Digitized Video Fluorescence Microscopy Studies of Adriamycin Interaction with Single P388 Leukemic Cells

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ABSTRACT

We have evaluated a new fluorescent method, the digitized video fluorescence microscopy technique, for the analysis of Adriamycin drug levels in single-cell suspension. This method uses a Leitz microscope equipped with an HBO 50 watt mercury source; the vertical body of the microscope is attached to an intensified silicon intensified video camera with its output coupled to a video cassette recorder and to an Apple II microcomputer equipped with a video image digitizer. Using this technique, we were able to corroborate previous findings of decreased uptake and increased efflux in resistant as compared to sensitive P388 leukemic cells. This instrument may have wide applications in the study of anthracycline cell interaction or of any other drug with fluorescent properties.

INTRODUCTION

Adriamycin is a cytotoxic antibiotic isolated from cultures of Streptomyces peucetius var. caesius. Its chemical structure consists of 2 components: a water-insoluble tetracycline aglycone (adriamycinone) which imparts the fluorescent characteristic of this drug; and a water-soluble basic reducing amino sugar (daunosamine) (1, 2). The pharmacokinetics of this drug has been studied with a variety of techniques including spectrofluorimetry, radioimmunoassay, and thin-layer and liquid chromatography (3–6). Although these techniques have been useful in measuring transport and tissue concentration of Adriamycin and its catabolites, they are incapable of monitoring drug transport in single-cell suspensions. Furthermore, because they require either large numbers of cells or the use of radiolabeled Adriamycin, they may not be suitable for directly studying the cells of individual leukemic patients. This communication describes a technique for the analysis of uptake and efflux of Adriamycin by single cells. The cells used for the study are the P388 leukemia, either sensitive or resistant to Adriamycin, but the technique is readily applicable to the study of cellular pharmacokinetics in circulating human neoplastic and normal cells as well as in cells from solid tumors.

MATERIALS AND METHODS

Cell Lines

P388 cells sensitive and resistant to Adriamycin were maintained by serial i.p. passages in DBA/2 mice. Ascitic cells were collected on Days 5, 6, and 7 after passage. Erythrocytes were removed by Ficoll-Hypaque density gradient sedimentation. The P388 cells were then washed and resuspended in Hanks' balanced salt solution without phenol red (at room temperature) at a final concentration of 10 × 10⁶ cells/ml. Cells were used immediately for the in vitro experiments.

Drugs

Adriamycin was purchased from Adria Laboratories and used at a concentration of 0.1 or 1.0 μg/ml.

Incubation Conditions

Sensitive or resistant P388 cells (5 × 10⁵ cells) were allowed to settle onto the surface of a glass coverslip previously coated with poly-L-lysine. This coating allows the cells to attach to the coverslip without impairing the viability of the cells or interfering with fluorescence measurements under incident light stimulation. The coverslip is inverted and placed so as to form the top of a chamber with openings designed to allow the inflow and outflow of media. Flow of the media was regulated by a motorized syringe pump; the temperature was maintained at 37° by placing the injection syringe in a heated block controlled by a Thermistemp (Yellow Springs Instrument). Flow rate through the chamber was 0.5 ml/min with the exception of the first 5 sec of the injection when the rate was 1 ml/min. This rapid initial flow was used to achieve a constant concentration of Adriamycin within the chamber within 1 sec. Since the chamber volume is 40 μl, the chamber fluid was exchanged at a rate of 12 times/min. When Adriamycin was used at a concentration of 1 μg/ml (2 × 10⁻⁴ M), measurements were obtained 30 sec after the initial injection followed by measurements at 1, 1.5, and 2 min. When Adriamycin was used at 0.1 μg/ml (2 × 10⁻⁵ M), readings were obtained at 20, 40, and 60 min; efflux was determined 5, 15, and 30 min postinjection of drug-free media. For every experiment, between 15 and 20 cells were analyzed. Most cells (>90%) exclude trypan blue at the end of the experiment despite this frequency of fluorescence stimulation. Death cells are excluded from the analysis.

Determination of Intracellular Drug Levels

The uptake and efflux of Adriamycin was quantitated by measuring the fluorescent intensity emitted by drug associated with the cells using a DVFM. This system consists of a Leitz microscope equipped with an HBO 50 watt mercury source; a vertical fluorescent illuminator with the appropriate filters is used for routine fluorescence studies. In this system, the most efficient excitation and emission filters for Adriamycin are 540 and 580 nm, respectively. An electronic shutter (Uniblitz Electronic, Rochester, N. Y.) was placed between the lamp source and the microscope. The shutter in the fluorescence activation light path is controlled by the computer and provides illumination times of 0.5 sec for each reading. This fast exposure minimizes and controls the photobleaching rate of Adriamycin. Chart 1 summarizes the mean rate of photobleaching of cells previously treated with Adriamycin after 2 sec of constant fluorescence stimulation. The vertical body tube of the microscope is coupled to a specially modified RCA TC-1040 intensified silicon intensifier target camera which distinguishes fluorescence at levels far below those of conventional microscopy. The gain of the camera was modified as follows. All nonessential automatic circuits were disabled. Manual control was set at maximum sensitivity. Autogain is in manual position but in full gain. Autoback and autotarget are in...
RESULTS

The initial association of Adriamycin with the cell is due to membrane adsorption of the drug. Early changes in fluorescence were not detectable when Adriamycin was used at a concentration of 0.2 μmol/ml; this early association was similar for both cell lines.

Time Course of Uptake and Efflux. A composite time course of uptake and net efflux of Adriamycin by sensitive and resistant P388 cells is shown in Chart 3. The drug was added to the chamber at zero time. Uptake was greater in the sensitive as compared to the resistant cell line so that by 60 min sensitive cells contained 30% more net drug than did the resistant cells. When incubation was prolonged beyond 60 min, many of the cells appeared nonviable as determined by trypan blue uptake. To assess drug efflux, the chamber was perfused with drug-free buffer at 37° after the initial 60 min of incubation with Adriamycin. Efflux over the first 30 min was first order in both cell lines, with an efflux rate constant k = -0.0495 ± 0.00592 in sensitive cells; k = -0.0242 ± 0.00592 in resistant cells (p = 0.0005) (Chart 4).

System Calibration

**Digisector.** The optimum settings for the width, brightness, and contrast knobs were set manually at the time the system became in operation. No changes have been made since.

**DVFM.** Instability of the mercury lamp source and lack of field fluorescence homogeneity are generally recognized sources of error in quantitative fluorescence techniques. Therefore, several steps were taken in order to minimize these problems. (a) The HBO 50 watt lamp is changed every 200 hr, and no experiments are carried out in the first 12 hr in order to allow for lamp stabilization. (b) A diffusion filter was placed between the lamp source and the microscope, and the illumination field was limited to 75% of the total in order to concentrate the fluorescent light; a special effort is made to calibrate the lamp manually after every change as closely as possible to the previous one. (c) Reference standards, including Fluoresbrite Fluorescent (rhodamine) microdisperse microspheres (diameter, 4.43 ± 0.31 μm) and a permanently stained slide with a homogeneous fluorescence preparation are run prior to the experiments in order to calibrate the gain if necessary. Generally, we have been able to keep the gain low (4) and constant, since daily controls showed less than 6% changes in fluorescence intensity; furthermore, the field is fairly homogeneous with a ±11% variation in the reading of the standards.

**RESULTS**

Initial Uptake. Chart 2 shows the initial Adriamycin uptake for the sensitive and resistant sublines. In both cell lines, a significant association of the drug with the cells was evident after only 30 sec of incubation when Adriamycin was used at a concentration of 0.1 μg/ml, the background was insignificant (<50), but when the concentration was increased to 1 μg/ml the level increased to 520 ± 70 (S.E.) brightness units and made the reading of the cell fluorescence somewhat difficult; for this reason, no comparative time course of uptake and efflux (using Gain 3) was done using this drug concentration.
Digital Video Fluorescence Microscopy

**DISCUSSION**

Adriamycin plays a dominant role in the treatment of leukemia and a variety of solid tumors in humans (7–9). However, as for all chemotherapeutic agents, a major limitation in its effective use is the acquisition of resistance by neoplastic cells (10, 14, 16). As the therapeutic target is intracellular, cellular uptake is a necessary factor in the biological and therapeutic effect of this drug. Thus, sensitive techniques based on the fluorescent properties of the drug have been developed to help elucidate the cellular pharmacokinetics of this drug (3–6, 7, 15, 20, 21).

Previous work (11–13) has suggested a generalized change in permeability properties of the cell membrane to account for the resistance of some malignant cells to the anthracyclines. Skovsgaard (28, 29) has suggested that resistance in an Ehrlich ascites tumor cell line was due to a lower influx and a higher efflux of the drug. Studies by Inaba and Johnson (17–19) in P388 leukemic cells indicated that, although there was a decreased uptake of the anthracyclines by their resistant sublines, resistance was primarily due to a diminished capability to retain the antibiotic within the cells, secondary to an active outward transport mechanism, and that any enhancement of this efflux process renders the cells highly resistant to the anthracyclines. In our study, a DVFM is applied to monitor drug uptake and efflux in single P388 cells. The data corroborate previous findings of increased uptake and decreased efflux in the sensitive lines as compared to the resistant leukemic cells. However, it is not possible to distinguish whether alterations in drug uptake in resistant cells are due to decreased binding, decreased influx, or increased efflux of the drug. It is possible not only that resistant cells have a decreased number of binding sites for Adriamycin but also that these sites may have a lower affinity for the drug, changes that could explain decreased net uptake as well as increased net efflux.

Although the DVFM is simple and sensitive, it does not differentiate between the fluorescence of Adriamycin or its catabolites. Also, like any other fluorescent technique, fluorescence quenching of the Adriamycin by DNA complicates interpretation of the data (15, 20); still, the results obtained indicate that this method permits the monitoring of drug levels in individual cells and may provide important data regarding kinetics of uptake and efflux, drug binding to intracellular sites, and the relationship between the above parameters and plasma pharmacokinetics. Thus, when applied clinically, this technique can provide the opportunity to follow intracellular drug levels in circulating tumor cells in vivo, i.e., leukemic cells isolated from patients treated with Adriamycin or other anthracyclines, to evaluate drug uptake into heterogeneous cells within a tumor population and to assess exit kinetics of a drug from tumor cells with correlation with plasma pharmacokinetics. Furthermore, this technique allows for the specific identification of cell type, offers the possibility for defining patterns of intracellular location of the different anthracyclines, and has a potential application in the study of other drugs that fluoresce or drugs which can be coupled to a fluorescent probe.

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