Influence of Microenvironmental pH on Adriamycin Resistance

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ABSTRACT

Resistance to Adriamycin (ADR) is frequently dependent upon enhanced efflux associated with the expression of the MDR1-encoded P membrane glycoprotein. Since enhanced expression of the MDR1 gene in ADR-resistant cells may be the result of spontaneous genetic mutation or amplification, it is presumed to be relatively stable and unalterable. Yet, reducing ADR efflux could increase sensitivity, and has been attempted using calcium channel blockers and other drugs. However, since the tumor cell microenvironment varies with respect to pH because of differences in vascularization, oxygenation, and metabolite clearance, the possibility exists that these factors could influence drug transport and the critical biochemical pathways which determine cytotoxicity, even in resistant cells.

Using flow cytometric analysis of ADR fluorescence, the influx and efflux of 10 μM ADR dissolved in MES buffer (pH 6.5) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 7.5 and 8.5) was measured in sensitive P388 and resistant P388/R84 cells in vitro. Substantially enhanced uptake of ADR was detected at alkaline pH in both cell populations, while the proportion of ADR-positive cells and the level of ADR uptake was decreased at lower pH. Acidification reduced ADR efflux, whereas alkalization increased efflux when the uptake pH was 6.5 or 7.5. At uptake pH 8.5, the pH of the external buffer had little effect, even in resistant cells. In resistant cells in an alkaline microenvironment, ADR transport and retention were superior to that observed in sensitive cells in an acidic microenvironment. No differences were observed in ADR transport when the transmembrane pH gradient was equilibrated. These observations are especially relevant to the effect of ADR on tumor cell subpopulations that are acidic, and in which drug diffusion is inefficient. Efforts to alkalize tumor cells prior to ADR therapy might reduce ADR resistance, even of genetic origin.

INTRODUCTION

Chemotherapeutic cytotoxicity depends upon many factors such as an adequate drug concentration in the tumor cell microenvironment, the cell cycle distribution, the rate of bidirectional drug transport across the cell membrane, relative rates of drug activation and deactivation, and the subsequent inhibition of cell reproductive processes, with or without lethal metabolic paralysis. Yet, even when these factors appear to favor drug cytotoxicity a variable proportion of cells usually survive drug treatment, particularly in solid tumors.

The most important reason for cell survival following cytotoxic drug exposure is the presence or development of genetic variants that are relatively drug resistant. Rapid mutation of malignant cells has been hypothesized to account for the development of resistant clones that finally emerge as predominant when sensitive cell subpopulations are eradicated during treatment (1). Apart from such spontaneous mutations, evidence exists for the transfer of drug-resistant genes between cells within a cell population (2); a mechanism that would also enhance survival of cell subpopulations within a tumor subjected to chemotherapy. Another important genetic mechanism of drug resistance is the development of pleiotropic drug resistance following drug exposure (3, 4). At the cellular level, this is characterized by the development of a wide range of cross resistance to anticancer drugs, usually of natural plant origin, that is induced by previous drug exposure. Activation of the multidrug-resistant gene (MDR1) is associated with the expression of a specific membrane glycoprotein of M, 170,000 (5, 6). The effect of this energy-dependent membrane pump protein is to enhance the efflux of intracellular drugs such as vincristine and ADR. Further studies have demonstrated that as the gene which encodes this protein is amplified, drug efflux is increased (7). The underlying mechanism by which this protein increases drug efflux has been elucidated in a number of studies that have demonstrated binding of vinblastine and anthracyclines to this P170 membrane glycoprotein (8, 9), and precipitation of the drug-protein complex by a monoclonal antibody to the P170 glycoprotein (10). Although expression of the P170 membrane glycoprotein has been demonstrated in selected patients with ovarian cancer (11), it is not yet known how frequently such expression occurs or is amplified in tumor cell populations during clinical chemotherapy.

Nevertheless, there is increasing interest in finding ways to reverse pleiotropic drug resistance by increasing drug uptake and reducing drug efflux. Specifically, ADR resistance has been diminished by the administration of verapamil, a calcium antagonist (12), and by certain drugs such as phenothiazines which bind to calmodulin, the calcium regulatory protein (13-15).

In evaluating the effect of drugs on ADR transport, the flow cytometric technique (16-20) has the advantage of predominantly measuring the free ADR in individual cells. This technique not only permits the detection of cells which contain measurable levels of ADR, but also the detection of cell subpopulations with different ADR levels (18, 19), and the detection of cells that do not contain free ADR.

In this study, we used flow cytometric analysis to measure the influence of extracellular pH (and intracellular pH) on the uptake and efflux of ADR in sensitive P388 and resistant P388/R86 cells (18, 19). These studies indicate that the microenvironmental pH influences the uptake of ADR, the proportion of cells that contain ADR, the relative intracellular ADR concentration, and the rate of efflux of ADR: all factors that can influence the cytotoxic process. Furthermore, to contrast the effects of intracellular and extracellular pH, the transmembrane pH gradient was equilibrated to determine the effect of intracellular pH on ADR influx and efflux.

MATERIALS AND METHODS

The P388/R84 and P388 murine leukemia cell lines were maintained as suspension cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum, antibiotics (penicillin and streptomycin), and 5% 2-mercaptoethanol. The cells were passaged every 2 to 3 days by diluting one third of the suspension culture with fresh medium. For determination of ADR uptake and efflux, log phase cells were removed from culture flasks, washed twice with ice-cold PBS, using centrifugation at 1,000 rpm for 5 min at 4°C. Equal numbers of cells were resuspended...
in three flasks containing prepared buffers of pH 6.5, 7.5, and 8.5. The pH 6.5 buffer was prepared by dissolving MES in RPMI so that the final buffer concentration was 25 mM MES. The pH was adjusted by adding HCl. The pH 7.5 buffer was prepared by dissolving HEPES in RPMI so that the final concentration was 25 mM HEPES. The pH was adjusted by the addition of NaOH. Finally, the pH 8.5 buffer was also prepared with HEPES in the same way as the pH 7.5 buffer, except that NaOH was added until the pH was 8.5. ADR stock solution (1 mM; Adria Laboratories, Inc., Columbus, OH) was added to each buffer to obtain a final ADR concentration of 10 μM. The final osmolarities of the three buffers were 307, 310, and 327 mOsm/liter; respectively.

Triplicate flasks containing P388 and P388/R84 cells in each of the three ADR-containing buffers were then transferred to a water bath at 37°C and the cells were allowed to incubate for varying time periods. Trypan blue viability exceeded 90% in all experiments during the selected time periods, which also correlated with viability by light scatter analysis (21). At predetermined time points, a small aliquot of cells was removed from each flask, washed twice in ice cold PBS, and resuspended in a small volume of ice-cold PBS prior to immediate flow cytometric analysis, using a FACS IV (Becton Dickinson) equipped with a 4-W argon laser at 488 nm. Using the well-established technique for measuring intracellular ADR by flow cytometry (16–20), cells were counted and analyzed by amplified measurement of forward-angle light scatter and simultaneous fluorescence, which distinguished cells that were fluorescence negative from those that were positive. Fluorescence emission was only collected beyond 520 nm using an appropriate band pass filter. At least 50,000 cells were measured during each sample analysis; and each sample was analyzed several times to ensure stability and reproducibility of the histograms. Data were collected on a PDP-11/34 computer (Digital Equipment Corporation) using NIH analytical software. Data analysis included measuring median ADR fluorescent intensity and counting the number of ADR-positive and -negative cells. Each complete experiment was repeated at least three times, but some variations in the time intervals made exact data comparisons impossible. Note that before these experiments were performed, drug-free cells were analyzed to determine the possible influence of autofluorescence. Any background fluorescence was then eliminated from the data analysis by establishing a fluorescence threshold for the presence of ADR, which in turn was correlated with an appropriate cell-light-scatter signal. In addition, FACS amplifier gain settings were standardized to ensure fluorescence comparability between all samples.

Initial uptake experiments monitored ADR concentration in cells after incubation for 15, 45, 75, 105, and 135 min, respectively. The effect of drug concentration was evaluated at ADR concentrations of 5, 10, and 50 μM respectively (data not shown). Comparable pH effects on ADR uptake were evident at all ADR concentrations, but 10 μM was arbitrarily selected for the complete range of experiments on influx and efflux described here.

The effect of pH 6.5, 7.5, and 8.5 (for 90 min at 37°C) on ADR uptake was then measured, and the cells were washed once in ice cold (4°C) matching drug-free buffers before being resuspended in warm (37°C) drug-free buffers of pH 6.5, 7.5, and 8.5, respectively. ADR efflux was then measured in each cell population after 30 min and 90 min, respectively, using flow cytometric analysis of residual cell fluorescence.

RESULTS

Experiments with sensitive and resistant cells were always done concurrently so that differences between cell lines could be evaluated under standardized conditions. However, when experiments were repeated several times, small variations in ADR uptake were sometimes detected that may have resulted from differences in cell growth phase at the time cells were harvested for experiments. Typical examples of data are therefore presented.

Flow cytometric analysis of ADR uptake in P388/S (Fig. 1, top) and P388/R84 (Fig. 1, bottom) cells after 105 min, is shown in Fig. 1. In these examples of fluorescence histograms, there is clear evidence of a considerable difference in the intensity of ADR fluorescence depending upon the pH of the drug-containing buffers in both sensitive and resistant cell populations. Furthermore, it is possible to discern two subpopulations that have differing fluorescent intensity among sensitive cells in a pH 6.5 buffer, and among resistant cells that were exposed to ADR in all three buffers.

Fig. 2 indicates the proportion of P388 cells (Fig. 2, top) and P388/R84 cells (Fig. 2, bottom) that have taken up ADR. The median level of ADR per cell is indicated by the median channel number (fluorescence intensity) of ADR uptake for the cell population. In these experiments, the proportion of P388 cells that take up ADR at pH 6.5 is lower than that at pH 7.5 and 8.5 and the median intensity increases significantly with increasing pH. However, in this particular experiment, the uptake of ADR by P388/R84 cells at pH 6.5 revealed a smaller proportion of ADR-positive cells, but with a comparable median fluorescent intensity. At pH 7.5, the median fluorescence remained the same, while the proportion of ADR-positive cells
Increased. After 105 and 135 min at pH 8.5, there were two distinctive subpopulations of differing fluorescence intensity. These subpopulations were analyzed separately and consisted of a minority population of relatively high median fluorescence, and a majority population of lower median fluorescence. However, when combined, these two subpopulations consisted of 100% of the cell population.

Fig. 3 reveals the influence of pH on the uptake and subsequent efflux of ADR in sensitive P388 cells (Fig. 3, top) and resistant P388/R84 cells (Fig. 3, bottom). After 90 min of incubation in a buffer of pH 6.5, ADR was taken up by nearly the whole sensitive cell population, but only by about 80% of the resistant cells. Cells were then transferred to buffers of pH 6.5, 7.5, and 8.5, and the efflux was measured at 30 and 90 min, respectively. After 30 min of efflux in a buffer of pH 6.5, the proportion of both P388 and P388/R84 cells that contained ADR was nearly halved; by 90 min the fraction of ADR-positive cells was halved again. The median intensity of ADR fluorescence was also approximately halved during this period, but remained constant as the fraction of ADR-positive cells declined.

Efflux of ADR was greater at pH 7.5 and 8.5. At efflux pH 7.5, in both sensitive and resistant cell populations, about half as many cells were positive for ADR as were seen at efflux pH 6.5, while the median intensity of fluorescence remained essentially unchanged. Within 30 min at efflux pH 8.5, there were again about half as many cells positive for ADR than were seen at efflux pH 7.5, and ADR efflux was greater still, being virtually complete by 90 min in both sensitive and resistant populations. Interestingly, at 30 min efflux was not diminished in P388/R84 cells at pH 8.5, since the median ADR concentration was similar to that found in the P388 cell population.

In Fig. 4, ADR uptake occurred at pH 7.5 and involved nearly 100% of both cell populations. However, the median ADR concentration was higher in P388 cells (Fig. 4, top). Differences in efflux were seen between sensitive and resistant cells. At efflux pH 6.5, it was evident that most P388 cells remained ADR positive, although there was a decline in the median drug concentration. In contrast, while many P388/R84 cells (Fig. 4, bottom) rapidly became ADR negative, the median ADR concentration declined by about a third and then stabilized.

At efflux pH 7.5, a similar response was seen. Only at efflux pH 8.5 did substantial differences occur between the sensitive and resistant cell populations. Although there was a decline in the number of ADR-positive P388 cells, there was a much greater reduction in the proportion of ADR-positive P388/R84 cells. Despite this, the median drug concentration in P388/R84 cells remained comparable to that in sensitive P388 cells. By 90 min, all ADR had been lost from resistant cells.

Finally, in Fig. 5, it is evident that all cells took up ADR at pH 8.5, although the median ADR concentration was lower in P388/R84 cells (Fig. 5, bottom). Relatively little efflux was seen at any pH among P388 cells (Fig. 5, top) compared to the efflux seen in P388/R84 cells. Furthermore, there was little effect of time on efflux in the sensitive population. Again, among resist-
INFLUENCE OF MICROENVIRONMENTAL pH ON ADR RESISTANCE UPTAKE

PH7.5 — pH8.5 — pH9.5 — pH6.5 — pH7.5 — pH8.5

Time, Minutes

Fig. 4. Top and bottom similarly represent the uptake of 10 μM ADR by P388 and P388/R84 cells after 90 min in a buffer of pH 6.5, 7.5, and 8.5, respectively. At this uptake pH there was greater contrast between sensitive and resistant populations, with many more sensitive cells retaining ADR than resistant cells. The median ADR concentration remained relatively high among resistant cells, although the relative number of ADR-positive cells declined substantially.

ant cells, the greatest effect on efflux was seen in the declining fraction of ADR-positive cells, with comparatively less effect on the median ADR concentration.

DISCUSSION

There is an assumption which is implicit in many pharmacological studies of chemotherapeutic drug action and resistance, that the degree of cytotoxicity is dependent upon stable and predictable biochemical pathways within tumor cells. Furthermore, it is presumed that intrinsic sensitivity and resistance is predominantly determined by variations in gene expression that occur as a result of a high spontaneous mutation rate in tumor cell populations (1). After all, variations in drug sensitivity occur much less frequently in more stable normal cells, which happen to be the specific site of toxicity. The greater predictability of the response of specific normal cells to cytotoxic drug action may not only result from more genomic stability, but also from a much better regulated cell microenvironment. Although the tumor cell microenvironment is known to be much more variable than that of normal cells, it is less clear to what extent this microenvironment can influence the degree of drug resistance, particularly when it is of genetic origin.

Despite known differences in the MDR1 expressed P-glycoprotein level in the P388 and P388/R84 cell lines (19), the data reveal differences in drug transport and efflux which are not easily explained simply on the basis of a modified efflux pump. In particular, it is now evident that the microenvironmental pH during the uptake of ADR influences subsequent efflux. Yet, it remains to be determined whether pH variation can also influence the effect of calcium channel blockers on ADR transport.

Other factors apart from the intracellular drug concentration influence the relative sensitivity of cells to anthracyclines: the capacity for DNA repair, the activity of DNA topoisomerase II and the activity of the redox cycle may have varying importance in different cell populations or subpopulations (22–25). However, the influence of pH on these factors was not measured. It remains possible that any change in the degree of ADR cytotoxicity induced by variations in the pH of the microenvironment may be modulated not only by changes in ADR transport, but also by the influence such variations in the extracellular pH may have on the intracellular pH, which could modulate such critical cell functions.

The flow cytometric method of analyzing ADR uptake and efflux, although predominantly measuring free intracellular ADR, is also an effective means to measure the heterogeneity of ADR uptake and efflux within a cell population. The measured heterogeneity of ADR transport suggests that relatively resistant cells exist within the sensitive P388 cell population, and that relatively sensitive cells exist within the resistant P388/
R84 cell population. Although enhanced efflux is not the only basis for ADR resistance, this interpretation is supported by evidence that intracellular ADR levels as measured by the flow cytometric method have been shown to correlate closely with subsequent cytotoxicity (26).

When evaluating these data, it is important also to exclude any potential artefacts or other factors that might have caused changes in cellular fluorescence. Since the fluorescence emission intensity of ADR is known to be influenced by DNA binding, it is important to assess an effect of pH itself on the proportion of ADR bound to DNA. In fact, no significant effect of pH within the range 6.4–7.8 has been observed on ADR binding to DNA (27). Neither was any effect of pH observed on the quantum efficiency of ADR during these experiments. Loss of cell viability and cell break down could also potentially distort fluorescence measurement, but the fluorescence signals were only measured from cells that generated an adequate light-scatter signal, which is an effective means of distinguishing viable from nonviable cells and of estimating cell volume (21). This procedure ensured that fluorescence was derived only from viable intact cells.

The data presented here provide information unavailable from standard pharmacological drug transport studies. In particular, the histograms reveal the proportion of ADR-negative cells (within the limits of detection); the proportion of ADR-positive cells; and the median fluorescent intensity, which is directly proportional to the intracellular drug level. The various pH levels were designed to model variations in the in vivo tumor cell microenvironment that might influence both ADR uptake and efflux, and hence cytotoxicity.

The data indicate that ADR efflux is most sensitive to an alkaline microenvironment when the uptake pH is acidic in both sensitive and resistant cells. In contrast, when ADR uptake occurs at pH 7.5, the proportion of ADR-positive cells falls more rapidly and the efflux is increased by an alkaline microenvironment (pH 8.5), especially in resistant cells. However, when the uptake of ADR occurs in an alkaline microenvironment (pH 8.5), efflux is delayed in both cell lines, even when the pH of the efflux microenvironment is also alkaline (pH 8.5). In fact, in an alkaline microenvironment, ADR transport and retention in resistant cells was superior to that observed in sensitive cells in an acidic microenvironment. Furthermore, no additional differences in influx or efflux were observed when the pH gradient across the cell membrane was equilibrated by the addition of ammonium acetate (data not shown).

These observations suggest the possibility that alkalinization of ADR prior to cell entry altered the drug in a way that was not achieved by the alkalinization of the intracellular compartment after drug entry. They also suggest that ADR efflux, which is only significantly enhanced by an alkaline microenvironment when the uptake pH is acidic or neutral, may be the result of an effect at the level of the cell membrane rather than on ADR itself. Interestingly, there is evidence that ADR uptake into unilamellar vesicle systems may be increased in response to transmembrane pH gradients (interior acidic) (28). Other studies have suggested that ADR uptake is dependent upon passive diffusion, which is influenced by the permeability of the cell membrane to unionized molecules (27). This might partly account for the influence of pH on ADR uptake, but probably does not explain the pH effects seen on energy-dependent ADR efflux.

Although it is not possible to explain all these pH effects on ADR transport, it is possible to consider the implications of such effects within tumors where the microenvironment varies with respect to pH. Inevitably, drug concentrations are higher near blood vessels within solid tumors, but because of variations in oxygenation and metabolite clearance, it is likely that this microenvironment is closer to neutral pH than more distant sites where drug concentrations are lower and the pH more acidic; conditions that favor cell resistance to ADR.

Clearly, the effects of pH on ADR transport support the conclusion that intrinsic drug resistance could be modified by changes in the tumor cell microenvironment. The alkalinization of tumors prior to ADR administration, if technically feasible, might lead to enhanced destruction of otherwise resistant cell subpopulations.

ACKNOWLEDGMENTS

The authors would like to express their sincere appreciation to Dr. Awtar Krishan who generously provided the P388 cell lines.

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