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MEMBRANE TRANSPORT, SULFHYDRYL LEVELS AND DNA CROSS-LINKING IN CHINESE HAMSTER OVARY CELL MUTANTS SENSITIVE AND RESISTANT TO MELPHALAN*

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Abstract—The mechanism of resistance to the alkylating agent melphalan was investigated in drug-sensitive and -resistant mutants of Chinese hamster ovary cells. Melphalan-resistant cells (Mel^R6), selected by a single exposure to melphalan, were 4.5-fold more resistant to drug than sensitive AUXB1 parental cells. Colchicine-resistant cells (CH^RC5), which are cross-resistant to melphalan, were 15-fold more resistant than wild type cells. The kinetic parameters for drug influx were not significantly different in sensitive and resistant cells. The steady-state drug level in both Mel^R6 and CH^RC5 cells was approximately 25 and 35% lower respectively than that in sensitive cells and this difference was accounted for by a more rapid rate of drug efflux from the resistant mutants. However, the level of drug resistance could not be explained entirely by this difference in drug transport.

Sulfhydryl group levels were elevated in both Mel^R6 and CH^RC5 cells relative to sensitive cells and these differences were statistically significant ($P < 0.001$). Furthermore, DNA interstrand cross-link formation was significantly lower in resistant cells than in sensitive cells. A similar rate of repair of DNA interstrand cross-links was observed in sensitive and resistant cells with the possible exception of a slower rate of repair in Mel^R6 cells. A higher level of DNA–protein cross-link activity, which may represent a mechanism for drug inactivation was observed in Mel^R6 cells.

These studies suggest that resistance to melphalan in Mel^R6 and CH^RC5 Chinese hamster ovary cell mutants is multifactorial involving lowered steady-state drug levels, enhanced drug efflux, elevated levels of sulfhydryl groups and decreased DNA interstrand cross-linking.

At least three possible mechanisms of resistance to alkylating agents have been described: (a) reduced membrane permeability to the drug [1, 2], (b) increased cellular concentration of protective agents such as thiols that spare critical target sites from lethal injury by alkylation [3–5], and (c) the presence of enzyme(s) either to circumvent a specific metabolic block or to enhance the capacity for repair of alkylated DNA [6, 7]. Resistance to nitrogen mustard appeared to be multifactorial in that both reduced drug transport and elevated sulfhydryl levels were noted in resistant L5178Y lymphoblasts [1]; others have also reported that reduced drug permeability alone is unable to account for the observed level of resistance [8, 9].

Several recent reports have described reduced drug transport as a mechanism of resistance to melphalan [10–12]. In a study of melphalan transport in drug-sensitive and -resistant L1210 leukemia cells Redwood and Colvin [12] demonstrated both a lower initial velocity of drug uptake and reduced drug levels in the steady-state in resistant cells. Furthermore, the alteration in melphalan transport in resistant cells was postulated to represent a specific mutation of amino acid transport system L [12], one of the two amino acid transport systems known to mediate melphalan influx [13–15].

In a similar but preliminary report, a leucine transport mutant of Chinese hamster ovary (CHO) cells 100-fold more resistant to melphalan than the parent line was isolated, in which it was also suggested that drug resistance may be due to a specific mutant affecting transport system L [10].

Factors other than transport have also been considered in studies of the mechanism of resistance to melphalan. Elevated levels of glutathione have been described in melphalan-resistant L1210 leukemia cells compared to the sensitive parent [16, 17]; however no difference in the repair of DNA interstrand cross-links was detected [18].

Elliott and Ling [11] have isolated and characterized several CHO cell mutants resistant to melphalan. A colchicine-resistant clone (CH^RC5) was cross-resistant to melphalan and the resistant phenotype appeared to correlate quantitatively with the presence of a high mol. wt glycoprotein in the plasma membrane [19]. The mechanism of resistance in CH^RC5 cells was attributed to reduced drug accumulation due to a plasma membrane alteration [11]. Stably resistant clones of melphalan-resistant cells were also isolated by a single exposure of CHO cells to melphalan, in order to obtain a drug-resistant clone with a minimal number of phenotypic changes. The Mel^R clones differed from the membrane mutant of the CH^RC5 type in that drug accumulation in the nuclear fraction was reduced suggesting that the mechanism of resistance was due to a nuclear alteration.

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We have investigated the mechanism of resistance to melphalan in the CH^RC5 and Mel^R clones of CHO cell lines. Evidence is presented that resistance to melphalan is multifactorial involving reduced steady-state drug levels, an enhanced rate of melphalan efflux, elevated sulfhydryl levels and a reduced frequency of DNA interstrand cross-linking.

MATERIALS AND METHODS

Drugs and chemicals. [^{14}C]Melphalan, L-*p*-(di-2-chloroethylamino)[^{14}C]phenylalanine (sp. act. 8 mCi/mmole) or L-*p*-(di-2-chloro[^{14}C]-ethylamino) phenylalanine (sp. act. 12.35 mCi/mmole) were prepared by M. Leaffer of the Stanford Research Institute (Menlo Park, CA); the radiochemical purity was 97% as determined by TLC on silica gel in *n*-butyl alcohol:acetic acid:water (7:2:1) or *n*-butyl alcohol:acetonitrile:water (5:5:2) [kindly supplied by Dr Robert R. Engle (Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD)]. Unlabeled melphalan (Alkeran) was provided by Dr J. R. MacDougall (Burroughs Wellcome and Co. Ltd, Lachine, Canada). [^{14}C]Thymidine (sp. act. 50 mCi/mmole), [^{3}H]thymidine (sp. act. 50–80 Ci/mmole) and DL-β-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) were obtained from New England Nuclear Co. (Boston, MA) and 2-(methylamino)isobutyric acid (methyl-AIB) from Sigma Chemical Co. (St. Louis, MO).

Cell lines and culture conditions. Melphalan-sensitive CHO cells (AUXB1), melphalan-resistant CHO cells (Mel^R1 and Mel^R6) and colchicine-resistant (CH^RC5) cells, which are cross-resistant to melphalan, were obtained from Dr V. Ling (Ontario Cancer Institute, Toronto, Canada). Mel^R1 and Mel^R6 cells were selected by a single exposure to melphalan [11] and were 3.5- and 4.5-fold more resistant respectively than AUXB1 parental cells. Since the results obtained with Mel^R1 and Mel^R6 were nearly identical, only the results with Mel^R6 are reported. CH^RC5 cells were selected for colchicine resistance in three successive steps from AUXB1 [20] and were approximately 15-fold more resistant to melphalan than parent cells. The dose–survival curves of all four cell lines have been published previously by Elliott and Ling [11].

The cell lines were maintained in monolayer culture in 75-cm^2 tissue culture flasks in alpha minimum essential medium (MEM) with 10% fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, NY), penicillin (10,000 U/ml) and streptomycin (10,000 μg/ml). For transport studies cells were harvested at 10×10^6–15×10^6 cells per flask using 0.25% trypsin, washed and resuspended in Dulbecco's phosphate-buffered saline (PBS) at a concentration of 1×10^6–3×10^6 cells/ml.

Transport studies. Transport studies were performed as described previously [1, 13, 21] by addition of melphalan to drug-sensitive and -resistant CHO cells suspended in Dulbecco's PBS. Incubations were terminated by rapid chilling to 4° and centrifugation through a layer of 0.25 M sucrose in Hopkin's vaccine tubes to remove extracellular radioactivity. The washed cells were solubilized in 0.5 N NaOH, and radioactivity was determined by liquid scintillation spectrometry.

Cell size was measured in a Coulter Model Z_{BI} electronic particle counter (Coulter Electronics, Hialeah, FL) calibrated with paper mulberry spores (mean cell diameter 12.5 μm) which was obtained from Coulter Diagnostics Inc (Miami Springs, FL). The cell volume (mean ± S.E.) obtained for parental AUXB1 cells was 1411 ± 29 μ^3, that for resistant Mel^R6 cells was 1694 ± 30 μ^3, and that for CH^RC5 cells was 1479 ± 39 μ^3. Cell/medium drug distribution ratios were based on the radioactivity calculated per cell volume relative to that of an equivalent volume of extracellular medium.

Uptake was corrected for rapid binding to the cell membrane by measuring the cell/medium distribution ratio of radiolabelled substrate at 4° at uptake times of less than 15 sec as described previously [21–23]. The correction factor (mean ± S.E.) for [^{14}C]melphalan, which was 0.47 ± 0.05 for the drug-sensitive cells, 0.66 ± 0.07 for Mel^R6 cells and 0.59 ± 0.04 for CH^RC5 cells, was routinely subtracted from the observed cell/medium ratio and was also used to correct initial uptake velocity data.

The kinetic parameters were derived from linear regression equations of Lineweaver–Burk plots [23, 24] and biphasic curves were corrected for two-component interaction by the method of Neal [23, 25]. Efflux studies were performed on cells suspended in Dulbecco's PBS as described previously [26].

Determination of sulfhydryl levels. Sulfhydryl levels were determined by the method of Sedlak and Lindsay [27] which detects thiol groups available to react with the reagent 5,5′-dithiobis(2-nitrobenzoic acid).

Alkaline elution analysis. For DNA cross-linking and repair studies CHO cells were labeled with [^{14}C]thymidine and grown overnight in monolayer culture in 25-cm^2 tissue culture flasks covered with 5 ml of alpha MEM without nucleosides containing 10% dialyzed FBS and [^{14}C]thymidine (0.01–0.02 μCi/ml) at a concentration of 1 μM. The label was removed and the cells grown for an additional 24 hr in complete alpha medium with 10% serum.

For DNA cross-linking studies, labeled cells were incubated for 1 hr at 37° in a monolayer in 25-cm^2 flasks with complete alpha medium and 10% FBS containing unlabeled melphalan. Cells were washed twice with cold Dulbecco's PBS, 3 times with cold calcium- and magnesium-free Hank's balanced salt solution containing 0.02% EDTA and gently scraped off the flask surface with a rubber policeman. Cells were irradiated on ice with 600 rads with a radioactive cobalt-60 source at a dose rate of 104 rads/min.

Drug-treated CHO cells were combined with L5178Y lymphoblasts that had been labeled overnight with 1 μM [^{3}H]thymidine (0.05–0.1 μCi/ml) in Fischer's medium with 10% dialyzed FBS, washed and irradiated on ice with 150 rads as earlier. ^{14}C-labeled CHO cells (2×10^5–3×10^5) and ^{3}H-labeled L5178Y cells (2×10^5–3×10^5) were suspended in a total volume of 15 ml and analyzed for cross-linking by a modification of the alkaline elution assay described by Kohn [2, 28].

For total cross-linking, cells were placed on 2-μm

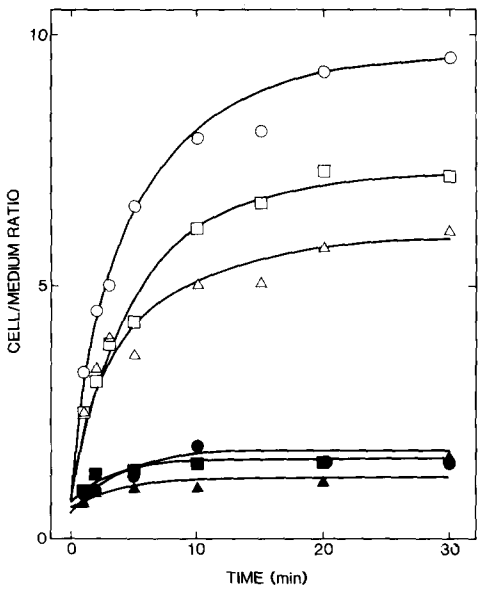

Fig. 1. Time course of the uptake of 100 μM [^{14}C]melphalan by sensitive and resistant CHO cells. Uptake is expressed as the cell/medium distribution ratio as described in the text. Uptake at 37° by AUXB1 (○), Mel^{R}6 (□) and CH^{R}C5 (△) cells, and at 4° by AUXB1 (●), Mel^{R}6 (■) and CH^{R}C5 (▲) cells.

polyvinylchloride filters (Millipore Corp., Bedford, MA) fitted on a polyethylene filter holder [Swinnex (Millipore Corp.)] modified to hold a volume of at least 20 ml [2, 28, 29]. Cells were washed with PBS, lysed with 5 ml of a solution of 2% sodium dodecyl sulfate (SDS), 0.02 M EDTA and 0.1 M glycine, at pH 10.0, washed with 5 ml of 0.02 M EDTA and eluted with a tetrapropylammonium hydroxide solution (pH 12.1–12.2) delivered to the filter by a peristaltic pump at a flow rate of approximately 35 μl/min. Fractions were collected at 90-min intervals, sampled and counted by liquid scintillation spectrometry.

For DNA interstrand cross-linking 0.8-μm polycarbonate filters (Nucleopore Corp., Pleasanton, CA) were used. After washing cells on the filter with PBS the lysing solution containing 0.5 mg/ml proteinase K (Boehringer-Mannheim, Dorval, Canada) was allowed to remain on the filter for 1 hr; the elution assay was continued as described earlier.

For DNA cross-linking repair studies, the drug was removed, the cells resuspended in full medium at 37° and cross-linking frequency determined at various time intervals.

The level of cross-linking, both total and DNA interstrand, was calculated from the elution profile as described by Kohn [28, 30].

RESULTS

Time courses of melphalan uptake by sensitive and resistant CHO cells

Time courses of uptake of 100 μM [^{14}C]melphalan by drug-sensitive AUXB1 cells and by drug-resistant Mel^{R}6 and CH^{R}C5 cells are shown in Fig. 1. Following an initial rapid component, which presumably represents rapid binding or association to the cell membrane [21–23], uptake of melphalan at 37° was linear for approximately 2 min, thereafter reaching steady-state levels by 20–30 min. The maximum cell/medium distribution ratio was approximately 10-fold for sensitive cells, 7-fold for Mel^{R}6 cells and 6-fold for CH^{R}C5 cells. The cell/medium distribution ratio at the steady-state in cells treated with 1 μM [^{14}C]melphalan was also lower in both resistant cell lines relative to the sensitive parent and these differences were statistically significant ($P < 0.01$). For all three cell lines drug uptake at 4° was markedly reduced reaching a plateau level of only one- to two-fold, indicating that melphalan uptake was strongly temperature-sensitive. To approximate initial uptake velocity conditions subsequent kinetic studies were terminated at 1 min.

Kinetic analysis of melphalan influx

Kinetic analysis of melphalan influx by sensitive and resistant CHO cells over a concentration range of 0.5–100 μM resulted in biphasic Lineweaver–Burk plots for all three cell lines, suggesting the involvement of two carrier-mediated transport systems. The kinetic parameters derived by linear regression analysis of each portion of the biphasic

Table 1. Kinetic parameters for melphalan influx in drug-sensitive and -resistant CHO cells*

Cell line	Low-affinity system		High-affinity system	
	K_m (μM)	V_{max} (amoles/cell/min)	K_m (μM)	V_{max} (amoles/cell/min)
AUXB1	49 ± 24†	209 ± 90	1 ± 1	7 ± 2
Mel^{R}6	23 ± 5	127 ± 22	1 ± 1	10 ± 3
CH^{R}C5	70 ± 19	386 ± 94	1 ± 1	3 ± 1

* AUXB1, Mel^{R}6 or CH^{R}C5 cells were incubated at 37° for 1 min with [^{14}C]melphalan at concentrations of 0.5–100 μM. The kinetic parameters were derived by linear regression analysis of Lineweaver–Burk plots; V_{max} was derived from the reciprocal of the y-intercept and K_m from the product of slope × V_{max}. The parameters were corrected for two-component interaction by the method of Neal [25] and represent the means ± S.E. of five to six determinations.

† The data were analyzed statistically by a two-tailed t-test comparing the significance of the difference of the means for the K_m and V_{max} of the sensitive and resistant cell lines. None of the kinetic parameters of the resistant cells was significantly different than those of the sensitive cells.

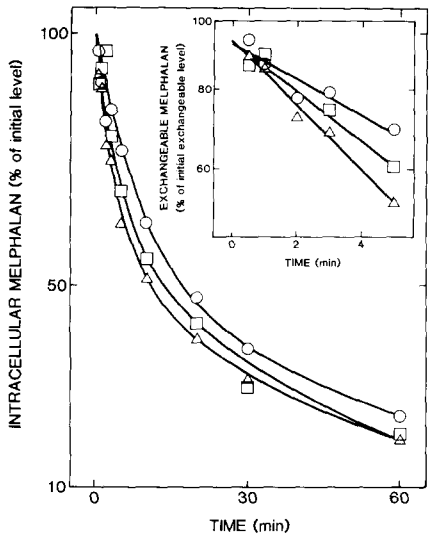

Fig. 2. Efflux of [^{14}C]melphalan from AUXB1 (○), Mel^{R}6 (□) and CH^{R}C5 (△) CHO cells. Cells were preincubated with 10 μM [^{14}C]melphalan for 10 min, then resuspended in transport medium at 37°. The intracellular drug concentration was determined in cell aliquots removed at the times indicated and the data are expressed as melphalan concentration as a per cent of the initial intracellular drug concentration plotted against the efflux time. The experimental points represent the mean of two to four determinations. Inset: A decay time analysis of the same data over the first 5 min of efflux is presented as a semilogarithmic plot of exchangeable intracellular melphalan concentration (expressed as a per cent of the initial exchangeable intracellular drug level) plotted against the efflux time. The exchangeable intracellular drug concentration was obtained by subtracting non-exchangeable drug from total intracellular melphalan as described previously [26].

Lineweaver–Burk plot and corrected for two-component interaction by the method of Neal [23, 25] are shown in Table 1. In the high-affinity system, the K_m, which is a measure of the binding affinity of the carrier for the drug, and the V_{max}, which is dependent on the number and/or mobility of carriers, were very similar in sensitive and resistant cells. Although some differences in the kinetic parameters were noted for the low-affinity system these were not statistically significant.

Table 2. Kinetic parameters for melphalan efflux from drug-sensitive and -resistant CHO cells*

Cell line	K (min^{-1}) (mean ± S.E.)	$t_{\frac{1}{2}}$ (min)	P†
AUXB1	0.028 ± 0.006	24.7	
Mel^{R}6	0.038 ± 0.005	18.1	NS‡
CH^{R}C5	0.051 ± 0.003	13.7	< 0.02

* AUXB1, Mel^{R}6 or CH^{R}C5 cells were incubated for 10 min at 37° with 10 μM [^{14}C]melphalan, washed, resuspended in transport medium and incubated at 37° for 60 min. Cell aliquots were removed at timed invervals and the intracellular drug content determined. A semilogarithmic plot of the time course of exchangeable drug remaining within the cell yields a linear plot from which the first-order efflux rate constant (K) may be derived from the negative slope of the regression line. The half-time ($t_{\frac{1}{2}}$) for efflux is calculated from the equation $t_{\frac{1}{2}} = \log_e 2/K$ as described previously [26].

† The data were analyzed statistically by a two-tailed t-test comparing the significance of the difference of slopes of the linear regression lines for melphalan efflux from drug-sensitive and -resistant cells.

‡ NS, not significant.

Chemical specificity of melphalan transport

Since melphalan is actively transported by two amino acid carrier systems in a variety of cells [13–15, 31, 32] the chemical specificity of melphalan influx was investigated in drug-sensitive AUXB1 and drug-resistant Mel^{R}6 cells. Uptake of melphalan was significantly inhibited by BCH, a specific inhibitor of system L, [33] and by sodium depletion. However, methyl-AIB, a specific antagonist of amino acid transport system A [34, 35], did not affect drug influx.

The relative contribution of the low- and high-affinity systems to melphalan influx in AUXB1 and Mel^{R}6 cells was determined by methods described previously [13, 14, 31]. At a concentration of 1 μM, melphalan transport was mediated 65–70% by the low-affinity system and 20–25% by the high-affinity system, whereas at 100 μM influx by the low-affinity system increased to 75–80% and that by the high-affinity system decreased to 10–15%.

Table 3. Relationship of melphalan transport to cytotoxicity in drug-sensitive and -resistant CHO cells*

Cell line	Extracellular melphalan concentration (μM)	Surviving cell fraction	Intracellular melphalan concentration (μM)
AUXB1	0.16	0.02	4.0 ± 0.1
Mel^{R}6	0.75	0.02	27.3 ± 0.6
CH^{R}C5	2.83	0.02	65.0 ± 3.3

* AUXB1, Mel^{R}6 or CH^{R}C5 cells were incubated at 37° for 1 hr in PBS containing 15 mM glucose and the extracellular concentration of [^{14}C]melphalan shown. Drug concentrations were used that produced an equivalent level of cell kill as determined from dose–survival curves published previously by Elliott and Ling [11], and the experimental conditions were identical to those used by Elliott and Ling [11]. The intracellular drug concentrations obtained represent the means ± S.E. of five to six determinations.

Table 4. Determination of sulfhydryl groups in melphalan-sensitive and -resistant CHO cells*

Cell line	Concentration of sulfhydryl groups (fmoles/cell)		
	Total	Non-protein	Protein-bound
AUXB1	23.0 ± 0.5	5.4 ± 0.3	17.7 ± 0.4
Mel^R6	34.9 ± 1.4†	12.0 ± 0.8†	22.9 ± 0.7†
CH^RC5	27.9 ± 0.5‡§	6.2 ± 0.3§‖	21.6 ± 0.6‡¶

* Total sulfhydryl group content was determined by treating aliquots of AUXB1, Mel^R6 and CH^RC5 cells with Ellman's reagent [27]. Non-protein sulfhydryl levels were measured on a TCA-soluble extract of each cell line. Protein-bound sulfhydryl levels were calculated by subtracting non-protein from total sulfhydryl levels. The data presented are the means ± S.E. of 6–12 determinations and were analyzed statistically by a two-tailed *t*-test comparing the significance of the difference of the means.

† Mel^R6 differed significantly from AUXB1 ($P < 0.001$).
‡ CH^RC5 differed significantly from AUXB1 ($P < 0.001$).
§ CH^RC5 differed significantly from Mel^R6 ($P < 0.001$).
‖ CH^RC5 was not significantly different than AUXB1.
¶ CH^RC5 was not significantly different than Mel^R6.

Efflux of melphalan from sensitive and resistant CHO cells

A time course of melphalan efflux from sensitive and resistant CHO cells at 37° is shown in Fig. 2. For all three cell lines efflux was linear for approximately 5 min, thereafter approaching a plateau level at 60 min with 25% of the initial radioactivity remaining in the AUXB1 cells and 20% remaining in the Mel^R6 and CH^RC5 cells.

A decay time analysis of melphalan efflux was performed over the first 5 min of efflux (Fig. 2 inset). The first-order efflux rate constant was greater and the $t_{\frac{1}{2}}$ shorter in resistant cells than in sensitive cells (Table 2), but these differences were only significant for CH^RC5 cells ($P < 0.02$).

Relationship of melphalan transport to cytotoxicity in drug-sensitive and -resistant CHO cells

The cytotoxic effect of melphalan derived from previously published dose–survival curves [11] was correlated with intracellular drug concentration in sensitive and resistant CHO cells (Table 3). Each cell line was treated with that concentration of melphalan which reduced the surviving cell fraction to 0.02 using drug levels that could be achieved clinically [36, 37]. Although the extracellular dose of drug used produced the same amount of cell kill, the intracellular melphalan concentration in the Mel^R6 and CH^RC5 cells was 7- and 16-fold greater respectively than that noted in the sensitive cells.

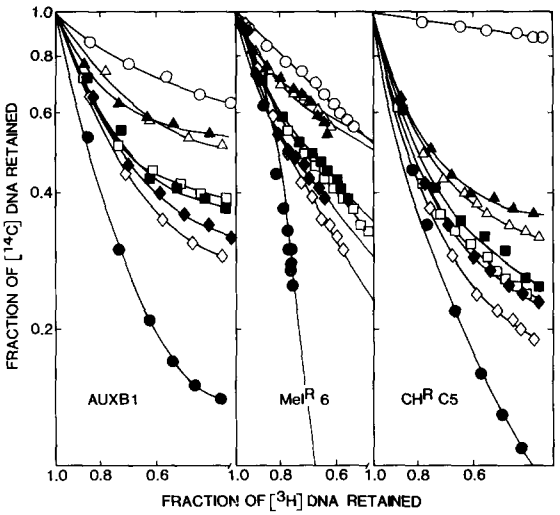

Fig. 3. Total DNA cross-linking in melphalan-sensitive and -resistant CHO cells treated with melphalan for 1 hr at 37° and assayed by alkaline elution after exposure of cells to 600 rads. Elution of [^{14}C]DNA from experimental cells is plotted against the simultaneous elution of [^{3}H]DNA from the internal standard cells as described in the text and previously [2, 28]. Cells were treated with melphalan at 0 μM (●), 20 μM (◇), 30 μM (◆), 40 μM (□), 60 μM (■), 80 μM (△) or 100 μM (▲). The control curve (○) consisted of cells not treated by drug or irradiation.

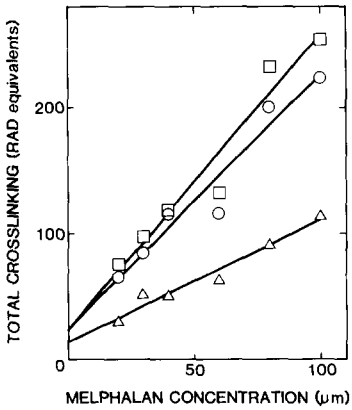

Fig. 4. Dose–response plot of total DNA cross-linking activity determined by alkaline elution in AUXB1 (○), Mel^R6 (□) and CH^RC5 (△) CHO cells treated with melphalan for 1 hr as described in the text and Fig. 3. The lines were determined by linear regression analysis. The linear regression equation for AUXB1 cells was $y = 2.05x + 27.25$ with a correlation coefficient of 0.99, that for Mel^R6 cells was $y = 2.33x + 23.03$ with a correlation coefficient of 0.97 and that for CH^RC5 cells was $y = 0.99x + 12.78$ with a correlation coefficient of 0.98.

Table 5. Properties of melphalan-sensitive and -resistant CHO cells*

Property measured	Mel^{R}6	CH^{R}C5
Colony survival†	4.5	15.0
Efflux rate constant†	1.4	1.8
Steady-state concentration‡	1.3	1.6
Total sulfhydryl groups†	1.5	1.2
DNA interstrand cross-linking‡	3.4	9.1

* The ratio of various properties of sensitive and resistant cells are presented. Colony survival is the ratio of D_{10} values [11]. Efflux rate constant is the ratio of the first-order efflux rate constants (*K*s) from Table 2. Steady-state concentration is the ratio of the cell/medium distribution ratio of drug at 30 min (Fig. 1). Total sulfhydryl groups is the ratio of total sulfhydryl levels (Table 4). DNA interstrand cross-linking is the ratio of slopes of dose–response curves for DNA interstrand cross-linking (Fig. 5).

† Resistant cells/sensitive cells.

‡ Sensitive cells/resistant cells.

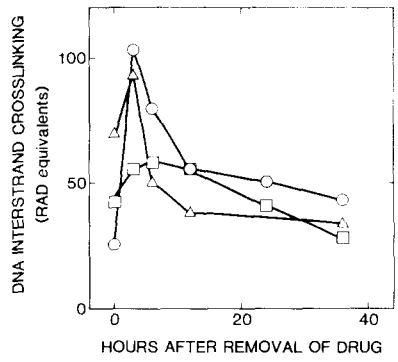

Fig. 6. Changes in DNA interstrand cross-linking following exposure of AUBX1 (○), Mel^{R}6 (□) and CH^{R}C5 (△) cells to 20 μM melphalan at 37° of 1 hr. DNA interstrand cross-linking measured by alkaline elution using proteinase K is plotted against time after removal of drug as described in the text.

Determination of sulfhydryl group levels in drug-sensitive and -resistant CHO cells

The concentration of total, protein-bound and non-protein sulfhydryl groups in melphalan-sensitive and -resistant cells was determined using the method of Sedlak and Lindsay [27]. Total and protein-bound thiol content was significantly higher in both resistant cell lines compared to the sensitive parental line (Table 4). However, non-protein sulfhydryl levels were significantly higher only in Mel^{R}6 cells.

DNA cross-linking in melphalan-sensitive and -resistant CHO cells

DNA cross-linking induced by melphalan was studied in drug-sensitive and -resistant cells using the alkaline elution procedure. Cells were incubated with various concentrations of melphalan at 37° for 1 hr. Some typical elution profiles for total cross-linking in AUXB1, Mel^{R}6 and CH^{R}C5 cells are shown in Fig. 3. Dose–response curves for total DNA cross-link activity in sensitive and resistant cells are presented in Fig. 4. Total cross-linking in AUXB1 and Mel^{R}6 cells was not significantly different; however a much lower level of cross-linking was observed in CH^{R}C5 cells and this difference was highly significant ($P < 0.001$).

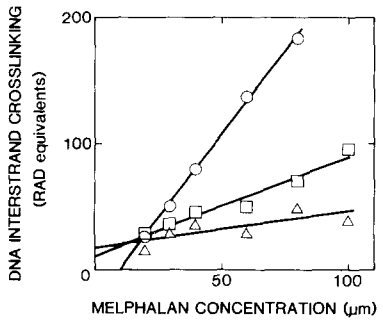

Fig. 5. Dose–response plot of DNA interstrand cross-linking measured by alkaline elution using proteinase K; AUXB1 (○), Mel^{R}6 (□) and CH^{R}C5 (△) cells were treated with melphalan for 1 hr as described in the text and Fig. 3. The lines were determined by linear regression analysis. The linear regression equation for AUXB1 cells was $y = 2.68x - 27.88$ with a correlation coefficient of 0.99, that for Mel^{R}6 cells was $y = 0.78x + 10.81$ with a correlation coefficient of 0.98, and that for CH^{R}C5 cells was $y = 0.29x + 16.34$ with a correlation coefficient of 0.80.

DNA interstrand cross-linking frequency was measured by exposing drug-treated cells to proteinase K, a procedure intended to eliminate DNA–protein cross-links (Fig. 5). The dose–response curves revealed that DNA cross-linking in both Mel^{R}6 and CH^{R}C5 cell lines was significantly lower than in melphalan-sensitive cells ($P < 0.001$). In one experiment, intracellular drug concentration was measured using [^{14}C]melphalan and these values were used in place of extracellular concentrations in a plot of DNA interstrand cross-linking; although this reduced the differences in the slopes somewhat, the differences between sensitive and resistant cells remained highly significant ($P < 0.001$).

DNA–protein cross-linking for the three cell lines was calculated by subtracting DNA interstrand cross-links. DNA–protein cross-linking was significantly higher in Mel^{R}6 resistant cells than in the other two cell lines ($P < 0.02$ or greater).

Repair of DNA interstrand cross-links in drug-sensitive and -resistant CHO cells

Repair of DNA interstrand cross-links in AUXB1, Mel^{R}6 and CH^{R}C5 cells was studied by incubating each cell line with 20 μM melphalan for 1 hr. Cells were washed to remove extracellular drug and then resuspended in fresh medium at 37° for 36 hr. Aliquots were removed at time intervals and DNA interstrand cross-links were determined by the alkaline elution method (Fig. 6). In all three cell lines there was an increase in cross-linking frequency with a peak level being reached at 3 hr for AUXB1 and CH^{R}C5 cells, and at 6 hr for Mel^{R}6 cells. Following this, cross-linking decreased in sensitive and resistant cells with little or no apparent difference in the pattern of DNA repair between AUXB1 and CH^{R}C5 cells but with possibly a slower rate of repair in Mel^{R}6 cells.

DISCUSSION

In this study we have compared the properties of AUXB1 cells, which are sensitive to melphalan, with those of two drug-resistant CHO cell mutants. The Mel^R6 resistant mutant, which was obtained by a single exposure of the parental sensitive line to melphalan, was approximately 4.5 times more resistant to melphalan than the parent line [11]. The CH^RC5 cell line was selected for colchicine resistance from AUXB1 cells by three successive treatments with colchicine; this line, which was cross-resistant to melphalan, was 15-fold more resistant to melphalan than the parent line [11, 20]. We have examined various properties of these cells in order to determine which mechanisms are involved in the development of resistance to melphalan.

Evidence was obtained that uptake of melphalan by AUXB1 and Mel^R6 cells was by an active carrier-mediated process involving two amino acid transport systems. Drug influx followed biphasic Michaelis–Menten kinetics and demonstrated chemical specificity qualitatively identical to that reported in several other tumor cell lines [13–15, 31, 32]. Evidence for active transport was that uptake proceeded 'uphill' against a concentration gradient and was strongly temperature-sensitive (Fig. 1).

Melphalan influx into resistant cells was similar to that in sensitive cells with no significant differences being noted in kinetic parameters. This finding differs from that of others who reported decreased melphalan influx by system L in drug-resistant L1210 cells [12] and CHO cells [10]. However, the steady-state levels of melphalan at extracellular concentrations of 1 and 100 μM were significantly lower in Mel^R6 and CH^RC5 cells than in AUXB1 parental cells (Fig 1). Furthermore, the rate of efflux appeared to be faster in resistant cells although this difference was statistically significant only for CH^RC5 cells. The changes in the steady-state drug level may be accounted for by these differences in drug efflux. A similar mechanism of resistance has been documented for other drugs such as daunorubicin [38, 39], adriamycin [40] and vincristine [39].

Although drug transport appears to be a factor in the development of resistance to melphalan in CHO cells, evidence obtained in this study suggests that other factors are also involved. If resistance to melphalan is dependent on differences in drug transport alone, then cell kill should be identical when the intracellular concentration is the same in sensitive and resistant cells. However, when each of the three cell lines was treated with that concentration of drug required to reduce surviving cell fraction to 0.02 (Table 3), the intracellular drug concentration in resistant Mel^R6 cells was 7-fold greater and that in CH^RC5 was 16-fold greater than that found in sensitive cells. The higher intracellular drug level associated with an equivalent degree of cell kill suggested that resistant cells tolerate higher drug levels. These observations clearly demonstrate that resistance to melphalan observed in Mel^R6 and CH^RC5 cells can not be accounted for by reduced drug transport alone.

The interaction of melphalan with the DNA of sensitive and resistant cells was measured using the alkaline elution procedure. Dose–response curves for total DNA cross-linking showed that only CH^RC5 cells exhibited reduced activity (Fig. 4). However, DNA interstrand cross-linking was significantly reduced in both resistant cell lines. Kohn and coworkers have found a correlation between DNA interstrand cross-linking and the cytocidal activity of several alkylating agents including melphalan [18, 41–43]. The lower level of DNA interstrand cross-linking induced by melphalan in Mel^R6 and CH^RC5 cells also appears to correlate with the reduced sensitivity of these cells to drugs.

A higher level of DNA–protein cross-linking was observed in Mel^R6 cells than in either sensitive cells ($P < 0.002$) or the more resistant CH^RC5 cells ($P < 0.02$). Thus, there was no apparent correlation between DNA–protein cross-link formation and cytocidal activity. Others have also reported no correlation between DNA–protein cross-links and sensitivity to melphalan in sensitive and resistant L1210 leukemia cells [18]. However, formation of DNA–protein cross-links may represent a mechanism of drug inactivation.

In a study of repair of DNA interstrand cross-links, activity in AUXB1 and CH^RC5 cells increased to a peak level 3 hr after removal of drug, probably as a result of further alkylation by mono-adducts and free drug (Fig. 6). Similar results have been reported for melphalan in other cells [44]. A small increase in DNA interstrand cross-linking was observed in Mel^R6 cells and peak activity was noted 6 hr after removal of drug. The small increment in cross-linking may be due to inactivation of mono-adducts by the higher level of non-protein sulfhydryl groups observed in Mel^R6 cells (Table 4); such a mechanism of mono-adduct inactivation has been postulated previously [42, 45].

A similar rate of removal of DNA interstrand cross-links was observed in AUXB1 and resistant CH^RC5 cells with possibly a slower rate of DNA repair in Mel^R6 cells (Fig. 6). However, if DNA repair was involved in the mechanism of resistance in Mel^R6 cells a more rapid, not a slower, rate of removal of cross-links would be expected. Precise statements about the mechanism of DNA repair are impossible to make since these curves represent a complex interaction of ongoing cross-link formation and removal of such ligands.

In an attempt to identify other possible causes of resistance to melphalan in CHO cells, the concentration of sulfhydryl groups was determined. The total thiol content in Mel^R6 cells was approximately 50% greater than that in sensitive cells whereas that in CH^RC5 cells was approximately 20% higher and both findings were statistically significant ($P < 0.001$). For Mel^R6 cells both protein-bound and non-protein sulfhydryl levels were elevated whereas for CH^RC5 cells only the protein-bound fraction was significantly increased. Elevated levels of thiol groups in cells resistant to alkylating agents have been reported previously [3, 4, 16, 17] and it has been suggested that the sulfhydryl groups may spare critical target sites such as DNA from alkylation. Thus the elevated sulfhydryl levels found in Mel^R6 and CH^RC5 cells probably account for part of the reduced DNA interstrand cross-linking and lowered

sensitivity of these cells to melphalan.

For both Mel^{R}6 and CH^{R}C5 cells decreased DNA interstrand cross-linking appears to account for the major portion of the increased resistance of these cells to melphalan (Table 5). Furthermore it appears that for Mel^{R}6 cells most of the decrease in DNA cross-linking can be accounted for by a lower drug accumulation and increased drug inactivation by sulfhydryl groups. On the other hand, for CH^{R}C5 cells, lower steady-state drug levels and elevated thiol levels appear to be insufficient to explain entirely the reduction of DNA interstrand cross-link activity. This would suggest that other mechanisms of resistance may be operative in these cells such as increased drug metabolism, increased hydrolysis of alkylating groups and/or decreased transport across the nuclear membrane.

Elliott and Ling [11] suggested that resistance to melphalan in Mel^{R}6 cells was due to a nuclear alteration and CH^{R}C5 cells represented a membrane mutant, characterized by decreased drug transport. Our results indicate that resistance to melphalan in Mel^{R}6 cells is characterized by decreased intracellular accumulation of the drug, elevated sulfhydryl levels and decreased DNA interstrand cross-linking. In CH^{R}C5 cells we have found not only a lower steady-state drug level but also an increased rate of drug efflux, elevated levels of sulfhydryl groups and reduced DNA interstrand cross-linking. Clearly resistance to melphalan appears to be multifactorial but it is not possible from this study to establish whether these changes result from a single pleitrophic mutation or from multiple mutations.

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