ISOLATION AND CHARACTERIZATION OF THE MURINE LYMPHOMA L5178Y CELL LINE HIGHLY RESISTANT TO 5-FLUOROURACIL

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5-Fluorouracil-resistant cell line designated as L5178Y/FU was established in this experiment. This is one of the colonies derived from the subculture which acquired resistance to 5-fluorouracil by passaging the L5178Y cells through ten successive episodes of culture in Fischer's medium containing 5-fluorouracil, in which each 5-fluorouracil treatment was followed by recovery intervals. The resistance of this line is about 80-fold that of the IC₉₉ of 5-fluorouracil, and also shows a cross resistance to both 5-fluorouridine and 5-fluoro-2'-deoxyuridine.

5-Fluoro-2'-deoxyuridine resistant subline designated as L5178Y/FUdR was also isolated from the subculture which acquired resistance to 5-fluoro-2'-deoxyuridine by using the same selection procedure. The resistance of this line is about 650-fold that of the IC₉₉ of 5-fluoro-2'-deoxyuridine, and shows a partial cross-resistance to 5-fluorouridine, but not to 5-fluorouracil.

Keywords—5-fluorouracil; 5-fluorouridine; 5-fluoro-2'-deoxyuridine; murine lymphoma L5178Y cell; resistance; cross-resistance

INTRODUCTION

The pyrimidine analog, 5-fluorouracil (FU), have been used extensively in the chemotherapy of certain types of malignant neoplasms since its introduction by Heidelberger *et al.*¹⁾ The mechanism of action of this analog, despite almost 30 years of intensive basic and clinical research, is not yet completely understood. For the studies on this problem, a pyrimidine analog resistant tumor line is useful. In a *in vitro* system, resistance toward its deoxyribonucleoside, 5-fluoro-2'-deoxyuridine (FUdR), develops relatively easily, and has been reported to become 500-fold resistant to the analog, but not a cross-resistant to FU.^{2,3)} However, in the FU-resistant tumor cell line established by Roosa *et al.*, the drug concentration required to inhibit the cell proliferation was 25-fold that for the sensitive cell line.³⁾ In a *in vivo*

experiment, a resistance to FU has also been reported to be as low as a level of pharmacological dose.⁴⁻⁷⁾ Such FU-resistant tumor lines with a low degree in resistance are useless to investigate mechanisms of cytotoxicity of FU. Therefore, it is the aim of this study to select FU-resistant subline. Development of resistance, and their cross-resistance to other analogs were further characterized.

MATERIALS AND METHODS

Materials—Fischer's medium, horse serum and penicillin-streptomycin solution were purchased from Grand Island Biological Co. (Berkeley, USA). N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). FU, FUR and FUdR were supplied by Mitsui Pharmaceuticals Inc. (Tokyo, Japan).

Cell Culture—A clone of murine lymphoma L5178Y cells, designated L5178Y/O, sensitive to pyrimidine analogs, was used in this study as the parental cell line for separating cell lines resistant to FU and its deoxynucleoside analog. A L5178Y/FUR subline established previously⁸⁾ is one of the colonies derived from a subculture which acquired resistance to FUR by passing the L5178Y cells through four successive episodes of culture in Fischer's medium containing FUR: each FUR treatment was followed by recovery intervals.

Cells were cultured in Fischer's medium supplemented with 10% horse serum, 100 $\mu\text{g}/\text{ml}$ streptomycin and 100 units/ml penicillin in a CO_2 incubator with a humid atmosphere of 5% CO_2 and 95% air as described by Fischer and Sartorelli.⁹⁾ Cultures were restarted from freezing stocks every six weeks.

Isolation Procedures of Resistant Cell Lines — FU and FUR resistant cell lines were established by a technique described in the selection of L5178Y/FUR subline previously.⁸⁾ Essentially, L5178Y cells were mutagenized by 3.0 μM MNNG for 24 hr. The mutagenized cells were treated by 24 hr exposure to analogs, thereafter, washed once, cultured in drug free medium until the cell proliferation has recovered. The resulting cells were exposed to a next higher concentration which was about three times the previous concentration. The above procedure was repeated whenever cells grew at the proliferation ratio of untreated cultures. Each of such procedure was termed as an episode of drug treatment. After the last episode, cells were cloned as follows; cloning with the soft agar technique employed 0.15% Nobles' agar in a medium consisting of nine volumes of Fischer's medium supplemented with 10% horse serum, antibiotics plus one volume of conditioned medium.

Sensitivity and resistance to FU or its nucleoside analogs were estimated by observing the effects on the proliferation of L5178Y cells as described previously. The antiproliferating activity of drugs were evaluated by the IC_{99} value, which is a concentration required for 99% inhibi-

tion on the cell proliferation of the control culture after a 4 day growth period. Resistance ratio, which was calculated on the basis of the IC_{99} value of resistant cell lines *versus* sensitive cell lines, was used as a parameter for evaluation of the resistance.

RESULTS AND DISCUSSION

Acquisition of FUR-Resistant L5178Y Cells

The L5178Y cells after mutation were exposed to 0.3 μM FUR for 24 hr, and then followed by

FIG. 1 Effects of 5-Fluorouracil, 5-Fluorouridine and 5-Fluoro-2'-deoxyuridine on the Proliferation of Four Sublines of L5178Y Cells

- (A) : 5-fluorouracil (FU),
 (B) : 5-fluorouridine (FUR),
 (C) : 5-fluoro-2'-deoxyuridine (FUdR),
 —●— : L5178Y/O,
 —□— : L5178Y/FU,
 —△— : L5178Y/FUR,
 —○— : L5178Y/FUR.

passage for 12 days in drug free medium. After this procedure, the treated cells were passaged through three successive episodes of culture in medium containing FUdR at increasing concentrations such as 1.0, 3.0 and 10 μM for a 24 hr interval; each FUdR treatment was followed by recovery intervals consisting of passage for 12, 10 and 7 days in the medium, respectively. Recovery from the 4th episode of FUdR treatment was clearly more rapid than that following the initial several treatments. Fifty five colonies were randomly picked up from colonies formed by the cloning method. Upon immediate testing, a clone of L5178Y/FUdR was selected among them because of the greatest ability to grow in medium containing 0.05 μM FUdR.

Acquisition of FU-Resistant L5178Y Cells

The mutant L5178Y cells were passaged through ten successive episodes of culture in a medium containing FU at increasing concentrations such as 1, 1, 1, 3, 10, 30, 100, 100, 300 and 1000 μM for 24 hr; each FU treatment was followed by recovery intervals consisting of passage for 8, 3, 3, 17, 15, 13, 14, 11, 13 and 15 days in the medium respectively. After the last episode, 113 clones were randomly picked up from individual colonies.

Seventy eight colonies among them were discarded because of low growth rate in mass culture in normal medium, and the rest were tested for

the ability to grow in medium containing 10 μM FU. As a result a clone designated as L5178Y/FU was selected for further studies because of the highest resistance to FU.

This subline and L5178Y/FUdR subline retained their respective levels of resistance after up to 6 months' passage in the absence of the drugs.

Cross-Resistance and Other Characteristics of Resistance Sublines

Fig. 1 shows the dose response curve of FU on the cell proliferation of L5178Y/FU subline. It can be seen that the IC_{99} values of L5178Y/O line for FU was 0.8 μM and that of L5178Y/FU subline was 65 μM , consequently, L5178Y/FU subline was about 80-fold resistant to FU compared with L5178Y/O line. This line of highly resistant cells, with the degree of resistance higher than any of subline reported elsewhere, was successfully established by mutation induced by MNNG and successive episodes of FU treatment. This subline was tested for a cross-resistance to both nucleoside analogs of FUR and FUdR. The IC_{99} values of FUR and FUdR for this subline are approximately 250 and 110-fold, respectively, of those needed for the parent line. Thus this subline exhibits cross resistance to FUR and FUdR at a higher degree than resistance to FU.

L5178Y/FUR subline, which acquired resistance by FUR treatment, was resistant to 24-fold

TABLE I. *Comparison of Sensitivity of 4 Sublines of L5178Y Cells on Cytotoxicities of 5-Fluorouracil and Its Nucleoside Analogs*

Cell line	Sensitivity (IC_{99} ; μM)		
	FU	FUR	FUdR
L5178Y/O	0.8	0.024	0.0023
L5178Y/FU	65 (81)*	6.0 (250)	0.25 (110)
L5178Y/FUR	3.0 (4)	0.57 (24)	0.014 (6)
L5178Y/FUdR	0.8 (1)	0.11 (5)	1.5 (650)

* Numbers in parentheses indicate a resistance ratio which was calculated on the basis of the IC_{99} versus of resistant cell sublines versus sensitive cell line (L5178/O).

increase over the IC_{99} of FUR, and showed a cross resistance to FU and FUdR as shown in Table I. But their cross-resistance was low in resistance ratio such as 4 for FU or 6 for FUdR. On the other hand, L5178Y/FUdR subline was 650-fold resistant to the cytotoxic effects of FUdR as compared with that of the parent line ($0.0023 \mu M$). Cross-resistance experiments with this subline revealed that the resistance to FUR was weak and that to FU was negligible.

The comparison of L5178Y/FU and L5178Y/FUdR on the development of resistance is of interest. The L5178Y/FUdR subline, which was selected by only four treatments with FUdR, required more than 650-fold as much for 99% inhibition on the cell proliferation as did the parent line. On the other hand, acquisition of resistance to FU needed ten treatments with FU, with a step-wise increment in the resistance, and eventually, the inhibitory concentration of FU for this line became 80-fold greater than that required for the parent line. It is evident that an establishment of a subline resistant to FU was more difficult. Difference in the cytotoxicity between FU and FUdR was examined previously,¹⁰⁾ and it was revealed that the inhibitory effect of FUdR by short exposure, even at a high concentration, disappeared within a week after removal of the drug from the culture medium. This fact is considered to be the reason why it is easier to establish the cell lines resistant to FUdR. On the other hand, the exposure to FU or FUR, even at a low concentration, caused a substantial and irreversible inhibition on cell proliferation. This might be one of many factors involved in the difficulty in the selection of FU-resistant tumor cells.

FU is thought to exert its cytotoxic effects by either or both of two separate biochemical mechanisms: The inhibition or alteration of RNA maturation and function by fluorouridine 5'-triphosphate (FUTP) incorporation into RNA,¹¹⁻¹⁴⁾ and the inhibition of DNA formation through the blockade of thymidylate synthetase by 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP).¹⁵⁻¹⁹⁾ These effects on DNA and RNA are also known to be exerted by FUdR and FUR,

respectively, which are rapidly converted *in vivo* and *in vitro* from FU.²⁰⁾ However, the relative importance of effects of DNA and RNA on the cytotoxic effect of FU remains unknown. By comparing the character of resistance to FU with that to FUR and FUdR, the relative importance of the DNA and RNA effects can be clarified. Observations on properties of development of resistance and cross-resistance in this experiment suggest that the cytotoxic effect of FU are similar to those of FUR rather than FUdR. Further studies on mechanism of resistance of those 3 sublines at enzyme level will make apparent the mechanism of cytotoxic effect of FU.

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