Resistance to Adriamycin: Relationship of Cytotoxicity to Drug Uptake and DNA Single- and Double-Strand Breakage in Cloned Cell Lines of Adriamycin-sensitive and -resistant P388 Leukemia

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ABSTRACT

Cloned lines of Adriamycin (ADR)-sensitive and -resistant P388 leukemia have been established from single cell cultures. A marker chromosome M1 was found in all cells in the heterogeneous resistant P388/ADR parental line as well as in the cloned resistant lines P388/ADR/3 and P388/ADR/7; a different marker chromosome M2 was present in the heterogeneous sensitive P388 parental line as well as the cloned sensitive line P388/4. Dose-survival studies showed that D0, the dose of Adriamycin reducing survival to 1/e (i.e., 37% of the initial population), was 33 ± 5 (SE) nm for sensitive P388/4 cells, 169 ± 17 nm for resistant P388/ADR/3 cells, and 336 ± 28 nm for the more resistant P388/ADR/7 cells. Drug uptake in sensitive P388/4 cells was 1.6-fold greater than in resistant P388/ADR/3 cells and 2.1-fold greater than in resistant P388/ADR/7 cells. The number of DNA single-strand breaks produced per μM Adriamycin was 131 ± 9 rad equivalents in sensitive clone 4 cells, 41 ± 8 rad equivalents in resistant clone 3 cells, and 33 ± 11 rad equivalents in resistant clone 7 cells. The number of DNA double-strand breaks produced per μM Adriamycin was 1721 ± 126 rad equivalents in sensitive cells, 117 ± 36 rad equivalents in resistant P388/ADR/3 cells, and 194 ± 16 rad equivalents in resistant P388/ADR/7 cells. Differences in drug uptake were insufficient to explain the higher incidence of DNA single- and double-strand breaks in sensitive cells. These findings strongly support the concept that resistance to Adriamycin in P388 leukemia cells is multifactorial; however, this study did not resolve whether these changes arise from a single pleiotropic mutation or from multiple mutations.

In sensitive P388/4 cells the number of DNA single-strand breaks formed could all be attributed to double-strand breaks. However, in both resistant cell lines the level of induction of single-strand breaks was in excess of that due to double-strand breaks, and this excess of single-strand breaks appeared to vary directly with the degree of resistance, being greater in the more resistant clone 7 cells than in the less resistant clone 3 cells. In both sensitive and resistant cell lines the ratio of true single- to double-strand breaks varied inversely with the concentration of Adriamycin. Finally, the cytotoxic activity of Adriamycin appeared to correlate more closely with formation of DNA double-strand breaks than with single-strand lesions.

INTRODUCTION

Resistance to Adriamycin has been attributed to the presence of an energy-dependent active extrusion pump in Adriamycin-resistant P388 leukemic cells (1–3) and in other anthracycline-resistant cells (4–8). Resistance to Adriamycin has also been reported in association with cross-resistance to other chemotherapeutic agents, a phenomenon referred to as MDR or pleiotropic drug resistance (9–15). Further, the pleiotropic drug-resistant phenotype appeared to correlate with the presence of a Mr 170,000 glycoprotein in the plasma membrane (10–13). Recently, the gene for this Mr, 170,000 or P-glycoprotein has been cloned (16). What remains unresolved is whether the complementary DNA for the P-glycoprotein is also the gene for MDR.

Reports of double minute chromosomes and homogeneously staining regions (17, 18) in some MDR cell lines suggested that gene amplification underlies this phenomenon. Direct evidence for the role of gene amplification in MDR was the finding of amplified DNA segments in Adriamycin- and colchicine-resistant Chinese hamster cell lines that were absent in drug-sensitive revertants (17). However, the nature of the proteins encoded by the amplified genes was not established although the Mr 170,000 glycoprotein and a Mr 19,000 cytosol protein frequently overproduced in MDR cells were possible candidates.

The above studies also did not resolve whether the changes in MDR cells were due to a single pleiotropic mutation or to multiple mutations (19). In a preliminary attempt to resolve this dilemma a study was undertaken on cloned cell lines of Adriamycin-sensitive and -resistant P388 leukemia to determine, as an initial step, if resistance to Adriamycin was due to single or multiple biochemical alterations. A second objective was to explore the relationship between cytotoxicity to Adriamycin and the formation of DNA single- and double-strand breakage.

MATERIALS AND METHODS

Drugs and Chemicals. [14C]Adriamycin labeled at the 14C position (specific activity, 11.4 or 16.9 mCi/mmol) was synthesized by M. Leaffer of the Stanford Research Institute, Menlo Park, CA, and was kindly provided by Dr. Robert R. Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The radiochemical purity was 98% as determined by thin-layer chromatography on silica gel in chloroform:methanol:acetic acid:water (40:10:3:1). [3H]Thymidine (specific activity, 50 to 80 Ci/mmol) and [3H]thymidine (specific activity, 50 to 80 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Proteinase K was purchased from E. Merck, Darmstadt, Germany, and tetrapropylammonium hydroxide was from the Eastman Kodak Company, Rochester, NY.

Cell Lines and Cultures. Adriamycin-sensitive and -resistant parental lines of P388 leukemia were originally established by Dr. Randall K. Johnson of the National Cancer Institute, Bethesda, MD, and were kindly provided by Dr. Robert R. Engle, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, and were kindly provided by the E. G. & G. Mason Research Institute, Worcester, MA. The cell lines were maintained in vivo by weekly transplantation of an inoculum of 105 cells i.p. in 6- to 8-week-old female DBA/2 mice. Suspension cultures in vitro were established by growing the cells in RPMI 1640 supplemented with 15% FBS (Grand Island Biological Company, Grand Island, NY). The cells grew exponentially with a doubling time of 11 to 12 h.

After suspension cultures had been established in vitro several clones of Adriamycin-sensitive and -resistant P388 leukemia were established by the limiting dilution technique using microtiter plates aimed to have single cells distributed in 1 of 5 wells with the cells suspended in RPMI 1640 enriched with 30% FBS. The plates were inspected microscopically on a daily basis to identify those wells containing a single cell. In a preliminary survey, the sensitivity of the cloned cell lines to...
Adriamycin was estimated by comparing incorporation of \(^{3}H\)thymidine in control cells to that in cells treated with 10 \(\mu\)M Adriamycin for 1 h. The cell lines selected for further study were designated as clone 4 of the heterogeneous sensitive parental line (P388) and clones 3 and 7 of the heterogeneous resistant parental line (P388/ADR). The cloned lines have been maintained in suspension cultures \textit{in vitro} in RPMI 1640 supplemented with 15% FBS.

Karyotypic analysis of the cloned and heterogeneous parental cell lines was performed. A marker chromosome M1 was present in each of 20 cells analyzed in the resistant parental line P388/ADR and also in the cloned resistant lines clones 3 and 7 (Fig. 1). A second marker chromosome M2, which was distinct from the M1 marker chromosome of resistant cells, was consistently found in all the cells of the sensitive parental line P388 and in the cloned sensitive line P388/4.

\textbf{Assay of Cytotoxic Activity.} The sensitivity of drug-sensitive and -resistant cells to Adriamycin was determined by the clonogenic assay of Chu and Fischer (20). Exponential phase cells at a concentration of 2 to 3 \(\times\) 10^6 cells/ml were treated with Adriamycin for 1 h at 37°C in RPMI 1640. The cloning efficiency of treated cells was determined at each drug concentration and surviving cell fraction was calculated. Linear regression analysis of the dose-survival curves was performed, the regression equation being in the form

\[ \log_{e} y = mx + b \]

where \(y\) is surviving cell fraction, \(x\) is dose of drug, \(m\) is slope of the regression line and \(b\) is the \(y\)-intercept. \(D_{0}\), the dose of drug reducing survival to 1/e, \(i.e., 37\%\) of the initial cell population, was derived from the negative reciprocal of the slope of the regression line as described previously (21-23). Statistical analysis of the dose-survival curves was performed by a \(t\) test comparing the significance of the difference of the slopes.

\textbf{Drug Uptake Studies.} Drug uptake studies were performed as described previously (24-26) by addition of \(^{3}C\)Adriamycin to drug-sensitive and -resistant P388 leukemia cells suspended in Dulbecco's phosphate-buffered saline. Incubations were terminated by rapid chilling to 4°C and centrifugation through a layer of 0.25 M sucrose in Hopkin's vaccine tubes to remove extracellular radioactivity. The washed cells were solubilized in 0.5 N NaOH and radioactivity was determined by liquid scintillation spectrometry.

Cell size was measured in a Coulter Model ZBI electronic particle counter (Coulter Electronics, Hialeah, FL) calibrated with paper mulberry spores (mean cell diameter, 12.5 \(\mu\)m) which was obtained from Coulter Diagnostics, Inc. (Miami Springs, FL) using methodology described previously (27). The cell volume obtained for sensitive P388/4 cells was 1225 ± 81 (SD) \(\mu\)l, that for resistant P388/ADR/3 cells was 1266 ± 80 \(\mu\)l, and that for resistant P388/ADR/7 cells was 1237 ± 93 \(\mu\)l.

\textbf{Estimation of DNA Single-Strand Breaks.} DNA single-strand breaks were measured by the alkaline elution technique of Kohn as described previously (19, 28, 29). The DNA of P388 leukemic cells was labeled with \(^{14}C\)thymidine by growing the cells overnight in RPMI 1640 containing 15% FBS and \(^{14}C\)thymidine (0.01 to 0.02 \(\mu\)Ci/ml) at a concentration of 1 \(\mu\)M. Cells were washed twice to remove exogenous label, resuspended in medium at a concentration of approximately 2 \(\times\) 10^6 cells/ml, and treated with Adriamycin for 1 h at 37°C. Approximately 5 \(\times\) 10^6 drug-treated cells were applied to an elution filter and washed twice with cold medium to remove Adriamycin.

\textbf{RESULTS}

Dose-Survival Curves of Adriamycin-sensitive and -resistant P388 Leukemic Cells. Dose-survival curves of Adriamycin-sensitive and -resistant P388 leukemic cells are shown in Fig. 2. The \(D_{0}\) for drug-sensitive P388/4 cells was 33 ± 5 \(\mu\)M, that for resistant P388/ADR/3 cells was 169 ± 17 \(\mu\)M, and that for resistant P388/ADR/7 cells was 336 ± 28 \(\mu\)M. Thus P388/ADR/7 cells were 10-fold more resistant to Adriamycin than P388/4 cells and 2-fold more resistant than P388/ADR/3 cells; in each case the difference was highly significant (\(P < 0.001\)). P388/ADR/3 cells were 5-fold more resistant than P388/4 cells and this difference was also statistically significant (\(P < 0.001\)).

Adriamycin Uptake by Drug-sensitive and -resistant P388 Leukemic Cells. Adriamycin uptake by drug-sensitive and -resistant P388 leukemic cells was determined in cells treated with 0.1 to 2 \(\mu\)M Adriamycin for 1 h at 37°C, under conditions identical to those used in the cytotoxicity studies; the results are presented in Fig. 3. Over the concentration range used, drug uptake in sensitive P388/4 cells was 1.6-fold greater than in resistant P388/ADR/3 cells (\(P < 0.001\)) and 2.1-fold greater than in resistant P388/ADR/7 cells (\(P < 0.001\)). Uptake in resistant clone 3 cells was 1.3-fold higher than that in resistant clone 7 cells and this difference was also statistically significant (\(P = 0.029\)).
Induction of DNA Single-Strand Breaks in Sensitive and Resistant P388 Leukemic Cells Treated with Adriamycin. DNA single-strand breaks induced in sensitive P388/4 cells by treatment with Adriamycin for 1 h were determined in the presence and absence of proteinase K by the alkaline elution procedure of Kohn et al. (28, 29), as illustrated in Fig. 4. The rate of elution of DNA, which is a function of the number of DNA single-strand breaks, increased proportionally as the dose of Adriamycin was increased from 0.1 to 2 μM. There was little difference in the number of single-strand breaks noted in the absence or presence of proteinase K.

From a standard calibration curve obtained by treating P388/4 cells with a dose range of ionizing radiation, the number of single-strand breaks induced in sensitive P388/4 cells by treatment with Adriamycin for 1 h was determined in the presence and absence of proteinase K by the alkaline elution method described in the text and previously (28—31). The number of breaks expressed as rad equivalents (dose of radiation producing an equivalent number of breaks) is plotted against extracellular concentration of Adriamycin. Each point represents the mean of at least 3 determinations; bars, SE. The lines were determined by linear regression analysis. The linear regression equation for single-strand breaks in sensitive P388/4 cells in the absence of proteinase K was $y = 131.3x + 106.3$ with a correlation coefficient of 0.988. The linear regression equation for resistant P388/ADR/3 cells was $y = 41.0x + 77.3$ with a correlation coefficient of 0.923, and that for resistant P388/ADR/7 cells was $y = 32.5x + 122.6$ with a correlation coefficient of 0.808.

DNA single-strand breaks induced by any agent may be expressed as the dose of radiation in rads producing an equivalent number of breaks (28—31). DNA single-strand breaks induced by Adriamycin in sensitive P388/4 cells and measured in the absence and presence of proteinase K were plotted as a function of Adriamycin concentration (Fig. 5). The slope of these curves represented the number of single-strand breaks produced in rad equivalent per μM Adriamycin concentration. For P388/4 cells...
the number of breaks in rad equivalents produced per μM Adriamycin was approximately 106 ± 10 (SE) rad equivalents in the absence of proteinase K, and that in the presence of proteinase K was approximately 131 ± 9 rad equivalents; the difference of 25 rad equivalents between these two curves is due to protein-associated strand breaks and was statistically significant (P < 0.001, one-tailed t test comparing the significance of the difference of slopes).

Since the difference in single-strand breaks induced by Adriamycin in the presence and absence of proteinase K was relatively small, all subsequent determinations of single-strand breaks were made in the presence of proteinase K. The number of single-strand breaks per μM Adriamycin produced in resistant P388/ADR/3 cells was 41 ± 8 rad equivalents, that in P388/ADR/7 cells was 33 ± 11 rad equivalents, and the difference was not statistically significant (Fig. 5). However, the level of single-strand breaks in sensitive P388/4 cells was 3.2-fold greater than that in resistant clone 3 cells and 4-fold greater than that in resistant clone 7 cells and in each case the difference was highly significant (P < 0.001).

Induction of DNA Double-Strand Breaks in Sensitive and Resistant P388 Leukemic Cells Treated with Adriamycin. The generation of DNA double-strand breaks in P388 leukemic cells exposed to Adriamycin was measured by the neutral elution technique as described in the literature (28, 32). The incidence of double-strand breaks per μM Adriamycin in sensitive P388/4 cells was 1721 ± 126 rad equivalents, that in resistant P388/ADR/3 cells was 117 ± 36 rad equivalents, and that in resistant P388/ADR/7 cells was 194 ± 16 rad equivalents (Fig. 6). The level of double-strand breaks in sensitive cells was approximately 15-fold greater than that in resistant clone 3 cells and 9-fold greater than that in resistant clone 7 cells; in each case the difference was highly significant (P < 0.001). Induction of double-strand breaks in the two resistant cell lines was not significantly different.

Formation of DNA Single- and Double-Strand Breaks as a Function of Intracellular Adriamycin Concentration. The high number of DNA single- and double-strand breaks in sensitive cells might be explained by the higher level of drug uptake observed in sensitive cells (Fig. 3). Accordingly a comparison of induction of single- and double-strand breaks in sensitive and resistant cells was determined at the same intracellular concentration of Adriamycin, in order to eliminate differences in drug uptake as a contributing factor.

Under these conditions the number of DNA single-strand breaks in rad equivalents produced per μM intracellular Adriamycin was 0.835 ± 0.085 (SE) rad equivalents in sensitive P388/4 cells, 0.450 ± 0.054 rad equivalents in resistant clone 3 cells, and 0.426 ± 0.152 rad equivalents in resistant clone 7 cells. Thus formation of single-strand breaks in P388/4 cells was 1.85-fold that in resistant P388/ADR/3 cells (P < 0.01) and 1.96-fold that in P388/ADR/7 cells (P = 0.046). The generation of single-strand breaks by Adriamycin in the two resistant cell lines was not significantly different.

The number of DNA double-strand breaks in rad equivalents produced per μM intracellular Adriamycin in P388/4 cells was 10.9 ± 1.2 (SE) rad equivalents, that in resistant clone 3 cells was 1.2 ± 0.4 rad equivalents, and that in resistant clone 7 cells was 2.5 ± 0.4 rad equivalents. Induction of double-strand breaks in sensitive cells was 8.9-fold greater than that in resistant clone 3 cells and 4.3-fold greater than that in resistant clone 7 cells; in each case the difference was highly significant (P < 0.001). The level of double-strand breaks in resistant clone 7 cells was 2-fold greater than that in clone 3 cells and although this difference was statistically significant (P = 0.037), the low incidence of double-strand breaks in both resistant cell lines raises doubts about the biological relevance of this finding.

Relative Production of DNA Double-Strand and Single-Strand Breaks in Sensitive and Resistant P388 Leukemic Cells Treated with Adriamycin. To determine the true frequency of single- and double-strand breaks induced by Adriamycin a formula was used which compared the observed values obtained with Adriamycin to the ratio of actual single-strand breaks and double-strand breaks induced by X-ray (30, 31). The formula takes into account that single-strand break frequency measured by alkaline elution includes both single-strand breaks arising from double-strand breaks (each double-strand break gives rise to two single-strand breaks) and true single-strand breaks. The formula for calculating the ratio of true single-strand breaks, s, to actual double-strand breaks, d, produced by Adriamycin is

\[ s = \frac{K_{RS}}{K_{RD}} \frac{\text{[SSB]}}{\text{[DSB]}} - 2 \quad (A) \]

where \( K_{RS} \) is the single-strand break frequency produced per unit of X-ray dose, \( K_{RD} \) is the double-strand break frequency per unit of X-ray dose, and [SSB] and [DSB] are the measured frequencies in rad equivalents of single- and double-strand breaks induced by Adriamycin in the presence of proteinase K. Values for the \( K_{RS} / K_{RD} \) ratio of actual single-strand breaks (excluding those arising from double-strand breaks) to double-strand breaks induced by X-radiation range from 10 to 40 in the literature (30, 31). Thus from the above equation, if Adriamycin produced double-strand breaks exclusively, \( s \) would equal 0, and the measured values for [SSB]/[DSB] for Adriamycin would fall between 0.05 and 0.2 for \( K_{RS} / K_{RD} \) values of 40 and 10, respectively, as illustrated by the broken lines in Fig. 7. In sensitive P388/4 cells almost all the experimental determinations of single- and double-strand breaks induced by Adriamycin fell within this range (Fig. 7). Conversely, for both resistant cell lines all the experimental measurements of single- and double-strand breaks fell outside of this range (Fig. 7B). For both resistant cell lines there appeared to be many single-strand breaks that could not be accounted for by double-strand breaks alone and this excess of single-strand breaks was greater in the
more resistant P388/ADR/7 cells than in the less resistant P388/ADR/3 cells. In both sensitive and resistant cells the s/d strand break ratio varied inversely with Adriamycin concentration (data not shown), a correlation recently observed by others (33).

Correlation of Cell Cytotoxicity with DNA Damage Induced by Adriamycin. Cell cytotoxicity as measured by the clonogenic assay of Chu and Fischer (20) was plotted on a log scale against DNA damage induced by Adriamycin on a linear scale as measured by the alkaline (28–31) and neutral (28–32) elution assays (Fig. 8). At the same level of cytotoxicity the frequency of SSB increased progressively from approximately the same level in sensitive P388/4 and resistant P388/ADR/3 cells to a higher level in the more resistant P388/ADR/7 cells (Fig. 8A). Conversely, for DSB the data for all 3 cell lines appeared to fall on the same plot, suggesting that cytotoxicity correlated more closely with DSB frequency (Fig. 8B).

DISCUSSION

Clones of Adriamycin-sensitive and -resistant P388 leukemia cells have been established by growing up lines from single cell cultures in microtiter plates using the limiting dilution technique. Dose-survival curves of these cloned lines demonstrated that drug-sensitive P388/4 cells were 5- and 10-fold more susceptible to the cytidial effect of Adriamycin than resistant P388/ADR/3 and P388/ADR/7 cells, respectively (Fig. 2).

Studies with [14C]Adriamycin showed that drug uptake by the sensitive cell line was 1.6- and 2.1-fold greater than that of the resistant clone 3 and clone 7 cell lines, respectively (Fig. 3). Although we did not study drug efflux in these cloned cell lines, others have demonstrated an energy-dependent active extrusion mechanism in the heterogeneous, Adriamycin-resistant parental line (1–3, 8, 33). Formation of DNA single-strand breaks as measured by alkaline elution technology was 3.2- and 4-fold greater in sensitive P388/4 cells than in resistant P388/ADR/3 and P388/ADR/7 cells, respectively (Fig. 5). Furthermore, generation of DNA double-strand breaks as measured by the neutral elution technique was 15- and 9-fold greater in sensitive cells than in resistant clone 3 and clone 7 cells, respectively (Fig. 6).

The higher incidence of DNA single- and double-strand breaks in sensitive cells could not be accounted for by the higher rate of drug uptake noted in those cells. In order to eliminate differences in drug uptake as a contributing factor to resistance, the rate of formation of DNA single- and double-strand breaks was determined at the same intracellular concentration of Adriamycin in sensitive and resistant cells. Under these conditions, the number of single-strand breaks was 1.85- and 1.96-fold greater in sensitive than in resistant clone 3 and clone 7 cells, respectively, and the level of double-strand breaks was 8.9- and 4.3-fold greater. These findings strongly support the concept that resistance to Adriamycin is multifactorial as suggested in other recent reports (34, 35). Although resistance to Adriamycin appears to be multifactorial, it was not possible from this study to establish whether these changes result from a single pleiotropic mutation or from multiple mutations.

Previous studies with L1210 leukemia have shown that the DNA single-strand breaks induced by Adriamycin are primarily protein-associated (30, 36), although at drug concentrations of 2.8 μM and greater, direct "nonenzymatic" breaks have also been observed (37). Our study with P388 leukemia differs from these previous reports, in that the DNA single-strand breaks formed were almost exclusively non-protein associated at drug concentrations of 0.2–0.8 μM.
concentrations of 0.1 to 2 μM. This finding suggests that P388 leukemia cells may be more vulnerable than L1210 cells to other lesions such as those induced by free radical formation (37).

The induction of single-strand breaks followed a dose-response relationship varying directly with Adriamycin concentration (Fig. 5). The positive y-intercept obtained in these plots directly reflects a threshold effect observed with the radiation calibration curve, in which no alteration in DNA retention or elution was observed until a dose of radiation of approximately 60 rads was exceeded (data not shown). This positive y-intercept could be eliminated by correction for the threshold effect observed with the radiation calibration curve. However, we prefer to present the raw data on single-strand breaks without any correction. Further, the comparisons made in this study between sensitive and resistant P388 cell lines involve slope differences, which would not be altered by such a correction.

The measured [SSB]/[DSB] ratio produced by Adriamycin in sensitive P388/4 cells was 0.202 ± 0.053 (SE), which was essentially identical to that reported previously for Adriamycin in L1210 cells (30). The single-strand breaks induced by Adriamycin in sensitive cells could be attributed entirely to those arising from double-strand breaks (Fig. 7A). Conversely, in resistant cells the frequency of single-strand breaks was greater than that attributable to double-strand breaks and this increase in single-strand breaks appeared to vary directly with the level of drug resistance, being greater in the more resistant clone 7 cells than in the less resistant clone 3 cells (Fig. 7B).

Finally, when cytotoxicity was plotted as a function of single-strand breaks 2 distinct curves were obtained for resistant clone 3 and clone 7 cells (Fig. 8A); the curve for sensitive cells was incomplete since much lower concentrations of Adriamycin were required to produce comparable levels of cell kill (Fig. 2). Conversely, a plot of cytotoxicity as a function of double-strand breaks appeared to follow the same relationship for all 3 cell lines (Fig. 8B). This finding suggests that at least for Adriamycin double-strand breaks appear to be a better index of cytotoxicity than are single-strand breaks.

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