Survival of Leukemic Mice

Table 1 shows the survival of leukemic mice that were treated with ACM in various schedules. When 5 mg/kg of ACM was administered 4 days after the inoculation of 10^7 leukemic cells, the survival after the initiation of therapy was 20.8 ± 1.8 days (mean ± SD), and %ILS was calculated as 31.6%. In mice that were administered 25 mg/kg of ACM on day 4, and mice that were administered 5 mg/kg of ACM daily on days 4, 5, 6, 7, and 8 (total dose of 25 mg/kg), the survivals were 15.6 ± 9.1 days and 12.0 ± 2.3 days, respectively. The major cause of death in these groups was the toxicity of ACM, such as gastrointestinal bleeding, rather than the advance of leukemia. If 5

Sensitivities of LCFU, CFU-S, and CFU-C to ACM

Figure 3 shows the dose–survival curves of LCFU in leukemic mice, and CFU-S and CFU-C in normal mice against ACM. They were suppressed in an exponential manner. Among them, LCFU was the most sensitive. CFU-S and CFU-C in spleen were more sensitive to ACM than those in bone marrow.

Changes in LCFU in Leukemic Mice Given the Administration of ACM

LCFU in the spleens of mice given the inoculation of leukemic cells increased rapidly and reached about 9 × 10^9 in surviving fraction on day 8 (Fig. 4). Thereafter, no significant increase was observed and mice died. On the other hand, in the group that received 5 mg/kg of ACM, LCFU was suppressed to about 2 × 10^{-2} on day 3, began to recover thereafter, and reached the level of day 0 on day 5 (Fig. 4). However, LCFU in the treated group never reached that in the sham-treated group in 12 days' observation.

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Recovery of CFU-S

untreated group

ACM

ACM injected group

Fig. 5. Changes in CFU-S after the administration of ACM at a dose of 5 mg/kg. CFU-S was suppressed up to 0.23 in bone marrow and 0.023 in spleen, but recovered within 5 days. Mean ± SD of 3 mice are shown.

The most troublesome dilemma in the treatment of acute leukemia lies in the fact that the antileukemic agents presently used have cytotoxic effects not only on leukemic cells but also on normal hematopoietic stem cells. Thus, the present study was designed to deter-

mg/kg of ACM was repeatedly injected on days 4, 7, 10, 13, and 16, the survival of mice was 33.8 ± 2.8 days and %ILS obtained 113.9%.

DISCUSSION

As indicated in Fig. 1, clinical signs of leukemia appeared after day 4 following the transplantation of leukemic cells. Namely, anemia progressed, leukemic cells appeared in peripheral blood, and the weights of spleen and liver increased, implying massive infiltration of proliferating leukemic cells into these hematopoietic organs. Taking account of the timing in the treatment of human acute leukemia, ACM was administered to mice at this stage of leukemia. According to the regrowth curves shown in Fig. 2, a single administration of ACM at a dose of 5 mg/kg resulted in about a 4-day (4th day injection) or 5-day (6th day injection) delay in the increase of leukemic spleen weights. These findings prove the effectiveness of ACM as an antileukemic agent.
mine the differences in the sensitivities to ACM between LCFU and normal hematopoietic stem cells and to establish the most appropriate regimen for the treatment of acute leukemia.

As shown in Fig. 3, LCFU was more sensitive to ACM at various doses than CFU-S and CFU-C, indicating the advantage of this agent in the treatment of leukemia. CFU-S and CFU-C in spleen were more sensitive than those in bone marrow. This result may be explained as follows. First, in terms of cell kinetics, splenic CFU-S has been described to proliferate more actively than bone marrow CFU-S. Because proliferating cultured cells were more susceptible to ACM than resting cultured cells (unpublished data), this result was considered to be quite reasonable. Next, pharmacokinetic studies revealed that ACM gathered in spleen more than in bone marrow. Owing to these reasons, splenic CFU-S was considered to be more sensitive to ACM than bone marrow CFU-S.

Changes in LCFU, CFU-S, and CFU-C following the administration of ACM were shown in Figs. 4, 5, and 6. The periods to reach nadirs were 1 day for CFU-C, 1 or 2 days for CFU-S, and 3 days for LCFU, respectively. Then, surviving fractions of these three units increased at a rapid rate from nadirs, and the doubling times were 7.8 hr for LCFU, 12 hr for CFU-C, and 15 hr for CFU-S. The generation times of CFU-C and CFU-S in mice have been reported to be 12–15 hr and 18 hr, respectively, and the rapid increase of surviving fractions of both units should be explained by "repopulation" mechanism. The generation time of LCFU has not been determined yet. However, that of leukemic cells in Rauscher murine leukemia virus induced erythroleukemia in BALB/c mice has been reported to be 7.9 hr. Therefore, the increase of surviving fractions of LCFU at a rapid rate should also be explained by "repopulation." Based on the differences in the sensitivities to ACM and recovery patterns between LCFU and normal hematopoietic stem cells, the effects of ACM on the survival of leukemic mice were investigated. In the group given a single injection of 5 mg/kg of ACM 4 days after the inoculation of leukemic cells, mice died from the advance of leukemia, and %ILS was only 31.6%. In contrast, in the group given a single injection of 25 mg/kg of ACM, most mice died from toxicity rather than from the advance of leukemia, although LD50 of ACM in B6C3F1 mice was 41.5 mg/kg. If 5 mg/kg of ACM, the dose which was not as effective against leukemia as a single dose but not as toxic against normal hematopoietic cells, was administered every 3 days (total dose of 25 mg/kg), the %ILS obtained was 113.9%. Although CFU-S and CFU-C did not recover completely 3 days after the injection of ACM at a dose of 5 mg/kg (Figs. 5 and 6), LCFU showed the nadir at that time (Fig. 4). The high %ILS obtained in the last group should be derived from these differences.

Further research, including a comparison with clinically useful and well studied anthracyclines such as ADM and DNR, should provide meaningful data for the establishment of the ideal regimen in the treatment of human acute leukemia.

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