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Effects on Leukemic Clonogenic Cells in Murine Myeloid Leukemia of 1-β-D-Arabinofuranosylcytosine and the Anthracyclines Adriamycin, Daunomycin, Aclacinomycin A, and 4′-Epidoxorubicin[1]

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ABSTRACT

M-3 murine myeloid leukemic cells undergo terminal divisions making colonies in methylcellulose culture and also renew themselves in methylcellulose and suspension; leukemic clonogenic cells are characteristic as stem cells. The effects of 1-β-D-arabinofuranosylcytosine and four anthracyclines (Adriamycin, daunomycin, aclacinomycin A, and 4′-epidoxorubicin) on M-3 leukemic clonogenic cells were studied. 1-β-D-Arabinofuranosylcytosine was effective in reducing primary and secondary colonies in methylcellulose and the growth of clonogenic cells in suspension. In contrast, the anthracyclines were not so effective in reducing secondary colonies in methylcellulose or clonogenic cells in suspension as to suppress primary colonies in methylcellulose. The results suggest that 1-β-D-arabinofuranosylcytosine but not the anthracyclines is effective for not only terminal divisions but also self-renewal of leukemic clonogenic cells. The study will be used as a practical screening test to examine the effects of antitumor agents on leukemic blast progenitors.

INTRODUCTION

A higher remission rate has been recently achieved in the treatment of AML[3] (1–3). The duration of the remission, however, still is not long enough to cure AML patients and many patients die of the relapse into leukemia. The clinical observation suggests that the present chemotherapy is effective in reducing leukemic cells but does not eradicate them.

Leukemic cells in AML patients are maintained by leukemic blast progenitors; they may renew themselves or undergo a determination-like process yielding cells capable of only limited terminal divisions (4). Terminal divisions can be assayed by colony formation in semisolid or viscid medium (5). Two approaches are now available to detect self-renewal: replating in methylcellulose culture (6); and suspension culture (7). Self-renewal capacity of leukemic blast progenitors is heterogeneous among the patients; it is highly correlated with the clinical outcome of the patients (8). Since self-renewal ability has been considered to be the biological nature of blast progenitors, the goal of AML treatment may be to eliminate self-renewal of blast progenitors and eradicate leukemic cells. From this point of view, it must be important to study the effects of antitumor agents on not only terminal divisions but also self-renewal of blast progenitors.

Based on the above concept, the effects of some antitumor drugs on blast progenitors from AML patients have been studied (9–11). The studies are of importance to find the correlations between *in vitro* sensitivity to drugs and clinical outcome. To screen the effective drugs on a large scale, the present study was directed to develop a more practical system which would contribute to an advance of the chemotherapy. As a model of AML, the murine myeloid leukemia line, M-3,[4] was chosen. We studied the biological nature of M-3 cells first. M-3 cells made colonies in methylcellulose culture and made secondary colonies by replating. Further, clonogenic cells showed exponential growth in suspension culture. Next, the effects of ara-C and the anthracyclines (ADM, DM, ACM, and 4′-epiDX) on clonogenic cells of M-3 cells in methylcellulose and suspension cultures were studied.

MATERIALS AND METHODS

Leukemic Cells. M-3 cell line, a gift from Dr. M. Bessho, has been established from leukemic cells in myeloid leukemia which arose in an RFM mouse 10 months after whole body X-irradiation of 3 Gy. Leukemic cells are like myeloblasts by Wright-Giemsa stain, positive for peroxidase and naphthol ASD-chloroacetate esterase staining, and negative for periodic acid-Schiff and naphthyl butyrate esterase staining. They have a marker chromosome, 2q−, which is specific for murine myelocytic leukemia (12). Leukemic cells have been maintained in α-MEM (GIBCO, Grand Island, NY) supplemented with 10% FCS (GIBCO) or frozen at −120°C until use.

Clonogenic Assay in Methylcellulose Culture. Leukemic cells (5×10^3) were plated in a Lux Petri dish (Miles Laboratories, Naperville, IL) in 1 ml of α-MEM with 0.8% methylcellulose (4000 cps; Wako Co., Osaka, Japan), 10% LCCM, (13) and 20% FCS. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After 7 days of incubation, colonies consisting of more than 50 cells were counted under an inverted microscope. The plating efficiency was shown as PE1.

Primary colonies were pooled, washed in α-MEM with 10% FCS twice, and then plated at a concentration of 5×10^3/ml in a Linbro microwell (Flow Laboratories, McLean, VA) in 0.1 ml of α-MEM with 0.8% methylcellulose, 10% LCCM, and 20% FCS. Secondary colonies of more than 50 cells were scored after 7 days. The secondary colony plating efficiency was shown as PE2.

Suspension Culture. Leukemic cells were cultured in suspension by the method of Nara and McCulloch (7) with a modification. Briefly, 5×10^3 cells were incubated in a Lux Petri dish in 3 ml of α-MEM with 10% LCCM and 20% FCS. In the experiment, low concentrations of cells were cultured and LCCM was used as a growth stimulating factor in contrast with the culture of leukemic cells from AML patients. At intervals, cells were harvested and counted and either served for clonogenic assay in methylcellulose culture or were recultured in suspension. The recovery of clonogenic cells were obtained by multiplying the plating efficiency in methylcellulose by the number of cells harvested from the suspension.

Assay of Normal Myeloid Progenitors. Normal myeloid progenitors (N-CFU) from RFM mice were assayed as described previously (14). Briefly, 5×10^4 marrow cells were cultured in 1 ml of α-MEM with

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[3] The abbreviations used are: AML, acute myeloblastic leukemia; ara-C, 1-β-D-arabinofuranosylcytosine; ADM, Adriamycin; DM, daunomycin; ACM, aclacinomycin A; 4′-epiDX, 4′-epidoxorubicin; α-MEM, α-minimal essential medium; FCS, fetal calf serum; LCCM, L-cell conditioned medium; PE, plating efficiency; N-CFU, normal colony forming units; D_{10}, dose required to reduce survival to 10% of control; SI, sensitivity indices.

[4] M. Maruyama, M. Bessho, and K. Hirashima. Establishment and characterization of a murine myeloid leukemia cell line, manuscript in preparation.

0.8% methylcellulose, 10% LCCM, and 20% horse serum (GIBCO). The colonies containing more than 50 cells were scored after 7 days.

Drug Sensitivity Tests. Drug sensitivity tests were done in both methylcellulose and suspension cultures. ara-C, a cell cycle specific drug, was added continuously in each culture at increasing concentrations. Although an inactivation of ara-C in cultures must be considered, continuous exposure was chosen to compare the data with those in human materials (9, 11). In methylcellulose, PE1 were determined after 7 days of culture. Then colonies were washed three times in α-MEM with 10% FCS and PE2 was assayed. In suspension, cells were harvested after 7 days of exposure to ara-C, counted, and washed three times and served for clonogenic assay. Anthracyclines, cell cycle nonspecific drugs, were added in cell suspensions in α-MEM with 20% FCS for 10 min. After three washings, cells were served for clonogenic assay and suspension culture at the same time. A 10-min exposure to the anthracyclines was considered enough to examine their effects of leukemic cells (9, 11).

Statistics. The data were shown as the mean ± SD of triplicate cultures. Each experiment was repeated at least three times. The dose-response curve for antitumor drugs was determined by the linear regression. D_{10} was obtained from the slope of the negative exponential survival curve.

RESULTS

Growth Patterns of M-3 Cells. When plated in methylcellulose, a statistically significant linear correlation was noted between the number of M-3 cells plated and colonies per dish [$P < 0.01$ (Fig. 1)]. PE1 was $676 \pm 71/5 \times 10^3$ cells and PE2 was $60.3 \pm 8.6/5 \times 10^3$ cells.

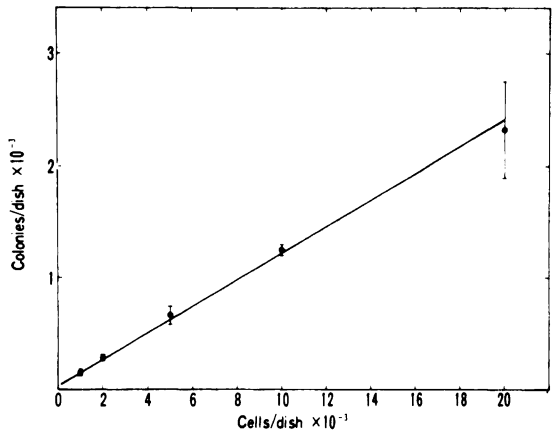

Fig. 1. Relationship between the number of M-3 leukemic cells plated and colonies formed 7 days after incubation in methylcellulose culture. A highly statistically significant linear correlation is observed ($r = 0.995$; $P < 0.01$). In this and succeeding figures, data are shown as the mean of triplicate cultures; *bars*, SD.

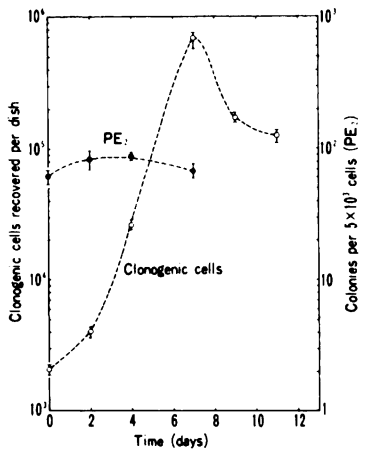

Fig. 2. Kinetic patterns of clonogenic cells recovered per dish after the suspension culture from day 0 to 11 days. Clonogenic cells showed exponential growth as long as 7 days and declined thereafter. PE2 from primary colonies after suspension culture was maintained meanwhile.

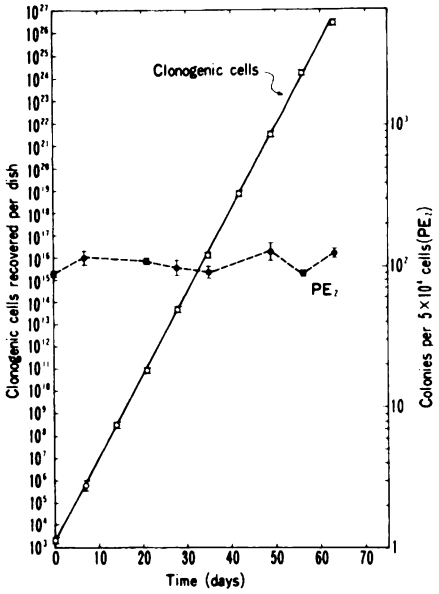

Fig. 3. Cumulative growth curve of clonogenic cells when M-3 cells were transferred every 7th day in suspension. The growth curve shows a significant exponential form ($r = 0.999$; $P < 0.01$). The doubling time was approximately 19 h. PE2 was maintained for 63 days.

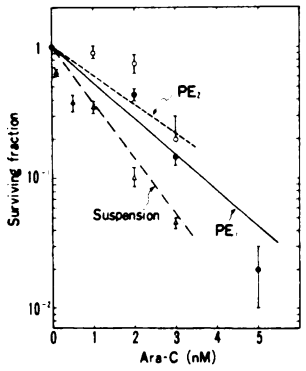

Fig. 4. ara-C survival curves for leukemic clonogenic cells in methylcellulose or suspension culture. Survival curves are a simple negative exponential ($P < 0.05$). Leukemic clonogenic cells are more sensitive to ara-C in suspension than in methylcellulose.

M-3 cells were cultured in suspension for 2 weeks (Fig. 2). Clonogenic cells had grown exponentially until day 7 and started to degenerate thereafter. PE2 was maintained for 7 days. PE2 was not determined after 7 days because of the degeneration of primary colony forming cells. M-3 cells were subcultured every 7th day, when clonogenic cells showed maximal growth and PE2 was maintained. At every subculture, clonogenic assay was carried out. Fig. 3 shows the exponential growth of clonogenic cells with a doubling time of approximately 19 h as long as 63 days; clonogenic cells continued to grow past 63 days. PE2 was maintained meanwhile.

Figs. 1–3 support that the M-3 leukemic cell population is maintained by leukemic clonogenic cells with high self-renewal capacity. Self-renewal was detected by secondary colony formation in a methylcellulose and suspension culture system. Since M-3 leukemic clonogenic cells are characteristic as stem cells, M-3 cells are considered to be suitable for studying the effects of antitumor drugs on malignant cell growth. LCCM enhanced the growth of M-3 cells in methylcellulose and sus-

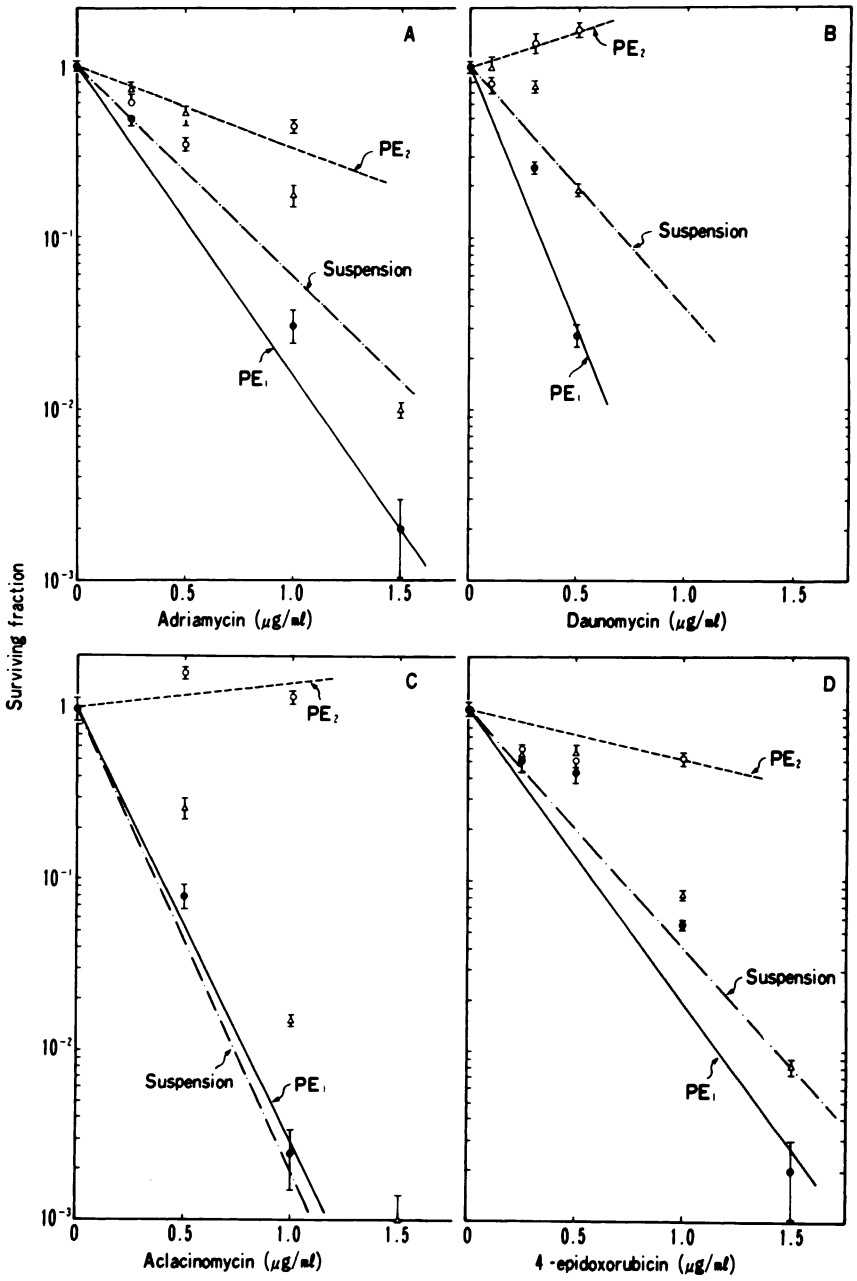

Fig. 5. Anthracycline survival curves. Leukemic cells exposed to ADM (*A*), DM (*B*), ACM (*C*), or 4′-epiDX (*D*) for 10 min were cultured in methylcellulose and suspension. The dose-survival curves for PE1 and clonogenic cells in suspension are simple negative exponentials ($P < 0.05$) but those for PE2 were not significantly linear ($P > 0.05$).

pension cultures, although they grew even without LCCM (data not shown).

Effects of ara-C and Anthracyclines on M-3 Cells. Fig. 4 shows the dose-survival curve for ara-C of PE1 and PE2 in methylcellulose and clonogenic cells in suspension culture. The curves were a simple negative exponential ($P < 0.05$). D_{10} values were 3.623 nM for PE1, 4.592 nM for PE2, and 2.381 nM for clonogenic cells in suspension.

Fig. 5 shows the effects of ADM, DM, ACM and 4′-epiDX. PE1 and clonogenic cells in suspension were suppressed by the drugs in a dose responsive manner ($P < 0.05$). D_{10} values of PE1 were 0.546 μg/ml for ADM, 0.321 μg/ml for DM, 0.384 μg/ml for ACM, and 0.366 μg/ml for 4′-epiDX. D_{10} values of clonogenic cells in suspension were 0.777 μg/ml for ADM, 0.717 μg/ml for DM, 0.351 μg/ml for ACM, and 0.723 μg/ml for 4′-epiDX. The dose-survival curves of PE2 were not significantly linear ($P > 0.05$); PE2 seemed to be suppressed slightly by ADM and 4′-epiDX but not suppressed by DM or ACM.

Effects of ara-C and Anthracyclines on N-CFU. To examine the hematotoxicity of ara-C and the anthracyclines, the effects of the drugs on N-CFU were studied. The dose-survival curves showed a simple negative exponential (Fig. 6). D_{10} values were 6.517 nM for ara-C, 3.307 μg/ml for ADM, 0.907 μg/ml for DM, 0.967 μg/ml for ACM, and 3.307 μg/ml for 4′-epiDX.

Table 1 summarizes D_{10} values for antitumor drugs and SI. SI were obtained by dividing D_{10} for N-CFU by D_{10} for leukemic clonogenic cells. The drug with higher SI may be used for the treatment of leukemia with less hematotoxicity. Among four anthracyclines, SI of ADM and 4′-epiDX were higher than those of DM and ACM.

Table 1 *D_{10} values for leukemic and normal myeloid clonogenic cells and SI of ara-C and the anthracyclines*

Drugs	D_{10} for L-CFU[a]	D_{10} for N-CFU	SI
ara-C	3.623 nM	6.617 nM	1.80
Anthracyclines			
ADM	0.546 μg/ml	3.307 μg/ml	6.51
DM	0.321 μg/ml	0.907 μg/ml	2.83
ACM	0.384 μg/ml	0.969 μg/ml	2.52
4′-epiDX	0.566 μg/ml	3.067 μg/ml	5.42

[a] L-CFU, leukemic colony forming units.

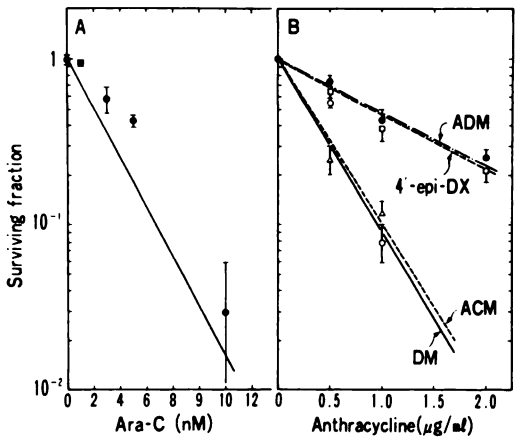

Fig. 6. Effect of ara-C (*A*) or anthracyclines (*B*) on normal myeloid progenitors (N-CFU) from RFM mouse. N-CFU were more sensitive to DM and ACM than to ADM or 4'-epiDX in a comparable dose.

DISCUSSION

We chose ara-C and anthracyclines for the study, since they have been studied well and were often used in the treatment of AML with good results (1–3). Clonogenic cells were more sensitive to ara-C in suspension than in methylcellulose culture, while they were not more sensitive to the anthracyclines in suspension than in methylcellulose culture (Figs. 4 and 5). The recovery of clonogenic cells after suspension culture is considered to reflect the self-renewal of leukemic blast progenitors (7). ara-C, therefore, is effective for not only terminal divisions but also self-renewal of leukemic clonogenic cells, whereas the anthracyclines are not effective for self-renewal. These data are comparable with those in human materials (11).

There are some differences between the biological nature of murine and human leukemic cells. The doubling time of M-3 leukemic clonogenic cells, 19 h calculated from Fig. 3, is much shorter than that of human leukemia which is 4–10 days (7). Further PE1 and PE2 of murine leukemic clonogenic cells are much higher than those of human leukemic blast progenitors (5, 6). Because of the differences, M-3 cells may not be a good model for human AML. However, the characteristics of clonogenic cells as "stem cells" can be detected easily in M-3 cells (Figs. 1–3). The fact that similar results were obtained between M-3 cells and human AML supports the usefulness of the present study as a screening test to determine the effects of antitumor drugs on leukemic blast progenitors.

Leukemic blast progenitors renew themselves in both methylcellulose and suspension cultures. PE2 and clonogenic cell recovery in suspension reflect the self-renewal capacity of progenitors (6, 7). A difference was noted between the dose-survival curves obtained in PE2 assays and those in suspension cultures of M-3 cells. Cell-to-cell interaction is important for the growth of progenitors (7, 15), and the toxicity of methylcellulose remains to be determined. The suspension culture system seems superior to the methylcellulose culture for the detection of the self-renewal of blast progenitors, although two cultures are complementary. Therefore, the effects of antitumor drugs on the self-renewal of clonogenic cells may be determined more clearly in suspension.

Four anthracyclines were studied. ACM and 4'-epiDX are newly developed anthracyclines and they are expected to be useful because of less cardiotoxicity, the major adverse effect of the anthracyclines (16, 17). The experiments on the anthracyclines were done on a weight basis, and SI were determined to compare the effectiveness (Fig. 6; Table 1). However, the differences of cellular drug uptake kinetics or intracellular drug behavior remain to be determined among the drugs. Further study will be necessary to discuss the superiority of the drugs.

Finally, the results show that there are differences of the effects on leukemic clonogenic cells among antitumor drugs. Studying many antitumor agents in a screening system reported in the present article will provide meaningful data for the establishment of the ideal regimen in the treatment of AML.

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