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Potentiation of Growth-inhibitory Activity of 9-β-D-Arabinofuranosyladenine by 2′-Deoxycoformycin in Human Cultured Cell Lines Derived from Leukemias and Lymphomas

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Growth-inhibitory activity of 2′-deoxycoformycin (DCF) and 9-β-D-arabinofuranosyladenine (Ara-A) used either singly or in combination was assessed in 30 human cultured cell lines (seven T-cell, nine B-cell, five non-T,non-B, and nine myeloid cell lines) derived from leukemias and lymphomas. DCF had little activity even at 100 μM on any of the cell lines, while Ara-A had an obvious inhibitory effect on them, especially on non-T,non-B cell lines at 10 μM or less. Lymphoid cell lines were apparently more sensitive to the combined use of Ara-A and DCF than myeloid cell lines. DCF potentiated the antiproliferative activity of Ara-A not only in T-cell lines with high adenosine deaminase (ADA) activity, but also in some other cell lines with low ADA activity. DCF was stable in the culture medium, but Ara-A in the medium containing cultured cells was rapidly inactivated. DCF completely inhibited the inactivation of Ara-A in the medium containing P12/ICH or NALM-6, but not in the medium containing Daudi. This suggests that there is some unknown mechanism(s) of inactivation of Ara-A other than ADA in Daudi, which was insensitive to Ara-A in the presence of 1 μM DCF. The capacity of DCF to inhibit degradation of Ara-A in the medium containing these cultured cells correlated with the level of Ara-A sensitivity potentiated by DCF. In all seven T-cell lines, seven of the nine B-cell lines, all five non-T,non-B cell lines, and only three of nine myeloid cell lines, the IC50 value for Ara-A decreased to 5 μM or less in the presence of 1 μM DCF. These results suggest that the combination of DCF and Ara-A may be effective against various types of lymphoid malignancies and some myeloid leukemias.

Key words: Deoxycoformycin — Arabinofuranosyladenine — Leukemia — Lymphoma — Drug sensitivity

2′-Deoxycoformycin (DCF), a nucleoside analog produced by *Streptomyces antibodicus*[1] or *Aspergillus nidulans* YK176-2,[2] is a potent tight-binding inhibitor of adenosine deaminase (ADA) (adenosine aminohydrolase; EC 3.5.4.4). Although DCF has not been proved to have antineoplastic activity in the murine tumor system,[3] it has been brought into clinical use in various lymphoid malignancies because of the association of a hereditary deficiency of ADA with lymphopenia and severe combined immunodeficiency disease, which led to the idea of pharmacologic inhibition of ADA by DCF as potential cytotoxic therapy of malignant lymphoproliferative diseases. The preliminary results have been encouraging in hairy cell leukemia,[4–6] adult T-cell leukemia/lymphoma (ATL),[7,8] acute lymphoblastic leukemia (ALL),[9–11] chronic lymphocytic leukemia (CLL),[12,13] mycosis fungoides,[14] and other lymphoid malignancies.[12,15] In Japan, the efficacy of DCF against ATL, a unique leukemia/lymphoma particularly resistant to present standard chemotherapy,[16–18] is anticipated.

The mechanism of the antineoplastic activity of DCF *in vivo* or *in vitro* has not been fully elucidated. However, it is generally accepted to be mainly mediated through the accumulation of adenosine analogs such as deoxyadenosine, adenosine or 9-β-D-arabinofuranosyladenine (Ara-A) after inhibition of ADA.[3,10,11,13] Ara-A is a functional analog of deoxyadenosine with established antiviral activity.[19,20] But the antineoplastic activity of Ara-A against human hematopoietic malignancies had been limited by rapid deamination to biologically inactive 9-β-D-arabinofuranosylhypoxanthine by ADA.[21,22] Potent inhibitors of ADA such as DCF have become available, and this has made it possible to evaluate the antitumor potential of Ara-A. Pilot clinical trials with the combination of Ara-A with DCF have yielded promising results.[23,24]

There have been few studies on the combined effect of DCF and Ara-A *in vitro* on cultured cell lines derived from human leukemias and lymphomas. We have con-

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ducted experiments *in vitro* to evaluate the combined effect of DCF and Ara-A on cell lines derived from human leukemias and lymphomas, and the results are presented here.

MATERIALS AND METHODS

Drugs DCF (YK-176: Lot No. AF-01) was obtained from The Chemo-Sero-Therapeutic Research Institute (Kumamoto). Ara-A was obtained from Mochida Pharmaceutical Co., Ltd. (Tokyo). They were dissolved in culture medium at an appropriate concentration just before use.

Cell lines Thirty human cultured cell lines derived from leukemias and lymphomas were used. The characteristics of these cell lines are shown in Table I.

The cells were cultured in a growth medium, RPMI-1640 (Boehringer Mannheim Biochem.) supplemented with 10% fetal calf serum (Hyclone Laboratories, a Division of Sterile Systems, Inc., Utah), 100 μg/ml aminobenzylpenicillin and 33 μg/ml gentamicin, in the floating state at 37°C under a humidified atmosphere in a 5% CO_2 incubator (Forma Scientific, MIP-3033). Exponentially proliferating cells were used for the experiments, which were carried out at least in duplicate.

Measurement of antiproliferative activity of DCF, Ara-A and DCF plus Ara-A The cells were suspended in fresh growth medium at an appropriate concentration (5×10^4/ml to 2×10^5/ml) according to the growth rate and cell density required for exponential growth in each cell line throughout the experiment. DCF and/or Ara-A diluted with the growth medium at various concentrations were added to a cell well (25820-24, Corning Laboratory Sci. Co.) containing the cells. The cells were cultured in the growth medium with or without the drug for 72 h in the CO_2 incubator, then the cell number was determined with a Coulter Multisizer (Coulter Electronics, Inc.). The growth inhibition rate of treated cells was expressed as a percentage of the growth of untreated cells as follows;

$$\frac{\text{experimental cell density} - \text{initial cell density}}{\text{control cell density} - \text{initial cell density}} \times 100\%.$$

IC50 value was calculated as the drug concentration which was required for 50% reduction of growth of treated cells as compared to that of control cells.

Measurement of ADA activity A known number of cultured cells ($1\times10^7/\mu$l) were washed in phosphate-buffered saline (PBS) and lysed by freezing (−80°C) and thawing (37°C) three times in PBS. After centrifugation at 2,000*g* for 10 min, the supernatant fluid was used as a crude cell extract for ADA activity. ADA activity was assayed by a modification of the method of Kalckar.[25] Briefly, ADA activity was measured in terms of the conversion of adenosine to inosine in the presence of the cell extract; the decrease in adenosine was followed spectrophotometrically in cuvettes maintained at 25°C by measurement of the decreased absorbancy at 265 nm. The assay was started at 25°C by the addition of 0.1 ml of cell extract to the reaction mixture containing 3.0 ml of 0.05 *M* phosphate buffer, pH 7.5, and 0.1 ml of 0.36 mg/dl adenosine (Sigma grade, Sigma). The enzymatic reaction was linear for at least several minutes. Therefore, the enzymatic activity was calculated from the maximum velocity of the reaction, and expressed as a unit which corresponds to micromoles of substrate converted per minute per mg of cell protein. Cell protein was determined by the method described by Lowry *et al.*[26]

Determination of DCF and Ara-A in the medium The stability of DCF and Ara-A in the medium with cultured cells was measured as a function of culture time. Three cell lines, P12/ICH (T-cell line), NALM-6 (non-T, non-B cell line) and Daudi (B-cell line), were used. To examine the stability of DCF, the culture medium containing 10 μM DCF with or without the cultured cells was incubated for 72 h at 37°C in the CO_2 incubator. The medium was harvested and the supernatant was obtained for determination of DCF after centrifugation at 1,500 rpm for 10 min. DCF was determined by high-performance liquid chromatography (HPLC) with an A-312 (ODS) column using 4% acetonitrile in 20 m*M* Tris-HCl buffer, pH 7.5, as a solvent. Other conditions were as follows: scale 0.01, 283 nm detection, 1 ml/min flow rate, and the sample was diluted with water.

To examine the stability of Ara-A, the culture medium containing 10 μM Ara-A and cultured cells in the presence or absence of DCF was incubated for 72 h at 37°C in the CO_2 incubator. The medium was harvested at a given time, and spun down at 1,500 rpm for 10 min to obtain the supernatant, which was then treated with the same volume of 25% trichloracetic acid, and the precipitate was removed by centrifugation at 10,000 rpm for 5 min. The acid-soluble supernatant was treated twice with the same volume of ether to obtain the aqueous fraction for determination of Ara-A content. Ara-A was determined by HPLC with a Nucleosil 10SA column using 0.1 *M* monopotassium phosphate solution as a solvent, 254 nm detection and a 1 ml/min flow rate.

RESULTS

Growth-inhibitory effect of drugs The growth-inhibitory effect of DCF was very weak, with IC50 values of more than 100 μM in all cell lines (Fig. 1). This indicates that the clinically achievable concentration of DCF, about 1 μM or less, has no significant growth-inhibitory effect on cultured leukemia and lymphoma cells. On the other hand, Ara-A had significant growth-inhibitory effects on

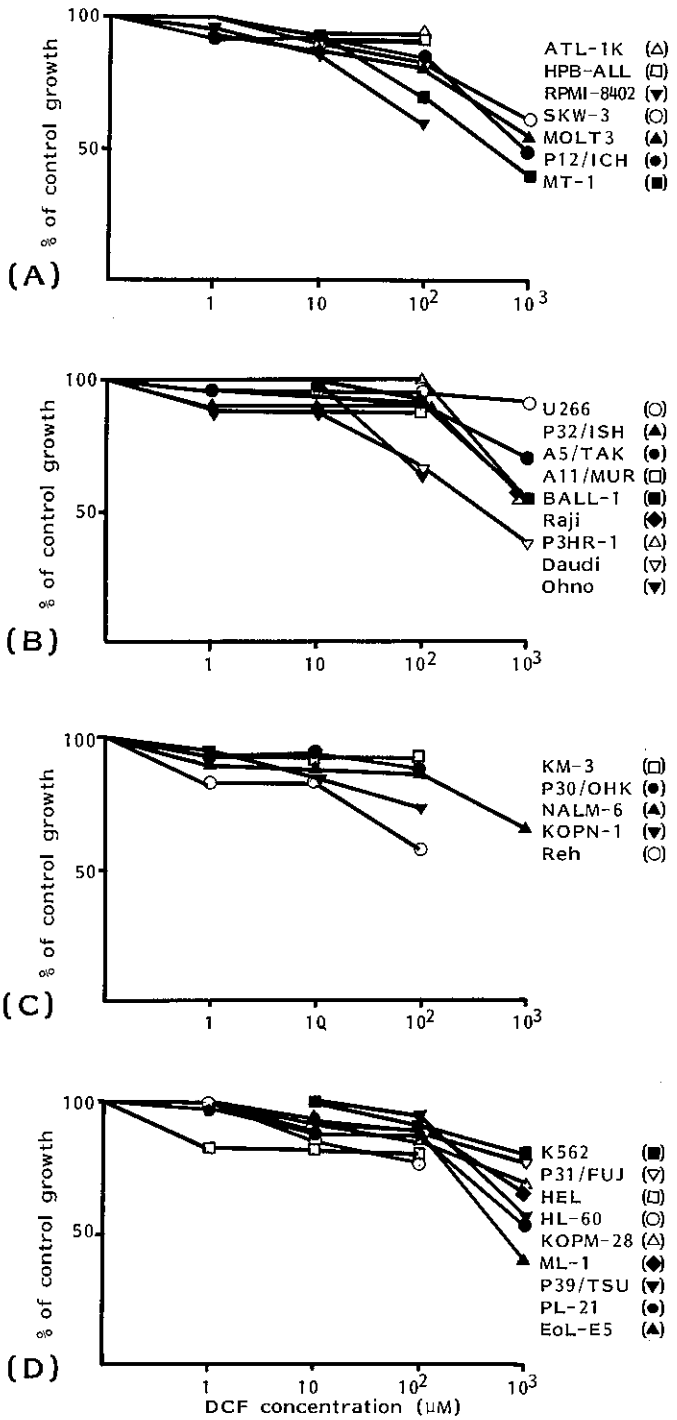

Fig. 1. Growth-inhibitory effect of DCF. Dose-response curves of DCF against various T-cell lines are shown in (A), those against B-cell lines in (B), those against non-T,non-B cell lines in (C) and those against myeloid cell lines in (D).

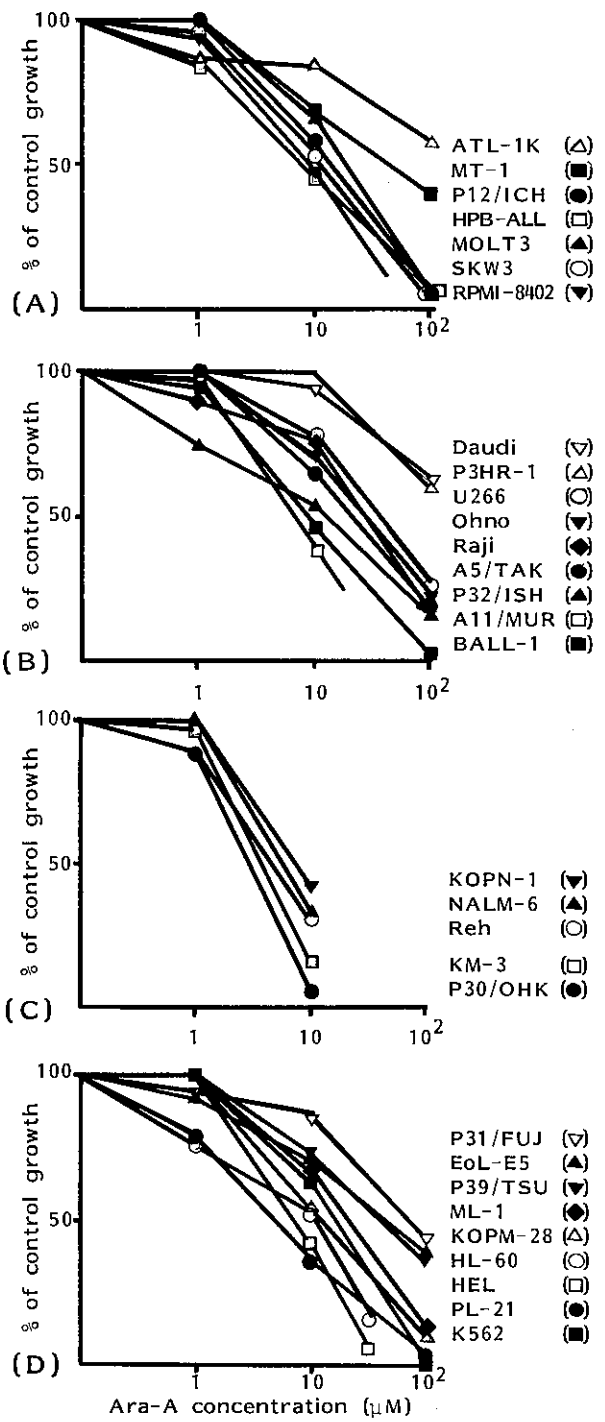

Fig. 2. Growth-inhibitory effect of Ara-A. Dose-response curves of Ara-A against various T-cell lines are shown in (A), those against B-cell lines in (B), those against non-T,non-B cell lines in (C) and those against myeloid cell lines in (D).

Table I. Increase in Ara-A Sensitivity in the Presence of DCF

	Origin	Doubling time (h)	IC50 (μM) for Ara-A		Degree of DCF potentiation[a]
			no DCF	1 μM DCF	
T-cell lines					
HPB-ALL	ALL	35	8.5	0.37	23
P12/ICH	ALL	27	15	3.2	4.7
MOLT3	ALL	23	18	3.0	6.0
RPMI-8402	ALL	25	7.8	3.5	2.2
SKW3	CLL	27	12	3.5	3.4
ATL-1K	ATL	43	110	2.0	55
MT-1	ATL	27	42	3.5	12
B-cell lines					
A5/TAK	B-L	23	21	1.9	11
Ohno	B-L	31	27	3.7	7.3
A11/MUR	B-L	27	64	5.0	13
BALL-1	B-L	20	8.4	2.7	3.1
U266	MM	35	35	3.8	9.2
P3HR-1	Burkitt	22	180	4.5	40
P32/ISH	Burkitt	25	12	1.4	8.6
Raji	Burkitt	20	26	11	2.4
Daudi	Burkitt	30	200	45	4.4
Non-T,non-B cell lines					
P30/OHK	ALL	30	2.9	0.68	4.3
NALM-6	ALL	18	5.6	2.0	2.8
KM-3	ALL	39	3.9	0.34	11
Reh	ALL	28	4.7	0.58	81
KOPN-1	ALL	27	7.2	3.4	2.1
Myeloid cell lines					
PL-21	APL	26	4.8	0.4	12
HL-60	APL	21	11	1.4	28
K562	CMLbc	24	17	4.7	3.6
KOPM-28	CMLbc	22	11	6.6	1.7
EoL-E5	EoL	23	44	24	1.6
P31/FUJ	AMoL	47	75	13	5.8
HEL	ErL	27	7.4	6.8	1.1
P39/TSU	RAEBT/AML	30	45	23	1.9
ML-1	AML	23	20	33	0.6

a) Degree of DCF potentiation was calculated as follows: IC50 of the cells for Ara-A measured in the absence of DCF was divided by that measured in the presence of 1 μM DCF.
ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; ATL, adult T-cell leukemia-lymphoma; B-L, B-lymphoma; MM, multiple myeloma; APL, acute promyelocytic leukemia; CMLbc, chronic myelogenous leukemia in blastic crisis; EoL, eosinophilic leukemia; AMoL, acute monocytic leukemia; ErL, erythroleukemia; RAEBT, refractory anemia with excess of blasts in transformation; AML, acute myelocytic leukemia. Original papers for the cell lines described above were listed in previous reports [29-33] except A11/MUR (unpublished), PL-21,[34] KOPM-28,[35] EoL-E5[36] and P31/FUJ.[37]

these cell lines as shown in Fig. 2. Non-T,non-B ALL cell lines were more sensitive to Ara-A ($P<0.01$) than T-, B- and myeloid cell lines. The IC50 was between 2.9 and 7.2 μM in non-T,non-B cell lines, while it was more than 10 μM in most of the other cell lines as shown in Table I.

As shown in Fig. 3, DCF at the concentration of 1 μM or less potentiated the growth-inhibitory activity of 10 μM Ara-A in many cell lines, especially in lymphoid cell lines. The DCF potentiation of Ara-A activity was concentration-dependent in T- and non-T,non-B cell lines, but not in other cell lines. The IC50 value for Ara-A

decreased to less than 4 μM (about 1 μg/ml) in all seven T-cell lines in the presence of 1 μM DCF, as shown in Table I. In non-T,non-B cell lines, 10 μM Ara-A alone had a marked antiproliferative activity. Thus, the combination of DCF at 1 μM or less and 5 μM Ara-A was tested for growth-inhibitory effect. DCF at 1 μM or less also potentiated the growth-inhibitory activity of Ara-A in all five non-T,non-B cell lines (Fig. 3). Three cell lines, Reh, KM-3, and P30/OHK, became extremely sensitive to Ara-A in the presence of DCF with IC50 values of less than 1 μM. The IC50 value for Ara-A also decreased to 4 μM or less in two other cell lines, NALM-6 and KOPN-1, in the presence of DCF (Table I). DCF at low concentrations (10 to 100 nM) also clearly potentiated the growth-inhibitory activity of Ara-A toward seven of the nine B-cell lines and three (HL-60, PL-21 and K562) of the nine myeloid cell lines tested (Fig. 3). The IC50 for Ara-A decreased to 5.0 μM or less in seven of the nine B-cell lines in the presence of DCF (Table I), whereas it did not decrease remarkably in two cell lines derived from Burkitt lymphoma, Daudi and Raji. The IC50 for Ara-A decreased to less than 5.0 μM in three (HL-60, PL-21 and K562) of the nine myeloid cell lines in the presence of DCF (Table I). Two (HL-60 and PL-21) of them were derived from acute pro-

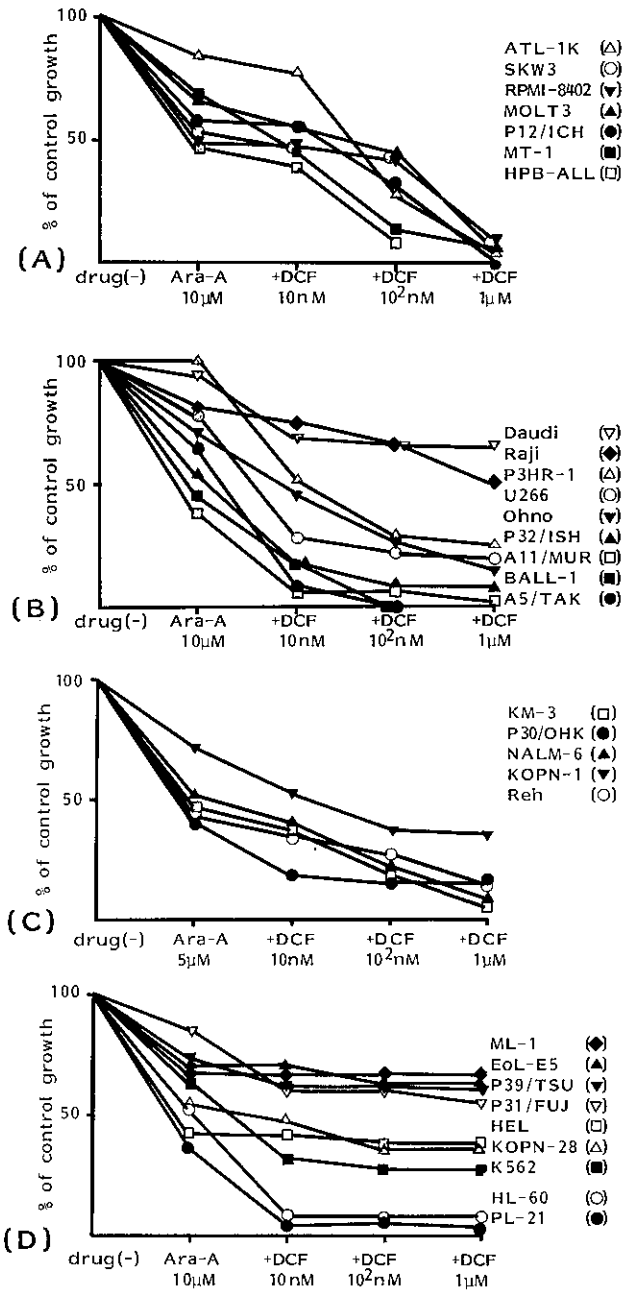

Fig. 3. Potentiation of growth-inhibitory activity of Ara-A by DCF. (A), T-cell lines; (B), B-cell lines; (C), non-T,non-B cell lines; (D), myeloid cell lines. Ara-A was used at 10 μM for experiments (A), (B), and (D), and 5 μM for (C).

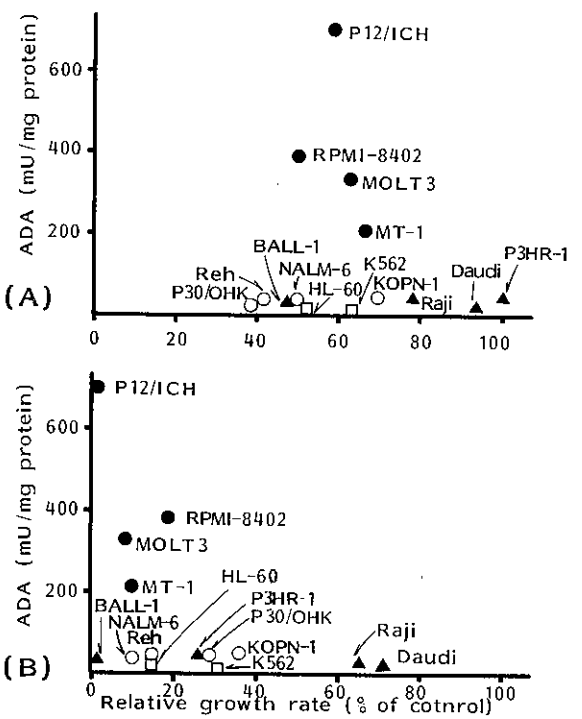

Fig. 4. Relation between growth-inhibitory activity of Ara-A and ADA activity of cells. Growth-inhibitory effect of Ara-A in the absence (A) or presence (B) of 1 μM DCF is shown in relation to ADA activity of the cells. Ara-A was used at 5 μM for non-T,non-B cell lines, and 10 μM for other cell lines. ●, T-cell line; ○, non-T,non-B cell line; ▲, B-cell line; □, myeloid cell line.

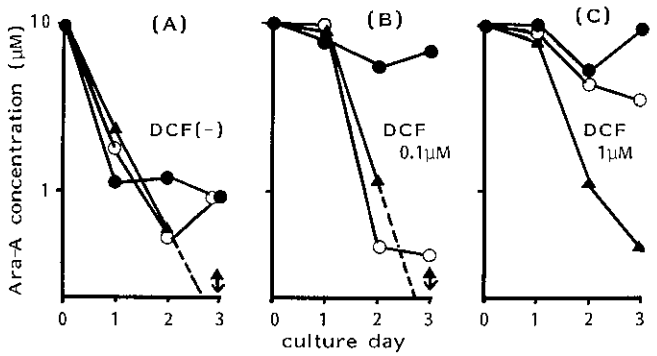

Fig. 5. Stability of Ara-A in culture medium. Stability of 10 μM Ara-A in the medium containing the following cultured cells was examined in the presence or absence of 0.1 and 1.0 μM DCF. ●, P12/ICH (T-cell line); ○, NALM-6 (non-T,non-B cell line); ▲, Daudi (B-cell line).

myelocytic leukemia (APL). The sensitivity of P30/FUJ increased significantly in the presence of DCF, but its IC50 still remained high (13 μM). The sensitivity of five other myeloid cell lines to Ara-A did not increase significantly in the presence of DCF. The degree of DCF potentiation did not correlate with the sensitivity of the cells to Ara-A, as shown in Table I.

Relationship between ADA activity and growth-inhibitory effect of drugs Fourteen cell lines (four T-cell lines, four B-cell lines, four non-T,non-B cell lines and two myeloid cell lines) were examined. As shown in Fig. 4A, the growth-inhibitory effect of 10 μM Ara-A was not associated with the ADA activity of cells. As shown in Fig. 4B, 1 μM DCF enhanced the growth-inhibitory activity of Ara-A in all four T-cell lines with high ADA activity (P12/ICH, RPMI-8402, MOLT-3, and MT-1) and in eight (BALL-1, P3HR-1, NALM-6, Reh, P30/OHK, KOPN-1, HL-60, and K562) of 10 cell lines with lower ADA activity, but not in two other cell lines with low ADA activity (Raji and Daudi). The degree of DCF potentiation of Ara-A activity in the cell lines appeared to be independent of ADA activity.

Stability of DCF and Ara-A in the medium DCF was stable in the medium with or without cultured cells during three culture days (data not shown). On the other hand, Ara-A was unstable when used singly in the medium containing cultured cells. More than 80% of Ara-A was lost during the first culture day (Fig. 5A). In the presence of 0.1 μM DCF, however, it was stable for three culture days in the medium containing P12/ICH (T-cell line) with high ADA activity (Fig. 5B), which increased in sensitivity to Ara-A in the presence of DCF. It was still unstable in the medium containing NALM-6 (non-T,non-B cell line) or Daudi (B-cell line) (Fig. 5B), both of which had low ADA activity. In the presence of 1 μM DCF, however, Ara-A became stable in the medium containing NALM-6 (Fig. 5C), which also increased in sensitivity to Ara-A in the presence of DCF. Ara-A was still unstable in the medium containing Daudi even in the presence of 1 μM DCF (Fig. 5C), and the IC50 of Daudi was high (45 μM) in the presence of DCF (Table I).

DISCUSSION

DCF had no significant growth-inhibitory activity *in vitro* toward any of the 30 cultured leukemia and lymphoma cell lines tested in this study. On the other hand, Ara-A possessed a clear growth-inhibitory activity against these culture cell lines, especially the non-T,non-B cell lines.

The combined effect of DCF and Ara-A was more marked in lymphoid cell lines than in myeloid cell lines. However, two myeloid cell lines, PL-21 and HL-60, that were derived from APL, became more sensitive to Ara-A in the presence of DCF, indicating that combined use of Ara-A and DCF may be effective against APL. Among the lymphoid cell lines, all T-cell lines having high ADA activity showed an apparent increase in sensitivity to Ara-A in the presence of DCF, which appeared to depend on the concentration of DCF. DCF at a concentration more than 0.1 μM can inhibit completely the degradation of Ara-A in the culture medium containing a T-cell line, P12/ICH with high ADA activity. The finding may explain the potentiation of Ara-A activity by DCF in T-cell lines. This also suggests that combination therapy with DCF and Ara-A may be effective against some T-cell leukemia-lymphomas *in vivo*. The majority of B-cell lines and all non-T,non-B cell lines tested were found to increase in sensitivity to Ara-A in the presence of DCF, even though their ADA activity was quite low. Degradation of Ara-A in the medium containing NALM-6 was prevented when DCF was present at 1 μM in the medium. These findings are interesting in view of the reports that DCF and Ara-A in combination have been effective against certain ALL.[22-24] On the other hand, some B-cell lines, especially Daudi and Raji cells, still gave high IC50s for Ara-A (more than 10 μM) even in the presence of 1 μM DCF. The finding that Ara-A was inactivated in the medium containing Daudi cells even in the presence of 1 μM DCF may explain why no significant increase in the sensitivity of the cells to Ara-A occurred in the presence of DCF. These results indicate that the capacity of DCF to inhibit degradation of Ara-A in the medium containing these cultured cells is correlated with the level of Ara-A sensitivity potentiated by DCF in the cells. This also suggests that ADA is usually responsible for the inactivation of Ara-A in the cells in

which the IC50 decreased to 5.0 μM or less in the presence of DCF, and that there is some unknown mechanism(s) of inactivation of Ara-A other than ADA in cells such as Daudi. Therefore, the potentiation of antineoplastic activity of Ara-A by DCF would not be expected in such cells even if a high concentration of DCF were present.

It has been reported that T-cell lines were far more sensitive to deoxyadenosine than B-cell lines *in vitro*.[27,28] In these reports, however, only a few T- and B-cell lines were examined. In this study using 30 leukemia and lymphoma cell lines, apparent differences in the sensitivity to Ara-A between T- and B-cell lymphoma lines could not be found. All seven T-cell lines, seven of the nine B-cell lines, all five non-T,non-B cell lines, and only three (two of them were derived from APL) of nine myeloid cell lines became sensitive to low levels (less than 5 μM) of Ara-A in the presence of 1 μM DCF. These results suggest that the combination of DCF and Ara-A may be effective against various types of lymphoid malignancies and perhaps against acute promyelocytic leukemia.

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