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Flow Cytometry Studies of Intracellular Adriamycin in Multicell Spheroids *in Vitro*[1]

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ABSTRACT

A marked gradient of Adriamycin uptake in cells of tissue-like multicell spheroids *in vitro* has been demonstrated by fluorescence photomicroscopy and flow microfluorimetry techniques. As expected, this gradient led to an increased net survival for cells from Adriamycin-treated spheroids relative to monolayers and markedly greater clonogenicity of central spheroid cells than external cells selected by fluorescence-activated cell sorting. Growth of cells as spheroids seemed to impart an additional degree of drug resistance relative to cells grown as monolayers, in that equal toxicity required greater intracellular fluorescence (and thus more Adriamycin) for the spheroid cells. The flow cytometry techniques thus provide a mechanism for quantification of Adriamycin penetration into spheroids and provide a method for selection of cells from various depths within the spheroid.

INTRODUCTION

Recent work has demonstrated the utility of FCM[3] techniques in monitoring intracellular Adriamycin content (4, 7, 8) and has related intracellular fluorescence to the toxic actions of the drug (5). Even within well-controlled *in vitro* exposure conditions, however, considerable heterogeneity of intracellular fluorescence can be observed within the cells of a population (5). A more complex situation can be anticipated *in vivo*, where dense cell packing, distance from the vascular supply, drug elimination, and other factors will influence drug availability. Thus, it seems likely that even greater heterogeneity of intracellular drug levels can be anticipated *in vivo*.

Adriamycin penetration into cells in a tissue-like situation can be studied *in vitro* using multicell spheroids as a model system (11). Previous studies with the V79 spheroid system have demonstrated that cell packing in the spheroid is very tight, as it is in tissues (5). Diffusion gradients of oxygen which are quantitatively similar to those *in vivo* are easily demonstrated (11), so it seems reasonable to assume that penetration of other metabolites or drugs will mimic the *in vivo* situation.

Previous results obtained with the V79 system (9), as well as another spheroid system, EMT6 tumor cells (12), indicated a marked diffusion gradient of Adriamycin within those spheroids. As expected, additional resistance to the drug due to its nonpenetration into the spheroid was observed (9, 12). Prior studies with the V79 spheroid system also showed that cells of spheroids were considerably more resistant to Adriamycin than were single cells (1). However, in those studies, very small spheroids of 6 to 10 cells were used; it thus seems unlikely that diffusion was a major limitation in those studies.

In the present report, we present quantitative measurements of Adriamycin localization in cells of V79 spheroids of various sizes and correlation of these FCM measurements with survival of the Adriamycin-treated spheroid cells. Additionally, we have utilized fluorescence-activated cell sorting to examine survival as a function of intracellular drug content (fluorescence) for cells from the external and internal regions of the spheroids.

MATERIALS AND METHODS

All FCM measurements used procedures identical to those described previously (5). Analysis of the FCM profiles and calculation of the mean fluorescence utilized the same techniques as those described in that paper (5). Due to obvious differences in response expected for different spheroid populations, all data presented are for a single experiment, chosen to be representative of 3 or more replicate experiments.

Our techniques for growing V79 Chinese hamster cells have been described in detail previously (2, 3, 11). In brief, spheroids are grown in a stirred suspension culture, initiated by seeding 10^4 cells/ml in a spinner flask, and with the cultures fed on Days 3, 5, and daily thereafter with minimal essential medium plus 5% fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.). Intact spheroids were exposed to Adriamycin by adding the drug to the culture medium. Adriamycin was freshly prepared from lyophilized stock immediately prior to each experiment.

Single-cell suspensions were prepared from spheroids by trypsinization using 0.25% lyophilized trypsin (Grand Island Biological Co.) in a citrate-0.9% NaCl buffer solution for 10 to 12 min at 37°, followed by repeated pipetting to ensure that a single-cell suspension resulted. Cells were then held at 4° until FCM analysis or sorting was complete. Cells from disaggregated spheroids were exposed to Adriamycin as monolayers. In all cases, the spheroids were trypsinized, cell counts were performed, and plates were seeded at a cell density (number of cells/ml of medium) equal to that of intact spheroids in spinner culture. A half-hr period was allowed for the cells to attach to the Petri dishes, at which time medium containing Adriamycin was added for the appropriate exposure time, and the monolayer was then trypsinized to form a single-cell suspension for FCM and viability studies.

Histological sections were prepared by exposing intact spheroids to the indicated concentration of Adriamycin, sharp freezing the spheroids, and sectioning the spheroids using a refrigerated microtome. Photographs were taken using a Zeiss photomicroscope equipped with an epifluorescent condensor, with excitation at 480 nm and fluorescence observed above 520 nm.

RESULTS

A qualitative impression of the diffusion rate of Adriamycin into V79 spheroids can be gained from Fig. 1, where spheroids were exposed to a relatively high drug concentration (to facilitate fluorescence photomicroscopy) and then frozen and sectioned at the indicated times. Diffusion of the drug into the V79 spheroids was obviously a fairly slow process, with only one to 2 cell layers brightly fluorescent after a 1-hr exposure. Interestingly, however, after 1 hr, most nuclei were somewhat fluorescent, and pyknotic cells throughout the spheroid were brightly fluorescent. The number of brightly fluorescent intact

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[3] The abbreviation used is: FCM, flow cytometry.
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cells, however, seemed to increase more slowly than for EMT6 spheroids (12). This was not a surprising result, insofar as the cell packing in the V79 spheroid is probably much tighter than in the EMT6 system.

A more quantitative approach to the diffusion of Adriamycin into V79 spheroids is shown in Chart 1, where cells from small or large spheroids were exposed to Adriamycin either as intact spheroids (*closed symbols*) or after disaggregation (*open symbols*). The mean fluorescence was plotted as a function of time for each population. It can be readily appreciated that the average intracellular drug content at any time was considerably greater in the disaggregated cells of the spheroids than in cells exposed as spheroids. Additionally, the mean intracellular fluorescence achieved for a given drug exposure was much greater in small spheroids than in larger spheroids. A somewhat unexpected result was also evident in Chart 1, as the cells from disaggregated large spheroids showed much less average uptake of Adriamycin than their counterparts from disaggregated small spheroids.

To examine the heterogeneity of Adriamycin content of the cell population, data were analyzed by determining the ratio of the fluorescence value exceeded by 2% of the population compared to that of the fluorescence value exceeded by 98% of the population (see Ref. 5). As would be expected, considerably more heterogeneity was seen in large spheroids (Chart 2*a*). Unlike the results obtained with cells from monolayers or disaggregated spheroid cells, heterogeneity in both small and large spheroids increased with increasing drug concentration.

A question of obvious interest is whether the decreased mean fluorescence of cells from an intact Adriamycin-treated spheroid is reflected by a decrease in intracellular Adriamycin content of all cells or is, instead, a result of the increased heterogeneity and relatively large number of cells containing only small quantities of drug. To address this question, each fluorescence profile was integrated, and the fluorescence of the "brightest" and "dimmest" 10% of the Adriamycin-treated cells from the intact and disaggregated spheroid populations was calculated and plotted in Chart 2*b*. The brightest cells were equally fluorescent regardless of the exposure condition; a marked difference in the fluorescence of the dimmest cells was, however, noted. The 10% level was not chosen on arbitrary grounds but, rather, because it represented the number of cells expected in the outer layer for spheroids of the size used in these experiments (2).

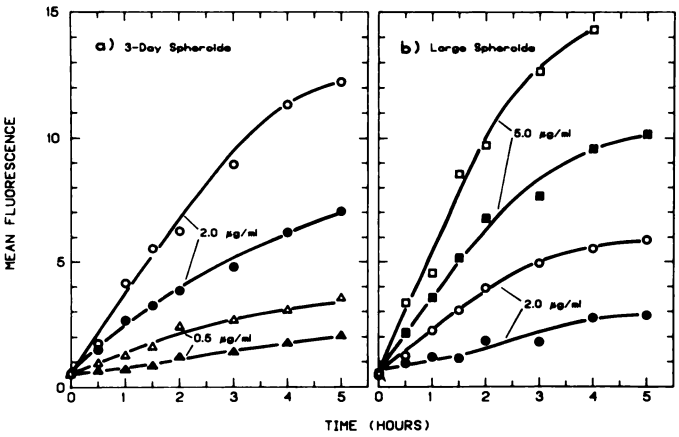

Chart 1. Adriamycin uptake as a function of drug concentration and time is shown for cells of small or large spheroids exposed to Adriamycin as intact spheroids (*closed symbols*) or after disaggregation of the spheroids to form a monolayer (*open symbols*). Note that the mean intracellular fluorescence was always greater for cells exposed as monolayers than for cells exposed as spheroids. Further, cells from large spheroids exhibited considerably less intracellular fluorescence.

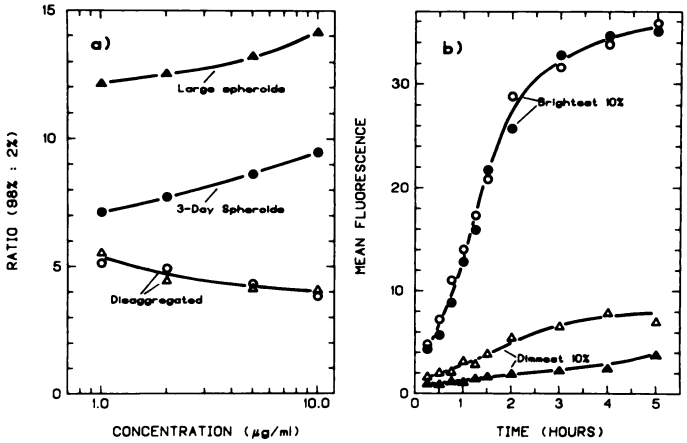

Chart 2. Heterogeneity of intracellular Adriamycin fluorescence is shown as a function of drug concentration for cells exposed as intact large or small spheroids (*closed symbols*) or after disaggregation into monolayers (*open symbols*). In *a*, heterogeneity increases as a function of drug concentration for cells exposed as intact spheroids, whereas a decrease is observed with increasing concentration when individual cells were exposed. Considerably more heterogeneity was seen for large spheroids. In *b*, when the mean intracellular fluorescence of the 10% of the cells having the most intracellular drug was compared after an exposure of 5 μg/ml, no difference was observed. Conversely, a large difference was noted in the 10% of the cells taking up the least Adriamycin.

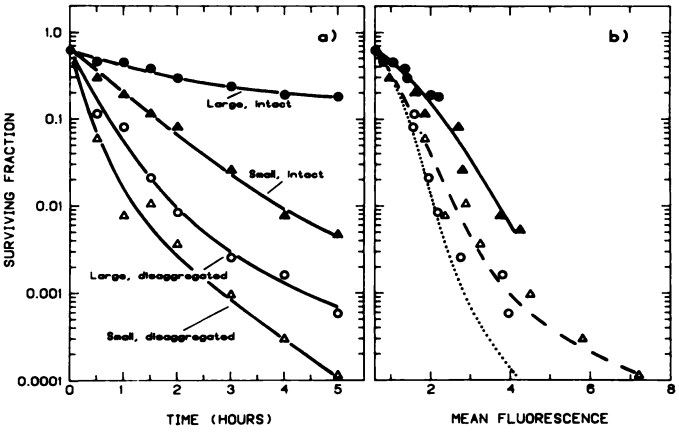

Chart 3. Toxicity of Adriamycin (1 μg/ml) to cells from small and large spheroids as a function of exposure condition and time. *Closed* symbols, cells exposed while in intact spheroids; *open* symbols, disaggregated cells from spheroids. In *b*, when survival was plotted as a function of mean intracellular fluorescence, more intracellular drug was required for a given level of toxicity in cells of intact spheroids than in disaggregated spheroid cells, and both appeared to require more drug than for cells grown as monolayers (···), where the *latter curve* was reproduced from Ref. 5.

The differential Adriamycin uptake observed for cells of intact and dissociated large and small spheroids correlates to some extent with the eventual cell survival (Chart 3*a*). As would be predicted on the basis of the intracellular drug levels (*e.g.*, Chart 1), large spheroids were, on the average, considerably more resistant to the Adriamycin treatment than were small spheroids, and cells of both were more resistant than if Adriamycin exposure occurred after separation of the spheroids into single cells. In agreement with the FCM data of Chart 1, cells from large spheroids were more drug resistant than were cells from smaller spheroids when both were exposed to Adriamycin as monolayers (Chart 3*a*).

When the same data were plotted with surviving fraction as a function of mean intracellular fluorescence (Chart 3*b*), a correlation was evident between toxicity and intracellular drug levels. Interestingly, the survival curves for intact spheroids always fell to the right of those for cells exposed after disaggregation, indicating that more intracellular drug (increased mean fluorescence) was required for equal toxicity when cells were exposed as intact spheroids. Likewise, the curve for cells from disaggregated spheroids indicated greater resistance than that of cells grown as monolayers (where the "control" curve has been reproduced from Ref. 5). The data in this chart represent the "minimum" response observed in numerous repetitions; when larger drug doses were used with large spheroids, additional killing was observed but only with a marked increase in the mean intracellular fluorescence (data not shown). Stated differently, as the heterogeneity of intracellular Adriamycin levels increased, the survival data fell even further to the right than the indicated lines of Chart 3*b*. Thus, V79 cells grown as spheroids were more resistant to Adriamycin than were cells grown as monolayers, and when treated as intact spheroids, these cells were even more drug resistant when survival was expressed as a function of either external drug concentration or mean intracellular fluorescence.

A better correlation between the intracellular drug level (fluorescence) and eventual cell survival should be obtainable if cells are sorted on the basis of their intracellular Adriamycin fluorescence. Using this approach, the mean intracellular fluorescence of subpopulations, selected in increments of 10% of the population, is plotted in Chart 4*a*. Again, population homogeneity was greatest (as was mean intracellular fluorescence) for dispersed cells from small spheroids (*open symbols*). Most heterogeneity was seen for cells of large spheroids that were intact when exposed to Adriamycin. When sorting windows were set to collect the dimmest 20% of the cell population, the next 20%, etc., the survival curves of Chart 4*b* resulted. These data again indicate a large degree of heterogeneity in the intracellular fluorescence level (and hence survival) of cells exposed as large intact spheroids, relative to dispersed cell populations. Additionally, for a given level of fluorescence, most toxicity was observed in the dispersed cells, and least, in the intact large spheroids.

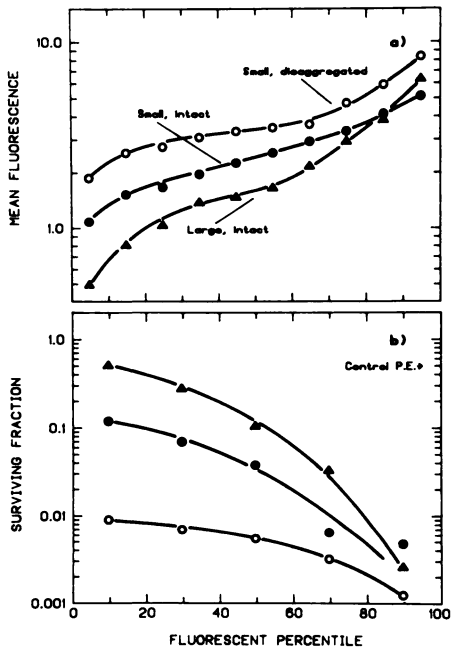

Chart 4. Adriamycin toxicity to sorted cells from spheroid populations. *a*, mean fluorescence of the dimmest 10% of the cells, the next 10%, etc. *Open symbols*, spheroid cells exposed to 1 μg Adriamycin for 2 hr as monolayers; *closed symbols*, cells exposed as intact spheroids. *b*, surviving fraction plotted for sorted cells. Thus, the first symbols represent the survival of the dimmest 20% of the cells, the next represent the next 20% of the cells, etc. Considerably less toxicity was noted for cells from large, intact spheroids, as expected.

DISCUSSION

The results presented in this paper show a marked diffusion gradient of Adriamycin into V79 spheroids. As would be expected, large spheroids appear quite Adriamycin resistant due to the relative inaccessibility of a large population of the cells of the spheroid. Additionally, however, growth of V79 cells as spheroids conferred a further degree of drug resistance; an analogous resistance to heat, radiation, and numerous other chemotherapeutic agents has been previously reported (3, 4, 6, 10, 11), and been attributed to growth under the conditions of close intercellular contact.

The heterogeneity of the observed intracellular Adriamycin levels was found to be a function of spheroid size, but even in large spheroids, intracellular fluorescence correlated well with cell survival from the various populations. Interestingly, cells from the external layer of large spheroids accumulated drug as rapidly as dispersed cells; only the internal cells were subject to additional diffusion limitations, as suggested previously using a different technique (9). It should be noted, however, that direct comparisons between spheroids and dispersed cells may be inappropriate. A number of factors may influence drug uptake, including cell geometry at the time of exposure, previous growth history, cell density, cell cycle position, and other factors (5). Thus, it is unclear whether the appropriate single-cell "control" for spheroids in suspension is a single-cell suspension or monolayer, or what cell density should be used. In all cases in the present report, the "control" curves for each chart were the same cell population (*i.e.*, cells from spheroids), exposed as single-cell monolayers rather than spheroids. Although trypsinization of the spheroids prior to Adriamycin exposure might be expected to alter their subsequent uptake of Adriamycin, we have not observed this in appropriate control experiments (5).

It is perhaps somewhat surprising that such a pronounced Adriamycin diffusion problem was evident in this spheroid system, particularly since Adriamycin shows activity against such a wide spectrum of solid tumors *in vivo*. Adriamycin treatment *in vivo*, however, usually involves numerous drug exposures, which would presumably eliminate some diffusion problems if progressive layers of the tumor were attacked by subsequent treatments. It must also be recognized that drug delivery through a uniform tissue mass *in vitro* may be quite different than *in vivo*, where the vascular system will be of critical importance.

An interesting corollary results from the present work. The marked diffusion gradient of Adriamycin into V79 spheroids should be exploitable as a technique for cell separation or selection. Since intracellular drug level is evidently a function of depth of the cell within the spheroid, relatively simple calculations based on the fluorescence profiles can be used to determine the location of cells collected in a sorting window based on degree of fluorescence. We are currently utilizing

this technique to select and study the internal noncycling and/or hypoxic cells from spheroids after treatment with Adriamycin and other cytotoxic agents. The technique is particularly attractive in this latter application, since the cells of interest can be selected on "negative" criteria; depending on exposure conditions, little if any Adriamycin reaches the cells, so their response to the other agents can be measured with minimal perturbation due to the selection procedure. Presumably, these techniques might be adaptable for transplantable tumors *in vivo*; such studies of tumor or spheroid repopulation after exposure to cytotoxic agents may provide a rationale for improved clinical therapy.

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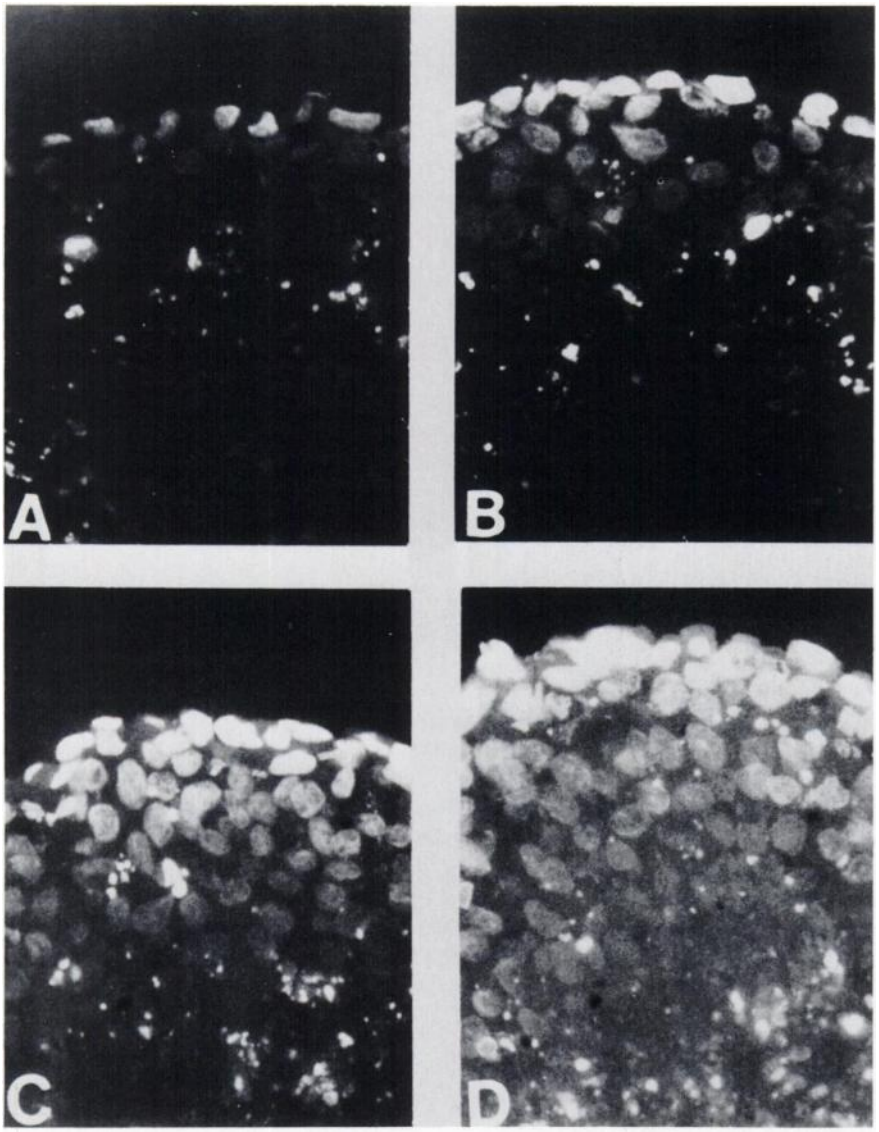

Fig. 1. Fluorescent photomicrographs of sections from spheroids exposed to 10 μg Adriamycin per ml for 10, 20, 30, or 60 min, respectively (*A* to *D*). Note that only external cells show high levels of intracellular Adriamycin, although fluorescence can be seen in all cells at the longest exposure time.

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