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EVIDENCE FOR A MEMBRANE SULFHYDRYL ASSOCIATED WITH RESISTANCE TO MELPHALAN IN A MURINE L1210 LEUKEMIA LINE

J. M. Fisher, A. S. Naujokaitis[1], Y. Uehara[2] and M. Rabinovitz

Laboratory of Medicinal Chemistry and Pharmacology, DTP, DCT,
National Cancer Institute, Bethesda, MD 20205

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SUMMARY: Murine L1210 leukemia cells, which display sensitivity or resistance to the chemotherapeutic alkylating agent, melphalan, are equivalently sensitive or resistant to the poorly permeable mercurial, p-chloromercuribenzenesulfonate. Cells of both lines do not differ in sensitivity to the sulfhydryl reagents, N-ethylmaleimide, iodoacetamide and iodoacetate or to the glutathione transferase substrates 1-chloro-2,4-dinitrobenzene and p-nitrobenzyl chloride. The results are interpreted in terms of the known biological reactivity of p-chloromercuribenzenesulfonate as a selective reagent for sulfhydryl groups of membrane proteins associated with monovalent cation permeability.

INTRODUCTION: The appearance of tumor cells resistant to a chemotherapeutic agent is a frequent outcome of treatment of a tumor-bearing host with a sub-optimal dose regimen. Such a line of murine L1210 leukemia cells resistant to melphalan[3] was developed at the Southern Research Institute [1]. This line of resistant cells has about twice the level of reduced glutathione [2], the principal free sulfhydryl component of cells. Indeed, there have been recurrent reports of increased levels in the free sulfhydryl content of tumor cells resistant to alkylating agents [3-7]. In view of the apparent critical role of cellular sulfhydryl groups in conferring resistance, we have compared the sensitivity of melphalan-sensitive and -resistant cells to inhibition by sulfhydryl reagents and substrates of glutathione transferase. The results indicate that melphalan resistance is associated with a membrane sulfhydryl group of limited accessibility to reaction with the poorly permeable mercurial, p-chloromercuribenzenesulfonate (PCMBS).

[1] Present address: Department of Medicine, Georgetown University Hospital, Washington, D.C. 20007
[2] Present address: Institute of Microbial Chemistry, 14-23, Kamiosaki 3-Chome, Shinagawa-ku, Tokyo 141, Japan
[3] Melphalan, L-phenylalanine mustard, L-PAM, p-di(2-chloroethyl)amino-L-phenylalanine

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MATERIALS AND METHODS:

Materials: RPMI 1630 medium and Dulbecco's phosphate buffered saline were obtained from the NIH Media Unit. Fetal bovine serum was purchased from Flow Laboratories, McLean, VA and gentamicin sulfate (Schering, 50 mg/ml) from MA Bioproducts, Walkersville, MD. Growth medium for primary culture contained 16.5% heat inactivated fetal bovine serum in RPMI 1630 medium together with gentamicin sulfate, 40 ug/ml, and mercaptoethanol, 50 um. Both bovine serum albumin, fraction V and its sulfhydryl modified derivative [8] were from Miles Laboratories, Elkhart, IN. Cell counts were determined by use of a Coulter counter, model ZBI with sizing by Coulter model C 1000 channelyzer. Beads of known diameter were also obtained from Coulter Electronics, Hialea, FL. Melphalan was obtained from Burroughs Wellcome, Research Triangle Park, NC, and its stock solutions were prepared in 75% ethanol containing an equivalent amount of hydrochloric acid.

Maintenance and Primary Culture of Murine L1210 Leukemia Cell Lines: The melphalan-sensitive L1210 line was obtained under contract from Mason Research Institute, Boston, MA and maintained by weekly intraperitoneal injections, as free cell suspensions of $1x10^5$ cells into female DBA/2 mice, with cell line renewal every 10-14 weeks.

The melphalan-resistant L1210 line was maintained in female DBA/2 mice by weekly intraperitoneal injections of $1x10^6$ (day 0) followed by a single intraperitoneal injection of melphalan at 7.5 mg/kg on day 2 in order to sustain the resistance. Male CDF/1 mice were used for the final transplant of each cell line before primary culture. All other procedures remained the same. These mice were sacrificed on days 6 or 7 and cells were removed by flushing with 6-10 ml of growth medium (less the inhibitory reagent) to be used in the individual experiment. The cells were washed three times in this medium by gentle centrifugation and resuspension, adjusted to a density of $2.5x10^5$/ml and 7 ml of the cell suspension were exposed to the reagent indicated for at least 40 hours. Individual initial and final cell densities were determined to estimate the growth fraction, $(N/N_0)-1$, from which the percent inhibition of growth was calculated.

Short-Term Exposure of Cells to Reagents: Cells were removed from the mouse and washed in either Medium I (Dulbecco's phosphate buffered saline, with bovine albumin, fraction V, final concentration 0.7% and glucose, 2.5 mg/ml or in Medium II, as above, but with sulfhydryl modified bovine albumin to prevent interaction of sulfhydryl reagents with the mercaptalbumin. The reagents were added as serial dilutions to Corning 25 cm^2 flasks containing 2.5×10^5 cells/ml in a final volume of 10 ml incubation medium and maintained at 37°C (except where noted otherwise) for the indicated time. The cell suspensions were removed to centrifuge tubes, chilled 5 minutes in an ice bath and the cells washed 3 times in growth medium. Exactly 7 ml of cell suspension were placed in a Corning 25cm^2 flask and the cells were grown at 37°C for the periods described in individual experiments (about 40 hours).

RESULTS AND DISCUSSION: L-1210 Cells sensitive or resistant to melphalan in vivo display this sensitivity or resistance when incubated with it for brief periods and then grown in primary culture (Fig 1, left panel). An equivalent degree of sensitivity or resistance was also observed after a brief incubation of these cells with PCMBS (Fig. 1, right panel). Both cell types did not differ in sensitivity to brief incubations with other sulfhydryl reagents, N-ethylmaleimide or iodoacetamide or to longer incu-

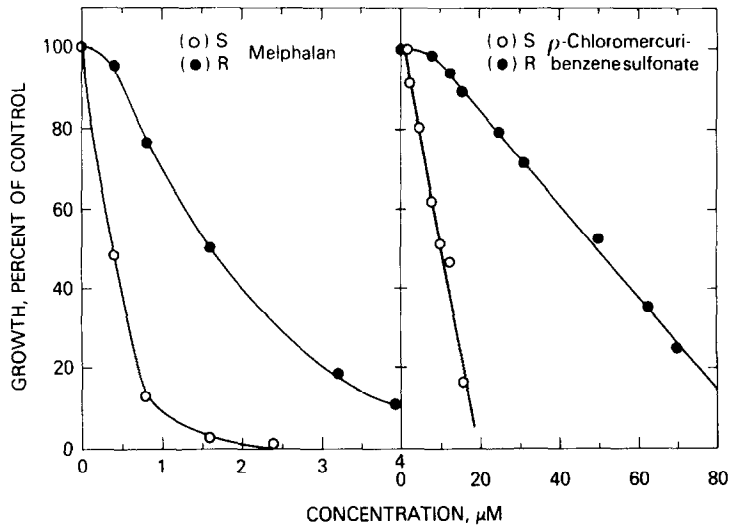

Fig. 1. Inhibition of Growth of Melphalan-Sensitive (S) and -Resistant (R) L1210 Cells by Melphalan (left panel) and p-Chloromercuribenzenesulfonate (right panel). Cells were incubated with the indicated compounds for 30 min. then washed three times and incubated for 40 hours as described in Methods and Materials under Short-Term Exposure of Cells to Reagents.

bation with iodoacetamide and iodoacetate (Table I). A slightly increased resistance is seen in the melphalan-resistant line to very brief (5 min.) incubations with N-ethylmaleimide. Both cell types were also equally sensitive to 1-chloro-2,4-dinitrobenzene and to p-nitrobenzyl chloride

TABLE I

Growth Inhibition of Melphalan-Sensitive and -Resistant Cells by Sulfhydryl Reagents.

Sulfhydryl Reagent	Exposure Time	ID_{50} Sensitive Cells μM	ID_{50} Resistant Cells μM
N-Ethylmaleimide	20 min	1.10	1.40
	5 min	1.60	2.60
	5 min (24°C)	1.10	1.70
Iodoacetamide	30 min	1.50	1.50
	42 hr	0.53	0.60
Iodoacetate	42 hr	7.80	7.50

Cells were incubated with several concentrations of the indicated sulfhydryl reagents under conditions as described in Materials and Methods. The concentration of each reagent resulting in 50 percent inhibition of growth was determined from its growth inhibition curve and is indicated as ID_{50}.

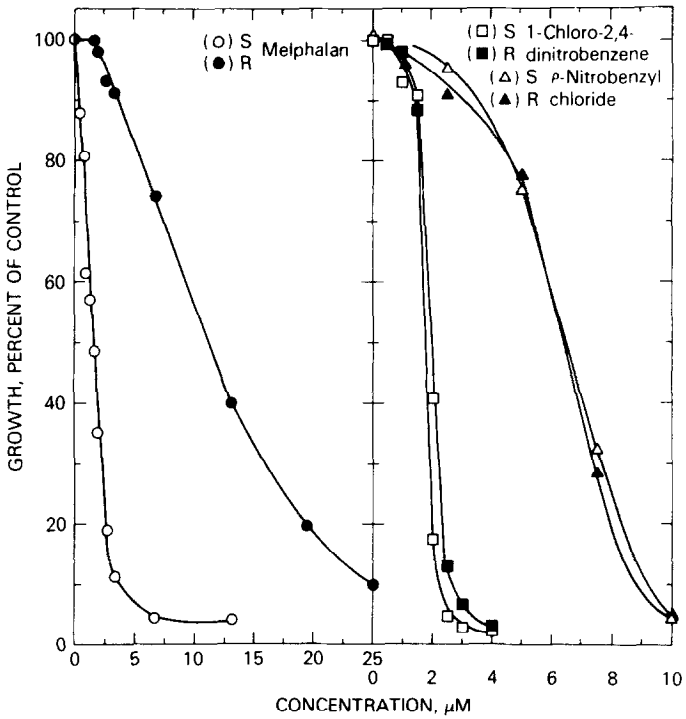

Fig. 2. Inhibition of Growth of Melphalan-Sensitive (S) and -Resistant (R) L1210 Cells by Melphalan and Substrates of Glutathione Transferase. Cells were incubated for 40 hours with the indicated concentrations of inhibitors in complete growth medium.

during prolonged exposure in growth medium (fig. 2, right panel). These compounds have been reported to be good substrates for one or the other of several isozymes of glutathione transferase [9]. The similar sensitivities of both cell types to reagents other than p-chloromercurbenzenesulfonate may be considered controls which serve to underscore the discriminatory character of the mercurial reagent. p-Chloromercuribenzenesulfonate (PCMBS) enters the erythrocyte membrane through a minor anionic channel [10] and reacts with sulfhydryl groups in its pathway, of which only a minority are associated with the mechanism for retaining passive control of sodium and potassium permeability [10-14]. These critical sulfhydryl groups are oxidized to disulfides during radiation [12,13], and both reactions result in sodium uptake, potassium release, swelling and lysis [10-14]. PCMBS causes similar membrane damage in Ehrlich ascites cells [15], murine L5178Y leukemia cells [16] and in our melphalan-sensitive and -resistant

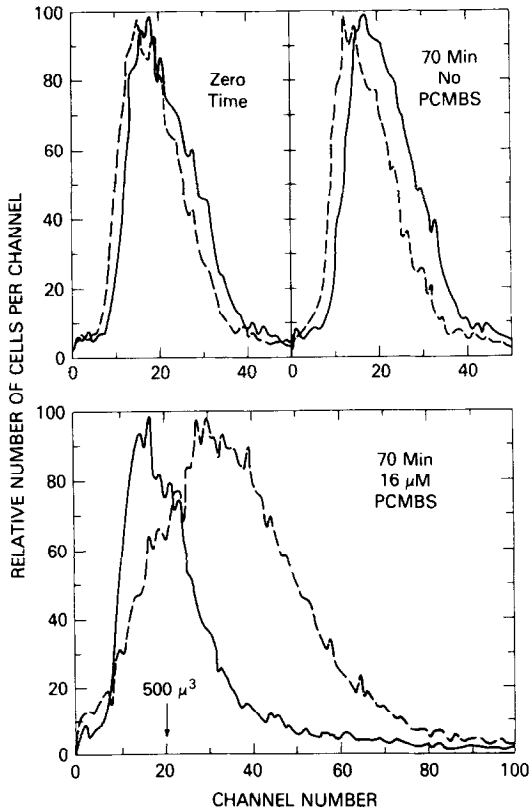

Fig. 3. The Plasma Membrane of Melphalan-Sensitive L1210 Cells is More Sensitive to Damage by p-Chloromercuribenzenesulfonate. The cells (---- Melphalan-Sensitive, —— Melphalan-Resistant) were incubated for 70 min. as described under Materials and Methods, Short-Term Exposure of Cells to Reagents, and then sized in a Coulter model C 1000 channelyzer. The increased cell volume of the sensitive cells indicates selective loss of permeability control.

L1210 lines. A selective effect on the melphalan-sensitive cells was observed at lower concentrations and earlier times (fig. 3).

The higher glutathione content of cells is associated with its increased transport to the exterior [17] via a pathway which could permit its interaction with either the incoming mercurial or with selective membrane sulfhydryl groups to form the mixed disulfide. It may be such a mixed disulfide that decreases the availability of selective membrane sulfhydryls to both melphalan and PCMBS. The reaction with sensitive sulfhydryl groups of proteins to form mixed disulfides has long been considered as a mechanism for the protection afforded by sulfhydryl compounds against radiation [18] and alkylating agents [19].

Repeated reports have indicated that a critical site for the cytotoxicity of alkylating agents may reside at the locus of the plasma membrane [20-22]. More recently, evidence was presented that nitrogen mustard inactivates membrane Na^+/K^+ ATPase [23], an enzyme also inhibited by PCMBS [24] and possibly having higher activities in tumor cells resistant to alkylating agents [25]. The differential reactivity of melphalan-sensitive and -resistant cells to a mercurial which reacts with sulfhydryl groups associated with monovalent ion movement indicates that this locus may be critical for the activity of melphalan.

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