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UPTAKE AND RETENTION OF ADRIAMYCIN AND DAUNORUBICIN BY SENSITIVE AND ANTHRACYCLINE-RESISTANT SUBLINES OF P388 LEUKEMIA

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Abstract—Sublines of P388 leukemia completely resistant to adriamycin (P388/ADR) or daunorubicin (P388/DAU) *in vivo* were studied *in vitro*. These sublines were more resistant to the cytotoxic effects of adriamycin (800-fold relative to sensitive parental cell line, P388/S) than to daunorubicin (18-fold for P388/ADR and 56-fold for P388/DAU). When the effects of the drugs on thymidine incorporation were compared *in vitro* in sensitive and resistant cells, it was observed that slightly higher levels of the drugs were required to inhibit nucleic acid synthesis in the resistant cells. The shift in inhibitory concentration was much less than the shift in cytotoxic concentration, particularly for adriamycin. The uptake and efflux of [G-^{3}H]daunorubicin and [14-^{14}C]adriamycin were studied. At low concentrations uptake of both drugs was impaired in the resistant sublines, whereas, at high concentrations a difference in uptake between sensitive and resistant cells was not evident. Resistance did not appear to be related to the difference in the rate of uptake. A markedly enhanced efflux of the drugs from the resistant cells was observed which correlated well with the difference in sensitivity of the sublines to adriamycin and daunorubicin. Enhancing the uptake of adriamycin by increasing the pH of the incubation medium and thereby increasing the proportion of non-ionized drug available for diffusion into the cells or by modifiying the cell membrane by the addition of Tween 80 failed to reverse resistance. The binding of daunorubicin to isolated nuclei from P388/S and P388/ADR cells was essentially similar. It is concluded that these anthracycline-resistant cell lines are resistant by virtue of decreased retention of the drugs.

The anthracycline antibiotics, daunorubicin and adriamycin, are effective in the treatment of a number of human tumors [1–3]. However, remissions or objective regressions of solid tumors, though dramatic, are often of short duration and resistance to subsequent treatment is observed [4].

Several studies have reported on the development and mechanism of resistance to anthracycline antitiotics in tumor cell lines. Danø developed sublines of Ehrlich ascites carcinoma resistant to daunorubicin [5] and adriamycin [6] using treatment *in vivo*. In these sublines, cross-resistance between anthracyclines and Vinca alkaloids was observed [7]. Evidence has been presented to suggest enhanced active outward transport of daunorubicin as a possible mechanism of resistance of a daunorubicin-resistant subline of Ehrlich ascites carcinoma [8]. Riehm and Biedler [9] developed resistance to daunorubicin in Chinese hamster cells *in vitro* and suggested decreased permeability of these cells to the drug as a mechanism of resistance. In addition, Kessel *et al.* [10] reported that the ability to retain daunorubicin *in vivo* was an important determinant of sensitivity to the drug in a number of tumor cell lines.

This paper describes studies of the mechanism of resistance of adriamycin- and daunorubicin-resistant sublines of P388 leukemia, a tumor highly sensitive to the anthracyclines [11]. The resistant sublines were completely refractory *in vivo* to maximally tolerated doses of adriamycin and daunorubicin [12]. The studies indicate that, although there is decreased uptake of anthracyclines by the resistant sublines, resistance is primarily due to a diminished capability to retain the antibiotics within the cell.

MATERIALS AND METHODS

P388 leukemia cell lines. Sublines of P388 leukemia resistant to adriamycin (P388/ADR) and daunorubicin (P388/DAU) were developed by treating $B6D2F_1$ mice bearing ascitic tumors with adriamycin or daunorubicin over successive transplant generations, as previously described [12]. Complete resistance *in vivo* to maximally tolerated doses of adriamycin and daunorubicin was evident after twelve transplant generations of exposure to the drugs. After eighty transplant generations of drug exposure, the adriamycin- and daunorubicin-resistant lines were split into two sublines; one was maintained in drug-treated mice and the other was transplanted without further drug treatment. Resistance proved to be stable for at least sixty transplant generations in the absence of drug. Resistant cells used in the present study were from sublines which were maintained in adriamycin- or daunorubicin-treated

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animals. However, treatment with the anthracyclines was discontinued one transplant generation prior to harvest of cells used in experiments described herein. The sensitive parental P388 leukemia (P388/S) was carried by weekly transplantation in untreated DBA/2 or B6D2F_1 mice. Sensitive and resistant cells were collected 7 or 8 days after transplantation and suspended in Eagle's minimum essential medium (MEM) containing 20% (v : v) fetal calf serum after removing erythrocytes by mild centrifugation.

Chemicals. [G-^{3}H]daunorubicin hydrochloride (14.1 mCi/m-mole) was a gift from Dr. Federico Arcamone, Farmitalia, Milan, Italy. [14-^{14}C]adriamycin hydrochloride (7.8 mCi/m-mole) was provided by Stanford Research Institute, Menlo Park, CA. Unlabeled adriamycin and daunorubicin were provided by the Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. The labeled compounds proved to be ⩾98 per cent chromatographically pure [silica gel thin-layer plates developed in chloroform–methanol–formic acid (75 : 25 : 2)] and radiochemically stable under the incubation conditions used in the experiments. [Methyl-^{3}H]thymidine (50.8 Ci/m-mole) was purchased from New England Nuclear (Boston MA). Tween 80 was purchased from Fisher Scientific Co. (Silver Spring MD).

Cytotoxicity assay in vitro. Cells (2×10^6/ml) were incubated in MEM in the presence of varying concentrations of adriamycin or daunorubicin for 1 hr at 37° in a shaking water bath. The cells were centrifuged, washed and resuspended in drug-free medium. These treated cells were inoculated i.p. into groups of eight B6D2F_1 mice (10^6 cells/mouse). The surviving fraction was determined by comparison of mean survival time of mice inoculated with drug-treated cells with that of mice inoculated with logarithmic dilutions (10^1 through 10^6) of cells incubated for 1 hr in the absence of drug [13].

Effects of drugs on DNA synthesis. Cells (2×10^6/ml) suspended in MEM were preincubated for 30 min at 37° in the presence of concentrations of adriamycin and daunorubicin between 0.1 and 100 μg/ml. [Methyl-^{3}H]-thymidine (0.1 μCi/2.0 pmoles/ml) was added followed by subsequent incubation for 30 min. Triplicate aliquots (0.25 ml) were placed on glass fiber filters (Whatman GF/C, 2.4 cm), washed twice with 3 ml of cold 5% trichloroacetic acid (TCA), followed by 2 ml ethanol and, finally, 1 ml ethanol–ether (1 : 1). The filters were placed in scintillation vials with 10 ml of scintillation fluid (LSC Complete, Yorktown Research, Yorktown, PA) and incorporated radioactivity was measured with a Beckman LS-250 liquid scintillation counter.

Cellular uptake and retention of labelled anthracyclines. Cells were suspended in MEM at 10^6 cells/ml and preincubated for 20 min at 37° in a shaking water bath. A solution of labeled anthracycline was added to give the desired final concentration. At appropriate time points triplicate 0.5-ml aliquots were removed and placed on glass fiber filters and washed twice with 3 ml of cold physiologic saline solution. When studying adriamycin, it proved necessary to centrifuge and resuspend the cells in drug-free medium prior to depositing them on the filters because free adriamycin was strongly adsorbed by the filters and gave unacceptably high background levels. The small degree of daunorubicin binding to the filters was determined by using cell-free blanks. Filters were placed in scintillation vials, the cells were solubilized by addition of 0.5 ml Protosol (New England Nuclear) and incubated at room temperature overnight prior to addition of 10 ml of scintillation fluid. Radioactivity was measured as stated above. The amount of drug taken up by the cells was expressed as pmoles/10^6 cells. Retention of labeled drug was measured by incubation of cells in the presence of drug for 1 hr at 37°, centrifugation, washing, and subsequent incubation in drug-free medium at 37° for an additional hour. Immediate binding of anthracyclines to cells, which was appreciable, was measured by exposing the cells to the labeled drug for 10 sec followed either by immediate deposition on filters or by dilution, centrifugation and incubation in drug-free medium for an hour. Sixty to seventy per cent of the adriamycin or daunorubicin that was immediately bound to the cell remained bound after a 1-hr incubation in drug-free medium.

Preparation of nuclei. Cells (2×10^8) were suspended in 7 ml of 2 mM $CaCl_2$ and incubated at 37° for 5 min. The swollen cells were broken by five strokes in a Dounce homogenizer. One ml of 2 M sucrose solution was added to the homogenate followed by centrifugation at 700 *g* for 10 min. The pellet was washed twice with 8 ml of 0.25 M sucrose, 2 mM $CaCl_2$ and resuspended in MEM. The nuclei appeared intact microscopically and the preparation contained less than 1 per cent intact cells.

RESULTS

Effects of adriamycin and daunorubicin on nucleic acid biosynthesis and cell survival of sensitive and resistant sublines of P388 leukemia in vitro. The sensitivity *in vitro* of P388/S, P388/ADR and P388/DAU cells to the cytotoxic effects of adriamycin and daunorubicin is shown in Fig. 1. The resistant sublines proved to be clearly less sensitive to the cytotoxic action of the anthracyclines than was P388/S. The LD_{99} (concentration which killed 99 per cent of the cells in 1 hr) of daunorubicin was 0.11 μg/ml for P388/S as compared to 2.0 μg/ml and 6.2 μg/ml for P388/ADR and P388/DAU, respectively. The resistant sublines were more resistant to adriamycin than to daunorubicin. The LD_{99} for adriamycin was 0.25 μg/ml for P388/S as compared to approximately 200 μg/ml for both resistant sublines. Resistance indices (ratios of LD_{99} values) for daunorubicin were 18 and 56 for P388/ADR and P388/DAU, respectively, and approximately 800 for adriamycin in both resistant sublines.

The relationship between concentration of adriamycin or daunorubicin and inhibition *in vitro* of thymidine incorporation into DNA was also studied. The concentrations of adriamycin which inhibited DNA synthesis by 50 per cent (IC_{50}) in P388/S, P388/ADR and P388/DAU cells were 2.5, 8.3 and 27 μg/ml respectively. The IC_{50} levels for daunorubicin in these sublines were lower: 0.8, 2.2 and 5.3 μg/ml respectively. For the sensitive cells, the IC_{50}

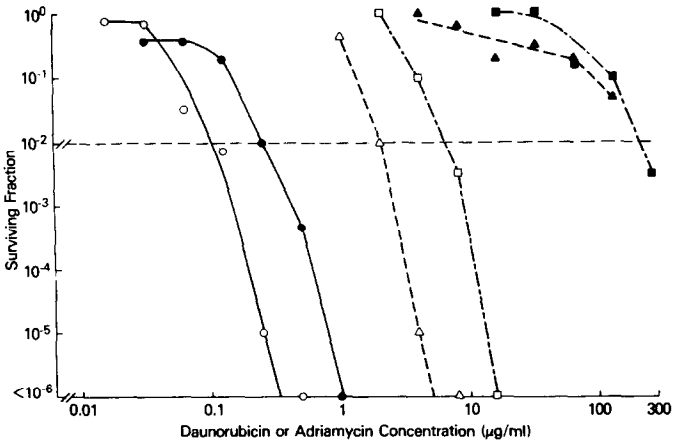

Fig. 1. Cytotoxicity *in vitro* of daunorubicin (open symbols) and adriamycin (closed symbols) to P388/S (○, ●), P388/ADR (△, ▲) and P388/DAU (□, ■) cells. Cells were incubated in the presence of drug for 1 hr and the surviving fraction was determined by bioassay *in vivo* as described in Materials and Methods.

values of both drugs were 7- to 10-fold higher than the LD_{99} levels. For the resistant sublines, the IC_{50} and LD_{99} levels for daunorubicin were approximately equal, whereas for adriamycin, DNA synthesis was inhibited by 50 per cent at concentrations an order of magnitude lower than the LD_{99}.

Uptake and retention of adriamycin and daunorubicin by sensitive and resistant sublines of P388 leukemia. The time courses of uptake and efflux of adriamycin by P388/S, P388/ADR and P388/DAU cells are shown in Fig. 2. An appreciable immediate binding of the drugs to the cells, as has been seen in other studies [14–16], was also evident in the sensitive and resistant P388 sublines. Uptake of the drugs by the sensitive cells was linear for 30 min at the concentrations tested; the rate of uptake slowed slightly thereafter but was linear from 30 to 120 min. Uptake was temperature dependent with no net uptake evident by cells kept in an ice bath. The immediate binding of the drugs to the cells was not dependent on temperature. Uptake of the drugs by P388/ADR and P388/DAU cells was essentially equivalent but was much lower than the rate of uptake by the sensitive cells. When cells which had been incubated in the presence of adriamycin or daunorubicin for 1 hr were washed and resuspended in drug-free medium at 37°, a portion of the drug which had been taken up flowed out of the cells; this efflux did not occur when cells were kept in an ice bath. The initial rate of efflux of daunorubicin from P388/S cells was greater than that of adriamycin. At 1 hr, 80 per cent of the adriamycin taken up was still retained by P388/S cells as compared to 55 per cent for daunorubicin. The proportion of drug retained by the resistant cell lines was much lower, about 20 per cent, and was actually less than the amount of drug which had originally bound to the cells (zero time).

The amounts of daunorubicin immediately bound, taken up in 1 hr, and retained after 1 hr of incubation in drug-free medium in all three cell lines increased linearly with increasing extracellular concentration

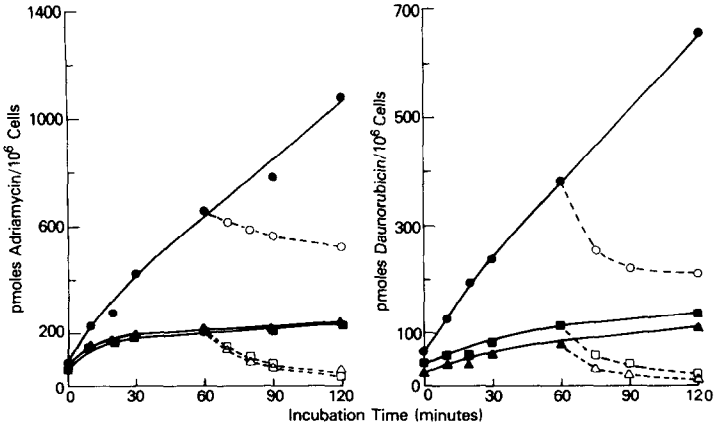

Fig. 2. Time course of uptake (solid symbols) and efflux (open symbols) of adriamycin and daunorubicin by P388/S (○, ●), P388/ADR (△, ▲) and P388/DAU (□, ■) cells *in vitro*. The concentration of adriamycin was 10 μg/ml and daunorubicin was 1 μg/ml. Cells were washed thoroughly and resuspended in drug-free medium after 60 min of drug exposure to measuse efflux. Each value represents the mean of three determinations with a coefficient of variation of less than 10 per cent.

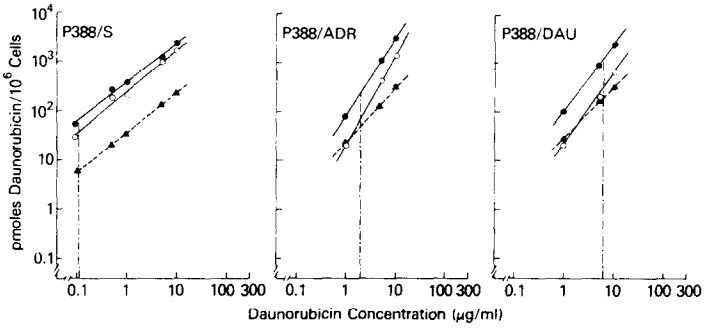

Fig. 3. Uptake and retention of daunorubicin by sensitive and resistant cell lines at varying extracellular concentrations of daunorubicin. Curves represent background due to immediate binding of drug to the cell (10-sec exposure, then incubation in drug-free medium for 1 hr, ▲----▲), uptake (1-hr incubation in the presence of the drug, ●——●), and retention (1-hr exposure to the drug, then 1-hr incubation in drug-free medium, ○——○). The vertical lines are drawn at LD_{99} concentrations as determined in Fig. 1. Each point is the mean of three determinations with a coefficient of variation of less than 10 per cent. The lines were drawn by regression.

of the drug (Fig. 3). The level of immediate binding was similar in the sensitive and resistant cells. The slopes of the uptake curves of the resistant cells were steeper than those of the sensitive cell line; uptake was equivalent or higher in the resistant cells at high daunorubicin concentrations ($>6\,\mu$g/ml), whereas at low concentrations the uptake was greater in the sensitive cells. The retention curve for sensitive cells closely paralleled the uptake curve with between 56 and 75 per cent of the daunorubicin retained at the various concentrations studied. In P388/ADR cells daunorubicin retention increased from 25 per cent at 1 μg/ml to 43 per cent at 10 μg/ml and in P388/DAU cells 21 and 27 per cent were retained respectively. The absolute levels of drug taken up or retained by the three cell lines did not correlate with equivalent cytotoxic concentrations of daunorubicin. At the LD_{99}, P388/S cells took up and retained 63 and 36 pmoles of daunorubicin; the values for P388/ADR cells at the LD_{99} were 246 and 72 pmoles and for P388/DAU cells, 1100 and 337 pmoles. The value that appeared to correspond with cytotoxicity was net retention (retention minus background level from immediate binding). An LD_{99} was reached in the resistant cell lines after concentrations had been reached at which retention exceeded background levels. The net retention of P388/S cells at the LD_{99} (0.11 μg/ml) was 31 pmoles as compared to 22 pmoles at the LD_{99} (2.0 μg/ml) in P388/ADR cells and 137 pmoles at the LD_{99} (6.2 μg/ml) in P388/DAU cells.

A similar comparison of the concentration dependence for immediate binding, uptake and retention of adriamycin is shown in Fig. 4. The sensitive cells took up less adriamycin than daunorubicin; however, adriamycin was retained with greater avidity. The per cent retention varied between 67 and 80 at the various concentrations evaluated. As with daunorubicin, the slope of the uptake curves in the resistant cells was steeper than in sensitive cells giving the greatest uptake deficit at low adriamycin concentrations. The greatest difference between daunorubicin and adriamycin was in retention. The levels of adriamycin retained by the resistant cells were below the background binding levels at all concentrations. This could account for the greater resistance of these cell lines to adriamycin. An LD_{99} for sensitive cells was reached at a concentration which gave a net retention of 18 pmoles adriamycin. In the resistant cell lines, however, equivalent cytotoxicity was obtained with very high concentrations of the adriamycin, at which the amount of drug retained was still below the background levels.

Effect of pH on the uptake, retention and cytotoxicity of adriamycin in sensitive and resistant cells. Adriamycin and daunorubicin are weak bases with pK_A values of approximately 8.2 [17]. Skovsgaard [17] demonstrated increased uptake of these drugs

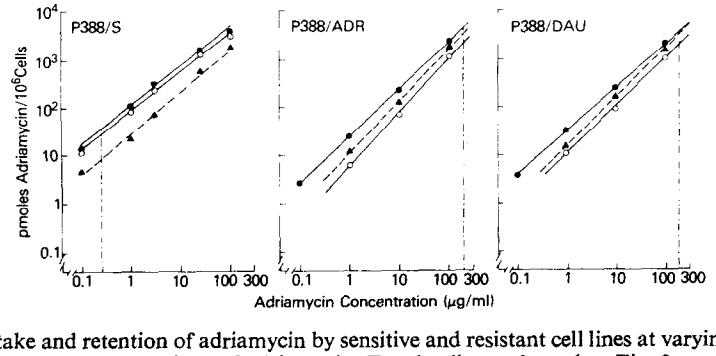

Fig. 4. Uptake and retention of adriamycin by sensitive and resistant cell lines at varying extracellular concentrations of adriamycin. For details, see legend to Fig. 3.

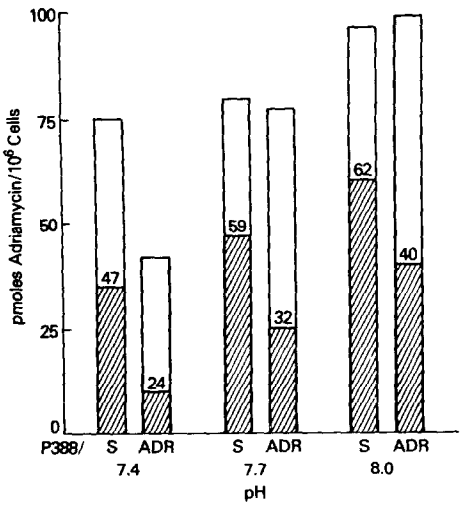

Fig. 5. Influence of the pH of the incubation medium on the uptake and retention of adriamycin by P388/S and P388/ADR cells. The cross-hatched plus open portion of the bars represent uptake over 20 min; the cross-hatched portions represent the amount of drug retained after 30 min in drug-free medium. Each value represents the mean of three determinations with a coefficient of variation of less than 10 per cent. The numbers are per cent of drug retained. Cells (10^6/ml) were incubated at 37° in Hank's balanced salt solution, the pH of which was adjusted by the addition of sodium bicarbonate. The pH of the medium changed by less than 0.1 pH unit during the course of the incubation.

by Ehrlich carcinoma cells when the pH of the incubation medium was increased, thus suggesting that the cells were more permeable to the non-ionized forms of the drugs. We evaluated the effect of pH on the uptake, retention and cytotoxicity of adriamycin in P388/S and P388/ADR cells to determine whether this manipulation could overcome the uptake and/or retention deficit and reverse resistance in P388/ADR cells. The effect of extracellular pH on uptake and retention is shown in Fig. 5. Using Hank's balanced salt solution buffered to pH 7.4, 7.7 or 8.0, it was found that the difference in uptake between sensitive and resistant cells evident at pH 7·4 was not observed at a higher pH. At pH 8.0 the uptake in sensitive and resistant cells was increased over that of sensitive cells at pH 7.4. On the other hand, greater retention of adriamycin by sensitive cells than by resistant cells was observed at each pH level. The cytotoxicity of adriamycin to P388/S and P388/ADR cells *in vitro* at pH 7.4 and 8.0 was determined by bioassay (Fig. 6). The cytotoxicity of adriamycin to the sensitive cells was enhanced at a higher pH; animals inoculated with cells exposed to 2 μg/ml of adriamycin for 20 min survived 6 days longer than animals inoculated with the same number of untreated cells, whereas at pH 8.0 the lifespan was increased by 17 days. Extracellular pH had no effect on the cytotoxicity of adriamycin to P388/ADR cells.

Effect of Tween 80 on uptake, retention and cytotoxicity of anthracycline antibiotics in sensitive and resistant cells. Tween 80 is a non-ionic surfactant which appears to increase permeability of the cell membrane and enhance uptake of a number of substances [18]. Tween 80 was shown [19] to enhance the uptake and potentiate the cytotoxicity of daunorubicin in resistant Chinese hamster cells. The effects of Tween 80 on the uptake and retention of adriamycin and daunorubicin by sensitive and resistant sublines of P388 leukemia are shown in Fig. 7. Although the detergent did not have a marked effect on uptake of either drug by P388/S cells, it clearly enhanced the uptake of both adriamycin and daunorubicin in the respective resistant sublines. The decreased retention of the anthracycline antibiotics in the resistant sublines was not affected by Tween 80. The only effect on drug retention was an enhancement of adriamycin retention by P388/S cells.

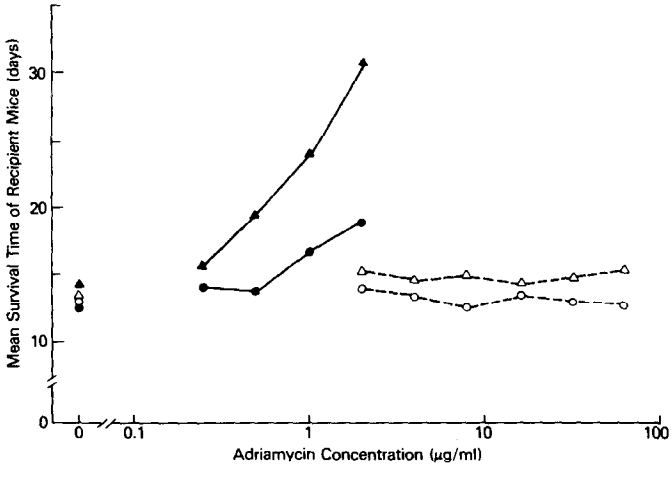

Fig. 6. Survival time of mice inoculated with P388/S (closed symbols) or P388/ADR (open symbols) cells which had been exposed *in vitro* to adriamycin at pH 7.4 (○, ●) or pH 8.0 (△, ▲). Cells (2×10^6/ml) were incubated at 37° in Hank's balanced salt solution containing various concentrations of adriamycin for 20 min. After washing and resuspension in drug-free medium, cells were inoculated i.p. (10^6 cells/mouse) in $B6D2F_1$ mice. Each point is the mean survival time of a group of eight recipient mice.

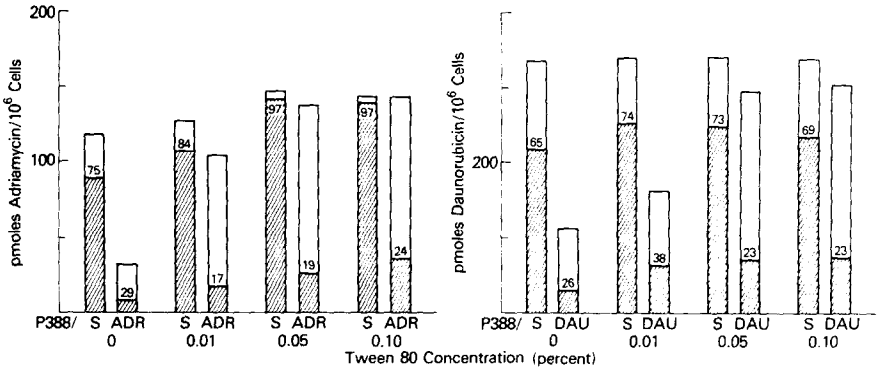

Fig. 7. Influence of Tween 80 on the uptake and retention of adriamycin and daunorubicin in sensitive and respective resistant cell lines. Cells were preincubated for 10 min at 37° in MEM with or without Tween 80 prior to addition of labeled adriamycin or daunorubicin. Cells were incubated in the presence of drugs for 1 hr to measure uptake (cross-hatched plus open portions of bars) and were washed and re-incubated in drug-free medium for another hour to measure retention (cross-hatched portion). Each value represents the mean of three determinations with a coefficient of variation of less than 10 per cent. Numbers are per cent of drug retained.

The influence of Tween 80 on the cytotoxicity of adriamycin to P388/S and P388/ADR cells is shown in Fig. 8. Tween 80 enhanced the cytotoxicity of adriamycin to both sensitive and resistant cells. P388/ADR cells were still 40-fold resistant to adriamycin in the presence of 0.10% Tween 80. Adriamycin, formulated in a solution containing 10% Tween 80, was tested against P388/ADR *in vivo* and, similar to results with adriamycin formulated in water, complete resistance was observed (data not shown).

Binding of [^{3}H]*daunorubicin to isolated nucleic from P388/S and P388/ADR cells.* In order to ascertain whether the differences in uptake and retention of anthracycline antibiotics in sensitive and resistant cells were due to alterations in drug binding at the nuclear level, the binding of [^{3}H]daunorubicin to nuclei isolated from P388/S and P388/ADR cells was compared (Table 1). Unlike uptake by whole cells, binding to isolated nuclei was instantaneous; the amount of daunorubicin bound in 10 sec was essentially identical to the amount bound after exposure to the drug for 30 min. The amount of daunorubicin bound by nuclei from P388/ADR cells was 10–17 per cent less than the amount bound by nuclei from P388/S cells. At the concentrations of daunorubicin used, the amount of drug bound to 10^6 nuclei was not dissimilar to the amount retained by P388/S cells, as shown in Fig. 3. However, binding by P388/ADR nuclei was much higher than the amount taken up or retained by whole P388/ADR cells at these concentrations, as shown by extrapolation of the curves in Fig. 3.

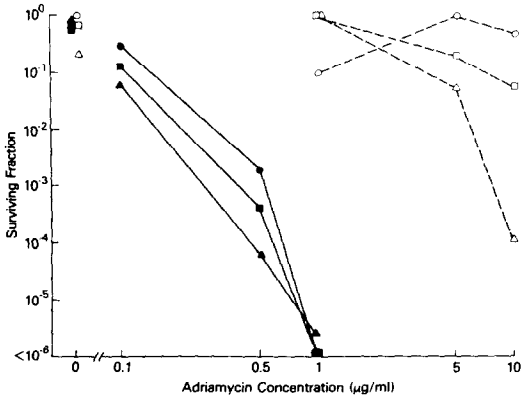

Fig. 8. Influence of Tween 80 on the cytotoxicity *in vitro* of adriamycin to P388/S (closed symbols) and P388/ADR (open symbols) cells. Cells (2×10^6/ml) were incubated at 37° for 1 hr in the presence of varying concentrations of adriamycin in the absence of Tween 80 (○, ●), or in the presence of either 0.05% (v/v) Tween 80 (□, ■) or 0.10% Tween 80 (△, ▲). The surviving fraction was determined by bioassay *in vivo* as described in Methods.

DISCUSSION

The sublines of P388 leukemia that are the subject of this study are essentially completely resistant to the therapeutic effects of adriamycin and daunorubicin *in vivo* [12, 20]. In addition, these sublines are cross-resistant to a broad spectrum of anthracycline derivatives, other chemically unrelated DNA binding agents, mitotic spindle poisons and protein synthesis

Table 1. Binding of [^{3}H]daunorubicin to nuclei isolated from P388/S and P388/ADR cells.*

Daunorubicin conc. (μg/ml)	Duration of incubation	Binding to nuclei (pmoles DAU/10^6 nuclei) P388/S	P388/ADR
0.05	10 sec	18.0±0.1†	15.0±0.8
	30 min	17.2±0.6	15.5±0.8
0.10	10 sec	30.6±0.2	27.8±0.7
	30 min	31.4±0.7	27.6±1.1

* Nuclei isolated as described in Materials and Methods were incubated in MEM at 37°.

† Values are mean±standard deviation of three determinations.

inhibitors; sensitivity is retained to alkylating agents and antimetabolites [20]. One obvious possibility to account for resistance to agents with such diverse mechanisms of action would be an alteration in the cell membrane which would impair the transport (or diffusion) of drugs into the cells. Such membrane alterations have been described for actinomycin D-resistant cell lines [21–23] and give rise to resistance to a spectrum of chemically unrelated cytotoxic agents [24].

Cell lines with acquired resistance to daunorubicin [4, 5, 8, 9, 19, 25, 26] and adriamycin [6, 25] have been studied by a number of investigators. The mechanism of resistance to daunorubicin of resistant Chinese hamster cells appears to be impaired uptake of the drug [19]. The characteristics of these cell lines are similar in many respects to a number of actinomycin D-resistant cell lines [19, 25] and reciprocal cross-resistance between the two drugs has been observed repeatedly [19, 20, 24, 27]. On the other hand, evidence has been presented which indicates enhanced efflux of daunorubicin from resistant sublines of Ehrlich carcinoma [8].

The studies with adriamycin- and daunorubicin-resistant sublines of P388 leukemia *in vitro* presented in this paper indicate that both uptake and retention of the anthracycline antibiotics are impaired in the resistant cells compared to the sensitive parental cell line. At first glance, the distinct difference in the uptake of the drugs by the sensitive and resistant cells shown in Fig. 2 would appear to be sufficient to account for the resistance of these cell lines to the anthracycline antibiotics. However, other evidence suggests that the decreased rate of uptake might not be the basis of resistance. First, the resistant sublines were clearly less sensitive to adriamycin than to daunorubicin, as shown in Fig. 1, yet the uptake of both drugs was decreased to a similar extent in the resistant cells. Second, parallel studies with actinomycin D [28] showed that the uptake of this agent was only slightly less in P388/ADR and P388/DAU cells than in P388/S cells, yet there was complete resistance *in vivo* and 1000- to 2000-fold resistance *in vitro*. Finally, the studies with actinomycin D showed a marked impairment of the retention of the drug by the resistant cells which corresponded with the degree of resistance. The differences in inhibition of thymidine incorporation by adriamycin and daunorubicin in sensitive and resistant cells appear to be related to the decreased uptake of the drugs, because there was a similar shift in the inhibitory potency of the two drugs which would correspond to the similar degree of impairment in uptake. Resistance to the DNA inhibitory effects of the anthracycline antibiotics did not reflect the differences between the drugs or the degree of resistance with respect to cytotoxic activity. It is important to note that the drugs, at extracellular concentrations high enough to result in uptake by the resistant cells, were apparently able to bind to DNA [29] and block thymidine incorporation. The binding of daunorubicin to isolated nuclei from P388/S and P388/ADR cells was quite similar, as shown in Table 1. These data indicate that resistance is probably not due to an alteration in the nuclear DNA or chromatin.

The data shown in Figs. 3 and 4 suggest that resistance to adriamycin and daunorubicin is most closely related to differences in retention of the drugs by the sublines. With daunorubicin, cytotoxicity to the resistant sublines was obtained at concentrations of drug which resulted in a positive net retention of the drug by the cells. This is identical to results which were obtained with actinomycin D [28]. The much greater relative resistance of these sublines to adriamycin was reflected in a greater impairment of drug retention. Even at concentrations of adriamycin which were cytotoxic to the resistant sublines, the retention following incubation in the presence of the drug for 1 hr and a subsequent 1-hr wash-out period was less than the background binding levels (as measured by exposure to the drug for 10 sec followed by incubation in drug-free medium for 1 hr). The extremely high concentrations of adriamycin which are lethal to the resistant cells (in excess of 200 μg/ml) could possibly bring about cell death by damaging the cell membrane or by some other mechanism rather than by binding to DNA and impairing template activity. This could explain why cytotoxicity was obtained even though retention of the drug was still less than background levels. No evidence was obtained which could explain how the level of drug retained by the resistant cells after 1 hr in drug-free medium could be less when cells were initially exposed to drug for 1 hr than when the initial exposure period was only 10 sec. The decreased rate of uptake by resistant cells at low concentrations of the drugs could very well be a reflection of an enhanced rate of efflux. The absence of a difference in uptake between sensitive and resistant cells at high concentrations could be due to saturation of the outward transport mechanism.

Skovsgaard [17] demonstrated in Ehrlich carcinoma cells that anthracycline antibiotics diffuse into the cells passively in their non-ionized forms and are actively transported out. Our data showing essentially nonsaturable uptake which was enhanced by elevated pH suggest passive diffusion in P388 leukemia cells also. We studied the effects of the metabolic inhibitors, sodium azide and 2,4-dinitrophenol, on uptake and efflux of daunorubicin by sensitive and resistant cells and were not able to show an effect in the presence of glucose (data not shown). In the absence of glucose, a condition in which metabolic inhibitors were shown to have an influence on uptake and efflux of anthracycline antibiotics in Ehrlich carcinoma cells [8, 17], we were unable to obtain evaluable uptake curves, probably due to clumping and instability of the P388 cell suspensions in those conditions. However, the decreased retention of the drugs in the resistant P388 sublines suggests the presence of an active efflux mechanism which is enhanced in the resistant sublines. This would be similar to the mechanism of resistance to daunorubicin in Ehrlich carcinoma sublines developed by Danø [8].

Efforts to reverse resistance by increasing the uptake of the drugs by either increased pH or addition of Tween 80 to the culture medium were not successful and add supporting evidence that resistance is due to decreased retention rather than

impaired uptake. Tween 80, however, did decrease the relative resistance of P388/ADR to adriamycin from 800- to 40-fold, about the level of resistance to daunorubicin. This effect on resistance might reflect the influence of Tween 80-induced membrane alterations on the efflux mechanism. However, the impaired drug retention in resistant cells was not markedly affected by Tween 80. On the other hand, studies with actinomycin D by Bowen and Goldman [30] suggest that Tween 80 might influence the binding of drugs to DNA as well as their transport into cells. Such an effect might explain the influence of Tween 80 on the relative resistance of these sublines to adriamycin.

REFERENCES

1. G. Bonadonna, G. Beretta, G. Tancini, C. Brambilla, E. Bajetta, G. M. DePalo, M. Delena, F. F. Bellani M. Gasparini, P. Valagussa and U. Veronesi, *Cancer Chemother. Rep.* (Part 3) **6,** 321 (1975).
2. R. H. Blum, *Cancer Chemother. Rep.* (Part 3) **6,** 247 (1975).
3. S. K. Carter, *J. natn Cancer Inst.* **55,** 1265 (1975).
4. B. J. Smith and D. Kundu, *Br. J. Cancer* **34,** 53 (1976).
5. K. Danø, *Cancer Chemother. Rep.* **55,** 133 (1971).
6. K. Danø, *Cancer Chemother. Rep.* **56,** 321 (1972).
7. K. Danø, *Cancer Chemother. Rep.* **56,** 701 (1972).
8. K. Danø, *Biochim. biophys. Acta* **323,** 466 (1973).
9. H. Riehm and J. L. Biedler, *Cancer Res.* **31,** 401 (1971).
10. D. Kessel, V. Botterill and I. Wodinsky, *Cancer Res.* **28,** 938 (1968).
11. A. Goldin and R. K. Johnson, *Cancer Chemother. Rep.* (Part 3) **6,** 137 (1975).
12. R. K. Johnson, A. A. Ovejera and A. Goldin, *Cancer Treatment Rep.* **60,** 99 (1976).
13. H. E. Skipper, F. M. Schabel, Jr. and W. S. Wilcox, *Cancer Chemother. Rep.* **35,** 1 (1964).
14. W. D. Meriwether and N. R. Bachur, *Cancer Res.* **32,** 1137 (1972).
15. K. Tatsumi, T. Nakamura and G. Wakisaka, *Gann* **65,** 237 (1974).
16. N. R. Bachur, M. Steele, W. D. Meriwether and R. C. Hildebrand, *J. med. Chem.* **19,** 651 (1976).
17. T. Skovsgaard, *Biochem. Pharmac.* **26,** 215 (1977).
18. E. R. M. Kay, *Cancer Res.* **25,** 764 (1965).
19. H. Riehm and J. L. Biedler, *Cancer Res.* **32,** 1195 (1972).
20. R. K. Johnson, M. P. Chitnis and A. Goldin, *Pharmacologist* **18,** 173 (1976).
21. D. Kessel and H. B. Bosmann, *Cancer Res.* **30,** 2695 (1970).
22. H. B. Bosmann, *Nature, Lond.* **233,** 566 (1971).
23. J. L. Biedler, H. Riehm, R. H. F. Peterson and B. A. Spengler, *J. natn Cancer Inst.* **55,** 671 (1975).
24. J. L. Biedler and H. Riehm, *Cancer Res.* **30,** 1184 (1970).
25. J. J. Wang, D. S. Chervinsky and J. M. Rosen, *Cancer Res.* **32,** 511 (1972).
26. K. Danø, S. Frederiksen and P. Hellung-Larsen *Cancer Res.* **32,** 1307 (1972).
27. R. Simard and R. Cassingena, *Cancer Res.* **20,** 1590 (1969).
28. M. Inaba and R. K. Johnson, *Cancer Res.*, **37,** 4629 (1977).
29. A. DiMarco and F. Arcamone, *Arzneimittel-Forsch.* **25,** 368 (1975).
30. D. Bowen and I. D. Goldman, *Cancer Res.* **35,** 3054 (1975).