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Comparison of Anthracycline Concentrations in S180 Cell Lines of Varying Sensitivity*

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Abstract—*Sublines of sarcoma 180 (S180) of varying sensitivity to adriamycin (ADR) have been selected in culture. The degree of resistance of these sublines ranged from 6- to 125-fold above that of parent S180 cells. ADR-resistant sublines demonstrated comparable degrees of cross-resistance to daunomycin (DNR), marcellomycin and AD 32, but each subline showed a uniform degree of tolerance toward actinomycin D and vincristine. Compared to the anthracycline-sensitive parent tumor, a 40% decrease in the intracellular steady-state level of [^{3}H]-daunomycin was observed in all sublines regardless of the degree of resistance. The level of cell-associated DNR and ADR observed after administration of equipotent concentrations of drug was different for each cell line and increased in proportion to the drug concentration. Thus, altered drug permeability appeared to be of minimal importance in the expression of high levels of resistance. In addition, the extent of DNR metabolism by the anthracycline-resistant sublines was not sufficiently different from that seen in parent S180 cells to account for the observed tolerance to these agents.*

INTRODUCTION

RESISTANCE to ADR, an anthracycline antibiotic used extensively in the treatment of a variety of malignant tumors [1], has been described in many cultured cell lines [2–9]. Reduced uptake of this agent, and its congener DNR, has been reported in anthracycline-resistant P388 leukemia [5–7], Ehrlich carcinoma [2, 3, 9–13] and Chinese hamster ovary cells [8, 14]. The mechanism of decreased net intracellular drug has been proposed to be the result of enhanced efflux of the drug by the resistant population [2, 6, 7, 9, 11, 12]. Mutually decreased drug permeability has also been found in vincristine-resistant cell lines which are cross-resistant to ADR and DNR [7, 14]. In this paper we report the development in culture of a series of S180 cell lines exhibiting progressive increases in their degree of insensitivity to ADR. These sublines were characterized with respect to cross-resistance to other chemotherapeutic agents and for permeability to ADR and DNR. In addition, the metabolism of [^{3}H]-DNR was also examined. The results indicate that reduced uptake or increased efflux of the anthracyclines are not the sole determinants of resistance in all of the sublines. Changes in drug metabolism also cannot account for the observed tolerance to the anthracyclines. Preliminary results of this study have appeared elsewhere [15].

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Abbreviations: S180, sarcoma 180; ADR, adriamycin or doxorubicin; DNR, daunomycin or daunorubicin; AD-32, *N*-trifluoroacetyl adriamycin-14-valerate; DNMOL, daunomycinol; DNMONE, daunomycinone; EMS, ethylmethane sulfonate; HPLC, high pressure liquid chromatography; ID_{50}, drug concentration at which half-maximal effect is achieved.

MATERIALS AND METHODS

Chemicals

ADR (NSC No. 123127) and DNR (NSC No. 82151) were the gift of Dr. John D. Douros of the Division of Cancer Treatment, National Cancer Institute. AD-32 and DNMOL were generously supplied by Dr. Mervyn Israel of the Sidney Farber Cancer Institute. Marcellomycin, bleomycin

sulfate and mitomycin C were kindly provided by Dr. Maxwell Gordon of the Bristol Laboratories (Syracuse, NY). Vincristine was purchased from Eli Lilly and Co. (Indianapolis, IN). Actinomycin D and 5-fluorouracil were obtained from Sigma Chemical Co. (St. Louis, MO). [14-^{14}C]-ADR (sp. act. 17 mCi/mmol) was the gift of Dr. John D. Douros of the National Cancer Institute. [^{3}H(G)]-DNR (sp. act. 1.3 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). EMS was obtained from Aldrich Chemical Co. (Milwaukee, WI). DNMONE was prepared by mild hydrolysis of the parent compound. This was accomplished by heating DNR to 60°C in 0.1 N HCl for 24 hr.

Stock drug solutions were prepared in 95% ethanol and stored in the dark at -20°C. The concentrations of anthracycline solutions were determined spectrophotometrically, and purity of these agents was ascertained by HPLC according to the method of Israel *et al.* [16]. Analysis was performed on a Dupont 830 chromatograph using a Waters μ-Bondapak phenyl reverse phase column. The mobile phase consisted of 30% CH_3CN in 0.05 M ammonium formate adjusted to pH 4.0 with formic acid. The column was monitored with a Perkin–Elmer 204-S fluorescence detector at an excitation wavelength of 485 nm and an emission wavelength of 595 nm.

Cell culture

S180 cells and ADR-resistant sublines were grown in Fischer's medium supplemented with 10% horse serum, 100 units/ml penicillin and 100 μg/ml streptomycin (Grand Island Biological Co., Grand Island, NY). For soft agar cloning, cells were diluted into medium containing 15% horse serum. Immediately before cloning, sterilized Noble agar solution (DIFCO Laboratories, Detroit, MI) was heated in a boiling water bath, diluted with medium and cooled to 37°C. The cell suspensions were mixed with the agar solution and incubated for 10–20 days, depending on the generation time of the cell line. The final agar concentration was 1.2 mg/ml.

Cytostatic activity in culture

S180 cells and ADR-resistant sublines were diluted with medium to give a cell density of 10^4 cells/ml. Appropriate dilutions of stock drug solutions were made in either 70% ethanol or sterile phosphate-buffered NaCl and added to cell suspensions. Cell numbers were determined in triplicate on a Coulter particle counter until the plateau phase was reached. For cloning experiments, cells were exposed to drug for 2 hr. Serial dilutions were then made in cloning medium. The concentration of drug which produced a 50% inhibition of cellular division or of cloning efficiency (ID_{50}) was determined from logarithmic plots of the percent of control growth or survival.

Development of resistant S180 sublines

ADR-resistant S180 sublines were selected by long-term exposure of cells to increasing concentrations of ADR in suspension culture. Treatment with ADR was begun at 0.1 μM, a concentration which inhibits the growth of S180 cells by about 5%. Cells which appeared to tolerate the doses of ADR employed were cloned as described above. Individual clones were isolated, cultured for 7 days in drug-free medium and tested for ADR resistance by measuring the ID_{50} in continuous culture or in the cloning assay as described above. The degree of resistance was defined as the ratio of the ID_{50} of each subline to that of parent S180 cells. Cells derived from isolated colonies which showed resistance (sublines A1 and A3) were maintained in suspension culture with non-inhibitory concentrations of ADR.

The mutagen EMS was also used to enhance the development of resistant populations employing the method of Friedrich and Coffino [17]. S180A3 cells were exposed to 2 mM EMS for 1 hr. The cultures were grown for 4 days and were then exposed for 1 hr to 1 μM ADR after which the cells were cloned in medium containing 2 μM ADR. Clones were isolated and tested for resistance to ADR by measuring the ID_{50} in continuous culture. The resulting resistant sublines (A5 and A10) were maintained in culture with ADR at a sub-inhibitory concentration. Prior to any experimentation with resistant cell lines, ADR present in the growth medium was removed by dilution of the drug by at least a factor of 100.

Uptake and efflux of [^{3}H]-DNR

Uptake and efflux of [^{3}H]-DNR was studied in cells in late exponential growth. Cells were resuspended at a concentration of 10^6 cells/ml in Hanks' balanced salt solution at pH 7.4 (Grand Island Biological Co., Grand Island, NY). After 15 min of incubation, [^{3}H]-DNR (final concentration 0.1 μM) was added and 0.2 ml samples of the cell suspension were withdrawn in duplicate over a period of 40 min. Samples were directly added to 1.0 ml of ice-cold 0.9% NaCl and centrifuged in a Beckman Microfuge B. Cell pellets were washed once with ice-cold 0.9% NaCl. Cells were lysed in 0.5 ml of distilled H_2O and transferred to scintillation vials and counted in Hydrofluor (National Diagnostics) using a Beckman LS 7500 liquid scintillation counter. Efflux was assessed by the method of Skovsgaard [12]. Zero time

uptake was obtained by immediate processing of the samples prior to transport occurring.

Levels of cell-associated [^{3}H]-DNR and [^{14}C]-ADR in proliferating S180 cell lines at a density of 10^5 cells/ml were also determined after a 2-hr exposure to the ID_{50} concentration of each drug. Samples (1.0 ml) were withdrawn in triplicate and treated as described above. Bound material was measured by determining radioactivity after exhaustive extraction of cell pellets with ethyl acetate:methanol (9:1,v/v).

Metabolism studies

ADR-sensitive and -resistant S180 cells in late exponential growth were exposed to [^{3}H]-DNR at concentrations of 0.25 and 0.50 μM respectively. At 0 and 24 hr, samples of 2×10^6 cells were collected by centrifugation, washed and lysed in distilled H_2O.

Prior to extraction with ethyl acetate and *n*-propanol (9:1,v/v) in the presence of 2% Na_2CO_3 (pH 9.0), unlabeled standards of DNR, DNMOL and DNMONE were added to the cell pellets. Four extraction volumes of 5 ml each were combined, dried under N_2 and resuspended in mobile phase prior to injection onto the HPLC column. Analysis by HPLC was performed as described above. Extraction efficiency was >98% for DNR and DNMONE and >95% for DNMOL.

RESULTS

Resistance of S180 sublines towards ADR

Table 1 shows the concentration of ADR producing 50% inhibition of (a) cell replication in the continuous presence of drug and (b) cloning efficiency after a 2-hr exposure to the anthracycline for S180 cells and ADR-resistant sublines. These data were derived from linear regression analyses of complete dose–response curves (not shown). A progressive increase in the degree of resistance to ADR was expressed by the S180 sublines A1, A3, A5 and A10. The degree of resistance of each subline was equivalent whether measured by growth in culture or by cell survival.

The doubling time of the cell population during exponential growth was progressively increased in sublines of increasing resistance to ADR. The mean population doubling time for parent S180 cells was 15.6 hr, while for the resistant sublines A1, A3, A5 and A10 the mean doubling times were 17.4, 20.2, 23.3 and 25.6 hr respectively. These values were statistically distinct for each subline ($P \leqslant 0.05$). It is difficult to conceive of a mechanism whereby drug sensitivity can be directly coupled to the cellular growth rate; in support of this, we have isolated a clone of S180A3 that maintained its 10-fold resistance to adriamycin but which had the normal doubling time of the parent S180 subline. Consequently, we do not believe that growth rate *per se* is an important facet of drug resistance. The median cell volumes of the ADR-resistant sublines, as determined with a Coulter particle counter equipped with a Channalyzer, were generally less than the median cell volume of the parent S180 cells; however, no correlation between cell volume and the degree of tolerance to ADR was observed. Thus the cell volumes of the S180A1, A3 and A5 sublines all were approximately 10% lower than the volume of parent S180 cells, while the difference in cell volume between S180 and S180A10 cells was 27%.

Cross-resistance to other chemotherapeutic agents

The sensitivity of the ADR-resistant sublines to the cytoxic action of other antineoplastic agents is shown in Table 2. The degree of resistance toward each agent is expressed as the ratio of the ID_{50} concentration of that agent for the subline under test compared to the parent S180 cells. The degree of resistance of the sensitive parent line is defined as 1.0.

Table 1. Degree of ADR resistance in S180 cell lines

	Growth in culture		Cloning	
Cell line	ID_{50}(μM)*	Degree of resistance†	ID_{50}(μM)	Degree of resistance
S180	0.35 ± 0.02‡	1.0	0.92 ± 0.08	1.0
S180A1	2.4 ± 0.1	6.7	4.8 ± 0.1	5.2
S180A3	3.9 ± 0.1	11.1	10.0 ± 0.9	10.9
S180A5	14.1 ± 0.1	39.8	19.9 ± 0.1	21.7
S180A10	43.4 ± 0.2	122.0	116.0 ± 1.0	127.0

* The concentration of ADR which resulted in 50% inhibition of growth or cloning efficiency (ID_{50}) for each cell line was calculated from linear transformation of dose–response curves.

† Degree of resistance is expressed as the ratio of the ID_{50} of each subline to the ID_{50} of S180 cells.

‡ ID_{50} ± the 95% confidence limit of the line at $y = 50$.

Table 2. Relative resistance of S180 sublines to cancer chemotherapeutic agents

Agent	Degree of resistance S180A1	S180A3	S180A5	S180A10
Daunomycin	—	10.8	76.9	154
AD-32	—	21.5	30	—
Marcellomycin	—	—	50	100
Actinomycin D	10.0	10.0	7.0	9.0
Vincristine	—	7.5	11.9	—
Mitomycin C	—	0.5	—	—
Bleomycin	—	—	0.3	0.8
5-Fluorouracil	—	—	0.8	—

The concentration of each agent which resulted in 50% inhibition of growth in culture in the continual presence of each agent was determined from linear transformation of dose–response curves as in Table 1. The degree of resistance relative to S180 cells was determined as the ratio of the ID_{50} of each subline to the ID_{50} of S180 cells.

The S180 sublines A3, A5 and A10 showed a progressive increase in tolerance to the anthracylines DNR, AD-32 and marcellomycin which paralleled the progressive increase in resistance to ADR. Because of limited solubility of AD-32, the ID_{50} of this agent was not determined for the S180A10 subline. The cytotoxic activity of actinomycin D and vincristine was also examined. S180A1 exhibited a 10-fold increase in resistance to actinomycin D, but tolerance toward this agent did not increase further in sublines A3, A5 and A10. Resistance to vincristine was also equivalent in the A3 and A5 sublines. No cross-resistance was observed toward 5-fluorouracil, mitomycin C or bleomycin.

Uptake and efflux of [^{3}H]*-DNR in S180 cell lines*

To determine whether alterations in transport and retention of drug was involved in the mechanism by which cells expressed their resistance to anthracyclines, the time course of [^{3}H]-DNR uptake and efflux was compared in each cell line at a concentration of 0.1 μM DNR.

The inset of Fig. 1 illustrates representative time courses for S180 sensitive and resistant sublines. The steady state after efflux decreased by 44% from that observed after drug uptake in sensitive cells and 40% in resistant cells. Zero-time uptake was equivalent in all cell lines and was proportional to the drug concentration administered. The zero-time uptake was also equivalent to that seen over 60 min at 0°C. This level was consistently about 10% of the steady-state level at all drug concentrations. At a level of 0.1 μM [^{3}H]-DNR the zero-time uptake was 2.0 pmol/10^6 cells. A steady-state concentration of 23 pmol/10^6 cells was achieved in S180 cells in approximately 20 min. This level corresponds to a concentration gradient (inside/outside) of 20-fold. A new steady state of 13 pmol/10^6 cells was attained in less than 5 min after dilution of the [^{3}H]-DNR with unlabeled DNR. For resistant cells, an uptake steady-state level of 16 pmol/10^6 cells was observed. This corresponds to a concentration gradient (inside/outside) of 16-fold. A new steady state of 11 pmol/10^6 cells was observed after efflux in resistant cells.

The upper panel of Fig. 1 shows a summary of the relative steady-state quantities of [^{3}H]-DNR after uptake and efflux in the anthracycline-resistant sublines expressed as a percent of the control S180 steady-state level. All of the resistant sublines showed decreased ability to transport and retain drug when compared with S180 cells. Approximately a 40% reduction in the steady-state level of [^{3}H]-DNR was observed in each anthracycline-resistant subline both before and after dilution of the radiolabeled compound when compared to that occurring in parent S180 cells, regardless of the degree of resistance. Although the difference between parent S180 cells and each anthracycline-resistant subline was statistically significant ($P \leqslant 0.01$), the resistant sublines were not significantly different from each other. When the observed steady-state levels of [^{3}H]-DNR were corrected for the differences in cell volume between S180 sublines the same conclusion was reached.

To further assess the impact of differences in anthracycline levels found in ADR-sensitive and -resistant S180 sublines, the amount of drug associated with cells at equipotent concentrations of anthracycline was studied. Cells were exposed to both [^{3}H]-DNR and [^{14}C]-ADR in concentrations proportionate to the degree of resistance and the amount of cell-associated drug in anthracycline-resistant cells was compared to that of sensitive parent cells. Each cell line showed a

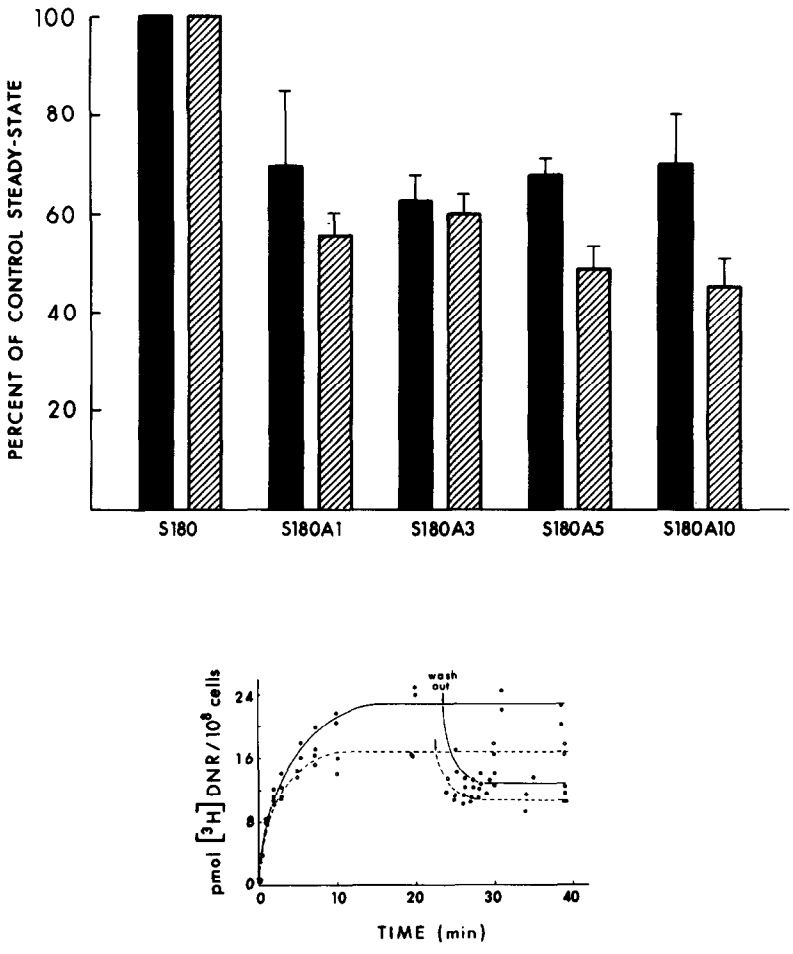

Fig. 1. Comparison of [^{3}H]*-DNR steady-state levels in sensitive and anthracycline-resistant* S180 *cell lines.* Solid boxes, *percent* S180 *steady-state level after exposure to* 0.1 μM *DNR;* hatched boxes, *percent* S180 *steady-state level after efflux. Inset: representative time courses of DNR uptake and efflux.* Solid circles, S180 *cells;* open circles, *drug-resistant subline (*S180*A3 used as an illustrative example). Arrow indicates time of addition of unlabeled drug.* Bars, *S.E. for* 2–4 *experiments.*

distinct level of drug at the concentration which resulted in equal inhibition of growth (Table 3). Moreover, the amount of both DNR and ADR increased in each cell line as a function of the concentration of anthracycline to which the cells were exposed. This indicates that a particular cellular level of drug is not associated with the degree of growth inhibition. Again, the amount of labeled material found associated with the resistant cell lines was about 40% less than the quantity which was found to be associated with S180 cells when the drug-sensitive cells were

Table 3. Drug content of S180 *cell lines at equipotent concentrations*

Cell line	Anthracycline	Concentration (μM)	Cell-associated radioactivity (pmol anthracycline/10^5 cells)	Cell-associated radioactivity after exhaustive extraction (pmol/10^5 cells)
S180	DNR	0.1	4.3 ± 0.3*	0.24 ± 0.4
S180A1		0.5	14.7 ± 1.1	1.9 ± 0.3
S180A3		1.0	29.8 ± 0.8	4.0 ± 0.6
S180A5		5.0	142.0 ± 4.9	6.6 ± 1.0
S180	ADR	0.07	0.58 ± 0.2	n.d.†
S180A3		1.0	6.6 ± 1.0	n.d.
S180A5		5.0	19.2 ± 2.5	n.d.

Cells were incubated for 2 hr at an extracellular drug concentration near the ID_{50} for each cell line.
*Mean ± S.E. for 4 determinations.
†n.d., not done.

exposed to an equal concentration of drug (data not shown). This result is in agreement with the findings reported in Fig. 1. Bound material which was not extractable with ethyl acetate:methanol was found to be present in relatively low concentrations in all cell lines (Table 3). Thus at the DNR ID_{50} value the resistant cells were found to contain from 5- to 30-times more bound [^{3}H]-DNR than sensitive cells. The amount of bound material was in general proportional to the drug concentration to which the cells were exposed. Bound material in S180 and S180A5 cells was approximately 5% of total cell-associated drug; in S180A1 and A3 sublines about 13% of cell-associated drug was non-extractable.

The amount of cell-associated [^{3}H]-DNR remained constant over periods of up to 72 hr in proliferating cultures of anthracycline-sensitive and -resistant cells exposed to concentrations of this agent which resulted in 10% inhibition of cell replication (data not shown), indicating that after the steady-state is reached the influx and efflux processes are in equilibrium, even if cell division occurs.

Metabolism of [^{3}H]*-DNR by S180 cell lines*

Differences in DNR metabolism between neoplastic cells susceptible and resistant to the anthracyclines have not been previously reported; however, it was possible that changes in production of the inactive aglycone or in the 13-alcohol derivative, the major metabolites of ADR and DNR, might be responsible for the observed resistance. Thus the metabolic conversion of DNR to these forms was studied in S180, S180A3 and S180A10 cells exposed to [^{3}H]-DNR for 24 hr. The concentration of drug to which S180 cells were exposed was 0.25 μM whereas A3 and A10 sublines were treated with 0.5 μM DNR. Under these conditions approximately equal intracellular levels of drug were achieved in all three sublines (Table 4).

Little net metabolism of [^{3}H]-DNR was observed in either S180 cells or the anthracycline-resistant cell lines over a 24-hr period. The net production of DNMONE and DNMOL represents the percentage of metabolite present after subtraction of the percentage present at zero-time. Such a calculation takes into account any impurity present in the labeled DNR and any structural change which was produced during the extraction procedure, either because of residual enzymatic activity during washing and handling of the cells or because of non-enzymatic conversion from the extraction process itself. No significant differences were found between parent S180 cells and anthracycline-resistant sublines A3 and A10. A significant decrease in aldo-ketoreductase activity from that of S180 cells was inferred from the metabolism in the two resistant sublines, however ($P \leqslant 0.05$); S180 cells produced 6.3% DNMOL over 24 hr, while the S180A3 and S180A10 sublines produced 1.3 and 0% (no net production), respectively, over the same period. The resistant sublines were therefore less able to convert DNR to DNMOL.

DISCUSSION

Sublines of sarcoma 180 cells have been selected in culture which show a progressive increase in resistance to adriamycin. The degree of resistance varied from 6- to 125-fold that of the parent cells, and was equivalent whether measured by inhibition of cellular replication in the continual presence of drug or by decreased cell survival after a 2-hr period of exposure to drug. In cloning experiments, effects of drug efflux should be maximized by dilution and growth of cells in

Table 4. Metabolism of [^{3}H]*-DNR in anthracycline-sensitive and -resistant cell lines*

DNR metabolite	Percent* S180	S180A3	S180A10
DNMONE	7.1 ± 0.7† (0.1)‡	4.6 ± 1.1 (0)	6.6 ± 1.1 (0)
DNMOL	8.5 ± 2.8 (6.3)	3.5 ± 0.5 (1.3)	1.8 ± 0.2 (0)

Cells were incubated for 24 hr at an extracellular concentration of 0.25 μM for S180 and 0.50 μM for S180A3 and S180A10 sublines. Cell-associated drug was extracted as described in the text. The procedure employed resulted in >98% recovery of DNR and DNMONE and >95% recovery of DNMOL.

* A minimum of 100,000 dpm was collected from the column at each injection, representing >90% of the injected material. One percent of the DNR present amounted to a minimum of 8500 dpm or 3.0 pmol.

† Mean ± S.E. for 4 determinations.

‡ Net production (% metabolite found − % metabolite present at zero-time).

drug-free medium, whereas in suspension experiments, since drug is continually present, a steady state will always be maintained and effects of efflux should be minimized. The equivalent degree of resistance observed with both assay systems implies that the factors important in resistance are operative during the steady-state exposure to the drug and are operative under conditions in which resistant cells can more efficaciously remove drug after the 2-hr exposure.

The S180 sublines developed were cross-resistant to three other anthracyclines (DNR, AD 32 and marcellomycin), with cells showing equivalent increases in tolerance to the anthracyclines as a group, even though the three agents tested have been tentatively assigned to different subclasses which possibly act through different mechanisms [18]. This result suggests that there is a common mechanism of resistance towards the anthracyclines as a class, which is possibly, although not necessarily, related to a common mechanism of action.

The anthracycline-resistant sublines also demonstrate some degree of resistance to vincristine and actinomycin D. A number of laboratories [2, 4–9, 11–14] have reported anthracycline-resistant cell lines which are mutually resistant to these two agents. A general decrease in intracellular levels due to a decrease in uptake and/or increase in drug efflux has been suggested to be the mechanism of resistance to actinomycin D, the vinca alkaloids and the anthracyclines [2, 4, 5, 14, 19, 20]. In the case of the S180 sublines reported here, the S180A1 line, which expressed a 6-fold increase in the degree of resistance to the anthracyclines, also showed a 10-fold increase in resistance to actinomycin D. However, even though the degree of resistance to the anthracyclines increased progressively in all of the other sublines (up to 125-fold in the S180A10 subline), the degree of resistance to actinomycin D and to vincristine remained constant at about 10-fold. This finding suggests that the higher degree of tolerance expressed by the S180A3, S180A5 and S180A10 sublines may be relatively specific for the anthracyclines and re-emphasizes our conclusion that resistance probably results from mechanisms other than through differences in net drug accumulation.

No increase in tolerance to the antimetabolite 5-fluorouracil [19], the bioreductive alkylating agent mitomycin C [21] or to bleomycin, an agent which has been shown to generate superoxide [22], was observed. This finding indicates that resistance to the anthracyclines is not a non-specific effect which is exhibited towards all drugs regardless of mechanism of action. It further suggests that a general ability to detoxify either oxygen-free radicals or activated alkylating agents is probably not involved in the resistance mechanism.

A decrease in cellular drug levels is the only mechanism of resistance which has been reported for the anthracyclines. In most studies with resistant cells, however, a set of cell lines expressing a graded response to drug action was not used, so it was only possible to compare drug levels between sensitive cells and a single resistant line [5–7, 23]. In the work presented here, levels of DNR were studied in sensitive and resistant cells under two conditions: the uptake and efflux of DNR was studied at the same concentration in all cell lines, and the drug levels of DNR were examined at concentrations of equal potency for each cell line. ADR-resistant cell lines all took up approximately 40% less drug than the sensitive cells at any given concentration, and contained 40% less drug after efflux of equilibrated DNR regardless of the degree of resistance to the anthracyclines. Furthermore, at equipotent concentrations of DNR or ADR different cellular levels were attained in each cell line, in direct proportion to the difference in concentration used. There was no one internal cellular level of drug which corresponded to 50% inhibition of survival in sensitive and resistant cells. In addition, the cellular drug levels did not change over time in either sensitive or resistant S180 cells. The amount of DNR which was non-extractable, and presumably bound to cellular material, was not decreased in resistant cells. At equipotent drug concentrations, the bound material was found to increase in each subline as the ID_{50} increased. This indicates that decreased drug retention is probably not responsible for resistance.

We have compared steady-state drug levels because the culture conditions in which the cells expressed resistance represent a closed system. Our results verify that once equilibrium is obtained it is constant, even while cells are dividing. In a comparison of uptake kinetics, we found that the K_m for transport of DNR for sensitive and resistant cells was similar: 120 μM and 80 μM respectively, far above the drug levels (0.01–0.1 μM) needed to cause cell kill (unpublished observations). Thus, although different kinetics are demonstrable, they most likely account only for resistance of the S180A1 cell line.

It was conceivable that resistance to the anthracyclines could be acquired by a change in metabolism. The aglycone is completely inactive when given directly, and the 13-alcohol is about one-half as active as intact DNR. The latter metabolite has been shown to be accumulated to a lesser extent than DNR itself, however, and may

be equally active at comparable intracellular drug levels [24]. Nevertheless, differences in the metabolism of DNR to DNMOL and DNMONE which could account for resistance were not seen in sublines A3 or A10 when compared to parent S180 cells. Metabolism was measured under conditions which yielded equal intracellular drug levels so that the amount of substrate would be constant. All of the S180 cell lines had little ability to metabolize DNR, in agreement with reports of low metabolism of this agent in other tissue culture cell lines [3, 25, 26]. A significant difference in the abilities of S180A3 and S180A10 to produce DNMOL as compared to the susceptible parent tumor occurred during the 24-hr period; thus resistant cell lines presumably had a lower level of aldo-ketoreductase activity than S180 cells. This finding cannot account for the expressed resistance, however, because the metabolite is about as potent as native drug inside the cell [24].

Because the degree of resistance to the anthracyclines observed in the A3, A5 and A10 sublines, and possibly in the A1 subline also, cannot be wholly accounted for by differences in the cell content of drug or in the metabolism of the anthracycline, other changes must be present which are responsible for the expressed resistance. It is quite possible that these unknown mechanisms of resistance have a direct bearing on the mechanism of action of these antibiotics.

We have recently demonstrated that adriamycin can be active without entering the cell [27]. Membrane interactions may therefore be critical to anthracycline cytotoxicity. For this reason, we are currently investigating membrane properties of anthracycline-sensitive and -resistant S180 cells.

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