MECHANISM OF ADRIAMYCIN RESISTANCE IN A SUBLINE OF MOUSE LYMPHOBLASTOMA L5178Y CELLS

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The biochemical mechanism of anthracycline resistance was studied with an adriamycin-resistant subline of mouse lymphoblastoma L5178Y cells. Both uridine and thymidine uptakes in the resistant cells were observed more resistant to adriamycin and daunorubicin than those in the parental cells. Aclacinomycin A exhibited the same degree of inhibition of nucleic acid syntheses in the sensitive cells and in the resistant cells. The resistance pattern observed by the inhibition of RNA and DNA syntheses seemed to parallel that by growth inhibition. No significant difference was demonstrated between the parental and resistant cells in the inhibition of RNA and DNA polymerase reactions with isolated nuclei. The uptake and retention of [^{3}H]adriamycin was observed significantly less in the resistant cells than in the sensitive cells. The results suggested that the adriamycin resistance may be due to alteration of the cytoplasmic mambrane and/or cytoplasm, resulting in decreased uptake and retention of the antibiotic in the resistant cells.

Anthracycline antibiotics, adriamycin and daunorubicin, are effective on a variety of human malignant tumors, which sometimes aquire resistance to the antibiotics. The mechanism by which human neoplasms become unresponsive to the drugs is not known. For the purpose of studying the mechanism of anthracycline resistance, an adraimycin-resistant mutant subline of mouse lymphoblastoma L5178Y cells has been isolated by the treatment of the culture with N-methyl-N'-nitro-N-nitrosoguanidine[1]. Since the resistant subline exhibits an unique cross resistance pattern with other tumor-inhibitory antibiotics, we have further investigated the biochemical mechanism of resistance, and the results are presented in this publication.

Materials and Methods

[^{3}H]Uridine (41.3 Ci/mmole) and [^{3}H]thymidine (56.9 Ci/mmole) were purchased from New England Nuclear, Boston, Mass.; [^{3}H]UTP (40.0 Ci/mmole) and [^{3}H]TTP (41.0 Ci/mmole) from Radiochemical Centre, Amersham, England. [^{3}H]Adriamycin (28.88 mCi/mmole; 49.8 μCi/mg), a product of Farmitalia, was generously given by Kyowa Hakko Co., Ltd., Tokyo. The radiochemical purity was higher than 98% by TLC.

Incorporation of [^{3}H]uridine and [^{3}H]thymidine were carried out by a method described previously[2], except that the substrate and drug were added to the culture medium simultaneously. Preparation of nuclei from L5178Y cells and DNA polymerase reaction with isolated nuclei followed the procedure described previously[3]. RNA polymerase reactions with isolated nuclei were performed according to the method of MARZLUFF *et al.*[4]. The incubation was carried out at 25°C for 30 minutes in the case of RNA polymerase reaction, and at 37°C for 20 minutes in DNA polymerase reaction.

Uptake and retention of [^{3}H]adriamycin in L5178Y cells:

The incorporation of adriamycin into the cells was measured after centrifugation or Millipore filtration. The cell suspension (3×10^6/ml) was incubated with [^{3}H]adriamycin at 4, 8 or 44 μg/ml in 0.5 ml of FISCHER's medium supplemented with 10% horse serum at 37°C for various periods. The mixture was then chilled in an ice bath, and diluted with 4 ml of PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO_4·

$12H_2O$ 2.9 g and KH_2PO_4 0.2 g in a liter of redistilled water). The cells were collected by centrifugation or by filtration, and the radioactivity was determined in a liquid scintillation counter. In the case of centrifugation, the pellets were washed twice with 4 ml of PBS and solubilized by incubating with 0.5 ml of protosol (New England Nuclear) for 1 hour at 50°C. In the case of filtration, the cells were collected on Millipore filters and washed 3 times with 3 ml of PBS. The 3 times washings were enough to remove unbound [^{3}H]adriamycin and gave constant values. For LINEWEAVER-BURK's plot studies, the incubation was carried out for 3 times in the presence of [^{3}H]adriamycin concentrations ranging from 1.6 μg/ml to 51.2 μg/ml, and the radioactivity collected on Millipore filters was determined. All the values were calculated with correction for those obtained in parallel mixtures, containing the same concentrations of [^{3}H]adriamycin, without incubation: *i.e.* 0 time.

For retention studies, [^{3}H]adriamycin was incorporated into the cells (3.2×10^6/ml) by incubating with [^{3}H]adriamycin at 5 μg/ml for the parental cells or 15 μg/ml for the resistant cells at 37°C for 20 minutes in 10% horse serum FISCHER's medium. The cells were then washed 3 times with PBS at 4°C, and suspended in the same medium: 2.1×10^6 cells/ml for the parental cells and 2.84×10^6 cells/ml for the resistant cells. The radioactivity, collected on Millipore filters, was determined after incubating at 37°C for various periods.

Results

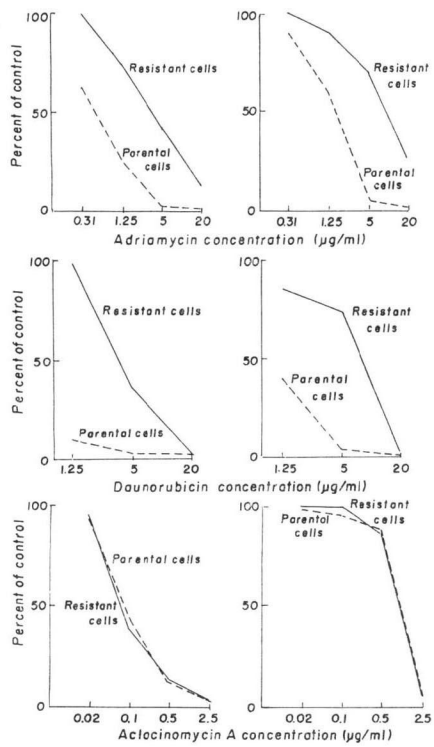
The Effects of Anthracycline Antibiotics on Nucleic Acid Syntheses in Intact Cells of Parental and Adriamycin-resistant L5178Y

RNA and DNA syntheses were studied by the incorporation of [^{3}H]uridine and [^{3}H]thymidine into a TCA-insoluble fraction of intact L5178Y cells of sensitive (parental) and resistant sublines. As illustrated in Fig. 1, both RNA and DNA syntheses in the resistant cells were observed more resistant to adriamycin and daunorubicin than those in the sensitive cells. By comparison of 50% inhibition, the degree of inhibition was 5~10 times greater in the parental cells than in the resistant cells. The same degree of inhibition by aclacinomycin A of nucleic acid syntheses was demonstrated in the sensitive and resistant cells. The resistance pattern, thus demonstrated by the inhibition of uridine and thymidine uptakes seemed to parallel that observed by the growth inhibition[1].

Fig. 1. Effects of anthacycline antibiotics on RNA and DNA syntheses in intact cells of parental and adriamycin-resistant L5178Y.

The left column: incoporation of [^{3}H]uridine (100% uptake=14,000 cpm/10^5 parental cells; 13,000 cpm/10^5 resistant cells).

The right column: incorporation of [^{3}H]thymidine (100% uptake=42,000 cpm/10^5 parental cells; 30,000 cpm/10^5 resistant cells).



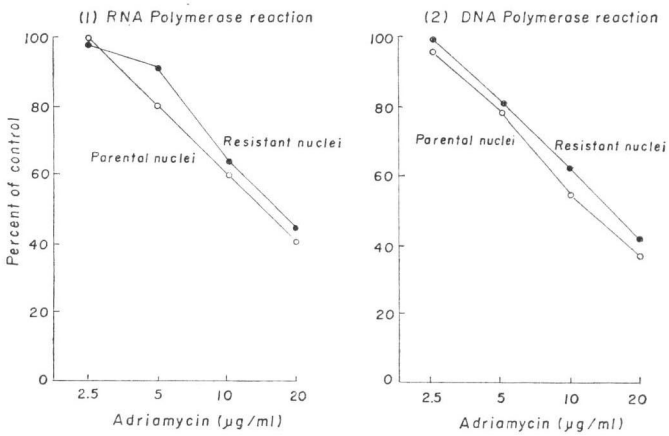
The Effect of Adriamycin on RNA and DNA Polymerase Reactions in Nuclei Isolated from Parental and Adriamycin-resistant Cells

The effect of adriamycin on RNA and DNA polymerase reactions were examined with isolat-

ed nuclei of the sensitive and resistant sublines. Both RNA and DNA polymerase reactions were blocked by the antibiotic, and no significant difference of the inhibition degrees was demonstrated between the parental and resistant cells (Fig. 2). The results indicated that the drug resistance may be absent in the nuclei; and the resistance, observed in the intact cells, may exist in the cytoplasmic membrane and/or cytoplasm.

Fig. 2. Inhibition by adriamycin of RNA and DNA polymerase reactions in nuclei isolated from parental and adriamycin-resistant L5178Y cells at various antibiotic concentrations.

(1) RNA polymerase reaction. [^{3}H]UMP incorporated into the parental nuclei was 1,375 cpm/2×10^6 nuclei, and that into the resistant nuclei 2,833 cpm/2×10^6 nuclei. (2) DNA polymerase reaction. [^{3}H]TMP incorporated into the parental nuclei was 1,756 cpm/10^6 nuclei, and that into the resistant nuclei 2,663 cpm/10^6 nuclei.

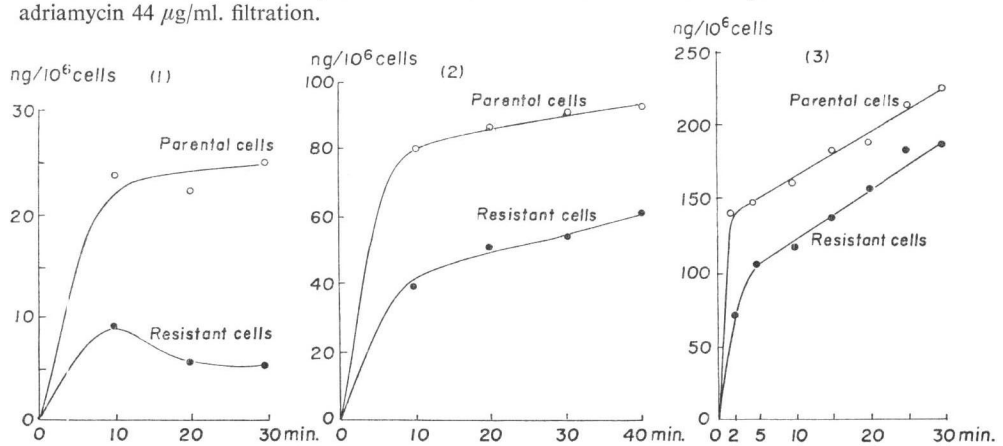


Accumulation (Uptake and Retention) of [^{3}H]Adriamycin in the Parental and Adriamycin-resistant Cells of L5178Y

The uptake and retention of [^{3}H]adriamycin was studied by filtration and centrifugation methods. The latter showed lower values than the former, probably because of leakage of [^{3}H]adriamycin from the cells during repeated centrifugations. The time dependency of accumulation of [^{3}H]adriamycin in the parental and resistant cells are presented in Fig. 3. The uptake of the antibiotic was observed higher in the sensitive cells than in the resistant cells. The difference in the accumulation seemed to reflect the sensitivity or resistance of the cells to the drug, and was dependent upon initial antibiotic concentrations in the medium: *i.e.* the difference was less as initial drug concentrations increased.

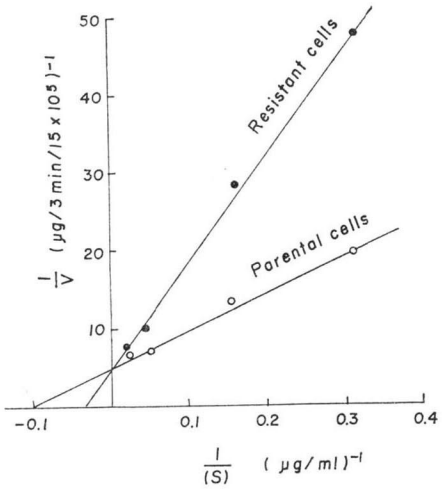
The LINEWEAVER-BURK plot of the initial rate of [^{3}H]adriamycin incorporation in the parental and

Fig. 3. Uptake of [^{3}H]adriamycin by parental and adriamycin-resistant cells of mouse lymphoblastoma L5178Y. (1) [^{3}H]adriamycin 4 μg/ml. centrifugation. (2) [^{3}H]adriamycin 8 μg/ml. filtration. (3) [^{3}H]-adriamycin 44 μg/ml. filtration.



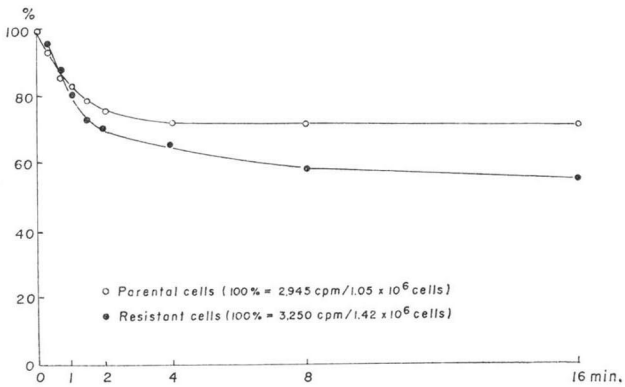
resistant cells showed linear relationship between 1/V and 1/S, where V is an initial rate of uptake of the drug and S is an initial antibiotic concentration. The apparent *Km* of adriamycin for the sensitive cells was approximately 1.8×10^{-5} M, and that for the resistant cells 4.8×10^{-5} M: *i.e.* the former bound adriamycin somewhat more tightly than the latter (Fig. 4).

Fig. 4. LINEWEAVER-BURK plot of the initial rate of uptake of adriamycin in parental and adriamycin-resistant L5178Y cells.



Since the accumulation of adriamycin in the cells could be due to differential rates of uptake and release by the cells and affinities for the chemoreceptors (DNA, tubulin *etc.*), the efflux of adriamycin from the cells was studied with the parental and resistant cells (Fig. 5). The resistant cells exhibited a slightly higher tendency to release the antibiotic than the sensitive cells. However, the difference seemed not to be high enough to cause, by itself, the difference of adriamycin accumulation in both cells (compare with Fig. 3). Therefore it is concluded that the adriamycin resistance may be attributed to both decreased incorporation and retention of the drug in the resistant cells.

Fig. 5. Efflux of [^{3}H]adriamycin from parental and resistant L5178Y cells.



Discussion

The biochemical mechanism of anthracycline resistance in mouse lymphoblastoma L5178Y cells has been studied in the current experiments. Since RNA and DNA polymerase reactions with nuclei isolated from the resistant cells are sensitive to adriamycin at the same level as those from the parental cells, and uptake and retention of [^{3}H]adriamycin are decreased in the resistant cells, the adriamycin resistance may be attributed to alteration of the cytoplasmic membrane and/or cytoplasm, resulting in decreased uptake and retention of the drug in the resistant subline of L5178Y cells.

The results seem to be in accord with those obtained in other 3 types of tumor cells, in which anthracycline resistance is due to permeability changes[5~10]. MEDOFF *et al.*[11] reported that amphotericin B induces sensitivity to actinomycin D in drug-resistant HeLa Cells. On the contrary, amphotericin B does not overcome the resistance in the adriamycin-resistant subline of L5178Y cells[1]. Therefore, if the cytoplasmic membrane is involved in the resistance, some mechanism, which is not affected by amphotericin B, may be the cause of this type of adriamycin resistance.

The resitant cell line used here has an unique cross resistance pattern[1]. It has been further confirmed by the sensitivity of nucleic acid syntheses in the intact cells (cf. Fig. 1), where the resistant cells show a cross resistance with daunorubicin but not with aclacinomycin A. The results suggest that the mechanism of drug uptake into the cells may have some different aspects among the anthracycline antibiotics.

There is another possibility that the resistance may be due to metabolic alteration in the resistant cells, with respect to inactivation or activation. It has been attempted without success to demonstrate enzymic activity of inactivating the antibiotic in extracts of the resistant cells (data are not shown).

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