Development and Characterization of a Melphalan-resistant Human Multiple Myeloma Cell Line\textsuperscript{1,2}

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ABSTRACT

We present data describing a human myeloma cell line (8226/LR-5) selected for resistance to melphalan which exhibits a 7-fold level of resistance to melphalan and is partially cross-resistant to other bifunctional alkylators and X-irradiation. Melphalan resistance is relatively unstable with a decrease in resistance observed within 17 weeks in the absence of drug. The resistance observed in this cell line is not mediated by reduced intracellular melphalan accumulation. DNA interstrand cross-linking at equivalent intracellular drug accumulation is significantly reduced in the resistant subline. Whether this reduction is the result of a decrease in the formation of this lesion or to an increased rate of removal of the lesion remains to be determined. Growth characteristics and cell cycle kinetics, including $S$ phase, were similar between sensitive and resistant cell lines. Intracellular nonprotein thiols were found to be significantly elevated in the resistant 8226/LR-5 cells; as cells revert or lose resistance, intracellular nonprotein sulhydryl levels decline. Prior treatment of the cells with buthionine sulfoximine significantly reduced nonprotein sulphydryl levels and enhanced melphalan cytotoxicity in both the sensitive and resistant cell lines. Thiols appear to play a role in mediating melphalan resistance.

INTRODUCTION

Chemotherapy continues, along with surgery and radiotherapy, to be an extremely effective modality of cancer therapy. The resistance of tumor cells to the cytotoxic effects of chemotherapy has been and continues to be a major obstacle to the successful treatment of human cancers since the modern era of cancer chemotherapy was introduced in the 1940s (1). Multiple myeloma is a B-cell neoplasm to which patients have a high initial degree of response to chemotherapy (2). The standard first-line therapy for this disease is treatment with alkylating agents such as melphalan or cyclophosphamide (3), and despite a 70–80% initial response rate to chemotherapy, all patients eventually relapse and succumb to this disease due to the emergence of drug-resistant tumor cells.

It is possible that several mechanisms may play a role in resistance to alkylating agents. These could include: (a) alterations in drug accumulation; (b) changes in subcellular distribution of drug which would alter drug concentration at the target; (c) differences in DNA repair capabilities; or (d) changes in cellular metabolic systems which would facilitate detoxification of the agent(s). Such mechanisms may be operating independently or in concert.

Current evidence suggests that the clinically effective alkylating agents exert their cytotoxic effects as the result of DNA adduct formation resulting in inter- and/or intrastand cross-links (4). Of interest, biochemical changes have been noted in tumor cells resistant to alkylating agents, which suggest a possible role for drug detoxification. Of particular relevance is the observation that elevations in glutathione and glutathione-based enzymes have been associated with drug resistance to several antineoplastic agents (5).

The in vitro model we are describing is a human myeloma cell line, 8226/LR-5, which was selected for resistance to melphalan and is cross-resistant to other bifunctional alkylating agents. Determining the mechanism of drug resistance to these agents could provide the basis for important advances in the treatment multiple myeloma.

MATERIALS AND METHODS

Cell and Culture Conditions. The RPMI 8226 human myeloma cell line was obtained from the American Type Culture Collection (Rockville, MD). It was originally obtained from the peripheral blood of a 61-year-old male with multiple myeloma (6). Cells were grown in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% (v/v) penicillin (100 units/ml), streptomycin (100 μg/ml), and 1% (v/v) L-glutamine (all from Grand Island Biological Supply Co., Grand Island, NY). Cells were maintained at 37°C in 5% CO₂–95% air atmosphere and subcultured every 6 days.

Drugs. Melphalan was obtained from Burroughs Wellcome, Research Triangle Park, NC. [3H]Melphalan (8 Ci/mmol) was obtained from SRI International (Menlo Park, CA). 4-Hydroxy cyclophosphamide was obtained from Dr. Robert Struck of Southern Research Institute, Birmingham, AL. cis-Platinum, methotrexate, and VP-16 were obtained from Bristol Myers Co. (Evansville, IN); mechlorethamine, dexamethasone, and gramicidin D were from Sigma Chemical Co. (St. Louis, MO); doxorubicin was from Adria Laboratories (Columbus, OH); vincristine was from Lilly Laboratories (Indianapolis, IN); 5-fluorouracil was from LyphoMed, Inc. (Rosemont, IL); ara-C (Cytarabine; Upjohn, Kalamazoo, MI). Drugs were prepared by dissolving in sterile phosphate-buffered saline, pH 7.4, or in the case of melphalan, acidified ethanol. The final concentration of ethanol was 0.01% when exposed to cells and was included in controls.

Selection of Melphalan-resistant Cells. RPMI 8226 (8226/S) cells were initially exposed to melphalan at a concentration of $1 \times 10^{-4}$ M, which represented the approximate IC\textsubscript{50} concentration. Fresh drug was added to the medium whenever it was changed (approximately 3 times weekly). The melphalan concentration was gradually increased to a final concentration of $5 \times 10^{-5}$ M (8226/LR-5) over a 47-week period. This concentration has been maintained for over 1 year. Cells were maintained in a drug-free medium for 1 week prior to drug sensitivity assays or drug accumulation studies.

Cell Growth Characteristics. For the determination of doubling time, cell growth curves were established for each cell line by plotting cell

\textsuperscript{1}The abbreviations used are: IC\textsubscript{50}, concentration resulting in 50% inhibition of cell growth; PBS, phosphate-buffered saline (5 mM KH\textsubscript{2}PO\textsubscript{4}, 150 mM NaCl, pH 7.4); MTTH, 3,4-dimethylethlazol)-2,5-diphenytrimetrazolium bromide; BSO, buthionine sulfoximine; NPSH, nonprotein sulphydryl; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate; PAG, 0.1% (v/v) glucose, 0.1 mM bovine serum albumin, sterile phosphate-buffered saline, pH 7.4; BCNU, N,N'-bis(2-chloroethyl)-N-nitrosourea.

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\textsuperscript{4}The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\textsuperscript{6}Preliminary results of this study were presented at the American Society of Clinical Oncology meeting, San Francisco, CA (1989).

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number versus growth time. Doubling time was determined directly from the linear portion of the plot. To assess possible differences in cell size, 8226/S and 8226/LR-5 cells were fixed in 1% PBS-buffered formalin, pH 7.5, and analyzed by flow cytometry (FAC Scan; Becton Dickinson, Rutherford, NJ). A 15-mW, 488 nm air-cooled argon laser was used for fluorescence excitation, and a 530 nm filter set with a band width of 30 nm was used for fluorescence detection. The instrument was calibrated by using Calibrite beads (Becton Dickinson) and a pool of normal lymphocytes stained with anti-human leukocyte antibody HLe-1 (Becton Dickinson Immunocytometry Division, Mountain View, CA). Ten thousand events per sample were collected. Data analysis was performed on a Model 310 Hewlett Packard 9000 series computer (Hewlett Packard, Mountain View, CA). The fraction of cells in S phase of the cell cycle was determined by using flow cytometry after propidium iodide staining of the cells (7).

Immunocytochemistry. Tumor cells were evaluated for expression of the plasma cell antigens PCA-1 (Coulter Immunology, Hialeah, FL) and Leu 17 (Becton Dickinson, Mountain View, CA). Expression of P- 170 was evaluated by using a panel of three monoclonal antibodies, C- 219, JSB-1, and MRK-16, using a previously described immunocyto- chemical method (8). By this same method, the monoclonal antibody Ki-67 (DaKopatts, Copenhagen, Denmark) was used to detect a nuclear proliferation antigen as a measure of proliferative activity of tumor cells (9).

In Vitro Cytotoxicity Assays. Two methods were used to assess chemosensitivity in the 8226/LR-5 and 8226/S sublines. A colorimetric assay based on the ability of viable cells to reduce the tetrazolium salt, MTT, to a blue formazan product was used to measure cytotoxicity following exposure to drug (10). Cells were plated into 96-well microtiter plates (Falcon; Becton Dickinson, Oxnard, CA) at 10,000 cells/well in 0.2 ml of medium with replicates of 8. Drug exposure was for either 1 h prior to plating the cells or continuously by incorporating the drug with the cells at the time of plating. After incubation for 4 days at 37°C, 50 μl of MTT dye (2 mg/ml) were added to each well and incubated for 4 h. Plates were then centrifuged at 500 x g for 5 min, medium was aspirated, and dimethyl sulfoxide was added to each well (100 μl). The plates were then mechanically agitated for 5 min and absorbance at 540 nm was determined on a microplate reader (Biomek 1000, Beckman Instruments, Palo Alto, CA). Data were expressed as the percentage of survival of control 8226 cells calculated from the absorbance (540 nm) corrected for background absorbance. The surviving fraction of cells was determined by dividing the mean absorbance values of the drug-treated samples by the mean absorbance values of untreated control samples. The IC50 for each particular drug was defined as the concentration of drug which reduces growth to 50% of untreated control cells and was calculated from linear transformation of the dose- response curves. A relative resistance index was expressed as the ratio of the IC50 of the resistant (8226/LR-5) cells to the IC50 of the sensitive (8226/S) cells.

The cytotoxic action of melphalan in the presence or absence of the chemomodifiers verapamil (6.6 μM) or ethacrynic acid (1 μM) was also studied. The dose-modifying factor was determined by dividing the IC50 of melphalan in the 8226/LR-5 cell line by the IC50 of melphalan plus verapamil or ethacrynic acid.

In addition to the MTT assay, a standard 2-layer, soft-agar culture system was used to assess drug cytotoxicity (11). Cells were exposed to melphalan continuously by incorporating it into the top layer of medium and agar and were plated in triplicate at a concentration of 20,000 cells/35-mm tissue culture dish. The cells were then incubated at 37°C under 10% CO2 and 90% humidified air to facilitate tumor colony formation. Tumor colonies greater than 60 μm in diameter were counted by using an automated inverted microscope (FAS-II Omnicon; Bausch & Lomb, Rochester, NY) 10–14 days after plating. The percentage of survival was based upon the ratio of the mean colony count of treated to control cells.

Radiation Sensitivity. The sensitivity of 8226 myeloma cells to the cytotoxic effects of X-rays was studied in soft agar. Cells were irradiated at room temperature with a Varian Clinac 4 4-MeV linear accelerator (Varian Associates, Palo Alto, CA) at a dose rate of 270 rads/min. Immediately after irradiation, the cells were placed in a 37°C incubator and survival was assessed 10–14 days later as described above. The mean lethal dose, Dn, which is defined as the dose of radiation required to reduce cell survival to 1/e (i.e., 37%) of the original value was calculated from the negative reciprocal of the linear portion of the survival curve. The quasithreshold dose, Dn, is a measure of the shoulder of the survival curve and was calculated by extrapolating the linear portion of the survival curve to the y-axis (12).

Cytogenetic Studies. Cultures were harvested for karyotypic analysis, slides were prepared, and staining for G-band and Q-band was performed as previously described (13). A minimum of 25 cells/cell line were analyzed, with results expressed according to ISCN recommenda- tions (14).

Protein Analysis. Cellular protein was labeled by incubating the cells for 18 h in the presence of 5 μCi/ml of [35S]methionine (specific activity, 10.75 mCi mmol; New England Nuclear) in RPMI 1640 medium (GIBCO) containing 10% of the normal concentration of methionine. The cells were fractionated and the plasma membrane isolation was performed as described (15). A representative portion of the whole cell, cytoplasmic, nuclear, and membrane fractions was utilized for gel electrophoresis. Samples were prepared for gel electrophoretic analysis by denaturing the protein in SDS sample buffer containing 2% SDS, 5% mercaptoethanol, 50 mM Tris-HCl (pH 7.2), 3% sucrose, and 0.01% bromophenol blue. Samples were boiled prior to loading onto a 7% polyacrylamide gel. Equal amounts of protein were loaded to each gel lane.

Western Blot Analysis for P-Glycoprotein. Plasma membranes from 2 × 106 cells were purified according to the method of Riordan and Ling (16). Polyacrylamide gel electrophoresis (50 μg protein/lane) was performed according to the method of Laemmli (17). Proteins were transferred from sodium dodecyl sulfate-polyacrylamide gels onto nitrocellulose filter paper according to the method of Towbin et al. (18). This blot was then probed with a [3H]Labeled monoclonal antibody, C- 219 (kindly provided by Dr. Victor Ling, Ontario Cancer Center, Toronto, Ontario, Canada), as described (19). Membrane preparations from the multidrug-resistant human myeloma cell line 8226/DDX40 were used as positive controls of P-glycoprotein expression.

Drug Accumulation and Efflux Studies. To determine cellular accumulation of [3H]melphalan in 8226/S and 8226/LR-5, cells were studied in the exponential growth phase. The cells were washed free of medium and resuspended at a concentration of 2 × 106 cells/ml in PAG transport medium. [3H]Melphalan was added to each tube of cells at 1, 10, or 50 μM, and the cells were incubated as a suspension submerged in a water bath at 37°C. At specific time points following the addition of [3H]melphalan, the cells were pelleted and washed twice with ipec PBS, pH 7.4. After the PBS wash, 1 × 106 cells were then aliquoted into scintillation vials and digested overnight with 1 n NaOH, followed by neutralization with 1 n HCl. Scintillation fluid was then added and [3H]melphalan was determined by liquid scintillation counting.

In addition to radiolabeled melphalan accumulation studies, HPLC analysis was also performed with the 8226/LR-5 and 8226/S cells following a 1-h incubation with 1 × 104 μM melphalan at 37°C in PAG medium to confirm results obtained above and to assess any changes in drug metabolism (20). The HPLC apparatus consisted of a Model 660 solvent programmer, two Model 6000A solvent delivery systems, and a Model 440 UV detector (Waters Associates). Chromatographic separations were accomplished by using a C18 Bondapak reversed- phase column and an isoteric mobile phase consisting of 2% acetic acid (pH 3.6) and acetonitrile (78/22, v/v) delivered at a flow rate of 1.5 ml/min. Detection was accomplished at 254 nm with quantitation based on peak height ratios compared to an internal standard.

NPSH assay. The nonprotein sulfhydryl content of the melphalan-resistant 8226/LR-5 subline and the drug-sensitive parental line, 8226/ S, was measured according to the method of Sedlak and Lindsay (21). A total of 5 × 106 cells were washed twice with ipec PBS, pH 7.4, and transferred to a microcentrifuge tube where they were lysed by sonication (Model 250 Branson sonifier, Danbury, CT). Cellular protein was precipitated by addition of 5% sulfosalicylic acid. The cell lysate was...
then centrifuged at 12,000 × g for 5 min at room temperature. A 1-ml aliquot of the supernatant was transferred to a tube containing 0.2 mM Tris buffer, pH 8.9. To each tube, 100 μl of 0.01 N 5,5'-dithiobis(2-nitrobenzoic acid) in absolute methanol were added. The contents were mixed and the absorbance of each sample was measured at 412 nm.

The concentration of NPSH in the sample was determined by comparing the absorbance reading of the sample to a standard curve constructed by using reduced glutathione. NPSH levels were analyzed 1 day post-

The elution procedure was carried out in the dark to prevent UV light-

DNA Alkaline Elution Assays. Approximately 1 × 106 drug-sensitive and -resistant cells were labeled with [14C]thymidine (0.1 μCi/ml; 55 mCi/mmol; ICN Radiochemical, Irvine, CA) and exposed for 30 min to melphalan (5 × 10⁻⁴ M; the IC₅₀ value for the 8226/S subline) at 37°C in PAG medium. After drug exposure, the cells were diluted and washed twice with cold medium and resuspended in complete RPMI medium containing 0.01 mM BSO.

Melphalan Cytotoxicity as a Function of NPSH Content. The cytotoxic effects of melphalan were studied in the two 8226 cell lines by using the MTT dye assay. Cytotoxicity was assessed in cells which had been depleted of glutathione by pretreatment with BSO as described above, as well as in cells not receiving such treatment.

RESULTS

Selection of Melphalan-resistant Clones. Melphalan-resistant 8226 myeloma cells were selected by continuously exposing cells to gradually increasing concentrations of melphalan. Despite the development of drug resistance, the 8226/LR-5 subline retained several biological characteristics of the sensitive parental line, 8226/S, as shown in Table 1. The cells of both sublines had a plasma cell morphology and expressed the plasma cell-associated antigens PCA-1 and high density CD38, characteristic of mature plasma cells (25). There was no difference in the expression of the nuclear proliferative antigen, Ki-67, with 98% of the cells in both the sensitive and resistant sublines expressing this antigen. The plating efficiency of both sublines in soft agar was similar as was the population doubling time. There was no difference between the sensitive and resistant cell lines when the percentage of cells in S phase of the cell cycle was compared.

This cell line (8226/LR-5) exhibits a 7-fold level of resistance to melphalan as compared to the parental cell line, 8226/S, and is cross-resistant to other bifunctional alkylators but not to natural products or antimetabolites (Table 2). The survival curves for 8226/LR-5 and 8226/S in the presence of melphalan are shown in Figs. 1 and 4. Comparing the IC₅₀ concentrations, a 6.8-fold level of resistance was observed for the 8226/LR-5 subline when the cells were continuously exposed to drug at 37°C. The degree of resistance was relatively unstable in the absence of drug, with the resistant cells losing some resistance when maintained in melphalan-free medium for 17 weeks (Fig. 4).

Cross-resistance Studies. As shown in Table 2, 8226/LR-5 demonstrated cross-resistance to a number of bifunctional alkylating agents, including methlolethamine, 4-hydroperoxyclophosphamide, and cisplatinum. This cell line was sensitive to natural products, including doxorubicin, VP-16, and the Vinca alkaloids, and was sensitive as well to antimetabolites. Verapamil or ethacrynic acid had no effect on melphalan cytotoxicity in the 8226/LR-5 subline. The addition of verapamil to natural products or antimetabolites (Table 2). The survival curves for 8226/LR-5 and 8226/S in the presence of melphalan are shown in Figs. 1 and 4. Comparing the IC₅₀ concentrations, a 6.8-fold level of resistance was observed for the 8226/LR-5 subline when the cells were continuously exposed to drug at 37°C. The degree of resistance was relatively unstable in the absence of drug, with the resistant cells losing some resistance when maintained in melphalan-free medium for 17 weeks (Fig. 4).

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Table 2 Cross-resistance patterns of human myeloma cell lines to various antineoplastic agents

Drug sensitivity testing was performed on several different classes of agents to determine the cross-resistance profile of the 8226/LR-5 cells. Exponentially growing cells were tested by using the MTT dye assay and results were confirmed by soft agar colony-forming assay. A minimum of 3 assays were performed for each drug. The IC50 refers to the drug concentration that results in a 50% reduction of dye reduction or colony formation.

<table>
<thead>
<tr>
<th>Agent</th>
<th>IC50 (M)°</th>
<th>8226/S</th>
<th>8226/LR-5</th>
<th>Degree of resistance°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melphalan</td>
<td>3.0 x 10^-6</td>
<td>20.5 x 10^-6</td>
<td>6.6</td>
<td></td>
</tr>
<tr>
<td>4-Hydroxy cyclophosphamide</td>
<td>2.3 x 10^-5</td>
<td>3.87 x 10^-5</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.2 x 10^-6</td>
<td>2.45 x 10^-6</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Nitrogen mustard</td>
<td>8.3 x 10^-7</td>
<td>2.23 x 10^-4</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>3.1 x 10^-8</td>
<td>1.9 x 10^-8</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Vinristine</td>
<td>1.6 x 10^-8</td>
<td>1.3 x 10^-8</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Vp-16</td>
<td>1.2 x 10^-7</td>
<td>8.5 x 10^-7</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>ara-C</td>
<td>3.7 x 10^-6</td>
<td>8.8 x 10^-7</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>2.32 x 10^-8</td>
<td>3.03 x 10^-8</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>6.0 x 10^-7</td>
<td>6.8 x 10^-7</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Gramicidin-D</td>
<td>0.03 x 10^-6</td>
<td>0.03 x 10^-6</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Dexamethasone°</td>
<td>0.073 x 10^-6</td>
<td>0.032 x 10^-6</td>
<td>0.4</td>
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</table>

° As determined by the MTT assay (25); continuous exposure.

Radiation Survival Studies. Radiation survival curves for the 8226/LR-5 and 8226/S cell lines are shown in Fig. 2. Individual survival points represent the mean ± SD for three experiments, each run in triplicate. The mean lethal dose, Do, was 160 rads for the 8226/LR-5 cell line compared with 80 rads for the 8226/S parental line. The quasithreshold dose, Dq, was 120 and 20 for the melphalan-resistant and -sensitive cell lines, respectively.

Cytogenetics. The range of chromosome counts was 58–67 in 8226/S, and 56–121 for 8226/LR-5 with near triploid modal populations of 61–62 (52%) and 63–66 (76%), respectively. Both cell lines had 11 identifiable structural abnormalities in common, including t(1;14)(p13;q32), del(2)(q35), t(3;?)(q29;?), t(5;6)(p13;p12), del(6)(q15), del(6)(q11), t(9;?) (p24;?), del(11)(q23), t(11;?)(p11;?), t(17;?) (q25;?), and HSR(21)(q22). 8226/S also had a deletion of chromosome 11 which the resistant line lacked, del(11)(p11). A representative G-banded cell from the 8226/S cell lines is provided in Fig. 3A. Additionally, there were two sidelines (sd1) recognized in the 8226/LR-5 which were not seen in 8226/S and which varied from the modal 8226/LR-5 population [i.e., sd1 lacked the t(15;16) and had a normal chromosome 15, while sd2 had an isochromosome of 7q instead of the del(7)(p13)].

Intracellular Melphalan Accumulation and Efflux Studies. In order to characterize the nature of melphalan resistance, the concentration-time course of [3H]melphalan accumulation was studied in the sensitive and resistant sublines. Table 3 demonstrates that the net accumulation of [3H]melphalan following a 1-h exposure at 37°C was unchanged between the two sublines. These findings were confirmed by HPLC analysis following a 1-h exposure to melphalan. In addition, there were no additional HPLC peaks associated with the 8226/LR-5 cells which would have suggested altered intracellular metabolism of melphalan (data not shown). Nonspecific surface absorption of [3H]
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Fig. 3. A, representative G-banded karyotype of 8226/S with unidentifiable markers (Umars) at the bottom left corner of the karyotype; B, inset, structurally rearranged chromosomes unique to 8226/LR5: t(4;7)(q335;?). del(7)(p13), der(16)t(15;16)(qI1;p13), and the i(7q) found in a sdl2.

Table 3 Accumulation of [3H]melphalan in sensitive (8226/S) and resistant cells (8226/LR-5)

<table>
<thead>
<tr>
<th>Drug concentration (μM)</th>
<th>Time (min)</th>
<th>8226/S</th>
<th>8226/LR-5</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>194 ± 9</td>
<td>208 ± 10</td>
<td>NS</td>
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<td></td>
<td>60</td>
<td>251 ± 112</td>
<td>196 ± 13</td>
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<td>10</td>
<td>30</td>
<td>633 ± 102</td>
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<td>60</td>
<td>558 ± 84</td>
<td>608 ± 50</td>
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<td>50</td>
<td>30</td>
<td>2549 ± 971</td>
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</tr>
<tr>
<td></td>
<td>60</td>
<td>2412 ± 452</td>
<td>2428 ± 326</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Statistical significance of the difference of the means as determined by 2-tailed Student’s t test.

*: NS, not significant.

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<table>
<thead>
<tr>
<th>Drug concentration (μM)</th>
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</thead>
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<tr>
<td>1</td>
<td>30</td>
<td>194 ± 9</td>
<td>208 ± 10</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>251 ± 112</td>
<td>196 ± 13</td>
<td>NS</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>633 ± 102</td>
<td>634 ± 136</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>558 ± 84</td>
<td>608 ± 50</td>
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</tr>
<tr>
<td>50</td>
<td>30</td>
<td>2549 ± 971</td>
<td>2390 ± 449</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>2412 ± 452</td>
<td>2428 ± 326</td>
<td>NS</td>
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</table>

*: NS, not significant.

melphalan was assessed by measuring the binding at 0°C after exposure to 1, 10, or 50 μM melphalan and immediately assay- ing for drug accumulation. A negligible amount of drug was determined to be bound under these conditions. The drug accumulation data are in contrast to that observed in our 8226/DOX subline which displays the MDR phenotype (26). In this cell line resistance to doxorubicin appears to be the result of a marked decrease in its accumulation (27).

Protein Analysis. As a first step in characterizing the 8226 melphalan-resistant subline, we analyzed the qualitative protein profiles in cell fractions enriched for cytoplasmic, nuclear, and membrane proteins. The molecular weight classes of the major membrane proteins are not significantly different when the sensitive and resistant cell lines are compared by 1-dimensional polyacrylamide gel electrophoresis. To determine if the resistant subline expressed the P-glycoprotein, Western blot analysis was performed on both the sensitive and resistant 8226 cells. Using the monoclonal antibody C-219, no evidence of P-glycoprotein expression was observed. This absence of P-glycoprotein was also noted by immunocytochemical measurement by using the antibodies C-219, JSB-1, and MRK-16. This has been further substantiated by Northern analysis of total cellular RNA, using the complementary DNA probe pCHP-1 which is specific for the mdr-1 gene (28) (data not shown).

NPSH Studies. The nonprotein sulfhydryl content of the 8226 cells was measured spectrophotometrically and was found
to be significantly elevated in the 8226/LR-5 cells elevated as compared to the 8226/S cells (P < 0.001; Student's t test). The melphalan-resistant subline contained 20.4 ± 0.8 nmol NPSH/10⁶ cells which compared to 12.1 ± 0.4 nmol/10⁶ cells in the drug-sensitive parental line (Table 4). For the purposes of maintaining drug resistance, the 8226/LR-5 cells are normally maintained in medium containing melphalan; however, when the 8226/LR-5 cells were kept in melphalan-free medium for 17 weeks, the NPSH content was reduced from 20.4 ± 0.8 nmol/10⁶ cells to 16.1 ± 0.8 nmol/10⁶ cells (P < 0.001; Student's t test). Interestingly, concurrent with the decline in NPSH, these cells became more sensitive to the cytotoxic actions of melphalan. Results revealed no statistical difference in cell size, as determined by flow cytometric analysis, between the 8226/S and 8226/LR-5 cells, which could have accounted for the observed difference in NPSH content. Protein concentrations were also identical between the cell lines (data not shown).

Depletion of Glutathione by Buthionine Sulfoximine. Buthionine sulfoximine, an inhibitor of the enzyme γ-glutamylcysteine synthetase, a key enzyme in the glutathione synthesis pathway, was used to deplete NPSH levels in the 8226 cells. BSO alone was relatively nontoxic; a concentration of 0.1 mM reduced the percentage of survival of 8226 cells from 100 to approximately 85%. Concentrations of 0.01 mM or less were nontoxic under the conditions used in these studies. Following a 24-h exposure to RPMI medium containing 0.1 mM BSO, NPSH values in both cell lines were reduced below the detectable limit of the assay (0.5 nmol NPSH). The depletion of thiols following exposure of the cells to BSO significantly enhanced melphalan toxicity in both the 8226/S and 8226/LR-5 cell lines (Fig. 4). Cytotoxicity was assessed by MTT assay following continuous exposure to drug in glutathione-depleted and nondepleted cells.

Alkaline Elution Studies. Melphalan is a bifunctional alkylating agent which reacts rapidly with nucleophilic sites in DNA to form monoadducts which are then slowly converted to DNA cross-links (29), the formation of which has been demonstrated to correlate with its cytotoxic effects (4, 30, 31). To measure the presence of DNA interstrand cross-links following melphalan treatment, alkaline elution experiments were performed by using a 30-min drug exposure and subsequent cell lysis with a solution containing proteinase K. This procedure removes most of the DNA-associated protein and thus any subsequent retention of [¹⁴C]DNA on the filter is assumed to represent DNA interstrand cross-links (23). Maximum cross-linking with melphalan has been observed between 6 and 12 h after drug exposure (4, 30, 31) and is presumed to represent the time required for conversion of drug-DNA monoadducts to interstrand cross-links. Therefore, we chose to study the presence of DNA interstrand cross-links present 6 h after exposure to melphalan. Immediately after a 30-min exposure of both cell lines to an IC₅₀ melphalan concentration for the 8226/S line, very few DNA cross-links were observed in either 8226 subline (Table 5). At 6 h after melphalan treatment, we observed a significant decrease (P < 0.002; Student's t test) in the number of DNA cross-links present in the 8226/LR-5 subline as compared to the 8226/S cells. As seen in Table 5, the 8226/S line had 176 ± 38 rad equivalents of DNA damage as compared to 68 ± 21 in the 8226/LR-5. Melphalan did not induce strand breaks in either cell line (data not shown).

Because radiation is used to introduce a controlled amount of DNA single strand breaks in order to quantitate the cross-link lesions, it was necessary to determine if both cell lines produced similar numbers of DNA single strand breaks when exposed to radiation under the conditions utilized in the cross-link assay. The formation of radiation-induced DNA single strand breaks was found to be identical between the two lines (data not shown).

DISCUSSION

In this study we have examined the biological characteristics and potential mechanisms of resistance in a human myeloma cell line selected for resistance to melphalan and displaying cross-resistance to other bifunctional alkylating agents. The parental cell line, RPMI 8226, was exposed to gradually increasing concentrations of melphalan resulting in a cell line, 8226/LR-5, which is approximately 7-fold resistant to melphalan. In addition to melphalan resistance, the 8226/LR-5 cell line displays cross-resistance to nitrogen mustard, 4-hydroperoxycyclophosphamide, and cis-platinum. It is sensitive to anthracyclines, Vinca alkaloids, epipodophyllotoxins, and the

Table 4 Nonprotein sulfhydryl content of 8226 human myeloma cell
NPSH levels in the 8226 myeloma cells were determined spectrophotometrically 1 day post-cell splitting by using the procedure of Sedlak and Lindsay (21). Doubling times and cell size were comparable among all lines. Data are presented as the mean ± SD of 4 experiments.

<table>
<thead>
<tr>
<th>Subline</th>
<th>-BSO</th>
<th>+BSO*</th>
</tr>
</thead>
<tbody>
<tr>
<td>8226/S</td>
<td>12.1 ± 0.4</td>
<td>ND³</td>
</tr>
<tr>
<td>8226/LR-5</td>
<td>20.4 ± 0.8</td>
<td>³</td>
</tr>
<tr>
<td>8226/LR-5 revertant</td>
<td>16.1 ± 0.8</td>
<td>ND³</td>
</tr>
</tbody>
</table>

* 1 mM BSO.
³ ND, not detected.
³ P < 0.001, Student's t test.

Table 5 Melphalan-induced DNA interstrand cross-links in 8226 myeloma cells
DNA interstrand cross-links were determined by the alkaline elution method in the 8226/S and 8226/LR-5 myeloma cell lines 6 h following a 30-min exposure to an IC₅₀ concentration of melphalan at 37°C. Results are presented as mean rad equivalents ± SD (n = 3-4).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>8226/S</th>
<th>8226/LR-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.0 ± 0.7</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>176 ± 38</td>
<td>68 ± 21²</td>
</tr>
</tbody>
</table>

² P < 0.001, Student's t test.
antimetabolites 5-fluorouracil and methotrexate. It displays a collateral sensitivity to ara-C, the significance of which is not yet understood. Both the 8226/S and 8226/LR-5 cell line are resistant to the effects of BCNU. The low level of drug resistance observed in these cell lines is not unlike alkylator-resistant cell lines reported by others (32, 33), where resistance is usually less than 10-fold. Examining the development of resistance to nitrogen mustard, cis-platinum, and BCNU, levels of resistance much greater than 10- to 20-fold were difficult to achieve regardless of the alkylating agent used. Low levels of drug resistance such as this may likely to be due to mechanisms which may be encountered in a clinical setting.

This cell line will be used as a model for acquired melphalan resistance as it occurs in myeloma cells. A potential criticism of this model, however, is the lack of stability, and this may reflect the means of selection in acquired resistance. We will address this by analyzing the role of the ras oncogene in conferring alkylating drug resistance. It is possible that in vitro acquired melphalan resistance may differ from a genetic mechanism such as oncogene activation. Recent studies indicate activated oncogenes may play a role in cellular resistance to chemotherapeutic agents. NIH3T3 cells transformed with activated (mutated) ras oncogenes display greater resistance to ionizing radiation and cis-platinum than nontransformed NIH3T3 cells (34, 35). Little is known about oncogenes activated in multiple myeloma; however, mutated ras oncogenes have been suggested to play a role in the genesis of multiple myeloma (36). In one study, 27% (12 of 43) of multiple myeloma patient samples tested positive for ras mutations at the time of diagnosis, while 46% (6 of 13) tested positive after treatment. ras mutations were found only in codons 12, 13, and 61 of N-ras, and codons 12 and 13 of K-ras. From these data it is possible to suggest that ras mutations are produced as a result of chemotherapy, or more likely, preexisting ras mutations may provide cells a growth advantage or drug resistance.

There were several chromosomal alterations unique to the resistant subline (Fig. 3B). However, it is not clear whether these clonal changes are directly related to the resistant phenotype. Of interest, chromosome 7 was frequently altered, a finding consistent with our earlier reports on anthracycline-resistant tumor lines (37).

Drug accumulation studies revealed no alteration in accumulation of [³H]melphalan over a 3-log concentration range in the 8226/LR-5 subline. Melphalan uptake into the cell is carrier mediated, either preferentially by the L-leucine amino acid transport system or by the system responsible for the uptake of neutral amino acids such as serine and cysteine (38). In light of our findings of similar melphalan accumulation in both the sensitive and resistant sublines, it does not appear that either of these systems has been altered in the 8226/LR-5 cells. This is in contrast to other reports demonstrating altered melphalan accumulation in resistant cells. Redwood and Colvin (39) have characterized alterations in melphalan uptake in L1210 cells made resistant to this drug, which appeared to be due to a mutation in the amino acid carrier system.

Decreased drug accumulation with enhanced drug efflux appears to be a primary mechanism of drug resistance associated with the multidrug resistance phenotype. In a prior study, analysis of a human myeloma cell line selected for resistance to doxorubicin, 8226/DOX, demonstrated a decreased drug accumulation secondary to increased drug efflux and the overexpression of the P-glycoprotein (26). In contrast to the MDR cell line, when the same 8226/S parental line was selected for resistance to the bifunctional alkylator melphalan, there was no evidence of altered drug accumulation and no expression of the P-glycoprotein.

Although the accumulation of [³H]melphalan was identical in these cell lines under the conditions used in this study, this finding does not rule out the possibility that there may be a differential distribution of melphalan within the cell. Melphalan may be bound to a subcellular compartment which would prevent its reaching the target and forming the lethal DNA crosslinks. Such a finding could account for the observed difference in DNA interstrand cross-links in the 8226/LR-5 cells. An isolated nucleus system in conjunction with alkaline elution assays will be used to address this question in future studies.

While the accumulation of melphalan in the 8226/LR-5 cells was unaltered from the sensitive parental line, the possibility exists of an increased metabolism of melphalan to its less toxic dihydroxy metabolite. Green et al. (40) observed no difference in melphalan accumulation between drug-sensitive and -resistant human ovarian carcinoma cell lines, but did observe that the cellular content of dihydroxy melphalan was elevated approximately 2-fold in the resistant line.

Elevations in glutathione and the associated glutathione-S-transferases have been reported in several cancer cell lines exhibiting resistance to alkylating agents and may account for at least a portion of resistance to these drugs (40--44). A previous study (44) demonstrated the ability of ethacrynic acid to reverse resistance to the alkylating agent chlorambucil in resistant tumor cells purportedly by inhibiting glutathione-S-transferase activity. We were unable to confirm these findings in the 8226/LR-5 line, thus bringing into question the role of the GST enzyme system in our cell line. Current studies are under way to assess the expression of glutathione S-transferase isoenzymes in the 8226/LR-5 line.

The addition of BSO to the melphalan-resistant 8226/LR-5 cell line resulted in a decrease of NPSH levels below the detection limit of our assay. When the cells were exposed to melphalan under these conditions, there was a marked increase in its cytotoxic effects in both the 8226/S and 8226/LR-5 cell lines, with a greater effect observed in the 8226/LR-5 cell line. Thus, in multiple myeloma, BSO appears to be acting as a chemoenhancer, increasing the efficacy of melphalan in sensitive as well as resistant myeloma cells. In addition, when the cells were placed in drug-free media for up to seventeen weeks, the level of melphalan resistance decreases in parallel with a decline in the NPSH content of the 8226/LR-5 cells. While this correlation alone is not definitive proof, when examined in conjunction with the BSO results, it does strongly suggest that thiols are indeed playing a role in melphalan cytotoxicity in this cell line. In light of these findings, a Phase II clinical trial in multiple myeloma patients is planned at our institution to study the use of BSO as a chemoenhancer of melphalan.

In addition to a role in drug detoxification, glutathione may also play a role in modulating the cellular response to drug- or radiation-induced damage by facilitating the repair of such lesions (45--47). Wellner et al. (45) demonstrated that human lymphoid cells were partially protected from the effects of radiation by the addition of glutathione monoethyl ester to the cell culture medium. Such effects were noted even when the ester was added after the insult, thus suggesting a role in the repair of radiation-induced DNA damage. Because thiols may spare critical target sites such as DNA from alkylation, the increased NPSH levels found in 8226/LR-5 could also account for the decreased interstrand cross-links observed.
MELPHALAN RESISTANCE IN HUMAN MYELOMA

Our assessment of DNA interstrand cross-linking in the 8226 cells was made at 6 h after drug removal, a point where the lesions are presumed to be at a maximum (4, 30, 31). Since the number of DNA interstrand cross-links is time dependent, our current findings do not ascertain whether the reduction in DNA interstrand cross-links observed in the 8226/LR-5 subline is due to a decrease in the formation (as a result of detoxification or altered drug distribution) or due to an increase in the removal of the lesions (i.e., increased DNA repair), or to a combination of the two processes. Hansson et al. (30) demonstrated a strong correlation between cytotoxicity and the area under the curve for DNA interstrand cross-links due both to melphalan and nitrogen mustard. Thus, the formation as well as the removal of these lesions are important in terms of the cytotoxicity of bifunctional alkylating agents.

Further studies are under way in our laboratories to determine the time course of the formation and removal of this lesion in the drug-sensitive and -resistant 8226 sublines as well as to assess the role of ras oncogene activation in conferring melphalan resistance.

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Development and Characterization of a Melphalan-resistant Human Multiple Myeloma Cell Line


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