STUDIES ON ACLACINOMYCIN A RESISTANCE IN MOUSE LYMPHOBLASTOMA

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(Received for publication February 25, 1980)

An aclacinomycin A-resistant subline of mouse lymphoblastoma L5178Y cells was isolated by successive treatment of tumor-bearing mice with the antibiotic. IC_{50} (50% growth inhibition) in culture was observed at a drug concentration of 0.22 μg/ml, which was *ca.* 11 times higher than IC_{50} for the parental cells. The resistant cell line exhibited cross resistance to mitomycin C, actinomycin D, macromomycin, auromomycin, vinblastine, cytochalasin B, and other anthracyclines: daunorubicin, adriamycin, 4'-O-tetrahydropyranyladriamycin, baumycins A1 and A2, aclacinomycins B and Y, MA144-S1, 1-deoxypyrromycin, cinerubin A, musettamycin, and pyrromycin. The 1-deoxy group of anthracyclines showed higher degree of cross resistance than the 1-hydroxy group. No significant cross resistance was found with bleomycin A_2, neothramycin and blasticidin S. The resistance to aclacinomycin A and cross resistance to adriamycin were also demonstrated by the method of uridine incorporation. The accumulation or retention studies with [^{3}H]adriamycin revealed that the resistance may be due to decreased uptake and increased efflux of the antibiotic in the resistant cells.

Aclacinomycin A is a new tumor-inhibitory antibiotic of the anthracycline group[1]. The drug is effective on leukemia L-1210 and P-388, sarcoma 180, 6C3HED lymphosarcoma and other transplantable tumors of animals; and exhibits less cardiotoxicity than adriamycin and daunorubicin[2]. The mechanism of action of aclacinomycin A is similar to that of adriamycin and daunorubicin. Aclacinomycin A produces a preferential inhibition of RNA synthesis over DNA synthesis by intercalating template DNA[3,4], and induces strand scission of DNA[5]. The interaction with tubulin, actin and heavy meromyosin has been also observed[4,6].

Human malignant neoplasms sometimes acquire resistance to the anthracycline group of antibiotics, and the mechanism of resistance is not known. For the purpose of studying the mode of anthracycline resistance, we have obtained an adriamycin-resistant subline of mouse lymphoblastoma L5178Y cells *in vitro*, and examined the mechanism of resistance, which may be attributed to permeability changes[7,8].

We have further isolated a resistant subline of L5178Y cells *in vivo* by administration of aclacinomycin A to mice bearing L5178Y tumor and studied the mechanism of resistance and cross resistance to other antitumor drugs. The results are presented in this publication.

Materials and Methods

[^{3}H] Uridine (40.8 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. [^{3}H] Adriamycin (28.88 mCi/mmol; 49.8 μCi/mg), a product of Farmitalia, was kindly given by Kyowa Hakko Co., Tokyo. The radiochemical purity was higher than 98% by TLC. Aclacinomycin A and related anthracyclines were generously supplied by Dr. T. OKI, Central Research Lab., Sanraku-Ocean Co., Fujisawa, Kanagawa. Adriamycin and mitomycin C (Kyowa Hakko Co.), bleomycin A_2 (Nippon Kayaku Co.), neothramycin and daunorubicin (Meiji Seika Kaisha, Ltd.), blasticidin S (Kaken Chemical Co.), vinblastine (Eli Lilly & Co.) and cytochalasin B (Adrich Chemical Co.) were also used. Other chemicals were of the highest grade commercially available.

CDF_1 (BALB/c×DBA/2) male mice, weighing 20~25 g, were supplied by the Institute of Medical Science, University of Tokyo.

Mouse lymphoblastoma L5178Y cells were generously given by Prof. SHIGEFUMI OKADA, Department of Radiation Biophysics, Faculty of Medicine, University of Tokyo in 1974. Since then the cells were cultured in FISCHER's medium supplemented with 10% horse serum. The cell number was determined by a Coulter counter.

Isolation of Aclacinomycin A-resistant Subline of L5178Y Cells

L5178Y cells of 2×10^6 were intraperitoneally inoculated into CDF_1 mice, which were then intraperitoneally injected with a PBS (phosphate buffered saline) solution of aclacinomycin A, 4 or 2 mg/kg/day for 7~9 days, starting 24 hours after tumor transplant. The cells harvested from the mice were transplanted into other CDF_1 mice, which were again treated with the antibiotic in the same way as above. After 5 successive transplant generations, the cells were inoculated into soft agar (0.15%) FISCHER's medium (10^5 cells/ml) supplemented with 15% horse serum and aclacinomycin A 0.4 μg/ml. About 2 weeks later, the colonies formed were transferred into 10% horse serum-FISCHER's medium. No antibiotics were added to the culture medium.

The aclacinomycin A-resistant cells were grown in the suspension cell culture as the parental cells. The cells of both cell lines could not be differentiated each other morphologically in GIEMSA staining.

Drug sensitivity of parental and resistant cell lines, [^{3}H] uridine incorporation, and uptake and efflux of [^{3}H] adriamycin were determined by the methods described previously[7,8].

Results

Isolation of Aclacinomycin A-resistant L5178Y Cells

First, we tried without success to obtain aclacinomycin A-resistant subline of L5178Y cells in culture in the presence or absence of N-methyl-N′-nitro-N-nitrosoguanidine. Then, a drug-resistant cell subline was isolated, after 5 successive transplant generations, by treatment of L5178Y tumor-bearing CDF_1 mice with aclacinomycin A, as described in Materials and Methods.

Effects of Aclacinomycin A on Growth of Parental and Resistant Sublines of L5178Y Cells

The growth of L5178Y cells was observed by counting the cell number in a Coulter counter. The doubling time of cells was *ca.* 11 hours for the parental cells, and *ca.* 18 hours for the resistant cells. Aclacinomycin A completely blocked growth of the sensitive cells, but not that of the resistant cells at an antibiotic concentration of 0.1 μg/ml. The time dependency of inhibition is illustrated in Fig. 1. IC_{50} (50% inhibitory concentration) of the drug was 0.02 μg/ml for the parental cell subline and 0.22 μg/ml for the resistant subline; *i.e.* the difference of sensitivity was *ca.* 11 fold (Table 1).

Drug Sensitivity of the Parental and Resistant Sublines of L5178Y

The effects of anthracyclines and other antitumor drugs on the growth of the aclacinomycin A-resistant and -sensitive cells are presented in Fig. 2 and Table 1. The resistant subline showed cross resistance to other anthracyclines. The 1-deoxy group of antibiotics (aclacinomycins B and Y, MA144-S1, 1-deoxypyrromycin, daunorubicin, adriamycin, 4′-O-tetrahydropyranyladriamycin, and baumycins A1 and A2) exhibited higher degree of resistance than the 1-hydroxy group of anthracyclines (cinerubin A, musettamycin, and pyrromycin). The cross resistance was observed with mitomycin C, actinomycin D, macromomycin, auromomycin, vinblastine, and cytochalasin B; it was not significant with bleomycin A_2, blasticidin S, and neothramycin.

Effects of Anthracyclines on RNA Synthesis in Intact Cells of Parental and Aclacinomycin A-Resistant L5178Y

RNA synthesis was studied by the incorporation of [^{3}H] uridine into a TCA-insoluble fraction of

Fig. 1. Effects of aclacinomycin A on the growth of parental and aclacinomycin A-resistant sublines of L5178Y cells.

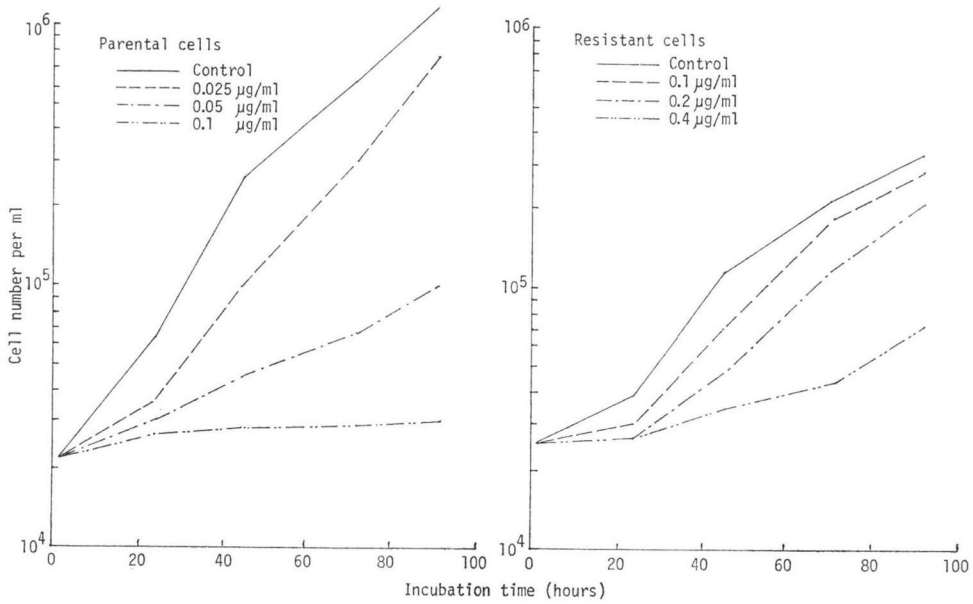
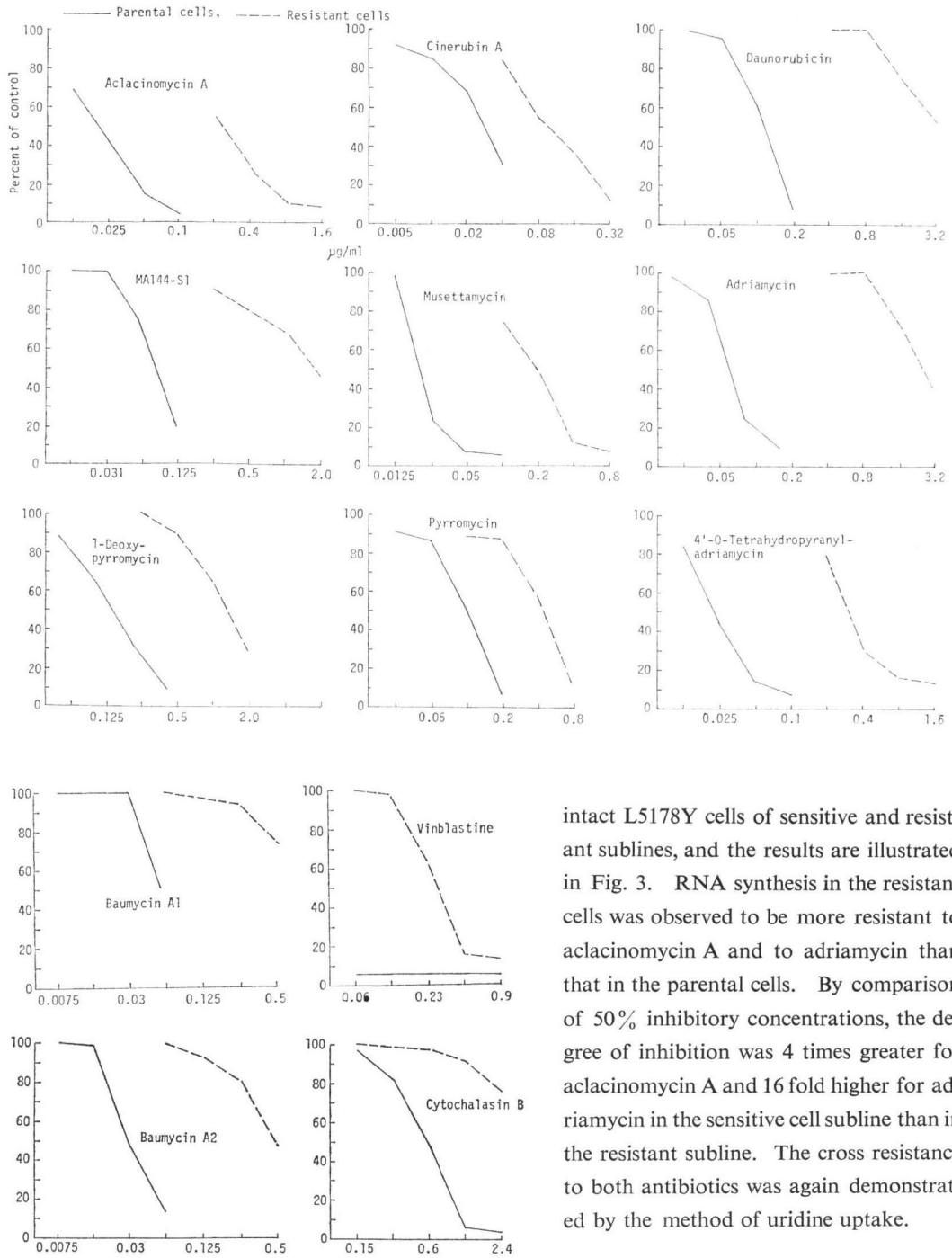


Table 1. Drug sensitivity of an aclacinomycin A-resistant subline of L5178Y cells in comparison with that of the parental cell line.

Antibiotics	IC_{50} (μg/ml)		Degree of* resistance
	Parental	Resistant	
Aclacinomycin A	0.02	0.22	11
〃 B	0.03	0.14	5
〃 Y	0.01	0.07	7
MA144-S	0.08	1.8	23
1-Deoxypyrromycin	0.14	1.3	9
Cinerubin A	0.03	0.1	3
Musettamycin	0.02	0.19	10
Pyrromycin	0.1	0.44	4
Daunorubicin	0.12	3.2	27
Adriamycin	0.06	2.5	42
4′-O-Tetrahydropyranyladriamycin	0.02	0.3	13
Baumycin A1	0.06	>0.5	>8
〃 A2	0.03	0.47	16
Mitomycin C	0.02	0.36	18
Actinomycin D	0.03	0.27	9
Macromomycin	0.06	0.45	8
Auromomycin	0.0013	0.016	13
Bleomycin A_2	2.1	3.1	1
Neothramycin	0.145	0.185	1
Blasticidin S	1.85	1.85	1
Vinblastine	<0.06	0.27	>5
Cytochalasin B	0.58	>2.4	>4

* The resistance is expressed as a ratio of IC_{50} values for resistant to parental cell line.
The cells were incubated with the drugs at 37°C for 3 days.

Fig. 2. Drug sensitivity of parental and aclacinomycin A-resistant sublines of L5178Y cells. The drug and the cells were incubated at 37°C for 3 days.



intact L5178Y cells of sensitive and resistant sublines, and the results are illustrated in Fig. 3. RNA synthesis in the resistant cells was observed to be more resistant to aclacinomycin A and to adriamycin than that in the parental cells. By comparison of 50% inhibitory concentrations, the degree of inhibition was 4 times greater for aclacinomycin A and 16 fold higher for adriamycin in the sensitive cell subline than in the resistant subline. The cross resistance to both antibiotics was again demonstrated by the method of uridine uptake.

Accumulation (Influx and Retention) of [^{3}H] Adriamycin in the Sensitive and Resistant Sublines of L5178Y Cells

Since [^{3}H] adriamycin was available and the aclacinomycin A-resistant L5178Y cells showed cross

Fig. 2. (continued)

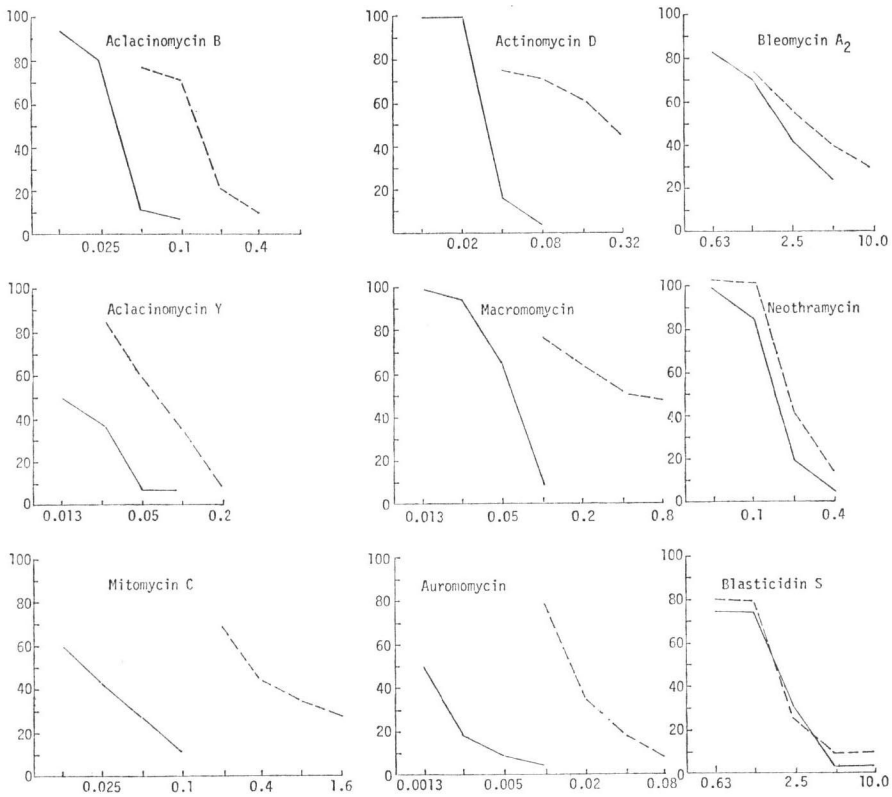
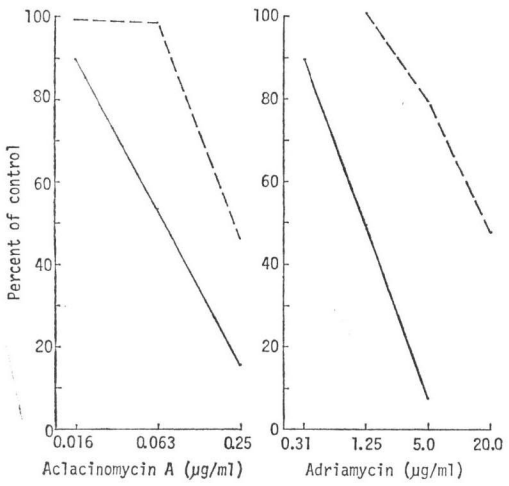


Fig. 3. Effects of anthracycline antibiotics on RNA syntheses in intact cells of parental and aclacinomycin A-resistant L5178Y.
—— Parental cells 100%=11,890 cpm/2×10⁵ cells
------ Resistant cells 100%= 6,892 〃



resistance to adriamycin, the influx and efflux were studied with adriamycin instead of aclacinomycin A. The uptake and retention of the [^{3}H] antibiotic in the cells was studied by the filtration method with or without washing by centrifugation. The time dependency of incorporation of [^{3}H] adriamycin into the sensitive and resistant sublines is presented in Fig. 4. The accumulation of the antibiotic was observed to be higher in the parental cells than in the resistant cells with washing the cells in buffered saline by centrifugation (Fig. 4B); but no significant difference was found between both cell lines in the absence of washing (Fig. 4A).

The LINEWEAVER-BURK plot of the initial rate of [^{3}H] adriamycin uptake in the parental and resistant cells revealed linear relationship between $1/V$ and $1/S$, where V is the initial rate of incorporation of the antibiotic and S the initial

Fig. 4. Uptake of [^{3}H] adriamycin by parental and aclacinomycin A-resistant sublines of L5178Y cells.
The cells were collected on glass fiber filters with (B) or without (A) washing the cells in phosphate-buffered saline by centrifugation.

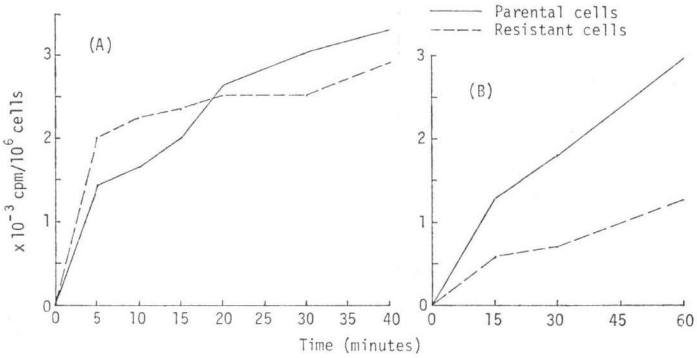


Fig. 5. The LINEWEAVER-BURK plot of the initial rate of [^{3}H] adriamycin uptake in parental and aclacinomycin A-resistant sublines of L5178Y cells.
The cells were incubated with various concentrations of [^{3}H] adriamycin at 37°C for 3 minutes.

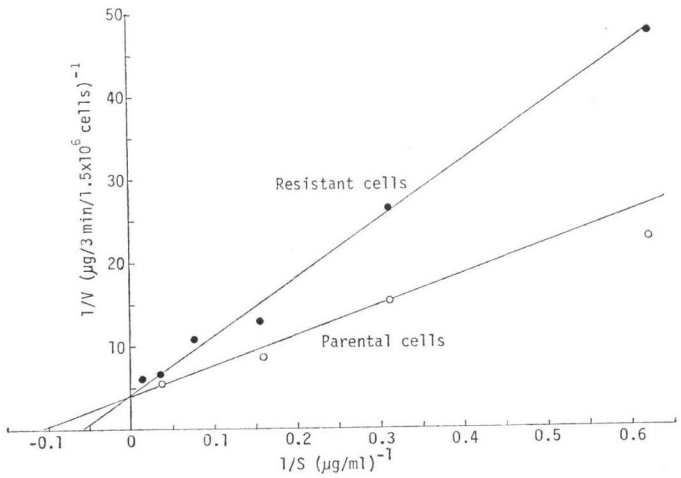
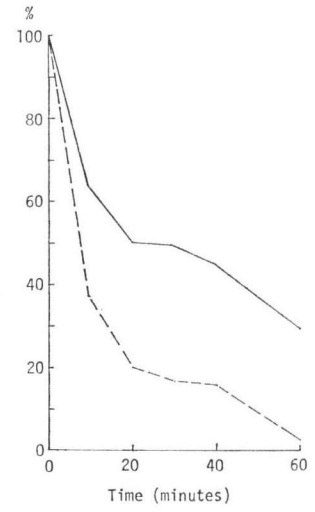


Fig. 6. Efflux of [^{3}H] adriamycin from parental and aclacinomycin A-resistant sublines of L5178Y cells.
The cells (1.9×10^6/ml) and [^{3}H] adriamycin (5.5 μg/ml) were incubated at 37°C for 60 minutes in FISCHER's medium with 10% horse serum, washed in phosphate buffered saline, and resuspended in the same fresh medium.
—— Parental cells 100%=3,026 cpm/10^6 cells
------ Resistant cells 100%=1,374 cpm/10^6 cells



drug concentration. The apparent *Km* of adriamycin for the sensitive cells was approximately 1.75×10^{-5} M, and that for the resistant cells 2.9×10^{-5} M: *i.e.* the former bound adriamycin somewhat more tightly than the latter (Fig. 5).

Since the accumulation of adriamycin in the cells could be due to differential rates of uptake and release by the cells and to affinities for the chemoreceptors (DNA, tubulin *etc.*), the efflux of the drug from the cells was examined with the sensitive and resistant cell lines (Fig. 6). The resistant cells exhibited a higher tendency to release the antibiotic than the sensitive cells.

Discussion

An aclacinomycin A-resistant subline of mouse lymphoblastoma L5178Y cells was obtained by successive treatment of tumor-bearing mice with the antibiotic. We failed to isolate aclacinomycin A-resistant L5178Y cells in culture in the current experiments; on the other hand we previously obtained an adriamycin-resistant subline of L5178Y cells *in vitro*[7]. This suggests that the drug-resistant cells are more easily isolated with adriamycin than aclacinomycin A. The difference for the two antibiotics to induce resistant tumor cells may be, at least partly, due to the mutagenicities of the two drugs: adriamycin is highly mutagenic[9] but aclacinomycin A is little mutagenic[10].

The resistant cells show a unique cross resistance pattern to other antitumor drugs. In the present study, the aclacinomycin A-resistant subline exhibited marked cross resistance to adriamycin. In the previous experiments[7], on the contrary, the adriamycin-resistant subline of L5178Y cells did not significantly show cross resistance to aclacinomycin A. Therefore, one-way cross resistance is demonstrated between adriamycin and aclacinomycin A. However, a number of resistant cell lines should be tested to support this conclusion. 4′-O-Tetrahydropyranyladriamycin, a new derivative[11], also exhibited cross resistance to aclacinomycin A.

The current studies with [^{3}H] adriamycin show that the resistance may be attributed to permeability changes: Decreased incorporation and increased release of the antibiotic in the resistant cells. However, the precise mechanism of resistance remains to be determined.

Acknowledgements

The current work was supported by a grant-in-aid from the Ministry of Education, Science and Culture, Japan. The authors express their deep thanks to Dr. HAMAO UMEZAWA, Institute of Microbial Chemistry for his kind advice and cooperation throughout the present study.

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