Role of Aldehyde Dehydrogenase in Cyclophosphamide-resistant L1210 Leukemia[1]

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ABSTRACT

A cyclophosphamide-resistant L1210 cell line has been shown to have unusually high aldehyde dehydrogenase activity. The sensitivity of this cell line to 4-methylcyclophosphamide and phosphoramide mustard *in vivo* and corresponding sensitivities *in vitro* indicate that 4-hydroxycyclophosphamide and/or aldophosphamide is the form in which cyclophosphamide reaches these tumor cells in mice and that intracellular aldehyde dehydrogenase activity is an important determinant of cyclophosphamide sensitivity in these leukemia cell lines.

INTRODUCTION

In a recent article (11), we described DNA cross-linking and cell viability studies in 2 lines of L1210 cells; one sensitive and the other resistant to cyclophosphamide and 4-hydroperoxycyclophosphamide. The fact that the resistance could be substantially reversed by pretreatment of the cells with a low concentration of disulfiram was interpreted as indicating that a higher activity of aldehyde dehydrogenase in the resistant line could account for their resistance to activated cyclophosphamide. Preliminary results have been presented in which a high aldehyde dehydrogenase activity also is associated with this line (6) of cyclophosphamide-resistant L1210 (12). A critical role for aldehyde dehydrogenase in determining the tissue selectivity of cyclophosphamide has been proposed earlier by Sladek (19) and Cox *et al.* (5). Although the intervention of this enzyme in the metabolism of activated cyclophosphamide in cell-free systems has been well-documented (7, 8), a clear demonstration of its physiological role has been lacking (13).

Cyclophosphamide metabolism involves activation of the oxazaphosphorine ring through hydroxylation at the 4-position (4-hydroxycyclophosphamide), ring opening to yield the aliphatic aldehyde aldophosphamide and a β-elimination reaction to release acrolein and phosphoramide mustard. Of this series of compounds, only phosphoramide mustard has alkylating activity at physiological pH (4).

While it is generally agreed that the liver is the major site of cyclophosphamide activation, and that phosphoramide mustard is the ultimate cytotoxic agent, there have been divergent views on the nature of the form in which the cytotoxic moiety reaches its target tissue (10). One school has favored the view that phosphoramide mustard leaves the liver as the cytotoxic form of cyclophosphamide, is transported in the blood stream, enters target tissues, and exerts its cytocidal effect through damage to DNA (21). The other hypothesis proposes that 4-hydroxycyclophosphamide is released from the liver, is taken up by target tissues, and releases phosphoramide mustard intracellularly.

This second view does not rule out the existence of the first mechanism, but believes it plays a minor role in the cytotoxic action of cyclophosphamide (17).

In the present studies, further properties of the cyclophosphamide-resistant L1210 cells are described and are discussed in terms of their significance to the therapeutic form in which cyclophosphamide is presented to target tissues.

MATERIALS AND METHODS

Female C57BL × DBA/2F₁ (hereafter called BD2F₁) mice (Cumberland View Farms, Clinton, TN) weighing 20 to 25 g were housed in plastic cages with food and water continually available. The L1210 lines were maintained by i.p. passage at weekly intervals. The resistant line, which is traceable to the original resistant line isolated by DeWys (6), does not require selective pressure of cyclophosphamide challenge to maintain its resistance. In the chemotherapy trials, treatments began 24 hr following implant of 10⁶ tumor cells. All agents were dissolved in 0.9% NaCl solution (saline) and administered at 0.1 ml/10 g body weight. Solutions of phosphoramide mustard were kept at 0° and administered within a few min of preparation. The antitumor activity of each agent was assessed by comparing the median survival time of the treated group with that of a control group of tumor-bearing mice. The results are expressed as a percentage increase in life span in groups of 5 mice/point, excluding long-term survivors.

4-Hydroperoxy- and 4-methyl-4-hydroperoxycyclophosphamide were prepared by ozonation of cyclophosphamide and 4-methylcyclophosphamide in the presence of hydrogen peroxide (14). 4-Hydroperoxycyclophosphamide was isolated as the crystalline solid (m.p. 108–110°). 4-Methyl-4-hydroperoxycyclophosphamide was isolated from the ozonation mixture by HPLC[2] on silica (Waters Associates Radial-pak in 60% chloroform:40% ethyl acetate) and its concentration determined by measuring its alkylating activity in a nitrobenzylpyridine assay (9) calibrated with 4-hydroperoxycyclophosphamide. The 4-methyl-4-hydroperoxycyclophosphamide was identified by mass-spectrometric identification of the cyanohydrin. Two mg of the hydroperoxide, dissolved in 0.5 ml of 0.1 M glycine buffer, pH 9.0, were treated sequentially with 50 mg of sodium bisulfite and 50 mg of sodium cyanide (each dissolved in separate 100-μl volumes of glycine buffer). After standing 15 min at room temperature (22°), the mixture was extracted with 2 ml of ethyl acetate. The organic phase was evaporated to dryness under a stream of nitrogen and the residue prepared for mass spectrometry by dissolving it in a small volume of chloroform; 4-hydroxycyclophosphamide was prepared by the reduction of 4-hydroperoxycyclophosphamide with sodium thiosulfate at 0° (22). Tritiated 4-hydroperoxycyclophosphamide (5 mCi/mmol) was prepared from [*chloroethyl*-³H]cyclophosphamide (Amersham International, Amersham, United Kingdom) in a microoozonation reaction in the presence of hydrogen peroxide (14). The 4-hydroperoxycyclophosphamide was isolated from the reaction mixture by preparative HPLC on silica (Supelcosil 5μ; Supelco, Inc., Bellefonte, PA) with a solvent composed of methylene chloride:ethyl acetate:methanol:water, 50:49.4:0.5:0.1, and detection by a refractive index detector (Waters Associates, Milford, MA). Analytical HPLC indicated that the radioactive hydroperoxide preparations contained 3 to 5% 4-ketocyclophosphamide.

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[2] The abbreviation used is: HPLC, high-pressure liquid chromatography.

Tissue culture studies were performed with L1210 cells originally derived from the tumors growing in mice. The cells were grown in Roswell Park Memorial Institute Medium 1640 supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, penicillin, and streptomycin. Both cell lines had doubling times of approximately 12 hr and are lethal to BD2F₁ mice when reintroduced i.p. *In vitro* cell survival studies used the method of Chu and Fischer (3).

Aldehyde dehydrogenase activity was determined by measurement of substrate-dependent reduction of NAD. Soluble extracts of liver and L1210 cells were produced by centrifugation (105,000 × g; 60 min; 4°) of homogenate or cells exposed to 3 cycles of freezing and thawing in 0.1 M potassium phosphate buffer, pH 7.4. The enzyme activity was measured immediately following preparation of the extracts. The assay contained 0.1 M potassium phosphate buffer (pH 7.4), 100 μM NAD, 1 mM aldehyde substrate, and approximately 100 μg of protein per ml. When mouse liver was the source of aldehyde dehydrogenase, pyrazole (100 μM) was added to inhibit alcohol dehydrogenase activity. NADH was measured by spectrofluorimetry (excitation, 350 nm; emission, 460 nm).

For studies of the metabolism of 4-hydroxycyclophosphamide, enzymically active extracts of cyclophosphamide-sensitive and -resistant L1210 cells were obtained by centrifuging (105,000 × g; 60 min, 4°) a suspension of cells (5 × 10⁷/ml) subjected to 3 cycles of freezing and thawing in 0.1 M potassium phosphate buffer, pH 7.4, 2 mM EDTA, and 1 mM 2-mercaptoethanol. The complete enzyme system, in a final volume of 100 μl, contained [³H]-4-hydroxycyclophosphamide (50 μM; 5 mCi/mmol), potassium phosphate buffer (0.1 M, pH 7.4), EDTA (2 mM), 2-mercaptoethanol (1 mM), NAD (0.1 mM), and cell extract (approximately 50 μg of protein). Following incubation at 37°, the mixture was cooled to 0°, and protein precipitated by the addition of zinc sulfate followed by barium hydroxide (20). The clear, neutral supernatant was injected directly onto the chromatograph.

The metabolites of 4-hydroxycyclophosphamide were separated by reverse-phase HPLC (Radial-pak C₁₈; Waters Associates) with a 40-min gradient of 10 to 40% methanol in 10 mM potassium phosphate buffer, pH 6.1, and 1.25 mM tetrabutylammonium sulfate at a flow rate of 1 ml/min; 1-ml fractions were collected and radioactivity measured by liquid scintillation spectometry. Metabolites were identified by cochromatography of authentic standards detected by their absorbance at 209 nm. Material which cochromatographed with authentic carboxyphosphamide (the gift of Dr. Arthur Myles, Collaborative Research, Inc., Lexington, MA) was additionally characterized by mass spectrometry of its methyl ester obtained by reacting the residue of chromatographic fractions, following solvent evaporation, with diazomethane in diethyl ether.

Mass-spectral anaysis was performed with a DuPont DP-102 GC/MS using chemical ionization with isobutane as the reagent gas; cyanohydrin derivative of 4-methyl-4-hydroxycyclophosphamide: m/z 291 (2Cl, [M + 1]⁺ minus hydrocyanic acid, relative abundance, 54); m/z 300 (2Cl, [M + 1]⁺ minus H₂O, relative abundance, 100); and m/z 318 (2Cl, [M + 1]⁺, relative abundance, 48); methyl ester of material cochromatographing with authentic carboxyphosphamide m/z 307 (2Cl, [M + 1]⁺, relative abundance, 100).

The measurement of DNA cross-linking in L1210 cells exposed to various alkylating agents was based on the alkaline elution assay (15). The method has been described previously (1).

RESULTS

The results of experiments to compare the antitumor effect of cyclophosphamide, 4-methylcyclophosphamide, and phosphoramide mustard on cyclophosphamide-sensitive and cyclophosphamide-resistant L1210 are summarized in Table 1. The choice of dosages used was based upon DNA cross-linking results in the sensitive tumor *in vivo* (data not shown) where each agent, at the given dose, produced approximately equal degrees of DNA cross-linking at 4 hr after administration. The present data

in Table 1 clearly indicate that the cyclophosphamide-sensitive cells are sensitive to all 3 agents since the life span is approximately doubled by each treatment. The cyclophosphamide-resistant tumor, while not responding to cyclophosphamide treatment, is as sensitive to 4-methylcyclophosphamide and phosphoramide mustard as are the sensitive L1210 cells.

In vitro studies were carried out in cyclophosphamide-sensitive and -resistant cells which were originally derived from those tumors growing in BD2F₁ mice. Chart 1 shows survival data for the sensitive and resistant lines in agar following exposure to either 4-hydroxycyclophosphamide or phosphoramide mustard. The cyclophosphamide-resistant cells are virtually unaffected by those exposures to 4-hydroxycyclophosphamide which are very toxic to the cyclophosphamide-sensitive cells. Thus, following a 30-min exposure to 50 μM 4-hydroxycyclophosphamide, the viability of the resistant cells is completely unaffected, while that of the sensitive cells is reduced 1000-fold. In contrast, the 2 cell lines are equisensitive to phosphoramide mustard. Except at very high concentrations of 4-hydroxycyclophosphamide (>150 μM), where toxicity unrelated to DNA cross-linking appears to

Table 1
Survival of tumor-bearing mice following treatment with cyclophosphamide, 4-methylcyclophosphamide, and phosphoramide mustard

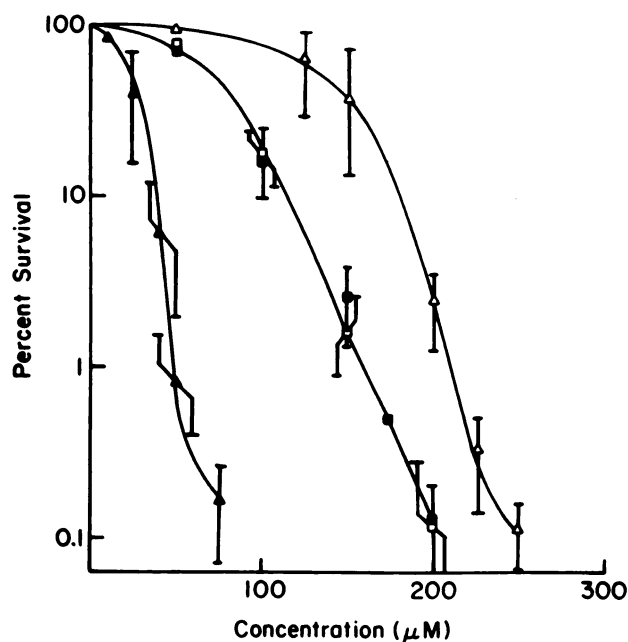| Treatment	Dose (mg/kg)	Tumor	% of increased life span
Cyclophosphamide	50	Sensitive	113
		Resistant	−10
4-Methylcyclophosphamide	80	Sensitive	107
		Resistant	90
Phosphoramide mustard	120	Sensitive	65
		Resistant	73

Chart 1. Survival of cyclophosphamide-sensitive and -resistant L1210 cells following exposure to 4-hydroxycyclophosphamide (△, ▲) and phosphoramide mustard (□, ■) for 30 min at 37°. The washed cells were plated in semisolid agar according to the method of Chu and Fischer (3). △, □, data from the resistant cell line; ▲, ■, data from the sensitive cells. Mean values are derived from 3 to 5 survival experiments. *Bars*, S.E.

emerge, *in vitro* DNA cross-linking data (Chart 2) parallel the survival results in showing no difference in phosphoramide mustard-induced DNA cross-links between the 2 cell lines but a striking lack of DNA cross-linking in the cyclophosphamide-resistant cells exposed to 4-hydroxycyclophosphamide. In contrast, exposure of cells to the 4-hydroperoxy derivative of 4-methylcyclophosphamide revealed that extensive DNA cross-linking is produced in both the cyclophosphamide-sensitive and -resistant lines. The results depicted in Chart 2 represent data from a single experiment. Similar results have been obtained from 2 other experiments, each using a different preparation of 4-methyl-4-hydroperoxycyclophosphamide.

Measurement of aldehyde dehydrogenase activity (Table 2) in soluble extracts of cyclophosphamide-sensitive and -resistant cells demonstrates the almost complete absence of activity in sensitive cells and a very high enzyme activity in extracts of cyclophosphamide-resistant cells. On a per-mg-protein basis, the activity in resistant cells is considerably higher than present in a soluble extract of mouse liver. Closely similar rates of NADH production were observed with either acetaldehyde, propionaldehyde, or 4-hydroxycyclophosphamide as substrates. Preliminary studies (12) indicate that the apparent K_m for 4-hydroxycyclophosphamide oxidation to carboxyphosphamide in extracts of cyclophosphamide-resistant cells is approximately 4 μM, a value consistent with the role of this activity in the prevention of phosphoramide mustard formation from activated cyclophosphamide.

The conversion of 4-hydroxycyclophosphamide to carboxyphosphamide by an extract of cyclophosphamide-resistant L1210 cells, and its dependence on added NAD, were followed by analysis of the components of reaction mixtures by HPLC using paired-ion chromatography. The substrate, [³H]-4-hydrox-

Chart 2. DNA cross-linking in cyclophosphamide-sensitive and -resistant L1210 cells following a 30-min exposure to 4-hydroxycyclophosphamide (△, ▲), 4-methyl-4-hydroperoxycyclophosphamide (○, ●), and phosphoramide mustard (□, ■). Treated cells were incubated for 3.5 hr in drug-free medium before cross-linking determination by alkaline elution. △, ○, □, resistant cells. ▲, ●, ■, sensitive cells.

Table 2
Aldehyde dehydrogenase activities

Extract	Activity[a] (nmol/min/mg protein)	No. of observations
Mouse liver	10.7, 12.3	
Cyclophosphamide-sensitive L1210	0.12 ± 0.01[b]	7
Cyclophosphamide-resistant L1210	24.3 ± 2.3	6

[a] Substrate was 1 mM acetaldehyde.
[b] Mean ± S.E.

ycyclophosphamide, was prepared by the reduction of [³H]-4-hydroperoxycyclophosphamide with sodium thiosulfate at 0°. Chromatography of the substrate revealed that 3% of the total radioactivity was not retained by the C_{18} column, and 5% appeared as 4-ketocyclophosphamide. The remaining radioactivity was concentrated in Fractions 24, 25, and 26 but with about 10% of the total being in Fractions 20 and 21 (Chart 3a). Both materials (Fractions 24 to 26, 20, and 21) appear to be related to 4-hydroxycyclophosphamide, since both react with semicarbazide and then chromatograph as the semicarbazone as a single chromatographic peak. The 2 materials [Fractions 24 to 26 (major) and 20 and 21 (minor)] are most likely *cis-* and *trans-*4-hydroxycyclophosphamide, respectively (2).

In the enzymic studies, chromatograms depicted in Chart 3a and c, indicate a mixture of compounds following 15-min incubation in the enzyme systems in the absence of NAD. An early runoff peak (Fractions 4 and 5) makes up about 3% of the total radioactivity, 4-ketocyclophosphamide (Fractions 14 and 15, designated "A") is present at about 10%, phosphoramide mustard (D) makes up 5 to 8%, and 4-hydroxycyclophosphamide (C) (Fractions 20 to 26) makes up the remainder except for an unidentified component (Fractions 29 to 31) which consistently accounts for about 5% of the total radioactivity. While there is no change in the composition of the sensitive enzyme reaction following incubation at 37° in the presence of NAD (Chart 3, a and b), the addition of NAD to the enzyme system derived from cyclophosphamide-resistant cells results in a mixture of products in which carboxyphosphamide predominates (Chart 3, c and d). The appearance of carboxyphosphamide is accompanied by a concomitant loss of 4-hydroxycyclophosphamide and a failure to produce phosphoramide mustard.

DISCUSSION

Cellular resistance to alkylating agents can be envisioned to result from changes in a number of the biochemical steps which lie between the initial entry of the agent into a cell and the ultimate death of the cell. Changes in the transport of agents into cells, their stability within the cell, and the repair of DNA damage have been proposed to account for decreased sensitivity of cell lines to alkylating agents. The results of the present studies can answer 2 important questions. (a) What is the form in which cyclophosphamide successfully interacts with tumor cells to exert its therapeutic properties? (b) What is the molecular nature of the resistance in cyclophosphamide-resistant L1210 cells?

The cyclophosphamide-resistant L1210 cells used in this study may be looked upon as a marker tissue with unique drug sensitivity whose viability and accumulation of DNA cross-links indicates which cyclophosphamide metabolite is transported to peripheral tissue to exert a therapeutic antitumor effect. From
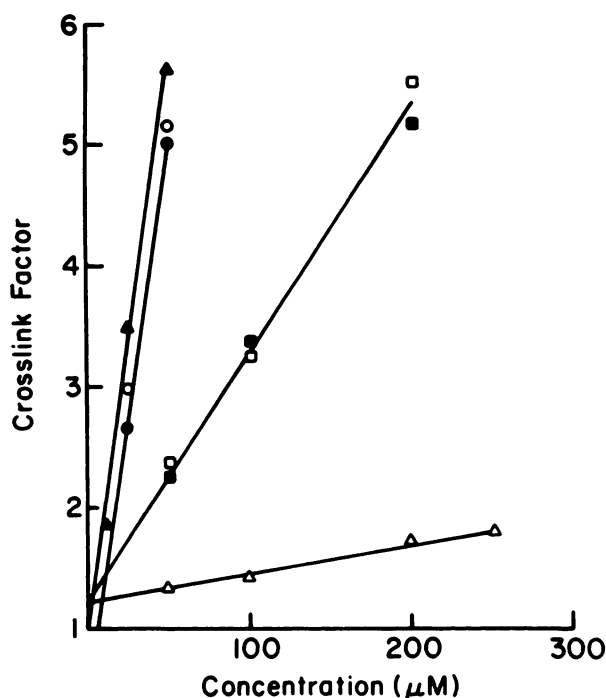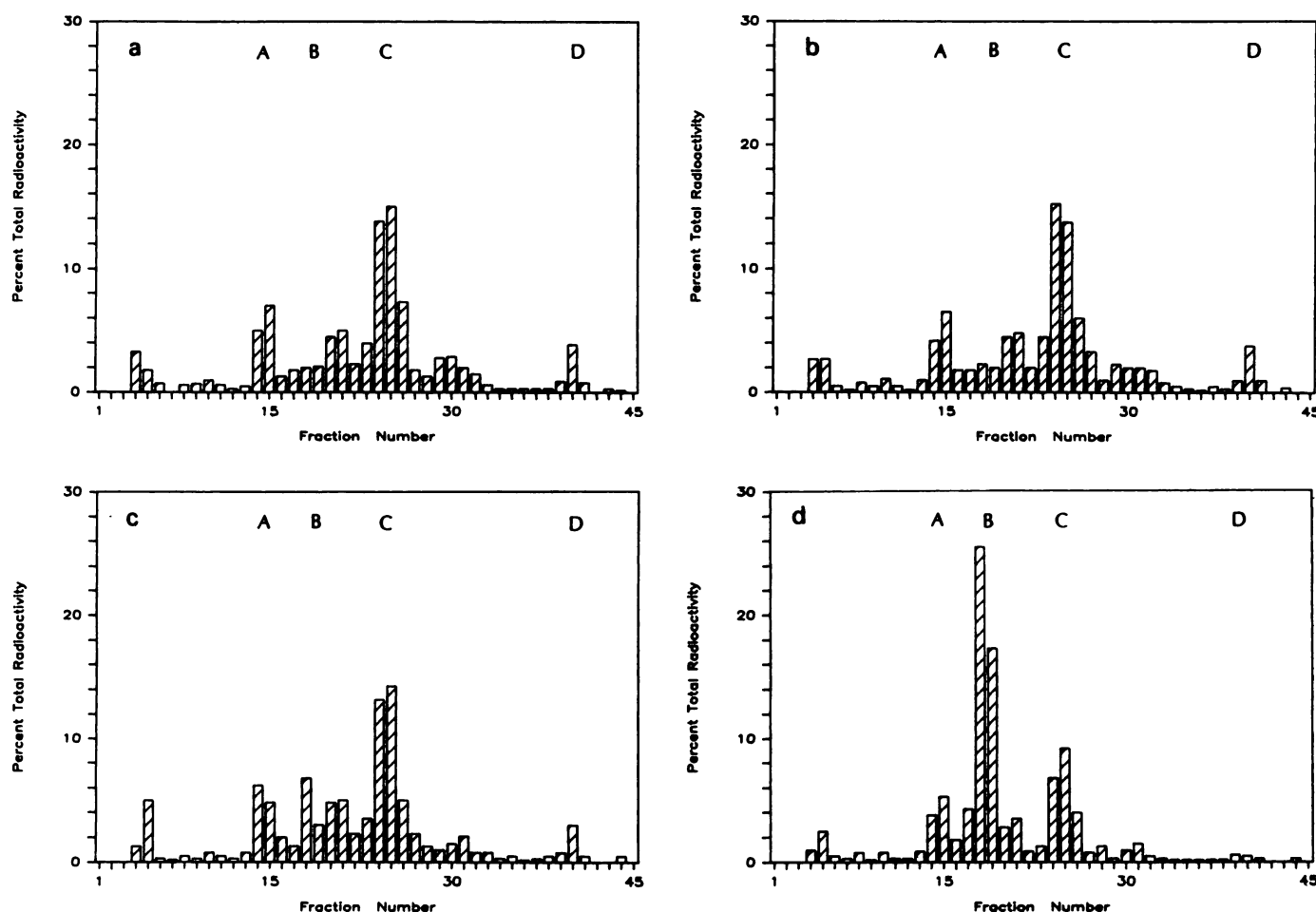
Chart 3. HPLC of the products of incubating [³H]-4-hydroxycyclophosphamide with soluble extracts of cyclophosphamide-sensitive or -resistant L1210 cells in the presence or absence of added NAD. *a*, extract of sensitive cells without added NAD; *b*, extract of sensitive cells with added NAD; *c*, extract of resistant cells without added NAD; *d*, extract of resistant cells with added NAD. Details are in "Materials and Methods," and components are identified in "Results."

the combination of *in vivo* and *in vitro* studies presented here, it is clear that 4-hydroxycyclophosphamide, and not phosphoramide mustard, is the cytotoxic form in which cyclophosphamide is presented to cyclophosphamide-sensitive and cyclophosphamide-resistant L1210 cells in mice. Since the cyclophosphamide-resistant cells are sensitive to phosphoramide mustard but cannot be eradicated by cyclophosphamide, it follows that phosphoramide mustard is not a significant entity in transporting the cytotoxicity of cyclophosphamide from its site of activation in the liver to L1210 cells in circulation and in peripheral tissues.

The proposed mode of cyclophosphamide resistance, an increased aldehyde dehydrogenase activity, is based upon: (a) our earlier demonstraton of the reversal of cyclophosphamide resistance *in vitro* by pretreatment of cells with a low disulfiram concentration (11); (b) the very high levels of aldehyde dehydrogenase within resistant cells; and (c) the lack of resistance to 4-methylcyclophosphamide. The sensitivity of both cell lines to 4-methylcyclophosphamide *in vivo* and their equal accumulation of DNA cross-links following incubation with 4-methyl-4-hydroperoxycyclophosphamide may be explained by the fact that, whereas 4-hydroxycyclophosphamide passes through an aldehyde intermediate and is thus susceptible to detoxification in cyclophosphamide-resistant cells, 4-methyl-4-hydroperoxycyclophosphamide ring opens to form a methyl ketone intermediate which is not a substrate for aldehyde dehydrogenase and is thus

not diverted to an inactive metabolite and can go on to produce phosphoramide mustard irrespective of the prevailing aldehyde dehydrogenase activity.

4-Hydroperoxycyclophosphamide appears to enter L1210 cells by active transport (18) and is probably quickly converted to 4-hydroxycyclophosphamide by glutathione peroxidase which is present in equal activities in both cyclophosphamide-sensitive and -resistant L1210 cells.³ The level of glutathione peroxidase within cells may prove to be a significant parameter in the use of 4-hydroperoxycyclophosphamide in *in vitro* situations such as pretreatment of leukemic marrow prior to autologous transplant (16).

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