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The Characterization of Highly Resistant Human Leukemic Cell Lines to 1-β-D-Arabinofuranosylcytosine

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Summary. **Two human myelomonocytic leukemia cell lines highly resistant to 1-β-D-arabinofuranosylcytosine (ara-C) were established. One line, designated KY-Ra, has been proliferating with 1×10^{-4} M ara-C for over one year. The other one, KY-Rb, has proliferated with 1×10^{-5} M N^4-behenoyl-1-β-D-arabinofuranosylcytosine (BH-AC) after it reached a proliferative capacity with 1×10^{-6} M ara-C.**

These two area-C-resistant cell lines have shown approximately 5,900 fold and 18,000 fold resistance to ara-C over the parental KY-821 cell line. The deoxycytidine kinase activity of each cell line was 0.39 (dCMP/min/μg protein) for KY-Ra, 0.26 for KY-Rb, 1.23 for KY-821. Deoxycytidine deaminase activities were almost identical. In drug accumulations, arac-C resistant cell lines showed a reduced uptake of ara-C during a 4 h exposure to 1 nM ara-C. After loading almost the same intracellular contents of drugs, ara-CTP accumulation decreased in two ara-C resistant cell lines, and the efflux rate was increased in KY-Ra.

These results indicate that the decreased deoxycytidine kinase activity, which resulted in a decreased ara-CTP accumulation, is one of major mechanisms of the resistance, and suggest the possibility that an increase of the outward flux of ara-C is one mechanism of resistance. These cell lines may be useful for studying the refractoriness encountered in ara-C administration.

INTRODUCTION

1-β-D-arabinofuranosylcytosine (ara-C), one of the most effective anticancer drugs, is widely used alone or in combination with other drugs. Moreover, this agent is used with low, conventional or high-dose administration regimens[1] in the treatment of acute leukemia.[2-5] Ara-C is an S phase-specific agent, whose cytotoxic activity is exhibited by converted form (ara-CTP). Ara-CTP exerts the cytotoxicity to inhibit DNA polymerase or to be incorporated into DNA.

Refractoriness to ara-C, however, is a frequent clinical problem. Although the metabolism of ara-C *in vitro* or *in vivo* has been recently resolved, the mechanism of resistance is incompletely documented. All pathways from influx to DNA incorporation have the possibility of accounting for the refractoriness.

As causes of refractoriness, a decrease in deoxycytidine kinase activity and an increase in cytidine deaminase activity[6] have been frequently documented, not only in clinical settings[7] but also *in vitro* experiments.[8-10] The other mechanisms postulated to account for refractoriness are: decreased ara-C influx,[11] increased ara-C efflux, a decrease in intracellular are-CTP generation and retention,[12] a decrease in ara-C incorporated in DNA,[13,14] removing ara-C from ara-C incorporated DNA,[15] an increase in dCTP pools,[16,17] the insensitivity of DNA polymerase to ara-C.[18] In this study, a leukemic cell line surviving at a 1×10^{-4} M concentration of ara-C was used. This drug concentration is similar to the serum concentration in high-dose ara-C regimen.[19] The mechanism of resistance to ara-C of these cell lines is discussed.

MATERIALS AND METHODS

Cell line

A leukemic cell line (KY-821) (provided by Dr. K Kishi, First Department of Internal Medicine, Niigata University School of Medicine), derived from an acute myelomonocytic leukemia patient, was established in 1982 and stored at −70℃ until use (unpublished data).

Two ara-C resistant cell lines (KY-Ra and KY-Rb)

used in this present study were isolated by gradually increasing extracellular concentrations of ara-C (every 3-4 weeks) from a 1×10^{-9} M concentration. After the concentration of ara-C reached 1×10^{-4} M, the ara-C resistant cell line (KY-Ra) was maintained in alpha-MEM containing the 1×10^{-4} M ara-C, 10% heat-inactivated fetal calf serum (IBL, Gunma, Japan), 1% nonessential amino acid solution (Gibco), 1% sodium pyruvate solution (Gibco), streptomycin sulfate (1 mg/ml) and penicillin G potassium (100 units/ml) for over 1 year. Another ara-C-resistant cell line (KY-Rb) was maintained in the same medium except for 1×10^{-5} M behenyoyl-ara-C, after it showed the capacity to survive in a medium containing 1×10^{-6} M ara-C. These cultures were incubated at 37℃ in a humidified atmosphere of 5% CO_2. Half of the medium was exchanged every one or two days.

Drugs

1-β-D arabinofuranosylcytosine (ara-C) and behenoyl-ara-C (BH-AC) were purchased from Nippon Shinyaku Company (Tokyo, Japan) and Asahikasei Company (Tokyo, Japan), respectively.

[^{3}H]-arabinofuranosyl cytosine (23 Ci/mmol) and [^{3}H]-deoxycytidine (18.8 Ci/mmol) were purchased from Amersham Radiochemical (Tokyo, Japan). Kanamycin and penicillin G were purchased from Meiji Seika (Tokyo, Japan).

Cytotoxic studies

KY-821 and two ara-C resistant cell lines showing logarithmic growth were used in all experiments. In determining the cytotoxicity of ara-C on the cell lines, cells were plated in flat-bottom trays (Falcon) at a concentration of 0.5×10^4 cell/100 μl with various concentration of ara-C. After 72 h, the numbers of cells were determined by the MTT assay.[20,21] Briefly, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (10 μl) was added to each well. After incubation at 37℃ for 3 h, acid-isopropanol (100 μl of 0.04 N HCl) was added to each well and mixed thoroughly. The plates were read on a Model 2550 EIA Reader (Bio-Rad). Data were expressed as percentage of survival of the control cells. The fifty percent inhibitory concentration was determined by liner regression analysis.

Morphological, cytochemical and differentiation induction studies

Cytocentrifuged smears of cells were stained with a May-Giemsa stain for morphological studies, and with peroxidase, chloroacetate esterase, and butyrate esterase for cytochemical studies.

In differentiation studies, these cells were cultured at a concentration of 1.0×10^5 cells/10 ml in the same medium as described above without ara-C, and were induced by phorbor 12-myristate 13-acetate (PMA) (1.4×10^{-8} M), retinoic acid (RA) (1.0×10^{-6} M) (Sigma Chemical Company) and dimethyl sulfoxide (1.3%) (Wako Pure Chemical Industries). After 72 h, cytospin preparations were made and stained with May-Giemsa for analysis. The percentages of mature myeloid cells (myelocytes to neutrophils) or monocytes were determined.

Immunofluorescence studies

These cells were analyzed by flow cytometry (Spectrum III, Ortho Diagnostic Systems, Raritan, NJ) after sequentilal staining with monoclonal antibodies and fluorescein isothiocyanate conjugated goat anti-mouse IgG antibodies (Coulter Immunology, Hialeath, FL). Monoclonal antibodies were: Leu-4 (CD3), Leu-12 (CD19), HLA-DR, (Becton-Dickinson, San Jose, CA), My4 (CD14), My7 (CD13), My9 (CD33), (Coulter Immunology, Hialeath, FL), and OKM1 (CD11), (Ortho).

Deoxycytidine kinase activity

Deoxycytidine kinase activity in the three cell lines was assayed by the Fisher's Method.[22] Cells from each line were homogenized with Tris buffer (20 mM, pH 8.0) and centrifuged at 20,000×g for 30 min. The supernatants of homogenized cells (100 μl) were adjusted to contain 10 μmoles of Tris-Cl, pH 8.0, 1.0 μmoles of $MgCl_2$, 0.5 μmoles of ATP, 10 μmoles of [^{3}H]-deoxycytidine and then incubated at 37℃ for 10 min. The reaction was terminated by heating for 1 min. The mixtures were diluted to distilled water (5 ml) and centrifuged at 1,500×g for 10 min. The supernatants were added on DEAE cellulose disc. The radioactivity of [^{3}H]-dCMP, which was converted from [^{3}H]-deoxycytidine, bound to DEAE cellulose disc, was determined.

Kinase activity is expressed as fmol dCMP/min/μg protein. The contents of the protein were determined according to the method by Bradford.[23]

Deoxycytidine deaminase activity

These cells were homogenized with a buffer (0.05 M Tris, 0.001 M EDTA, 0.002 M dithiothreitol) and

centrifuged at 10,000 × 2, for 10 min. The supernatants (300 μl) were incubated with 10 μm moles of [^{3}H]-deoxycytidine at 37℃ for 30 min. The reaction was terminated by the addition of cold 0.1 N HCl (0.2 ml). The mixture was added to a column (3.5 ml bed volume) of "DOWEX AG 50W-X4" resin pretreated with distilled water. The column was washed with distilled water (4 ml) to elute deoxyuridine but not deoxycytidine. Aquasol 2 (10 ml) was added and the radioactivity was determined by liquid scintillation counter. The deaminase activity is expressed as units described by Steuart et al.[6]

Drug accumulation and efflux studies

Cells of each cell line were incubated in the presence of 1 nM [^{3}H]-ara-C at a concentration of 1×10^6 cells/10 ml. After 4 h, cells were harvested, washed with PBS, and then the radioactivity of the pelleted cells was determined by liquid scintillation counter. In determining the efflux of [^{3}H]-ara-C from the three cell lines, cells (1×10^6 cells/10 ml) were preincubated for 1 h with [^{3}H]-ara-C at various concentrations determined in the preliminary experiments, in which these three cell lines showed almost the same magnitude of drug accumulation. After this incubation, cells were harvested, washed with PBS and then replated in a drug-free medium at a concentration of 1×10^6 cells/10 ml. Then, after 30 min incubation, cells were harvested and radioactivity was determined as described in drug accumulation studies.

Ara-C incorporation into DNA

In determining ara-C incorporated into DNA, the method by Ross et al. was used with some modification.[24] The cells with the same sequential treatment as described in "efflux studies" were harvested, washed with cold PBS, and then incubated in 5 ml hypotonic solution (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$) for 15 min on ice. Then, the pelleted cells were washed with 5 ml hypotonic solution (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.2% Triton X) once. The cells were collected by centrifugation (3,000 rpm for 5 min at 4℃) and resuspended in 5% cold trichloroacetic acid (3 ml) for 10 min followed by collecting on glass fiber filters (934-AH; Whatman Chemical). After these filters were washed with 1% trichloroacetic acid (5 ml), 95% ethanol (20 ml) and dried, their radioactivities were determined.

Initial influx of ara-C

100 μl-aliquots containing 1×10^6 cells were suspended to 10 μl-aliquots of 10 nM ara-C in 0.4 ml microtubes (Bio Plastics Co. Japan). After incubation for 20 sec, 200 μl-aliquots of a mixture of 20% olive oil and 80% Di-N-butyl phthalate (Wako Pure Chemical Co. Osaka, Japan) were added and centrifuged at 10,000 rpm for 1 min (4℃) to separate cells from the medium. Pelleted cells were lysed overnight with tissue solubilor. The radioactivity of the cells was then determined by liquid scintillation counter.

Analysis of Ara-C Metabolites

The procedures as described elsewhere[25] were used with modification. The cells with the same sequential treatment as described at "efflux studies" were harvested and washed with 0.9% NaCl solution. The cell pellets were treated with 50 μl of 60% ice-cold perchloric acid. After 30 min of mixing, the perchloric acid mixtures were centrifuged to separate the acid-soluble and acid-insoluble portions. The supernatants (acid-soluble fraction) were neutralized with 5 N KOH and 5 N KH_2PO_4 solutions, and loaded into a DEAE-Sephadex A-25 column (2.5 ml bed volume) pretreated with ammonium formate (pH 6.5). Then the columns were washed serially with 5 ml of distilled water, 0.1 M, 0.2 M, 0.45 M, and 0.65 M ammonium formate solution to elute ara-C, ara-CDP choline, ara-CMP, ara-CDP, and ara-CTP, respectively. The fractions were mixed with Aquasol 2 (10 ml) and counted by liquid scintillation counter.

Statistics

The significance of the difference was determined by Wilcoxson's Test.

RESULTS

Morphology

These three cell lines showed a morphology identical to myeloblasts, which had round nuclei with a few nucleoli and basophilic cytoplasma (Fig. 1). A small number of spontaneously differentiated cells were seen.

Differentiation studies

These three cell lines induced by the same chemical

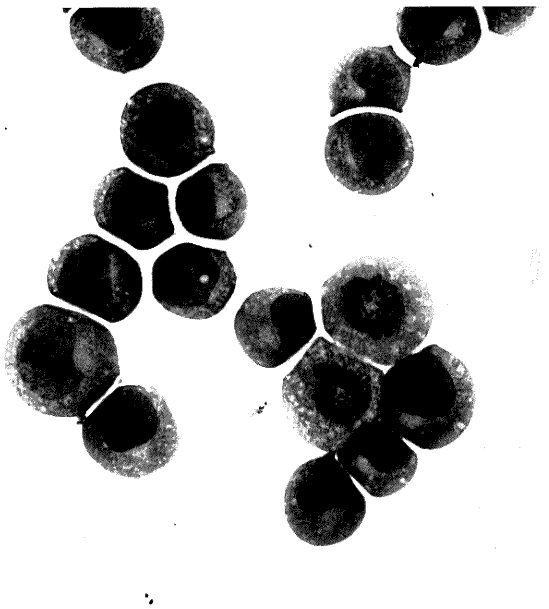

Fig. 1. Cytocentrifuged smear of KY-Ra (May-Giemsa ×430).

agents were differentiated. PMA, DMSO, RA induced cells to differentiate to monocytic cells, myelocytic cells, and myelocytic cells, respectively. The populations of the differentiated cells also varied. Ara-C resistant cells showed more resistance for differentiation by some chemical agents (DMSO, RA) than parental cells. (Table 1)

Cytochemistry

These three cell lines were negative for peroxidase, and positive for chloroacetate esterase and butyrate esterase. (Table 1)

Immunofluorescence studies

Leu-4 (CD3), Leu-12 (CD19) of the lymphocyte cell markers were negative or minimal on these three cell lines. My7 (CD13) and My9 (CD33) of the myeloid cell markers were positive on them. My7 reacted with 23% of the parental cells, and reacted with 94% of KY-Rb and 100% of KY-Ra. (Table 1)

Table 1. Biological features of KY-821, KY-Rb and KY-Ra

	KY-821 (parent)	KY-Rb	KY-Ra
Morphology	>95% myeloblasts	>95% myeloblasts	>95% myeloblast
Differentiation study			
Response to RA			
mature myeloid cells (%)	82.7±16.9	71.0±16.4	25.7±12.0
Response to DMSO			
mature myeloid cells (%)	98.3±0.5	68.0±23.3	62.0±29.7
Response to PAM			
mature monocytes (%)	97.0	96.3±3.9	96.3±2.1
Cytochemistry*			
Peroxidase	(−)	(−)	(−)
Chloroacetate esterase	(+)	(+)	(+)
Butyrate esterase	(+)	(+)	(+)
Phenotype (%)			
My 4 (CD14)	5	6	2
My 7 (CD13)	23	100	94
My 9 (CD33)	77	99	100
OKM 1 (CD11)	8	4	1
Leu 4 (CD3)	1	1	0
Leu 12 (CD19)	1	1	1
HLA-DR	1	1	1

* (−): negative, (+); positive

Cytogenetic studies

In trypsin Giemsa staining, KY-821 had 64 to 79 chromosomes (mode of 72). KY-Rb and KY-Ra had 58 to 69 chromosomes (mode of 62), 60 to 67 chromosomes (mode of 64). (Fig. 2) There were no homogeneously staining regions (HSR). In Giemsa staining, double minute chromosomes (DMs) were shown. KY-811, KY-Rb and KY-Ra had an average of 4.2 of DMs, 29.0 of DMs and 1.2 of DMs, respectively.

Cytotoxic studies

As shown in Fig. 3, the sensitivdties of the three cell lines to ara-C were different. The resistance of KY-Ra and KY-Rb were approximately 18,000 fold and 5,900 fold more than the parent cell line (KY-812), respectively. (Table 2) KY-Rb showed a plateau pattern of survival percentage at between 10^{-6} M to 10^{-4} M concentrations. The precise mechanism of the plateau pattern is uncertain. The maintaining of KY-Ra in a drug-free medium for three months showed no change in the resistance.

Deoxycytidine kinase activity

Table 3 shows the deoxycytidine kinase activity of the three cell lines. Ara-C resistant cell lines showed a reduction in deoxycytidine kinase activity (KY-Ra; 0.39 fmol dCMP/min/μg protein, KY-Rb; 0.26) in comparison to the parental cell line (KY-821; 1.23).

Deoxycytidine deaminase activity

Deoxycytidine deaminase activities of the three cell lines were almost the same (Table 3). The differences were not statistically significant.

Drug accumulation

The parental cell line (KY-821) showed approximately 3 times and 10 times more drug accumulation than that of KY-Rb and KY-Ra at 4 h incubation, respectively (Fig. 4A). The drug accumulation of KY-821 increased with incubation time. KY-Ra showed a mild drug accumulation not related to the incubation time. Twenty-four hours incubation of KY-Ra with

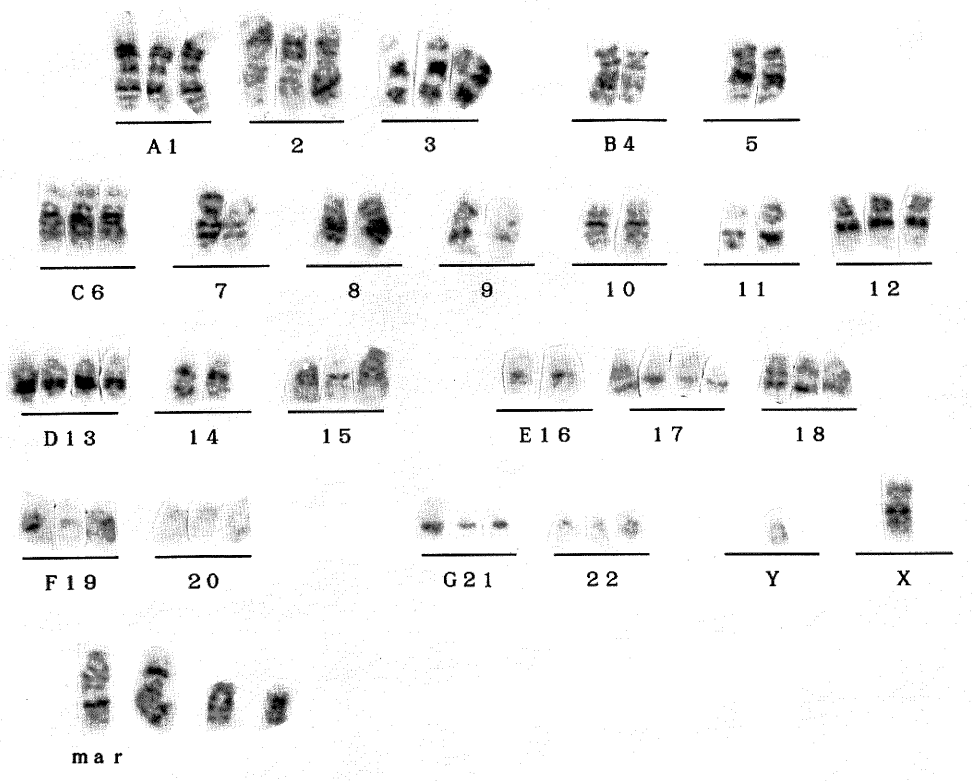

Fig. 2. Karyotype of a hypotriploid KY-Ra cell.

drugs showed almost the same drug accumulation as that of the first 4 h incubation (data not shown). The pattern of drug accumulation of KY-Rb showed an intermediate pattern between that of KY-821 and KY-Ra. These differences between these three cell lines were statistically significant.

Efflux of drugs

After loading the same magnitudes of intracellular contents of drugs by incubating cells with various contents of drugs, the efflux of ara-C was determined. After 30 min incubation in a drug-free medium, large amounts of drugs from KY-Ra were lost. The percentages of the efflux of drugs were approximately 48% (KY-821), 57% (KY-Rb), and 96% (KY-Ra) at 30 min (Fig. 4B). The differences in efflux rate between KY-Ra and the other two cell lines were statistically significant.

Ara-C incorporation into DNA

The percentage of ara-C incorporated into the DNA of the initial intracellular ara-C were 6.5% for KY-821, 3.2% for KY-Rb, 1.2% for KY-Ra.

Initial influx of ara-C

The uptakes of ara-C into cells for 20 sec were 2.2 $\pm$0.4 (fmol/10^6 cells) for KY-821, 2.8$\pm$0.8 for KY-Rb, 2.2$\pm$0.1 for KY-Ra, respectively. The values were not statistically significant.

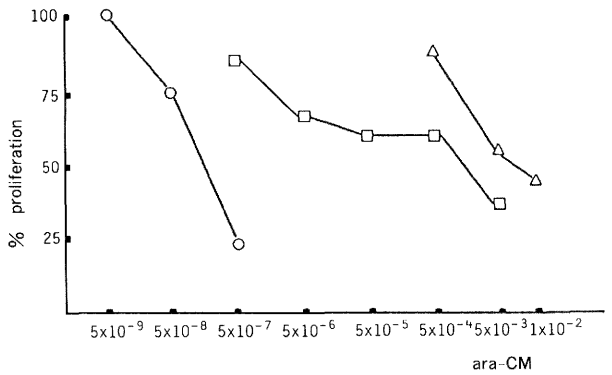

Fig. 3. Cytotoxic effect of ara-C on KY-821 (○), KY-Rb (□) and KY-Ra (△). Cells were treated with drugs for 72 h, and then cell numbers were determined by MTT assay. Cytotoxicity is expressed as percentage of the untreated cells. Values of each point represent the average of four separate experiments. The SD of each points are within 20.6%.

Analysis of ara-C metabolites

The percentage of phosphorylated ara-C in parental cells was greater than ara-C resistant cells. The percentage of ara-CTP was 48.2% of the acid-soluble fraction in the parental cells. In ara-C resistant cells, the percentage of ara-CTP was 3.7% for KY-Rb, and 0.7% for KY-Ra, respectively (Table 4).

DISCUSSION

Ara-C resistant human leukemic cell lines, designated KY-Ra and KY-Rb, are here reported. These two ara-C resistant cell lines and the parental cell line showed almost the same features in regard to morphology, cytochemistry, phenotype and karyotype. The cytochemical studies demonstrated the three cell lines to be positive for chloroacetate esterase and butyrate esterase. This result indicated that the three cell lines showed myelocytic and monocytic differentiation. The results of immunofluorescence studies also revealed that these three cell lines showed the myelocytic and monocytic phenotypes to be positive with the My7 and My9 markers. In differentiation studies, however, ara-C resistant cell lines showed resistance for differentiation by certain chemical agents (RA, DMSO). It might indicate the relationship between the resistance to ara-C and the resistance for differentiation by some chemical agents, because the resistance for differentiation positively

Table 2. Sensitivity of KY-821, KY-Rb and KY-Ra to ara-C. Values represent the concentration of ara-C to produce 50% inhibition of cell growth. Relative resistance is calculated as value of IC_{50} of cells/value IC_{50} of parental cells

cells	ara-C (IC 50, M)	relative resistance
KY-821	$4.56\times10^{-7}\pm1.32\times10^{-7}$	1
KY-Rb	$2.69\times10^{-3}\pm1.70\times10^{-3}$	5,899
KY-Ra	$8.60\times10^{-3}\pm2.31\times10^{-3}$	18,860

Table 3. Deoxycytidine kinase activity and deoxycytidine deaminase activity of KY-821, KY-Rb and KY-Ra

cells	kinase activity (fmol dCMP/min/μg protein)	deaminase activity (units)
KY-821	1.23$\pm$0.35	7.3$\pm$1.7
KY-Rb	0.26$\pm$0.03	7.0$\pm$1.9
KY-Ra	0.39$\pm$0.06	4.8$\pm$0.8

coincided with the resistance to ara-C in these three cell lines.

The KY-Ra line exhibited the resistance to ara-C in a 1×10^{-4} M concentration, which was clinically observed during high-dose ara-C administration.[19] The *in vitro* models reported previously did not reach the resistance to ara-C in high concentrations, which was observed during the high-dose ara-C regimen. The leukemic cell line reported in this present study showed a high resistance to ara-C. This high resistance may coincide with the clinical refractoriness encountered during the high-dose ara-C regimen. Many mechanisms rendering leukemic cells resistant to ara-C have been either documented or speculated on in *in vivo* and *in vitro* settings.[6-17] The decreased deoxycytidine kinase activity has especially been demonstrated as a cause of resistance to ara-C in many reports.[7-10] In *in vitro* experiments using murine neoplasms, the loss of this enzyme was considered a main mechanism.[8] In regard to human leukemia, Bhalla et al. reported an ara-C resistant human leukemic cell line which showed a highly reduced deoxycytidine kinase activity compared with the parental cell line.[9] In addition, Tattersall et al. described in clinical research that some cases in leukemic patients displayed a decrease in deoxycytidine kinase activity.[7] Ara-C resistant cell lines reported here also showed a reduced deoxycytidine kinase activity. The deoxycytidine deaminase activities were almost the same levels in these cell lines. The resistance for ara-C in these cell lines may be attributed to the reduced activity of deoxycytidine kinase. In the drug accumulation study, these three cell lines showed different drug accumulation values during 4 h exposure to 1 nM ara-C. There was a converse relationship between the accumulation of ara-C and the resistant level of cells to ara-C. On analysis of the ara-C metabolites, ara-CTP accumulation in parental cells was much greater than ara-C resistant cells. These findings indicate that decreased kinase activity, which resulted in decreased drug accumulation and decreased ara-CTP accumulation, is one mechanism of ara-C resistance. However, it is impossible to explain the high resistance of these cells only by the reduced deoxycytidine kinase activities, because the degree of decrease of the kinase activity is smaller than that of previous reports.[9,10] It may indicate the presence of other mechanisms of resistance.

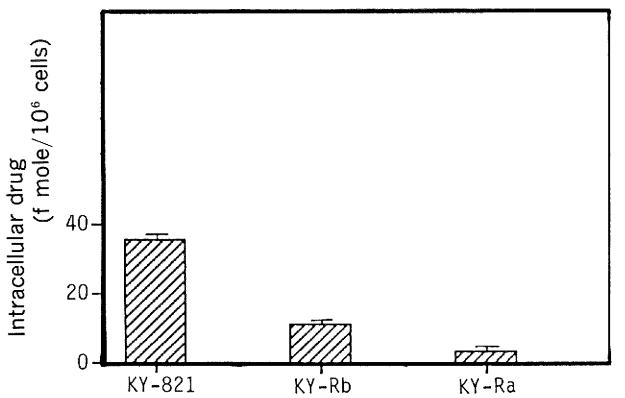

Fig. 4A. Intracellular accumulation of ara-C into KY-821, KY-Rb and KY-Ra. Values are expressed as f mole per 1×10^6 cells. Each value is the mean of three separate experiments±SD.

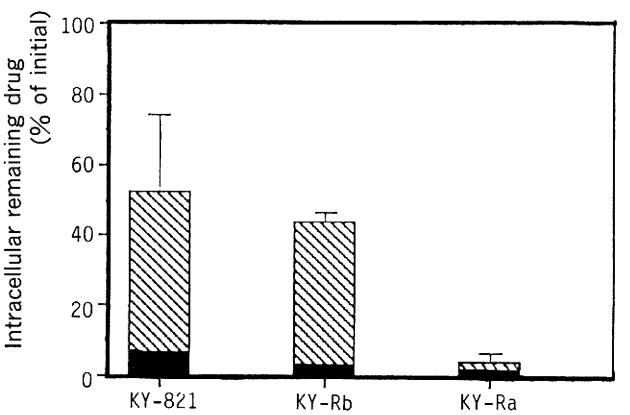

Fig. 4B. Efflux of ara-C from KY-821, KY-Rb and KY-Ra. KY-821, KY-Rb and KY-Ra were preincubated for 1 h with 2 nM, 5 nM and 10 nM [^{3}H] ara-C, respectively. After preincubation, the averages of initial intracellular radioactivity of KY-821, KY-Rb, KY-Ra were 1405.4 dpm/1×10^6 cells, 1709.2 dpm/1×10^6 cells, 2658.9 dpm/1×10^6 cells, respectively. Values are expressed as the percentage of initial intracellular radioactivity. Each value is the mean of the three separate experiments±SD. The black area represents the percentage of ara-C incorporated into DNA.

Table 4. Analysis of ara-C metabolites. Values are expressed as percentages of acid-soluble fractions. Each value is the mean of two or three experiments

	KY-821 (%)	KY-Rb (%)	KY-Ra (%)
ara-C	37.3	92.4	95.5
ara-CDP choline	0.5	0.5	0.6
ara-CMP	4.3	2.1	0.8
ara-CDP	9.7	1.3	2.4
ara-CTP	48.2	3.7	0.7

When nucleoside permeants are metabolized intracellularly, there are many problems in determining the transport process.[26] For a study of the efflux process, non-metabolized nucleoside, ATP-depleted or kinase deficient cells should be employed to eliminate the intracellular metabolism.[26] The same problems as described above also occur in an ara-C efflux study. However, it is possible to study the efflux process of ara-C in KY-Rb and KY-Ra, because intracellular metabolism of ara-C was suppressed and intracellular metabolites of ara-C at initial time were almost the same in both cell lines. In this efflux study, KY-Ra showed a high efflux rate in comparison with KY-Rb. It was also demonstrated that ara-C incorporated into DNA had minimal influence on the ara-C efflux in these cell lines. Therefore, there is the possibility that the increased efflux of ara-C is one mechanism encouraging the cells resistant to ara-C in KY-Ra. However, it is impossible to clarify the transport process in high concentrations of ara-C, because the percentage of intracellular ara-CTP in KY-Ra increases when extracellular ara-C concentration is high (data not shown). Further examination is required to clarify the ara-C resistance in these cell lines. Thus, these ara-C resistant cell lines are useful to study the refractoriness encountered during high-dose ara-C administration, the reversal of resistance, and the effect of new therapeutic agents.

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