Cancer Chemother Pharmacol (1990) 26: 198–204

Cancer Chemotherapy and Pharmacology

Slow penetration of anthracyclines into spheroids and tumors: a therapeutic advantage?

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Received 23 October 1989/Accepted 18 January 1990

Summary. Despite clear evidence that the effective penetration of the anthracycline antibiotics into experimental tumors or multicell spheroids is poor, these drugs exhibit clinical activity against a variety of solid tumors. In an attempt to understand this apparent contradiction, we used the Chinese hamster V79 spheroid system and flow cytometry techniques for intra-spheroid pharmacological studies of doxorubicin and daunomycin. Our results indicate that the slow delivery of the anthracyclines to the inner cells of spheroids is due to the rapid binding of the drug by cells in the outer layers. After exposure, the anthracyclines are retained much more effectively when cells remain in intact spheroids than when the spheroids have been dispersed, resulting in considerably more cytotoxicity in situ. This result indicates a need for considerable caution in attempting to predict the anti-tumor efficacy of drugs by using either conventional cell-culture systems, spheroids that have been disaggregated immediately post-exposure, or excision assays of tumors from experimental animals. Furthermore, our results suggest the need for a critical evaluation of the significance of the multidrug resistance (MDR) phenotype for cells surrounded by other drug-containing cells as opposed to single cells in drug-free culture medium.

Introduction

The anthracycline antibiotics were first developed as antineoplastic agents in the 1960s and are now widely used due to their activity against a variety of solid and hematological malignancies [16]. The continued use of the best-known derivative doxorubicin (Adriamycin) may be somewhat surprising, in view of its rather severe acute (alopecia, vomiting, myelosuppression) and late (cardiac) toxic effects. These, however, can be minimized with appropriate dose scheduling. Perhaps even more surprising is laboratory evidence suggesting that the anthracyclines should be of limited potential: tumor cells efficiently develop resistance to this family of drugs [1, 3, 14, 16, 22]; furthermore, the anthracyclines are one of the few classes of antineoplastic agents that penetrate very poorly into solid tissue masses ([7, 10, 12, 13, 15–21, 30; reviewed in [17]). Clearly, these inconsistencies between clinical and laboratory observations suggest that our understanding of the anti-tumor activity of the anthracyclines is incomplete.

We [7, 8], like other investigators [13, 17–21, 24–26, 30–32], have often touted the value of spheroids as a good model for evaluating drug penetration, based in part on results obtained using doxorubicin. Admittedly, it has often been hard to rationalize the poor delivery of this drug with its readily demonstrated clinical efficacy; at least in our own case, we have generally adopted the fairly trivial explanation that clinical response is based not on a single treatment but, rather, on repeated drug exposures over the course of time.

Our recent interest in multifraction treatments of the spheroid system with common chemotherapeutic agents (including the anthracyclines) led to some rather unexpected observations that stimulated the present studies. Spheroid regrowth and repopulation generally proceeds quite rapidly following non-ablative drug exposures. However, with the anthracyclines we have found that the net number of surviving cells often *decreases* during the interval between the termination of one treatment and the initiation of the next (manuscript in preparation). That observation may not be overly surprising; doxorubicin intercalates into cellular DNA and remains for long periods of time (a phenomenon observed and described by radiotherapists as a "recall" reaction; see [2, 4, 5]). If a drug is not rapidly cleared from the cell, a potential for continuing toxicity obviously remains [35].

The question addressed in the current report was whether the post-exposure environment of the cell could influence the retention of anthracyclines and their associated cytotoxicity (essentially, an evaluation of post-exposure pharmacology at the cellular level). Clearly, one

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might expect quite a different rate of elimination of the drug if the cell is surrounded by other drug-containing cells, as opposed to the more typical in vitro case in which an isolated cell sits in a sea of culture medium that provides a high intracellular-to-extracellular drug concentration gradient. An obvious extension of the significance of these results concerns the role of the multidrug resistance (MDR) phenomenon (activated drug efflux) in cases in which the immediate extracellular environment is another tumor cell rather than culture medium.

Materials and methods

Cell line and culture techniques. Chinese hamster V79-171b lung cells were used exclusively in these studies. The cells were routinely maintained as monolayers on plastic petri dishes with biweekly subcultivation in Eagle's minimal essential medium supplemented with 10% fetal calf serum. To initiate spheroid growth, asynchronous cells were removed from the plastic growth dishes by trypsinization. Spinner culture flasks (250 ml) were then inoculated with 2×10^6 cells in 150 ml medium plus 5% serum and were stirred at 180 rpm; the gas phase comprised 5% CO_2 in air. During the growth of the spheroids, the medium was first changed on day 3 and then daily thereafter (spent medium was completely removed and replaced with fresh medium by sedimentation of the spheroids to the bottom of the flask, followed by removal of the medium by aspiration). Spheroids were used after 7–12 days of growth, which produced spheroids with a diameter of 400–800 μm.

Drugs and treatment. Doxorubicin and daunomycin were purchased from Sigma Chemical Co. (St. Louis, Mo.) and stock solutions were prepared using sterile water. The desired drug concentration was achieved by dilution into the normal growth medium of the spheroids. The clinical formulation of etoposide (Bristol Laboratories of Canada, Toronto, Ontario) was diluted directly into spheroid growth medium as required. Drug exposures lasted for either 1 or 2 h; for "delayed assay" experiments, the drug-containing medium was removed at the end of the exposure period by sedimentation of the spheroids followed by removal of the medium by aspiration, two washes, and the addition of fresh growth medium. Additional washes were done hourly for at least the first 6 h post-exposure.

Cell-sorting procedures. Our cell-sorting techniques are based on the general principles of slow diffusion and the rapid, essentially irreversible binding of Hoechst 33342 as it penetrates into the spheroid mass. The external cells of the spheroids fluoresce very brightly, whereas little stain reaches (and is bound by) the innermost cells (staining at the outside of the spheroid is >100-fold that obtained deep in its interior; e.g. [7]). When the stained spheroid is reduced to a monodispersed single-cell suspension, a fluorescence-activated cell sorter can be used asceptically to recover individual cells containing predetermined levels of the Hoechst stain (therefore, cells that were at any desired depth within the spheroids at the time of staining). The sorted cells are then placed in normal culture dishes for determination of their viability by the conventional clonogenicity assay [8–10].

Our cell sorter is a dual laser Becton Dickinson FACS 440 (Becton Dickinson Immunochemistry Systems, Mountainview, Calif.). Hoechst 33342 (Sigma) was used at 2 μM by direct addition of the dye to the drug-containing flask for the final 20 min of drug exposure. At the end of drug exposure, excess drug and stain were removed by aspiration after the spheroids had sedimented to the bottom of a collection tube. After three washes, the spheroids were reduced to a single-cell suspension using 0.25% trypsin at 37° C for 10–12 min with continuous agitation. The stained, disaggregated single-cell suspension was then resuspended in a mixture of growth medium and trysin ($\geq 3:1$, v/v) and maintained at 4° C to inhibit stain and drug efflux during the sorting procedure (typically <1 h).

The primary laser was operated at 400 mW and 488 nm, with monitoring of forward scatter and 90° scatter signals. Additionally, the intracellular levels of the anthracyclines were monitored in the actual cells sorted by fluorescence intensity measurements, using the 488-nm line for excitation and emission through a 550-nm longpass filter [7, 10, 23]. The UV laser was operated at 40 mW power with the 350- to 360-nm lines, and the Hoechst emission was monitored through a (449 ± 10 nm) bandpass filter. The cell population was defined on the basis of the forward scatter signal; the resulting signals of Hoechst intensity and 90° scatter were processed through matched logarithmic amplifiers, and the ratio of these signals was used to generate a "Hoechst concentration" profile, which was integrated to establish ten windows of equal cell numbers. Both the actual number of cells desired per petri dish for the clonogenicity assay and the number of replicate samples were preprogrammed. The FACS was thus essentially used as a micromanipulator to introduce the desired number of cells into sorting tubes; these were then poured and rinsed into growth dishes for the clonogenicity analysis [9].

The data reported are from representative experiments that were replicated 2–5 times. Each survival point represents a minimum of 400 colonies; thus, the accuracy (based on Poisson statistics) of any of the survival data is within an expected variance of 5% (or less) of the plotted value, or about the size of the plotting symbols used. This expected level of reproducibility has also been experimentally confirmed for our sorting techniques [9], using independent exposures to doxorubicin. We did not "average" our data for different spheroid populations due to the fact that anthracycline uptake and toxicity is a critical function of spheroid size and growth fraction; differences between experiments are thus more an indication of spheroid variability than of uncertainty in the assay technique.

Experiments to assess the toxicity of Hoechst 33342, alone or in combination with the anthracyclines or etoposide, were negative. With the anthracyclines, this was easier to assess, since *either* the Hoechst or the anthracycline fluorescence gradient (or both) can be used for subpopulation separation and analysis [7]. No additional toxicity was observed after Hoechst staining compared to anthracycline treatment alone. Additionally, from a technical point of view, addition of Adriamycin does not interfere with Hoechst-based sorting due to either quenching or energy transfer mechanisms, presumably because these factors are small and quite constant throughout the spheroid (doxorubicin gradients of ≤5-fold are observed) relative to the several hundred-fold range of Hoechst intensities. Detailed studies of this question have previously appeared [7, 27]. In the case of etoposide, selective interactions in the subpopulations of cells could not be identified as easily, since the subpopulations could not be studied without the Hoechst staining. In this case, our controls were exposed to etoposide at 2–3 different levels of toxicity, and the Hoechst stain concentration was then escalated to determine the level at which significant additional killing occurred. Hoechst concentrations at least 10-fold higher than those typically used were required to produce any additional cell killing in the etoposide samples, and concentrations 20-fold higher than the norm were needed to produce additional toxicity in the anthracycline-treated spheroids.

Cell-distribution analysis. To estimate cell kinetic changes in the control and drug-treated spheroids, DNA distributions of the cell subpopulations were determined using the method of Vindelov [36]. Essentially, this consists of a single-step nucleus preparation/ethidium bromide staining protocol, which enables individual cell nuclei to be assayed for their DNA content and, thus, their position in the cell cycle. Since the V79 cells exhibit karyotypic instability as the spheroids enlarge (like many tumor cells), we presented the DNA distribution data only as repetitive histograms for qualitative analysis (in our opinion, no satisfactory algorithms are available for quantitative determination of subpopulation percentages in perturbed, karyotypically unstable cell populations).

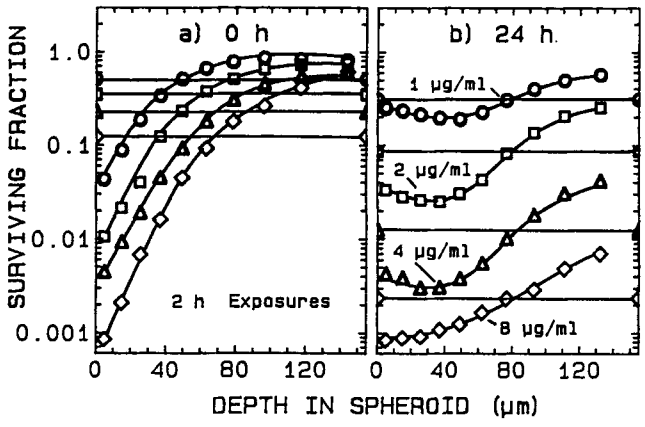

Fig. 1. Survival of cells recovered from increasing depths within spheroids with a diameter of 655 ± 14 (SEM) µm immediately after a 2-h exposure to the indicated concentrations of doxorubicin (*panel a*) or 24 h after terminating the drug exposures (*panel b*). Note the marked decrease in survival in most cell subpopulations when the cells remained in situ for 24 h

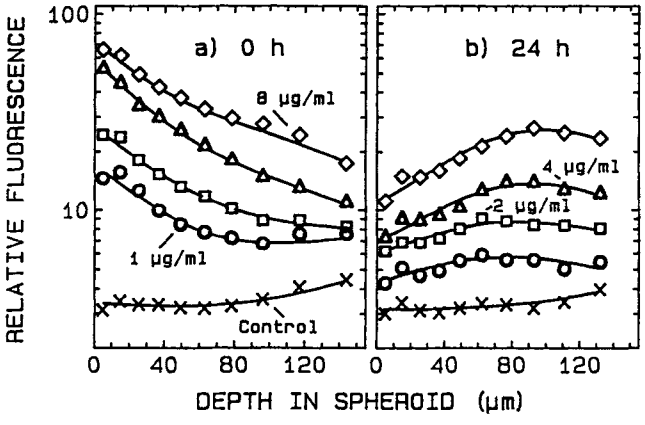

Fig. 2. Doxorubicin fluorescence in cell subpopulations (the same cells assayed for cytotoxicity in Fig. 1) assayed by flow cytometry immediately following exposure (*panel a*) or 24 h after terminating the exposure (*panel b*). The drug was effectively retained in the cells located deeper in the spheroid

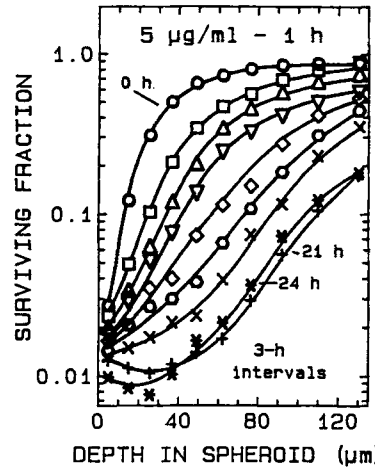

Fig. 3. Survival of cells from spheroids with a diameter of 752 ± 27 (SEM) µm exposed to 5 µg/ml doxorubicin for 1 h, showing the effects of location in the spheroid and assay time post-exposure. Cell viability decreased progressively for at least the first 21 h post-exposure

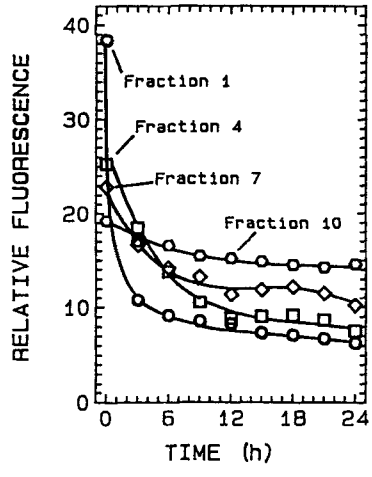

Fig. 4. Doxorubicin retention in selected subpopulations of cells from the spheroids shown in Fig. 3. Note that the outermost cells (*Fraction 1*) showed a rapid loss of drug (fluorescence); within the 1st h, less drug was retained than in the innermost (*Fraction 10*) cell population. In contrast, the latter cells showed only a small decrease in fluorescence over the 24 h interval

Results

In terms of either cytotoxicity (Fig. 1) or intracellular drug levels (Fig. 2), the effects of a 2-h exposure to doxorubicin were quite different, depending on whether the spheroids were assayed immediately after exposure (panel a, each figure) or left intact for 24 h prior to assay (panel b). As has repeatedly been observed in this and other spheroid systems, two factors tend to make the internal cells resistant to anthracyclines: limited drug penetration (as indicated in Fig. 2) and, additionally, cell-cycle redistribution and microenvironmental influences that markedly reduce the efficacy of the delivered drug. Interestingly, however, the rather large differential in achievable cytotoxicity between the external and internal cells was reduced when drug toxicity was assayed 24 h after drug exposure rather than immediately post-exposure (cf. panel b and panel a in Fig. 1).

At higher drug concentrations, a considerable increase in cytotoxicity to the innermost cells was noted when assay was delayed 24 h; concomitant with this, the actual quantity of intracellular drug *increased* in these cell subpopulations relative to the immediate post-exposure assay. Conversely, in the outermost cell populations, intracellular drug levels decreased dramatically over the 24 h interval. At higher drug levels, virtually the same fraction of viable cells was observed near the spheroid surface at either 0 or 24 h, whereas at lower drug concentrations, a net increase in survival was observed with the 24 h post-exposure "repair" time.

A more detailed analysis of the pharmacokinetic changes occurring during the first 24 h after doxorubicin

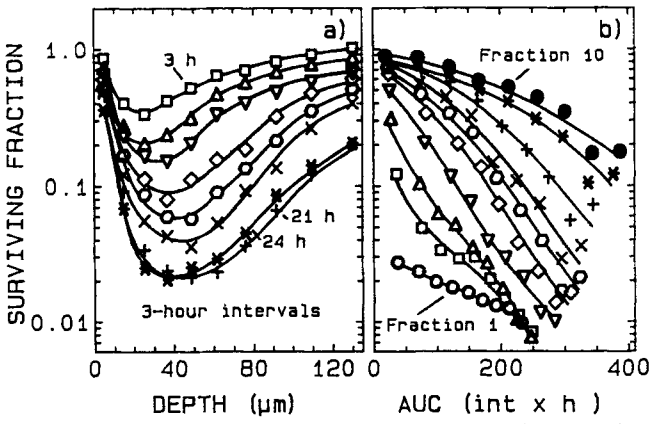

Fig. 5. Surviving fraction of cells in the spheroids shown in the previous two figures, now plotted relative to the survival measured immediately after drug exposure (*panel a*) or as a function of integrated intensity (int) drug exposure as estimated by the AUC of doxorubicin fluorescence as shown in Fig. 4. Both panels indicate that the greatest changes with time occurred in cells midway through the spheroid rim

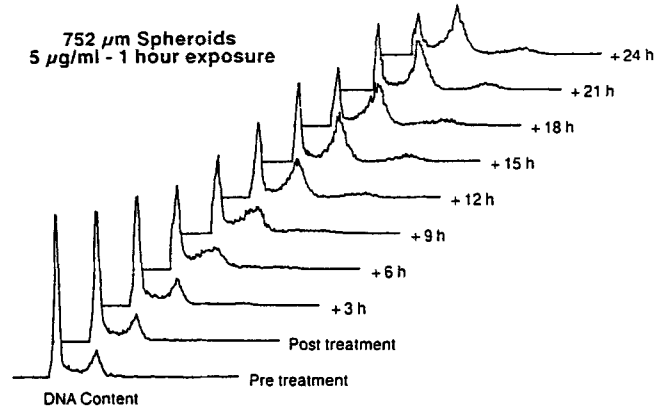

Fig. 6. Histograms showing DNA content of the doxorubicin-treated spheroid population characterized in the previous three figures. Note the drug-induced "G_2 block" evident 24 h after termination of the drug treatment

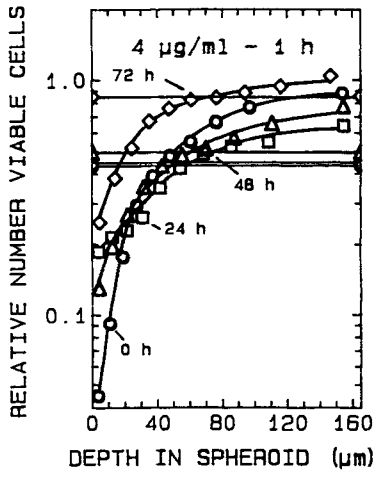

Fig. 7. Relative number of viable cells recovered from doxorubicin-treated spheroids, as a function of time after treatment and depth in the spheroid. The starting spheroid population was 407 ± 11 (SEM) μm in diameter. Unlike those in the previous figures, these data were plotted after normalizing for the increasing number of cells per spheroid during the treatment-to-assay interval. At 24 (□) and 48 (△) h, the number of viable cells increased in the outer layers and decreased near the middle of the spheroid. By 72 h, viable cells had increased in number throughout the spheroids

exposure is shown in Figs. 3–5, in which a 5-μg/ml exposure to doxorubicin for 1 h was evaluated at 3-h intervals for the first 24 h post-exposure. In both the outermost and innermost cell populations, a progressive decrease in the surviving fraction occurred as the assay interval was increased to 21 h (Fig. 3). The greatest effect was observed in cells midway through the viable rim of the spheroid; the viability of cells that would have had a high probability of survival had they been isolated and assayed for clonogenicity immediately after drug exposure decreased by almost two decades when they were left in the intact spheroid for 24 h prior to assessment of clonogenicity (replating in fresh culture medium under monodispersed conditions). The corresponding changes in the intracellular drug levels over this period are shown in Fig. 4. For clarity, data for only four of the subpopulations are included; as would be expected, decreases in intracellular drug levels observed in the outermost (fraction 1) cell population were much more rapid and dramatic than those seen in the innermost (fraction 10) cells.

The impact of these time-dependent changes in intracellular drug levels on the fraction of viable cells found in each cell subpopulation can perhaps be more clearly seen with the data plotted as shown in Fig. 5. The left panel of this figure shows the time-dependent decreases in surviving fraction (ratio of survival at the assay time to that immediately post-exposure) as a function of cellular position in the spheroid. Only moderate changes in the relative viability were noted in the outermost and innermost cell subpopulations, but the dramatic decrease in survival for cells midway through the viable rim is more clearly shown in Fig. 5a. To estimate the pharmacological basis of this response, Fig. 5b shows cell survival, fraction by fraction, plotted as a function of the AUC of Fig. 4 (that is, the product of the relative fluorescent intensity of the cell populations and the time at that level). Although it may seem surprising that a better pharmacological correlation between exposure duration and survival was not apparent, it must also be remembered that the subpopulations of cells differ not only in their drug uptake and retention but also in their inherent resistance to doxorubicin. Thus, the external, rapidly cycling cells (fraction 1) contained few resistant cells; in contrast, virtually 100% of the cells in fraction 10 were doxorubicin-resistant (the near parallelism of the curves may indicate a similar degree of resistance). Populations of intervening cells between the outer- and innermost subpopulations showed varying degrees of sensitivity and resistance.

The changes in cell kinetics suggested by the data of Figs. 3–5 were evaluated using flow cytometric analysis of cellular DNA content (Fig. 6). During the 24-h study period, an obvious redistribution of cells through the cell cycle occurred; a significant fraction of the diploid cells accumulated in G_2 phase 24 h after doxorubicin exposure. Similarly, the small tetraploid cell subpopulation also

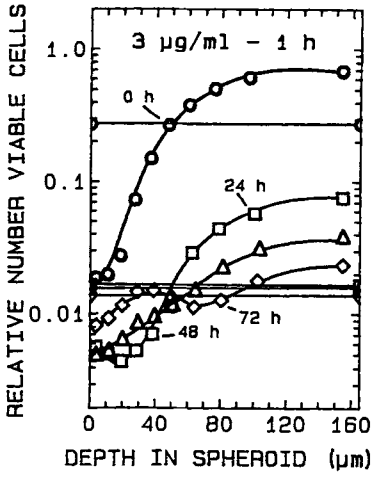

Fig. 8. Relative number of viable cells recovered from daunomycin-treated spheroids, as a function of time after exposure and depth in the spheroid. Although the overall toxicity of this treatment was not substantially different from that shown in Figs. 7 or 9, markedly different post-treatment growth kinetics were observed. "Recovery" was apparent only in the outermost cells at the longest observation time

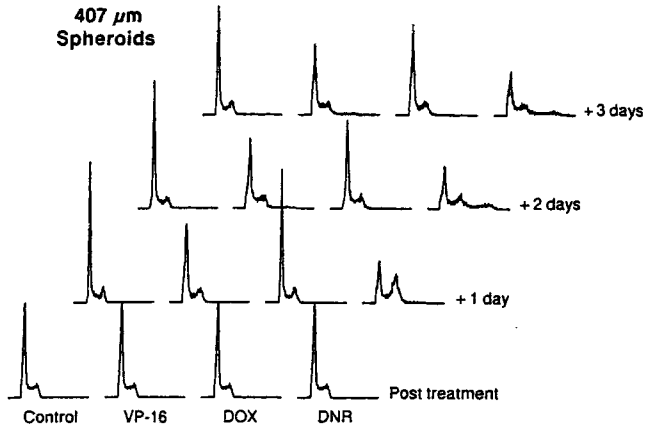

Fig. 10. Histograms showing DNA content of the spheroid population described in Figs. 7–9 and including distributions for untreated (*Control*) spheroids followed for the same 72 h interval. A slight increase in S-phase cells, relative to the G_1 peak, can be seen for the etoposide (*VP-16*)- and doxorubicin (*DOX*)-treated cells. The greatest perturbations were noted in the daunomycin (*DNR*)-treated spheroids

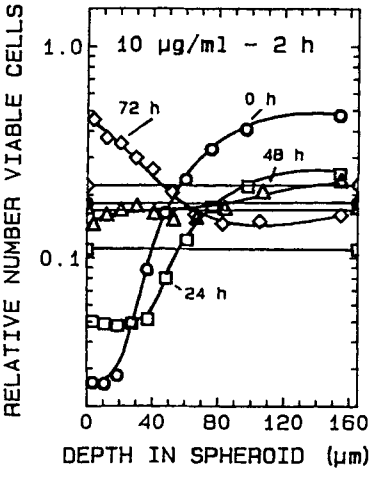

Fig. 9. Relative number of viable cells recovered from etoposide-treated spheroids, as a function of time of exposure and depth in the spheroid. Unlike the results obtained with anthracyclines (Figs. 7 and 8), the number of viable cells began increasing immediately in the outermost cell subpopulations; cell transit to the internal regions, however, appeared to be delayed

showed a G_2 block, which appeared to be maximal at 21–24 h. This high degree of cell-cycle arrest suggested that repopulation within the first 24 h was likely to play only a minor role in the overall response documented in the previous figures.

Similar observations over a longer period after spheroid exposure to doxorubicin, daunomycin and etoposide (another agent with a similar pattern of toxicity through the spheroids and with some degree of similarity in terms of effects on topoisomerase II; see [29, 33, 38]) are shown in Fig. 7–10. A somewhat smaller spheroid population (diameter, 407 ± 11 µm) was chosen to maximize the role of progression. After doxorubicin exposure, progressive changes in the number of viable cells recovered per sort fraction (as a function of depth in the spheroid) occurred during the post-exposure period (Fig. 7). Both repopulation and regrowth of the spheroids took place; at the termination of the experiment, the spheroids exceeded 600 µm in diameter. The data are plotted to indicate the increasing number of viable cells found in each fraction. Clearly, the most relevant feature to be seen in Fig. 7 is the rapid increase in the number of viable cells in the outermost one or two cell subpopulations of the spheroids, with the opposite effect noted for the first 48 h post-exposure in the innermost cell population (a net decrease in surviving cells). At 72 h, all cell subpopulations showed an increase in the number of viable cells (repopulation), yet none had fully recovered to pre-exposure numbers.

An equitoxic dose of daunomycin produced different results in the same spheroid population (Fig. 8). Progressive *decreases* in the number of viable cells in each fraction occurred over the following 3 days; again, the effect was considerably more dramatic in the innermost cell subpopulations. Over the same period, the spheroid diameters decreased from an initial size of 407 µm to a final size of 245 ± 14 µm. In the case of etoposide (VP-16), the post-exposure survival kinetics were more straightforward (Fig. 9). A progressive increase in the relative number of viable cells occurred during the first 3 days post-exposure, although, interestingly, repopulation appeared to be confined largely to the external regions of the spheroids, with less increase in the relative number of viable cells occurring in the innermost cell subpopulations. The spheroids first decreased to a minimal diameter of 300 ± 17 µm at 48 h, then regrew to a diameter of 350 ± 34 µm by 72 h.

For a better understanding of these kinetic changes and repopulation events, the DNA distributions observed over this time course are shown in Fig. 10, contrasted to control (untreated) values from the same cell subpopulation observed over the same 3-day period. Etoposide produced only very subtle changes in DNA distribution; a small increase in proliferating (S-phase) cells, however, can be deduced with numerical techniques. Similarly, doxorubi-

cin produced a small increase in the proliferative (growth) fraction. The greatest perturbation was seen with daunomycin; a very pronounced G_2 block was seen 1 day following exposure, and the perturbed DNA distribution obviously persisted throughout the entire period of exposure. We repeatedly observed a greater cytostatic effect for daunomycin relative to doxorubicin at equitoxic doses.

Discussion

As indicated in the introduction to this paper, the efficacy of the anthracycline antibiotics as anticancer agents would not be predicted based on the propensity of tumor cells to develop resistance to these agents and the poor delivery/distribution of these drugs in solid tumors. Contrary to the position that we and others have previously adopted, the present data suggest that slow (effective) drug diffusion and active efflux mechanisms may not necessarily be a therapeutic disadvantage in tumors in vivo.

Both the cellular membrane and nuclear DNA are targets for anthracycline binding [3, 12, 20, 34]; in terms of the reversibility of that binding, the available evidence suggests that DNA binding is likely to be the more stable method of retention of these drugs [16]. Since this propensity for binding presumably decreases the net diffusion rate of anthracyclines, it is not hard to envisage that this may indeed be a therapeutic advantage; not only is *inward* diffusion relatively slow, but so is *outward* diffusion. Thus, if therapeutic doses of the anthracycline derivatives can be delivered to the innermost cell subpopulations of a solid tumor, it would seem reasonable to expect that the ability of these agents to remain longer within the tumor cells may in fact markedly increase their cytotoxic effects. Qualitatively, this can be envisaged simply in terms of the concentration gradients produced: as the drug is delivered, the concentration gradient is very high from the drug reservoir (the blood supply in vivo or the culture medium in vitro) into the tumor or spheroid; once the drug has been removed, a much lower concentration gradient (inward to outward) is established and, as a consequence, outward diffusion probably occurs even more slowly than inward diffusion. Similarly, the dynamic changes in the concentration gradients must be considered during the "washout" phase; as drug effluxes into the reservoir (culture medium or blood supply), the highest intracellular drug concentration occurs in cells midway through the tumor cord or spheroid rim (e.g. Fig. 2b), with the net result that diffusion from those cells then continues to be "inward" towards the cells containing lower drug concentrations as well as "outward" toward the reservoir.

Clearly, timing is of crucial importance. This is also true at the cellular level; as the "contact time" increases for intracellular anthracyclines, a different subcellular drug distribution is expected. This has implications both for drug toxicity and for flow-cytometry-based measurements of intracellular drug levels. Our previous characterization of the subcellular deposition of doxorubicin in V79 cells showed that most of the retained drug was within the nucleus after about 2 h, and virtually all of the retained drug was intranuclear when those cells had been incubated in drug-free medium for >1 h [11]. Since the fluorescence of doxorubicin is quenched upon the drug's intercalation into cellular DNA, we did not convert the flow cytometry signals into quantitative estimates of intracellular drug concentration. It should be noted, however, that the flow cytometry data offers a *conservative* estimate of the anthracycline concentrations in the inner cells of spheroids with time: increasing fluorescence despite the greater quenching due to intercalation is clear evidence of increasing intracellular drug levels.

Although the spheroid system would seem to be ideal for more quantitative diffusion studies, it is, unfortunately, a highly dynamic system (not unlike a tumor). Thus, in addition to changing drug levels, kinetic changes in spheroid cell subpopulations continuously occur [6]. Cell division is normally restricted to the outermost regions of the spheroids, and these cells then progress inward to become the innermost (non-cycling, quiescent) population. If the growth rate is slowed, the environmentally severe, nutrient-deprived internal conditions in the spheroid can rapidly lead to cell death, even in the absence of any direct cytotoxic interaction between delivered agents and those innermost cell subpopulations. Although killing quiescent cells may be therapeutically useful, it is nonetheless a complicating factor in attempts to evaluate direct interactions between delivered chemotherapeutic agents and cell subpopulations of interest.

As we have shown in previous work [7, 8], the resistance of the innermost cell subpopulations of spheroids to the anthracyclines (even delivered at doses comparable to those achieved in the outermost cells; see Fig. 5b) is significant. Coupled with drug delivery problems in situ, in some cases the net resistance is even greater than that reported for MDR-positive cells. Since recent reports have implicated a role of hypoxia in gene amplification [28, 37], we would speculate that the internal microenvironment of the spheroid (and tumor?) may even promote drug resistance, thereby exacerbating an unfavorable therapeutic situation. It should be noted, however, that these conclusions rest largely on immediate post-exposure assays of drug toxicity, which clearly underestimate the net efficacy of the anthracyclines. This, in turn, highlights an important limitation of single-cell systems for "drug screening" and also has implications concerning the value of excision assays in experimental tumor (or spheroid) systems. Moreover, our results indicate the need for a critical evaluation of the role of overexpressed p-glycoprotein (enhanced active drug efflux) in a three-dimensional system; i.e. is the enhanced ability to transport drug out of a cell as relevant to the cell in the middle of a tumor mass as it is to that in the middle of a petri dish?

Acknowledgements. This work was supported by Medical Research Council of Canada grant MA-10238 and by USPHS grant CA-37775. I thank Denise McDougal, Sandra Vanderbyl and Nancy LePard for their expert technical assistance.

References

1. Bradley G, Juranka PF, Ling V (1988) Mechanism of multidrug resistance. Biochim Biophys Acta 948: 87
2. Cassady JR, Richter MP, Piro AJ, Jaffe N (1975) Radiation-Adriamycin interactions: preliminary clinical observations. Cancer 36: 946
3. Deffie AM, Alam T, Seneviratne C, Beenken SW, Batra JK, Shea TC, Henner WD, Goldenberg GJ (1988) Multifactorial resistance to Adriamycin: relationship of DNA repair, glutathione transferase activity, drug efflux and P-glycoprotein in cloned cell lines of Adriamycin-sensitive and -resistant P388 leukemia. Cancer Res 48: 3595
4. Donaldson SS, Glick JM, Wilbur JR (1974) Adriamycin activating a recall phenomenon after radiation therapy. Ann Intern Med 81: 407
5. Durand RE (1976) Adriamycin: a possible indirect radiosensitizer of hypoxic tumor cells. Radiology 119: 217
6. Durand RE (1976) Cell cycle kinetics in an in vitro tumor model. Cell Tissue Kinet 9: 403
7. Durand RE (1981) Flow cytometry studies of intracellular Adriamycin in multicell spheroids in vitro. Cancer Res 41: 3495
8. Durand RE (1986) Chemosensitivity testing in V79 spheroids: drug delivery and cellular microenvironment. J Natl Cancer Inst 77: 247
9. Durand RE (1986) use of a cell sorter for assays of cell clonogenicity. Cancer Res 46: 2775
10. Durand RE (1989) Distribution and activity of antineoplastic drugs in a tumor model. J Natl Cancer Inst 81: 146
11. Durand RE, Olive PL (1981) Flow cytometry studies of intracellular Adriamycin in single cells in vitro. Cancer Res 41: 3489
12. Egorin MJ, Hildebrand RC, Cimino EF, Bachur NR (1974) Cytofluorescence localization of Adriamycin and daunorubicin. Cancer Res 34: 2243
13. Erlichman C, Vidgen D (1984) Cytotoxicity of Adriamycin in MGH-U1 cells grown as monolayer cultures, spheroids and xenografts in immune-deprived mice. Cancer Res 44: 5369
14. Gerlach JE, Kartner N, Bell DR, Ling V (1986) Multidrug resistance. Cancer Surv 5: 25
15. Inoue S, Ohnuma T, Takaoka K, Suzuki Y, Kaneko M, Safirstein R, Holland JF (1987) Effects of doxorubicin and cisplatin on multicellular tumor spheroids from human lung cancer. Cancer Drug Deliv 4: 213
16. Kaye S, Merry S (1985) Tumour cell resistance to anthracyclines. A review. Cancer Chemother Pharmacol 14: 96
17. Kerr DJ, Kaye SB (1987) Aspects of cytotoxic drug penetration, with particular reference to anthracyclines. A review. Cancer Chemother Pharmacol 19: 1
18. Kerr DJ, Wheldon TE, Kerr AM, Freshney RI, Kaye SB (1986) The effect of Adriamycin and 4'-deoxydoxorubicin on cell survival of human lung tumour cells grown in monolayer and as spheroids. Br J Cancer 54: 423
19. Kerr DJ, Wheldon TE, Kerr AM, Kaye SB (1987) In vitro chemosensitivity testing using the multicellular tumor spheroid model. Cancer Drug Deliv 4: 63
20. Kerr DJ, Wheldon TE, Russell JG, Maurer HR, Florence AT, Halbert GW, Freshney RI, Kaye SB (1987) The effect of non-ionic surfactant Brij 30 on the cytotoxicity of Adriamycin in monolayer spheroids and clonogenic culture systems. Eur J Cancer Clin Oncol 23: 1315
21. Kerr DJ, Wheldon TE, Hydes S, Kaye SB (1988) Cytotoxic drug penetration studies in multicellular tumour spheroids. Xenobiotica 18: 641
22. Kramer RA, Zakher J, Kim G (1988) Role of the glutathione redox cycle in acquired and de novo multidrug resistance. Science 241: 694
23. Krishan A, Ganapathi R (1979) Laser flow cytometry and cancer chemotherapy: detection of intracellular anthracyclines by flow cytometry. J Histochem Cytochem 27: 1655
24. Kwok TT, Twentyman PR (1984) The response to cytotoxic drugs of EMT6 cells treated either as intact or disaggregated spheroids. Br J Cancer 51: 211
25. Nederman T, Carlsson I, Malmqvist M (1981) Penetration of substances into tumor tissue – a methodological study on cellular spheroids. In Vitro 17: 290
26. Olive PL (1986) Patterns of mutagen binding and penetration in multicell spheroids. Environ Mutagen 8: 705
27. Olive PL, Chaplin DJ, Durand RE (1985) Pharmacokinetics, binding and distribution of Hoechst 33342 in spheroids and murine tumours. Br J Cancer 52: 739
28. Rice GC, Ling V, Schimke RT (1987) Frequencies of independent and simultaneous selection of Chinese hamster cells for methotrexate and doxorubicin (Adriamycin) resistance. Proc Natl Acad Sci USA 84: 9261
29. Ross WE (1985) DNA topoisomerases as targets for cancer therapy. Biochem Pharmacol 34: 4191
30. Soranzo C, Ingrosso A (1988) A comparative study of the effects of anthracycline derivatives on a human adenocarcinoma cell line grown as a monolayer and as spheroids. Anticancer Res 8: 369
31. Sutherland RM (1988) Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 240: 177
32. Sutherland RM, Eddy HA, Bareham B, Reich K, Vanantwerp D (1979) Resistance to Adriamycin in multicellular spheroids. Int J Radiat Oncol Biol Phys 5: 1225
33. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226: 466
34. Tritton TR, Yee G (1986) The anticancer agent Adriamycin can be actively cytotoxic without entering cells. Science 217: 248
35. Twentyman PR (1979) Timing of assays: an important consideration in the determination of clonogenic cell survival both in vitro and in vivo. Int J Radiat Oncol Biol Phys 5: 1213
36. Vindelov LL (1977) Flow microfluorometric analysis of nuclear DNA in cells from solid tumors and cell suspensions. A new method for rapid isolation and staining of nuclei. Virchows Arch [Cell Pathol] 24: 227
37. Young SD, Marshall RS, Hill RP (1988) Hypoxia induces DNA over-replication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci USA 85: 9533
38. Zwelling LA (1985) DNA topoisomerase II as a target of antineoplastic drug therapy. Cancer Metastasis Rev 4: 263