Hyperthermia, Adriamycin Transport, and Cytotoxicity in Drug-sensitive and -resistant Chinese Hamster Ovary Cells

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ABSTRACT

Adriamycin cytotoxicity and membrane permeability to Adriamycin were studied at elevated temperatures in a drug-sensitive Chinese hamster ovary cell line and in a pleiotropic drug-resistant mutant to determine whether hyperthermia can overcome this form of acquired drug resistance. In drug-sensitive cells Adriamycin cytotoxicity, measured by colony survival studies, increased at temperatures as low as 38°C, and at 43°C, the combined effect of Adriamycin and hyperthermia exceeded the predicted additive effect by a factor of 10. There was a marked increase in the rate of Adriamycin uptake between 37°C and 45°C. Although the rate of Adriamycin efflux was also increased, intracellular drug levels at equilibrium were higher at elevated temperatures. The magnitude of the increase in intracellular drug levels at elevated temperatures was insufficient to account for the larger increase in cytotoxicity observed. We were unable to increase membrane permeability to Adriamycin or to increase Adriamycin cytotoxicity in the drug-resistant Chinese hamster ovary cell line by the use of hyperthermia; however, the drug-resistant cells were not cross-resistant to hyperthermia. Therefore, heat may be effective against residual tumor cells which are resistant to chemotherapy.

INTRODUCTION

The combination of regional hyperthermia with systemic chemotherapy has potential in cancer treatment primarily because local heat delivery may provide intensification of cytotoxic activity within a defined target region. This may lead to an improved therapeutic ratio by conferring on chemotherapy the geographical specificity which is inherent to radiation therapy but which is at present only achievable with cytotoxic drugs when they are administered either topically or intraarterially. There is also evidence to suggest that the effects of hyperthermia and cytotoxic drugs may prove to be complementary. Tumor cells located in less well-vascularized regions of a tumor may be relatively resistant to systemic chemotherapy because they are exposed to lower concentrations of drug. Hyperthermia, however, kills cells most efficiently in the low pH environment of the tumor core. Furthermore, the temperature achieved in poorly vascularized regions of the tumor may be higher because of less efficient cooling by circulating blood. Although chemotherapy and hyperthermia are already being used together in the clinic (1), there is insufficient information about the interaction between these two modalities at the cellular level for the planning of optimal treatment regimens.

The interaction of hyperthermia and Adriamycin has been previously studied by other workers (2–5). Adriamycin and simultaneous 43°C hyperthermia have been shown to be synergistic in CHO3 cells in vitro and in EMT6 cells both in vitro and in vivo. The mechanism thought most probable is that heat causes a transient increase in membrane permeability to Adriamycin, and limited studies of intracellular Adriamycin levels were consistent with this hypothesis (2). Acquired drug resistance in certain systems is due to a decrease in membrane permeability to cytotoxic drugs (6–9). We have therefore studied the cytotoxicity of Adriamycin and membrane permeability to Adriamycin at elevated temperatures in a drug-sensitive CHO cell line and in a pleiotropic drug-resistant mutant in order to determine whether hyperthermia can overcome this form of acquired drug resistance. We have considered whether changes in membrane permeability are sufficient to account for changes in the cytotoxicity of Adriamycin at elevated temperatures.

MATERIALS AND METHODS

Tissue Culture. Drug-sensitive CHO cells (Aux B1) and colchicine-resistant CHO cells (CH5 C5) (10), which are cross-resistant to Adriamycin, were grown in monolayer in 75-cm² plastic tissue culture flasks (Falcon; Becton-Dickinson Canada, Inc., Mississauga, Ontario, Canada), at 37°C under 5% CO2 in α-MEM (Gibco Canada, Burlington, Ontario, Canada), supplemented with 10% FBS and 1% penicillin (50 units/ml-streptomycin (50 µg/ml), both from Flow Laboratories, Mississauga, Ontario, Canada. All uptake studies were carried out using cells grown to confluence and incubated for 24 h at 37°C with fresh culture medium. The cells were harvested with citrated phosphate-buffered saline (0.14 M NaCl-0.01 M sodium phosphate 0.015 M sodium citrate), washed by centrifugation, and resuspended in PBS containing 1% BSA (Sigma Chemical Co., St. Louis, MO) and 10 mM glucose at pH 7.4.

Colony Survival Experiments. Cells were harvested as above and resuspended in α-MEM containing 10% FBS and 20 mM HEPES. Aliquots (0.1 ml) of cells were added to 0.9 ml of Adriamycin (Adria Laboratories of Canada, Ltd., Mississauga, Ontario, Canada) solution (prewarmed at the incubation temperature) in culture medium in screwtopped polystyrene tubes. The absorbance, measured at 480 nm using a spectrophotometer, of a freshly prepared solution of Adriamycin did not change after a 60-min incubation in the polystyrene tubes, indicating that the drug does not bind to these tubes, in agreement with other findings (11). The tubes were incubated in a temperature-controlled water bath (Haake D3; Haake, Saddle Brook, NJ) at temperatures ranging from 37°C–47°C. Under these conditions 1 ml of aqueous solution reached a temperature within 0.1°C of the water bath temperature within 3 min. Tubes were removed from the water bath at different time intervals and then centrifuged (2 min, 1000 x g), washed once, and resuspended in culture medium. The cells were carefully mixed before diluting to the appropriate concentration and plating in tissue culture-coated Petri dishes. The Petri dishes were incubated at 37°C in an atmosphere of 5% CO2 for 7 days. The plates were washed with PBS, fixed with 95% ethanol, and stained with methylene blue before counting macroscopic colonies. Control plating efficiencies were greater than 50%. The percentage of survival was expressed as the mean number of colonies obtained relative to the mean number of colonies obtained in the control. Two hundred cells per plate were seeded in the control, but where levels of survival were uncertain, cells were plated at more than one density to ensure that countable colonies would be obtained, and the results were corrected accordingly. We have previously demonstrated that, in this system, there is linearity between the number of cells plated and colonies formed over the range of 10 to 10⁶ (data not presented).
Measurement of Adriamycin Influx. ¹⁴C-labeled Adriamycin (specific activity, 44.9 μCi/mg) was a generous gift from Maurice Leaffer of SRI International, Menlo Park, CA. We detected no aglycone or other impurities by thin-layer chromatography using the solvent system chloroform:methanol:water (80:20:3).

Freshly harvested CHO cells were resuspended at 10⁷ cells/ml in PBS containing 1% BSA and 10 mM glucose at room temperature. We found that BSA was an adequate replacement for FBS in transport experiments, by carrying out parallel Adriamycin uptake experiments in buffers containing either albumin or serum (data not presented). Adriamycin uptake in the presence of 1% BSA was identical to that in the presence of 10% FBS.

Aliquots (100 µl) were placed in glass tubes and preheated for 2 min in a circulating water bath to allow them to reach the incubation temperature before the addition of Adriamycin. The temperature of the cells was monitored with a 24 gauge hypodermic Thermistor temperature probe (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) and was found to reach the temperature of the water bath within 30 s. At time zero, 100-µl aliquots of Adriamycin solution, previously equilibrated at the incubation temperature, were added to the cells, and the suspension was mixed and incubated for the required time. To stop influx, 4 ml of ice-cold buffer were added, and the cells were centrifuged (1 min, 1000 × g) and washed 3 times with ice-cold buffer. The final dry pellet of cells was solubilized with 1% sodium dodecyl sulfate, and the liquid scintillation cocktail ScintiVerse II (Fisher Scientific Co., Montreal, Quebec, Canada) was added. The radioactivity was determined using an LKB Model 1218 Rackbeta liquid scintillation counter equipped with a dpm calculation program (Fisher Scientific Co.). Drug uptake data are expressed as dpm rather than as intracellular concentration because the drug binds to the nucleus and is not evenly distributed in the cell (12). For this reason and also because we measured no change in cell volume after 60 min at temperatures ranging from 37°C-45°C, Adriamycin uptake was not normalized with respect to cell volume at elevated temperatures.

Measurement of Adriamycin Efflux. Freshly harvested cells (10⁷/ml) were preloaded with [¹⁴C]Adriamycin (5 µg/ml) for 30 min at 37°C in PBS containing 1% BSA and 10 mM glucose at pH 7.4. The cells were then centrifuged (2 min, 1000 × g) and washed 3 times with ice-cold buffer. For determination of efflux, cells were resuspended in ice-cold Adriamycin-free PBS-BSA-glucose, and 100-µl aliquots were put in glass tubes. Three-tenths ml of buffer (prewarmed at the incubation temperature) were added, and the cells were incubated at temperatures ranging from 37°C-45°C for varying times. To stop efflux, cell suspensions were centrifuged (1 min, 1000 × g) in 4 ml of ice-cold buffer. The radioactivity was determined in the cell pellet. The zero time point represents the Adriamycin content in the cells prior to efflux, and each time point is expressed as a percentage of this point.

RESULTS

Fig. 1 shows the effect of 20-min exposures to temperatures at 1°C intervals in the range 37°C-47°C on cell survival with and without Adriamycin (1 µg/ml), in the drug-sensitive cell line Aux B1, and in the resistant cell line CHPC5. One-hundred percent survival of drug-sensitive cells was observed in the absence of Adriamycin at temperatures from 37°C-41°C, but a gradual decrease in survival was found from 42°C-44°C (Fig. 1a). In the presence of Adriamycin, cell survival decreased progressively from 60% at 37°C-5% at 43°C. At each temperature within this range, we observed more cell killing than would be expected if the effects of hyperthermia and Adriamycin were simply additive. A 20-min exposure to 40°C alone was nonlethal, and a 20-min exposure to Adriamycin at 37°C gave 60% cell survival, but a 20-min exposure to 40°C and Adriamycin gave a cell survival level of 34%, indicating that the nonlethal temperature treatment potentiates the effect of Adriamycin. A 20-min exposure to 43°C without Adriamycin gave a cell survival level of 80%, and a 20-min exposure to drug alone at 37°C gave 60% cell survival. If the 2 modalities acted independently, we would therefore predict that exposure to 43°C with Adriamycin would give a cell survival level of 48%, equal to the product of the 2 independent survival fractions, but the observed level of cell survival was 5%, indicating that the 2 agents are synergistic. At 45°C, cell survival decreased by approximately 2 logarithms regardless of whether Adriamycin was present or not, since the heat treatment alone is highly lethal at this temperature.

In the presence of Adriamycin, cell survival at 37°C was higher in the drug-resistant cells than in the drug-sensitive cells (Fig. 1b). Unlike the drug-sensitive cells, however, there was no evidence of any increase in the cytotoxicity of Adriamycin as temperature increased from 37°C-47°C.

Heat sensitivity was compared between the drug-sensitive and resistant cells at 43°C and 45°C, and survival curves together with confidence intervals calculated using the method described in Ref. 13 are shown in Fig. 2. The response of the drug-resistant cells to hyperthermia was similar to that of the drug-sensitive cells, since the confidence intervals overlap at most points on the curves. It appears, therefore, that the pleiotropic drug-resistant phenotype confers no protection against heat.

Fig. 3 shows time courses for uptake of [¹⁴C]Adriamycin at 37°C in drug-sensitive and -resistant cells. In both cell types uptake increased rapidly for about 20 min and then reached approximate steady-state levels, although uptake did continue to increase very slowly with time up to 60 min. In drug-resistant cells, Adriamycin uptake increased initially at a slower rate than in drug-sensitive cells and reached a lower steady-state level. After 30 to 60 min, the level of Adriamycin in drug-resistant cells was approximately 60% of that in drug-sensitive cells.

Fig. 4 shows the 15-min uptake of [¹⁴C]Adriamycin by drug-sensitive and -resistant cells as a function of Adriamycin concentration at temperatures from 31°C-50°C. Uptake increased with concentration over the range of 0.1 to 20 µg/ml in both types of cells. In drug-sensitive cells there was a marked increase in Adriamycin uptake with temperature (Fig. 4a). At 10 µg/ml, uptake at 45°C was 1.8 times higher than that at 37°C. The inset shows the lower and clinically more relevant concentration range in greater detail. At 1 µg/ml, uptake at 43°C and 45°C was, respectively, 1.26 and 1.4 times greater than at 37°C.
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Fig. 2. Heat sensitivity of drug-sensitive and drug-resistant cells. Heat survival curves are shown for Aux B1 cells at 43°C (•) and 45°C (○) and for CH°°C5 cells at 43°C (O) and 45°C (D), in 1 ml of α-MEM plus 20% FBS and 20 mM HEPES. These data are typical of those obtained in 6 separate experiments. Points, mean given for 2 estimations. Confidence intervals on the line of best-fit for the data were obtained using the method described in Ref. 13. These are vertical confidence intervals, and their right boundary ends at the last data point.

Fig. 3. Time course for [MC]Adriamycin uptake. Reaction mixtures contained 10^6 drug-sensitive (•) or drug-resistant (○) cells and Adriamycin (1 μg/ml) in 0.2 ml of PBS-1% BSA-10 mM glucose at 37°C. Points, mean for 3 estimations; bars, SD.

Less Adriamycin uptake occurred in drug-resistant cells than in drug-sensitive cells (Fig. 4b). The volume of the drug-resistant cells, measured using a Coulter Counter attached to a pulse height analyzer, was approximately 7% larger than that of the drug-sensitive cells and therefore did not account for the difference in drug uptake between these two cell types. The effect of temperature on Adriamycin uptake in drug-resistant cells was much less marked than in drug-sensitive cells. Uptake values at 37°C, 40°C, and 43°C were similar, but there was a very slight increase in uptake at 45°C. At a drug concentration of 10 μg/ml, uptake at 45°C was 1.25 times higher than that at 37°C, but at 1 μg/ml, no difference was detected. In both cell types the marked increase in Adriamycin uptake at 50°C correlated with a high number of dead cells (35 ± 5%) measured by trypan blue exclusion.

We wished to determine whether the 20 to 30% increase in Adriamycin uptake from 37°C–43°C in drug-sensitive cells was sufficient to account for the 10-fold decrease in cell survival from 37°C–43°C. Since drug uptake increases approximately linearly with increasing drug concentration we are able to increase uptake at 37°C simply by manipulating external drug concentrations. We have therefore described the dependence of cell survival on Adriamycin concentration in drug-sensitive and drug-resistant cells after 20 min at 37°C (Fig. 5). There was a log-linear decrease in cell survival over a range of 1 logarithm as Adriamycin concentration increased from 0 to 5 μg/ml in drug-sensitive cells. Increasing the drug concentration by 30% (from 1 μg/ml to 1.3 μg/ml) decreased cell survival by 10% at most, showing that the increase in uptake observed at 43°C is insufficient to account for the 10-fold increase in cytotoxicity.

The level of cell survival was higher in drug-resistant cells than in drug-sensitive cells (Fig. 5). In drug-resistant cells,
were incubated for 20 min at 37°C. Points, mean for 2 estimates; bars, SD.

Mg of Adriamycin per ml in 1 ml of MEM plus 10% FBS and 20 mm HEPES

cells. The cells were preloaded with 5 μg/ml. Figure 6 shows the effect of temperatures from 37°C-45°C on

above that concentration cell survival decreased slowly to a level

Adriamycin concentrations up to 2 μg/ml were nonlethal, and

above that concentration cell survival decreased slowly to a level

62% with 5 μg/ml.

Fig. 6 shows the effect of temperatures from 37°C-45°C on

the efflux of Adriamycin from drug-sensitive and -resistant
cells. The cells were preloaded with 5 μg of Adriamycin per ml, the
cells were washed, and efflux was measured in the absence of
extracellular Adriamycin. In drug-sensitive cells there was an
initial rapid rate of efflux during the first 3 min, after which
efflux occurred at a much slower rate. In drug-resistant cells
there was an initial rapid rate of efflux during the first 5 min,
after which a plateau was reached. In both cell types, the initial

rates of efflux were equivalent at 40°C, 43°C, and 45°C, but

they were higher than those at 37°C. In the absence of drug
influx, the Adriamycin content after 10 to 15 min was about
10% lower in the heated cells than in the cells at 37°C, and it was
about 15% lower in the drug-resistant cells than in the
normal cells. Despite the increase in efflux at higher temperatures
the saturation level of drug in the cells increased with

Figure 5 shows the intracellular content of [14C]Adriamycin

in drug-sensitive and -resistant cells after 30-min exposures to

temperatures ranging from 31°C-50°C, at which time Adriamycin
uptake has reached approximate steady-state levels for both
cell types (Fig. 3). In drug-sensitive cells, the saturation
level of drug was dependent on temperature and increased
steadily from 37°C-50°C, and the final intracellular concentra-
tion of Adriamycin was 1.1 times higher at 43°C than at 37°C.
In drug-resistant cells a smaller change in steady-state levels of
Adriamycin was detected over the same temperature range.

### DISCUSSION

We have observed a synergistic effect between Adriamycin
and hyperthermia in drug-sensitive Aux B1 cells. Heat
potentiates the effects of Adriamycin at temperatures as low as 38°C,
and by 40°C, there is a 2-fold increase in cytoxicity. Body
temperatures in this range commonly occur in cancer patients,
and the effect may therefore contribute significantly to the case-
to-case variations in efficacy and toxicity observed in clinical practice. Synergism between hyperthermia (42°C-43°C) and
Adriamycin in Chinese hamster cells has been previously de-
scribed (2), and we have shown that at 43°C the combined effect
of hyperthermia and Adriamycin exceeds the predicted additive
effect by a factor of 10.

We have found a measurable increase in rates of Adriamycin
uptake at temperatures as low as 40°C. The rate of efflux of Adriamycin is also increased at elevated temperatures, but this

effect is less important than the increase in influx, so that
intracellular levels of Adriamycin at equilibrium are increased
at 40°C and above. These findings are in agreement with an
earlier less detailed report of higher Adriamycin content in cells
incubated with drug at 43°C for 1 h than at 37°C. In that study
we showed a 26% increase in Adriamycin uptake at 43°C
to explain the increase in cytotoxicity. In drug-sensitive cells
we observed a 26% increase in Adriamycin uptake at 43°C

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Adriamycin content (dpm)</th>
<th>Aux B1</th>
<th>CH&lt;sup&gt;+&lt;/sup&gt;C5</th>
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<tr>
<td>37</td>
<td>301.7 ± 25.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>164.9 ± 27.6&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>172.6 ± 38.3</td>
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<td>43</td>
<td>332.0 ± 39.2</td>
<td>186.2 ± 25.3</td>
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<tr>
<td>45</td>
<td>347.3 ± 37.9</td>
<td>187.5 ± 21.3</td>
<td></td>
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<tr>
<td>50</td>
<td>357.5 ± 58.5</td>
<td>234.8 ± 72.8</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> Mean ± SD for 9 estimations from 3 separate experiments.
<sup>b</sup> Mean ± SD for 24 estimations from 8 separate experiments.
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compared to 37°C, but this was associated with a 10-fold decrease in cell survival. A similar change in intracellular Adriamycin levels at 37°C, however, decreased cell survival by only 10%. Thus the synergism observed is not adequately explained by the change in membrane permeability. There is in fact no clear evidence that Adriamycin has to gain access to the intracellular compartment to produce cell death. We have previously shown a lack of correlation between cytotoxicity and intracellularly bound Adriamycin in drug-sensitive CHO cells, suggesting that nuclear-bound Adriamycin may not be primarily involved in Adriamycin-induced cell death (12), and other workers have reported that Adriamycin can be cytotoxic without entering cells (14).

The pleiotropic drug resistance of the CH⁵C5 line is associated with the appearance of a high-molecular-weight cell surface glycoprotein which is thought to decrease membrane permeability to a number of cytotoxic agents (6, 15). We have confirmed that both equilibrium levels of Adriamycin and the initial rate of Adriamycin uptake are lower in drug-resistant cells than in drug-sensitive cells. In CH⁵C5 cells we have observed little change in the rate of Adriamycin influx at elevated temperatures up to 45°C, although there may be a small increase in equilibrium intracellular levels of the drug. Likewise we observed little increase in Adriamycin cytotoxicity in the drug-resistant C5 CHO cell line at temperatures up to 45°C. We found, however, that the drug-resistant phenotype confers no cross-resistance to hyperthermia, so that heat may have value in eradicating resistant subpopulations of tumor cells which have survived chemotherapy.

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REFERENCES

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