Flow Cytometric Monitoring of Cellular Anthracycline Accumulation in Murine Leukemic Cells

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ABSTRACT

Cellular accumulation of daunorubicin (DNR), N-trifluoracetyl-adriamycin-14-valerate, and THP-Adriamycin (THP-ADR) in doxorubicin sensitive and resistant murine leukemia P388 cells was studied with laser excited flow cytometry. Appearance of DNR fluorescence in P388/S cells was rapid in contrast to that of P388/R cells. A comparison of P388/S and P388/R cells incubated for 20–30 min showed that DNR fluorescence in P388/R cells was one-sixth that of P388/S cells. In contrast, the difference between fluorescence value of P388/S and P388/R cells similarly incubated with N-trifluorocetyl-adriamycin-14-valerate or THP-ADR was less than 2-fold. Chlorpromazine, verapamil, and trifluoperazine increased the cellular accumulation and cytotoxicity of DNR and THP-ADR, but had no major effect on N-trifluorocetyl-adriamycin-14-valerate fluorescence or cytotoxicity in P388/R cells. Fluorometric and soft agar assays confirmed the data on the effect of these modulators on drug accumulation obtained by the more rapid method of laser flow cytometry.

INTRODUCTION

Doxorubicin (ADR) and daunorubicin (DNR) are important anti-tumor agents with dose-limiting cardiotoxicity as one of the major limitations to their continued clinical use (1). Several new anthracyclines with reduced cardiotoxicity have been synthesized recently (2, 3). AD 32, an Adriamycin analogue, earlier synthesized by Dr. Israel and his colleagues, differs from ADR in rapid cellular transport as well as in its lack of binding to nuclei or chromatin (4, 5). THP-ADR, synthesized by Umezawa et al. (3), has reduced cardiotoxicity and is undergoing clinical trials (6, 7). In contrast to ADR, THP-ADR cellular transport is rapid (8), and most of the drug fluorescence is localized in the cytoplasm (microsomes) with uniform light staining of nuclei (4).

Several recent studies have shown that cellular resistance to cancer chemotherapeutic drugs such as ADR can be compromised by exposure of cells in vitro to anticalmodulin drugs (e.g., phenothiazines) or calcium channel blockers (e.g., verapamil) (9–11). It has been suggested that these drugs reduce drug efflux from the resistant cells, thereby enhancing their drug sensitivity.

Anthracyclines such as doxorubicin are fluorescent, and we have earlier shown that argon laser excitation in a flow cytometer (cell sorter) can be used for rapid detection and quantitation of cellular anthracycline fluorescence in a heterogeneous cell population (12, 13). In a recent study we have used this method (laser excited flow cytometry) to monitor the effect of phenothiazines on retention of doxorubicin in drug sensitive and resistant P388 leukemia cells (14, 15). Observations from this study confirmed the earlier data of Ganapathi and Grabowski (9), Ganapathi et al. (10), and Tsuruo et al. (11) and further showed that phenothiazine effects on drug efflux are cell cycle stage and cell proliferation status related. Rapidly proliferating cells or cells in the late S, G2/M part of the cell cycle were more sensitive to phenothiazine effects than were non-cycling cells or cells in the G1 or early S part of the cell cycle.

Anthracyclines such as ADR, DNR, AD 32, and THP-ADR differ markedly in their cellular transport and binding characteristics (4, 8, 16, 17). In the present study, we have used multiparameter laser flow cytometry (for identification of cell populations based on their cellular drug fluorescence), fluorometric assay (for quantitation of cellular drug content), and soft agar assays (for cytotoxicity) to compare the effect of phenothiazines (chlorpromazine and trifluoperazine) and calcium channel blocker (verapamil) on the cellular accumulation of these important anthracyclines in P388 drug sensitive and resistant cells.

MATERIALS AND METHODS

Suspension cultures of P388 cell line and its doxorubicin resistant sub-line (P388/R84) were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, penicillin, and streptomycin. In soft agar assays, the ID50 for the P388/S and P388/R84 cells was 0.05 and 4.8 µg/ml of ADR, respectively.

Doxorubicin hydrochloride (ADR, NSC-123127) was obtained from Adria Laboratories, Inc., Columbus, OH. Daunorubicin hydrochloride (DNR, NSC-821151) was obtained from Ives Laboratories, Inc., New York, NY. AD 32 (NSC-246135) was obtained from the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. THP-ADR (Theprubicin) was obtained from Dr. H. Umezawa, Institute for Microbial Chemistry, Tokyo, Japan.

Stock solutions of ADR, DNR, and THP-ADR were prepared in Ca2+ and Mg2+ free Hanks’ balanced salt solution. A stock solution of AD 32 was prepared in 100% ethanol. Fresh dilutions of the drugs were prepared in normal saline before each experiment.

Phenothiazines, chlorpromazine (Thorazine), and trifluoperazine (Stelazine) were obtained from Smith, Kline, and French Laboratories, Carolina, PR. Verapamil (Calan) was obtained from Searle Pharmaceuticals Inc., Chicago, IL. Fresh serial dilutions from the stock solutions were made in normal saline before addition to the cultures.

Flow Cytometry. Cell suspensions incubated in vitro with anthracyclines with or without the addition of phenothiazines or verapamil were analyzed for their cellular fluorescence in a Coulter Electronics Epics V cell sorter interfaced to a MDADS data acquisition and analysis system. Fluorescence emission (above 530 nm) and forward angle light scatter were collected, amplified, and scaled to generate multiparameter histograms. A minimum of 10,000 cells was analyzed for each histogram generated. Details of our analytical methods have been described earlier (13, 14).

For generation of two parameter histograms based on cellular drug fluorescence and length of incubation (time), cell suspensions were directly mixed with the drug containing medium in the sampling cuvet of the flow cytometer (Coulter Epics V), maintained at 37°C with constant stirring. Final drug concentrations used were AD 32 (1.4 µM), THP-ADR (3 µM), and DNR (3.5 µM). For drug combination experiments P388/S and P388/R cells were incubated with AD 32 (1.4 µM), DNR (3.5 µM), or THP-ADR (3 µM) for 30–60 min at 37°C, with or without the addition of CPZ (5–100 µM), TFP (5–100 µM), or VPL (2–100 µM).
ANTHRACYCLINE TRANSPORT MODULATION

Fluorometric Assay. Cells incubated with DNR (3.5 μM) or THP-ADR (3 μM) for 1 h or AD 32 (1.4 μM) for 30 min were washed in cold saline (3 times), and the cell pellets were resuspended in an acid-alcohol reagent containing 95% ethanol, 1 N hydrochloric acid, and distilled water in a 5:2.8:1.6 ratio (18). The supernatant recovered after sonication and centrifugation of the cell suspension was analyzed in a Aminco-Bowman spectrofluorimeter. The excitation and emission wavelengths used were: AD 32, 476 and 560 nm; DNR, 460 and 560 nm; and THP-ADR, 470 and 570 nm, respectively.

The drug concentration in the sample was derived from a standard curve based on known anthracycline concentration in the solvent.

Soft Agar Clonogenic Assays. Cells exposed to the various drug combinations for 1 h were washed twice in drug-free Hank's balanced salt solution, resuspended in S-MEM (Gibco 320-1385) (2x) medium, and mixed with an equal amount of 0.6% soft agar at 45°C. The S-MEM medium was supplemented with fetal calf serum (15%), 2-mercaptoethanol, and antibiotics, penicillin, and streptomycin. Soft agar containing plastic dishes were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide, and 90% nitrogen for 7 days. Details of our soft agar culturing methods have been described earlier (19). Colonies larger than 50 μm in size were counted from triplicate plates for determination of mean number of colonies + SD per plate.

RESULTS

Comparison of Cellular Fluorescence of DNR, AD 32, and THP-ADR. Two-dimensional flow histograms (of cellular drug fluorescence versus time in min) shown in Fig. 1 are of P388/S and P388/R cells incubated with DNR (3.5 μM), AD 32 (1.4 μM), or THP-ADR (3 μM) for 30 min. On the abscissa of each figure, the amount of cellular drug fluorescence as measured after excitation with the 488-nm argon laser line is recorded on a linear scale (64 channels), whereas on the ordinate, length of incubation in min (10 min span) is shown. The height of the figure indicates the number of cells recorded at a given time with the fluorescence of a particular channel value.

Fig. 1, A, E, and I, records cellular drug fluorescence of P388/S cells within the first 10 min of incubation in the drug containing medium, whereas Fig. 1, B, F, and J are of the same culture between 20 to 30 min of incubation. In P388/S cells incubated with 3.5 μM DNR (Fig. 1, A and B) cellular drug fluorescence gradually increases, reaching a maximum after approximately 20 min of incubation (Fig. 1B). At least four distinct populations (arrows) on the basis of their DNR fluorescence can be identified. In P388/R (Fig. 1, C and D), the appearance of fluorescent cells is slower than that of P388/S cells (compare Fig. 1, A with C). After 30 min of incubation, cellular DNR fluorescence in P388/R cells is still less than that of P388/S cells and mostly confined to a single peak (compare Fig. 1, B with D).

Fig. 1, E to H, shows the accumulation of AD 32 (1.4 μM) cellular fluorescence in P388/S and P388/R cells. In contrast to cells exposed to DNR and shown in Fig. 1A, the cellular accumulation of AD 32 is much more rapid, and maximum

![Fig. 1. Cellular fluorescence histograms of P388/S (AB, EF, and IJ) cells incubated, respectively, with DNR (3.5 μM), AD 32 (1.4 μM), or THP-ADR (3 μM) for 30 min. CD, GH, and KL are of P388/R cells similarly exposed to DNR, AD 32, or THP-ADR. On the X-axis cellular drug fluorescence is recorded on a linear scale of 64 channels. The Y-axis records times 0 to 10 min. Height of the histogram records number of cells in a given channel.](image-url)
cellular drug fluorescence is reached within the first 10 min of incubation. Fig. 1F shows that between the next 20 to 30 min of incubation, there is no further increase in cellular drug fluorescence. In contrast to P388/S cells incubated with DNR and shown in Fig. 1B, where at least four subpopulations can be identified on the basis of their drug fluorescence, cells exposed to AD 32 show only two major subpopulations (arrows).

A comparison of Fig. 1G (P388/R cells incubated with AD 32) with cells incubated with DNR (Fig. 1C) shows that AD 32 fluorescence appears rapidly in the resistant cells, and there is a gradual increase in intracellular drug fluorescence within the first 10 min. During the next 20 to 30 min of incubation (Fig. 1F), there is no major increase either in the number of fluorescent cells or in the amount of per cell drug fluorescence. This is in sharp contrast to cells incubated with DNR, where the resistant cells show a gradual increase in the number of fluorescent cells recorded within the first 10 min (shown in Fig. 1C). Cells incubated with AD 32 are on the average more fluorescent than cells incubated with DNR for the same length of incubation [note that the AD 32 concentration (1.4 μM) used for incubation is less than one-half that of DNR (3.5 μM) or THP-ADR (3 μM)].

Fig. 1J to L, is of P388/S and P388/R cells incubated with THP-ADR (3 μM) for 0 to 10 (Fig. 1, I and K) or 20 to 30 min (Fig. 1, J and L). Cellular fluorescence in P388/S cells incubated with THP-ADR was lower than that of cells incubated with DNR or AD 32, and maximum cellular fluorescence is reached within the first 10 min of incubation. No major increase in cellular fluorescence is seen after the first few min of incubation (compare Fig. 1, I and J). This stands in sharp contrast to cells exposed to AD 32 or DNR in which a rapid increase in the amount of cellular fluorescence within the first 10 min of incubation is seen.

In P388/R cells incubated with THP-ADR (Fig. 1, K and L) the accumulation of cellular drug fluorescence was gradual, and the number of fluorescent cells increased slowly within the first 30 min of incubation. This is in contrast to P388/S cells incubated with AD 32 and shown in Fig. 1G, where the maximum number of fluorescent cells is rapidly reached within the first few min of incubation. In contrast to P388/S cells incubated with DNR (Fig. 1B), cells exposed to THP-ADR show a unimodal distribution (Fig. 1J) and, unlike the former, most of the cells are within a single peak of fluorescence. After 1 h of incubation, cellular fluorescence of P388/S cells incubated with THP-ADR was 2- to 3-fold higher (based on peak fluorescence channel number) than that of P388/R cells (data not shown).

Effect of Transport Modulators on Drug Fluorescence, DNR. In cells incubated with CPZ, TFP (5–10 μM), or VPL (2–100 μM) alone, no major effect on forward angle light scatter signal was detected. At higher concentrations of CPZ or TFP (100 μM), a reduction in narrow angle light scatter, indicating cell shrinking and damage to the cell membrane, was evident. Cells incubated with CPZ, TFP, or VPL (100 μM) and excited with 488 nm argon laser line did not emit any fluorescence. Fig. 2 is of P388/S and P388/R cells incubated with DNR (3.5 μM) alone (Fig. 2, A and E) or in the presence of 100 μM CPZ (Fig. 2, B and F), TFP (Fig. 2, C and G), or 20 μM VPL (Fig. 2, D and H). On the abscissa of these figures, cellular drug fluorescence is recorded, whereas on the ordinate forward angle light scatter (which approximates cell size) is indicated. Height of bars indicate number of cells recorded in a particular channel. As seen in Fig. 2, A to D, co-incubation of P388/S cells with CPZ or TFP (5–100 μM) or VPL (20–100 μM) did not cause any major increase in DNR cellular fluorescence (PFCV 12 versus 13 to 18). In contrast, P388/R cells which have normally reduced cellular drug fluorescence (PFCV 1) due to rapid efflux (Fig. 2E), significantly increased cellular drug fluorescence (PFCV 11 to 14) on co-incubation with CPZ, TFP (100 μM), or 20 μM VPL (Fig. 2, F, G, and H).

N-Trimfluoroacetyl-Adriamycin-14-Valerate. Histograms in Fig. 3 are of P388/S and R cells incubated with AD 32 (1.4 μM) alone (Fig. 3, A and E) or in combination with CPZ (100 μM); Fig. 3, B and F), TFP (100 μM; Fig. 3, C and G), or VPL (20 μM; Fig. 3, D and H). Unlike DNR (Fig. 2), cellular fluorescence of AD 32 in P388/S and R cells was approximately similar (Fig. 3, A versus E, PFCV 28 versus 26). Co-incubation of P388/S or R cells in the presence of high CPZ or TFP concentrations (100 μM) reduced the amount of cellular drug fluorescence (PFCV 4 to 7) and light scatter, whereas lower concentrations (5–10 μM) had no effect. VPL (up to 100 μM) had no major effect on intracellular AD 32 fluorescence of P388/S or R cells (compare Fig. 3, A with D and E with H; PFCV 31, 29).

These observations are in contrast with those on DNR described in Fig. 2, where coincubation with CPZ, TFP, or VPL significantly increased drug fluorescence in P388/R cells without making any difference to cellular drug fluorescence of P388/S cells.

4-O-Tetrasdihydropyranadriamian. Histograms in Fig. 4 are of P388/S and R cells incubated with THP-ADR (3 μM for 1 h) and analyzed with or without co-incubation with CPZ (100 μM), TFP (100 μM) or VPL (20 μM). P388/S cells incubated with THP-ADR (Fig. 4A) had a unimodal cellular drug fluorescence.
Fig. 3. Record cellular drug fluorescence of P388/S or P388/R cells incubated for 1 h with DNR (3.5 μM) or THP-ADR (3 μM) or AD 32 (1.4 μM) for 30 min. Fluorescence is recorded on the X-axis, while light scatter (approximate cell size) is recorded on the Y-axis. A and E are after incubation with anthracycline alone, while B, F, G, D, and H are after co-incubation with CPZ, TFP (100 μM), or VPL (20 μM) and the anthracycline. Peak fluorescence channel values are indicated above each major peak.

Fig. 4. Record cellular drug fluorescence of P388/S or P388/R cells incubated for 1 h with DNR (3.5 μM) or THP-ADR (3 μM) or AD 32 (1.4 μM) for 30 min. Fluorescence is recorded on the X-axis, while light scatter (approximate cell size) is recorded on the Y-axis. A and E are after incubation with anthracycline alone, while B, F, G, D, and H are after co-incubation with CPZ, TFP (100 μM), or VPL (20 μM) and the anthracycline. Peak fluorescence channel values are indicated above each major peak.

Fluorometric Assays. Data in Table I show that P388/S cells incubated with DNR (3.5 μM) for 1 h had approximately 6-fold higher DNR content (2.0 μg/5 x 10^6 cells) than did P388/R cells (0.35 μg/5 x 10^6 cells) treated similarly. In P388/S cells exposure to high CPZ or TFP concentrations (100 μM) resulted in a loss of DNR fluorescence (presumably by causing cell shrinkage and damage to the cell membrane), whereas lower concentrations (5–10 μM) had no significant effect on DNR retention. In contrast, a significant increase in DNR (3.5 μM) retention was seen in P388/R cells by co-incubation with CPZ, TFP (10–100 μM), or VPL (2–20 μM).

AD 32 retention was 1.38 and 1.31 μg per 5 x 10^6 P388/S and R cells, respectively. Co-incubation with CPZ, TFP, or VPL, especially at higher concentrations, reduced AD 32 content in P388/S cells. P388/R cells were relatively more resistant, and at low TFP or VPL concentrations (2–20 μM), an apparent increase in AD 32 retention of P388/R cells was observed.

THP-ADR accumulation in P388/S cells (2.7 μg/5 x 10^6 cells) incubated for 1 h (3 μM) was 7-fold higher than that of P388/R cells (0.37 μg/5 x 10^6 cells). Co-incubation with 10 μM of CPZ or VPL (2 or 20 μM) did marginally increase (less than 20%) accumulation of THP-ADR in P388/S cells. However, in P388/R co-incubation with CPZ, TFP (10–100 μM), or VPL (2–20 μM) increased the accumulation of THP-ADR by 4- to 11-fold.

Soft Agar Assays. Data in Fig. 5 are from colony forming assays using DNR (0.1–1 μM), AD 32 (6.9 μM) or THP-ADR (0.75 μM) alone or in combination with CPZ (1–10 μM) or TFP (20–200 μM) in P388/R cultures.

DNR (Fig. 5A). In P388/R cells exposure to CPZ concentra-
with SD (bars) based on counting of the total number of surviving colonies from chlorpromazine (1-10 μM) or VPL (1-100 μM). Data presented are the mean cytotoxicity of AD 32, as shown in Fig. SB.

with CPZ (10 μM) and DNR concentrations of 0.1 or 1 μM. As shown in Fig. 5C, CPZ effects on the cytotoxicity of THP-ADR in P388/R cells.

tions of higher than 10 μM was cytotoxic. In cells incubated with CPZ (10 μM) and DNR concentrations of 0.1 or 1 μM, the number of surviving colonies was 86 and 42%, respectively, of cells incubated with either drug alone. As shown in Fig. 5, the synergistic effect of VPL on DNR cytotoxicity in P388/R cells was more pronounced than that of CPZ described above.

AD 32 (Fig. 5B). In contrast to the enhanced (CPZ) or synergistic (VPL) cytotoxicity of DNR seen in P388/R cells (Fig. 5A), neither of these agents had any major effect on cytotoxicity of AD 32, as shown in Fig. 5B.

THP-ADR (Fig. 5C). The CFA data from P388/R cells incubated with THP-ADR (0.75 μM) alone or in combination with CPZ (1-10 μM) or VPL (1-100 μM) were similar to those of cells exposed to DNR. As shown in Fig. 5C, CPZ effects were marginal, whereas VPL had a pronounced synergistic effect on the cytotoxicity of THP-ADR in P388/R cells.

DISCUSSION

Data in the present study confirm our earlier observation showing that laser flow cytometry can be used for rapid analysis of anthracycline transport modulator effects in drug sensitive and resistant cells (12-14). This method offers a means to study drug accumulation in individual cells and thus identify sub-populations in a heterogeneous tumor on the basis of drug transport (influx, efflux, and retention) characteristics in individual cells. The laser activated cell sorting capability further allows one to collect sub-populations of interest for other morphological or biochemical studies.

Thus one sees not only major differences between the cellular drug fluorescence profile of the three anthracyclines but in certain cases (such as cells incubated with DNR for 20–30 min), several subsets differing in their cellular fluorescence content can be readily identified. In an earlier study, we have shown that cell cycle position is a major determinant for the modulating effect of chlorpromazine on doxorubicin accumulation (14, 15). In contrast to DNR, cellular fluorescence distribution of AD 32 is homogeneous, and only two sub-populations can be identified in cells incubated for 20–30 min. Experiments are under way to further identify these subsets on the basis of differences in their anthracycline fluorescence. As shown in Fig. 1, P388/S and R cells incubated with DNR for 20–30 min differ markedly in their cellular fluorescence content. In contrast P388/S/R cells incubated similarly with AD 32 do not show any differences of similar magnitude in their fluorescence content. In spite of this lack of pronounced difference, P388/R cells are as resistant to AD 32 as they are to ADR or DNR. Thus, at least for AD 32, drug transport and retention may not be a major determinant of cytotoxicity in P388/S and R cells. AD 32 is also unique in that CPZ, TFP, or VPL has no major effect on either its cellular retention or cytotoxicity. These observations confirm data of Ganapathith et al. (10), who reported that TFP does not potentiate cytotoxicity or drug retention of AD 32 in P388 cells.

However, it is important to bear in mind that AD 32 differs from DNR and THP-ADR in its lack of DNA binding and rapid influx. In a cell damaged by exposure to high levels of CPZ, AD 32 will rapidly diffuse out of the cell (as none of it binds to DNA) in contrast to THP-ADR or DNR which, in spite of the membrane damage, will continue to give fluorescent signals due to their nuclear binding. The rapid influx of AD 32 may also explain why the efflux system in P388/R cells may not operate against cellular accumulation of this drug and why the efflux blocking agents are ineffective.

Several studies have shown that rapid drug efflux may be responsible for the lack of drug sensitivity in certain drug resistant cells (20, 21). Co-incubation of cells with phenothiazines or calcium channel blockers will inhibit drug efflux and thus confer drug sensitivity on otherwise resistant cells (9–11, 22). In most of these studies, soft agar assays have been used to monitor the modulating effect of the phenothiazines or calcium channel blockers on drug efflux. However, the soft agar assays take a minimum of 7 days to yield results, and in most clinical situations one needs more rapid access to data. Laser excited flow cytometry as shown in the present communication provides a fast method for monitoring of these effects and, in the three drugs screened, it provided data comparable to those obtainable by the fluorometric and soft agar clonogenic assays.

We have recently used this method to screen tumor cells from patients for their anthracycline transport characteristics and their sensitivity to various drug transport modulators (23). Studies are under way to correlate flow cytometric and cytotoxicity data from soft agar assays and clinical response.

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