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Advances in analytical cellular pathology. G. Burger et al, eds.

THE EFFECT OF DEPLETION OF GLUTATHIONE ON THE TOXICITY OF DOXORUBICIN IN FRIEND LEUKEMIA CELLS WITH ACQUIRED RESISTANCE AS MEASURED WITH FLOW CYTOMETRY

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INTRODUCTION

Anthracyclines are thought to act via the formation of free radicals (1-3). Free radicals are detoxified by intracellular redox buffers such as glutathione (GSH), a tripeptide (Glu-Cys-Gly) which provides equivalents for the enzymatic reduction of electrophilic toxic compounds in the cell. The aim of this study was to see to what degree GSH levels in anthracycline-sensitive Friend leukemia cells (FLC) and a resistant subline (ARN15) were of importance to anthracycline toxicity. For this purpose, the glutamylcysteine synthetase, an enzyme in the GSH pathway, was specifically inhibited using buthionine sulfoximine (BSO). Inhibition of the GSH pathway via BSO should lead to decreased levels of GSH produced.

Intracellular GSH in vital cells was determined with flow cytometry using o-phthaldialdehyde (OPT), which forms a fluorescent adduct with GSH (4). The effects of decreased GSH levels on the toxicity of doxorubicin were determined by monitoring growth of the cells in presence of doxorubicin with and without BSO.

MATERIAL AND METHODS

Cells. A doxorubicin (DOX)-sensitive Friend murine erythroleukemia cell line (FLC) and multidrug-resistant subline (ARN15) were kept in log-phase growth in Dulbecco's modified Eagle's medium (DME, Gibco, Eggenstein, FRG), supplemented with 10% fetal calf serum, in 5% CO_2/95% air at 37°C.

Cell staining. DOX-sensitive FLC or resistant ARN15 cells were incubated at $1x10^6$ cells/ml in 2 ml of DME in 24-well culture plates for 24 h in the presence of BSO. Staining of the cells for GSH and protein-SH groups was done according to the methods of Treumer and Valet (1986) (2), with modifications. Briefly, 1 ml of cells at 10^6 cells/ml was resuspended in ice-cold Hank's medium without serum, pH 7.4, and incubated with 5 μl of a 25 mM solution of o-phthaldialdehyde (OPT) in dimethylformamide (DMF) for 10 min on ice. Simultaneously, 20 μl of 2 mg/ml propidium iodide (PI) in DMF was added to stain the nuclei of dead cells.

Flow cytometry. Four parameters, cell volume and three fluorescence emissions of each cell, were simultaneously collected as list mode data using a Fluvo-Metricell flow cytometer (HEKA-Elektronik, Lambrecht/Pfalz, FRG). The sheath fluid was 5 mM HEPES buffered saline (HBS), pH 7.35. The cell volume was measured from the change of electrical resistance through a 90 μm x 90 μm cylindrical orifice. Excitation between 358 and 378 nm was accomplished by epi-illumination of the cells through an oil-immersion objective (x100, numerical aperture 1.30, Zeiss, Oberkochen, FRG) with an HBO 100 watt high pressure mercury arc lamp (Osram, Munich, FRG). Three fluorescence emissions were recorded, corresponding to protein-SH (414-445 nm), GSH (456-503 nm) and PI (>545 nm).

Two-parameter histograms of fluorescence at two different emission wavelengths enabled the separation of cells into vital and dead populations (Figure 1a), due to the differential staining of DNA of dead cells with PI. Vital cells were then used for the calculation of intracellular GSH concentration (Figure 1b).

Growth Assay. DOX-sensitive FLC or resistant ARN15 cells were incubated at $2x10^5$ cells/ml in 2 ml of DME in 24-well culture plates for 72 h, in presence of DOX and/or BSO. Cells were counted before and after the incubation period with a Coulter counter Model A. Growth is expressed as the ratio of experimental to control growth.

Chemicals and drugs. Doxorubicin hydrochloride (Adriblastin^R, Farmitalia) at 2 mg/ml was dissolved in sterile saline. Buthionine sulfoximine (Sigma, Deisenhofen, FRG) at 1 mg/ml was dissolved in HBS.

RESULTS

Intracellular levels of GSH were found to be elevated (170% of FLC) in DOX-resistant ARN15 cells (1.67±0.47 relative units) as compared to the sensitive parent cell line FLC (0.97±0.24). BSO has been reported to be a selective inhibitor of γ-glutamylcysteine synthetase (3). Incubation of cells with BSO for 24 h produced a maximal decrease in GSH of 80% in ARN15 (see Figure 2).

Buthionine sulfoximine was used to study the effects of GSH depletion on the toxicity of DOX in the sensitive and resistant cells (see Table 1 and Figure 3). After 72 h, growth of the FLC was significantly inhibited by DOX in all concentrations tested (IC_{50}=0.01 μg/ml). ARN15 cells were up to 1,000-fold resistant (IC_{50}=10 μg/ml). However, when BSO was present, the resistance to DOX was decreased an average of 10-fold (IC_{50}=1 μg/ml). BSO had no effect on the toxicity of DOX in FLC.

TABLE I. DOXORUBICIN IC_{50} IN FLC AND ARN15 CELLS

Cell line	DOX only	+ BSO 25 μg/ml	+ Verapamil 10 μg/ml
FLC (sensitive)	0.01 μg/ml	0.01 μg/ml	0.02 μg/ml
ARN15 (resist)	10.00 μg/ml (x1,000 resist)	1.00 μg/ml (x100 resist)	0.15 μg/ml (x15 resist)

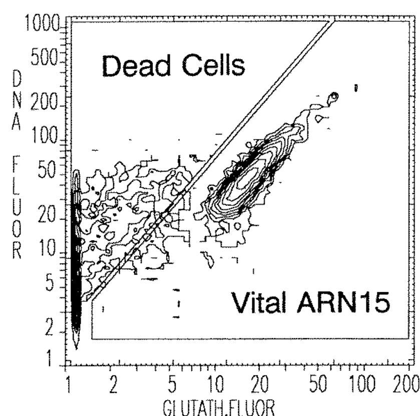

Fig. 1a. Two-parameter histogram of OPT-stained ARN15 cells, showing GSH-specific fluorescence versus PI staining.

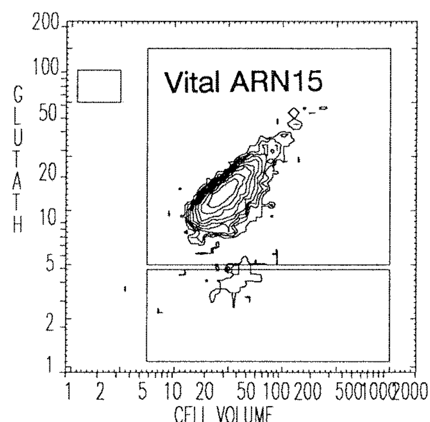

Fig. 1b. Two-parameter histogram of OPT-stained ARN15 cells showing GSH specific staining of vital cells.

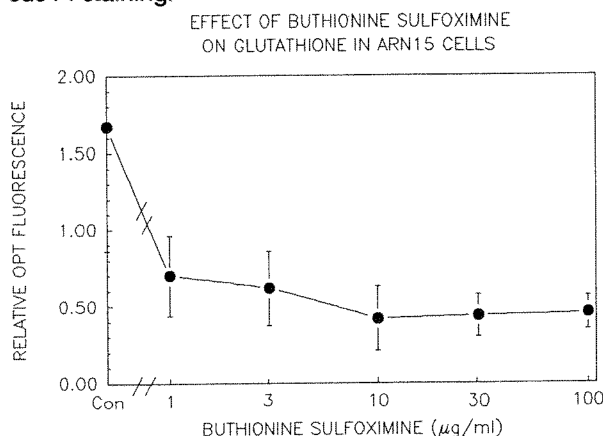

Fig. 2. Effect of 24-h incubation with BSO on intracellular GSH concentration in ARN15, as measured with OPT. Values are means of GSH-specific fluorescence/volume.

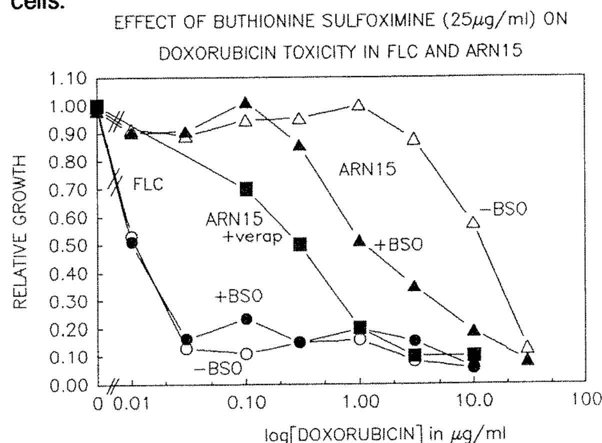

Fig. 3. Effect of BSO on DOX-mediated inhibition of growth of FLC and ARN15 cells. Cells were incubated for 72 h with DOX, with or without BSO (25 μg/ml).

CONCLUSIONS

Depletion of GSH to 20% of control values leads to an order of magnitude increase of the sensitivity of ARN15 cells to doxorubicin. Significant resistance remains in the ARN15 cells, suggesting additional mechanisms of resistance are also present. Verapamil, reported to decrease multidrug resistance by inhibition of efflux (5,6), increases sensitivity in these cells by almost two orders of magnitude. Simultaneous incubation with verapamil and BSO might induce a complete reversal of resistance. Such combined regimens are now being investigated.

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Supported by DFG SFB 324, project G6, and the German-American Cooperative Project in Cancer Research.

ADVANCES IN ANALYTICAL CELLULAR PATHOLOGY

Proceedings of the First Conference of the European Society for Analytical Cellular Pathology, Schloss Elmau, F.R.G., 12–17 November 1989

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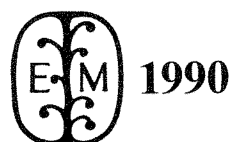

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