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Effects of amino acids on the transport and cytotoxicity of melphalan by human bone marrow cells and human tumor cells*

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Summary. *In human tumor cells freshly obtained from patients with breast cancer, ovarian cancer, or adenocarcinoma of unknown etiology and in normal human bone marrow cells, the cell-to-medium ratio (intracellular/extracellular concentration) in vitro of 5.42 μM melphalan rose rapidly to levels of 6–17 after 35 min at 37 °C in Dulbecco's phosphate-buffered saline containing bovine serum albumin and glucose. Only patient C (breast cancer) had received chemotherapy. In all cells studied, L amino acids (1 mM) such as leucine, glutamine, tyrosine, and methionine reduced the cell-to-medium ratio of melphalan at 3 and 35 min. There was a good correlation between the reduction of melphalan transport at 35 min in the heterogeneous nucleated bone marrow cell population by amino acids and their effect on melphalan cytotoxicity in the CFU-C system. Aminoisobutyric acid (AIB), a specific substrate of the A system of amino acid transport, at a concentration between 1 and 50 mM had no significant effect on melphalan uptake at 3 min in any of the human cells studied except those of patient C. At 35 min AIB (10 or 50 mM) significantly reduced the intracellular melphalan concentration in normal bone marrow cells and tumor cells from patients B and C. At 2 mM, 2-aminobicyclo-(2, 2,1)-heptane-2-carboxylic acid (BCH), a specific substrate of the L system of amino acid transport, reduced the cell-to-medium ratio to 70% of control at 3 and 35 min in human bone marrow cells. In tumor cells from patients A, B, D, and F, 2 mM BCH had no significant effect on melphalan uptake at 3 min; it slightly decreased uptake in tumor cells from patient C. At 35 min, 2 mM BCH significantly reduced melphalan transport in tumor cells from patients C and F only.*

The lack of a BCH-suppressible component to melphalan uptake into human tumor cells freshly obtained from previously untreated patients contrasts with the presence of this component in murine L1210 leukemia cells, murine P388 leukemia cells, and human tumor cell lines. This suggests that minor differences in melphalan transport may exist amongst species and also between human tumor cells which are freshly obtained and cell lines maintained in culture.

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Introduction

The uptake of melphalan is by active, carrier-mediated transport in many murine and human cell culture lines. The presence of amino acids in the culture medium reduced intracellular melphalan in murine leukemia cells [17–20]. In these cells, melphalan appears to be transported by two separate neutral amino acid transport systems, one of which is a BCH-sensitive, sodium-independent L (leucine preferring) system and the other a BCH-insensitive, sodium-dependent L system, where BCH is 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid, a specific substrate of the L system [15]. In L5178Y lymphoblast and LPC-1 plasmacytoma cells, two neutral amino acid transport systems also seem to be implicated: an L and an ASC (alanine, serine, cysteine-preferring) system [1, 7, 8]. In L1210 leukemia cells, L5178Y lymphoblasts, human peripheral blood lymphocytes, and MCF-7 human breast cancer cells a component of melphalan transport is reduced by the presence of BCH in the medium [2, 8, 16]. In murine bone marrow cells, the failure of 2 m*M* BCH to alter melphalan cytotoxicity in the CFU-C assay suggests that this cell type lacks a high-affinity BCH-sensitive leucine transport system [16]. This investigation of the transport and cytotoxicity of melphalan was carried out to correlate results found in freshly obtained human tumor cells and normal human bone marrow cells with those found in murine cells and human cell culture lines.

Materials and methods

Materials. Unlabeled and [chloroethyl-^{14}C]melphalan (10.9 mCi/mmol) were kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Silver Spring, Md (NSC No. 8806). Unlabeled melphalan solutions were prepared daily in 75% ethyl alcohol containing an equimolar concentration of hydrochloric acid. Further dilutions were made in aqueous medium immediately prior to use, to minimize hydrolysis. Radiolabeled melphalan was dissolved in absolute ethanol and stored at −20 °C. Thin-layer chromatography revealed that the drug was stable for weeks at this temperature.

Human bone marrow cells were aspirated into a heparinized syringe from the posterior iliac crest of 25 volunteers following informed consent. Results of melphalan transport and cytotoxicity in normal human bone marrow

cells were pooled because individual results were similar. Human tumor cells were obtained from patients at the time of diagnostic procedures such as thoracentesis or paracentesis. These cells were utilized for experiments within 24 h. Trypan blue viability was always greater than or equal to 95%. Tumor results are presented individually.

Bovine serum albumin was obtained as serum fraction V from Miles Laboratories (Elkart, Ind). Phosphate-buffered saline (Dulbecco's modification) and preservative-free heparin were purchased from GIBCO Canada (Burlington, Ont., Canada). All amino acids were L amino acids except for glycine (Sigma, St. Louis, Mo) and the racemic mixture, DL-2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), obtained from Calbiochem Behring (La Jolla, Calif). The silicone oil (specific gravity 1.045 at 25 °C, viscosity 70 centistokes at 25 °C) was purchased from Harwick Chemical Corporation (Cambridge, Mass). [Carboxy-^{14}C]inulin (17.1 mCi/mmol) and tritiated water (0.5 mCi/ml) were obtained from Amersham Corporation (Oakville, Ont., Canada). Ficoll–hypaque reagent came from Pharmacia Fine Chemicals (Dorval, Que., Canada).

Methods. Normal human bone marrow was aspirated into a syringe, where it was mixed with preservative-free heparin to a concentration of 100 units heparin/ml marrow. It was then layered onto 4 ml Ficoll–Hypaque reagent and centrifuged for 40 min at 500 *g* at room temperature. The interface was removed, washed, and suspended in PAG (Dulbecco's modification: phosphate-buffered saline, pH 7.4 + 0.1 m*M* bovine serum albumin + 0.25% glucose). Tumor cells were obtained by centrifugation (10 min at 250 *g*) of tumor effusions. Red blood cells were removed by ammonium chloride lysis except in patient F's sample, from which red blood cells were separated out by centrifugation (10 min at 330 *g*) on Ficoll–Hypaque reagent at room temperature.

The ammonium chloride lysis procedure was carried out as follows: (a) 20 ml ammonium chloride-lysing buffer at 4 °C (155 m*M* NH_4Cl, 10 m*M* $KHCO_3$ and 0.1 m*M* ETDA) was added to the cell pellet and left at 0 °C for 3.5 min, then (b) an equal volume of 2X McCoy's was added, (c) the cells were centrifuged, and (d) they were washed twice in PAG. The percentage of tumor cells on slides stained with Wright's and Giemsa stains was determined by a cytopathologist. Bone marrow or tumor cells at concentrations of 2×10^6 cells/ml in PAG were preincubated with different concentrations of amino acids for 15 min at 37 °C. Melphalan (5.42 μ*M*) was then added and the cells incubated further at 37 °C for 3 and 35 min. Studies with melphalan alone were performed at 1, 3, 9, and 35 min in selected cases. At these time points, aliquots of the incubation mixture were layered in microcentrifuge tubes containing 1 ml Versilube F-50 silicone oil. The tubes were spun at 12 000 *g* for 1 min at room temperature. Cell pellets were solubilized in 0.2 *N* NaOH overnight at 0–4 °C and then neutralized with an equivalent volume of 1 *N* HCl [15]. To this was added 10 ml liquid scintillation cocktail, and the samples were counted in a Searle Mark III liquid scintillation spectrometer with correction for quenching. The percentage of total radioactivity representing unhydrolyzed melphalan was determined by thin-layer chromatography of the cell extract of 35-min control samples on cellulose plates using (65 : 1 : 34) isopropyl alcohol: formic acid: water [14].

Nonspecific adsorption of labeled melphalan was estimated by measuring drug binding at 0–4 °C following exposure of the cells to melphalan for up to 35 min. Also, to aid in the determination of the amount of extracellular melphalan trapped in the cell pellet, cells were incubated with ^{14}C-inulin. Intracellular water was estimated using tritiated water [6, 22].

Kinetic studies were performed in tumor B utilizing 3, 6, 12, 33, 50, 75, and 100 μ*M* melphalan. Quadruplets of uptake at 2 min after drug addition were determined. The K_m and V_{max} were calculated from the Lineweaver-Burke plot ($1/v$ vs $1/s$). A similar K_m result was obtained with a plot of $[s/v]$ vs [s]. Linear regression analyses of either line revealed that the best fit was achieved with 3- to 75-μ*M* concentrations ($r \geq 0.9958$).

To determine the effect of ammonium chloride lysis on cells, murine P388 leukemia cells were obtained from the peritoneal cavity of CD2F, mice 5 days after the implantation of 2 million cells. The P388 cells were separated from contaminating red blood cells by either ammonium chloride lysis or centrifugation on Ficoll–Hypaque as described above. Experiments with melphalan alone or in the presence of 2 m*M* BCH were carried out as described above, except that the BCH was added simultaneously with the melphalan.

For the melphalan cytotoxicity studies with normal human bone marrow cells, the CFU-C assay was utilized as previously described with minor modifications [12]. Cells (2×10^6) were incubated at 37 °C in PAG with 1.2 μ*M* melphalan for 35 min. This concentration of melphalan consistently reduced colony growth to 4% of control in ten different normal human bone marrow samples. Cells were preincubated with amino acids for 15 min prior to the addition of melphalan in the test samples. To determine the cytotoxic effect of melphalan on freshly obtained human tumor cells, the double-layer culture system, the human tumor stem cell assay (HTSCA), was utilized [9, 13]. The concentration of melphalan was initially 1.2 μ*M*, but was increased to 6.1 m*M* because of a lack of sensitivity of most tumors to the initial concentration. The conditions of the incubation were otherwise the same as with bone marrow cells. Following the 35 min incubation the cells were washed twice with PAG and cultured by the techniques above. The plates were incubated at 37 °C in a 7% CO_2 humidified atmosphere. Colonies (>20 cells for CFU-C and >40 cells for HTSCA) usually appeared within 1–2 weeks for normal bone marrow cells and 2–5 weeks for tumor cells. All test samples were performed in duplicate.

Statistical analysis was performed utilizing Student's *t*-test.

Results

Normal human bone marrow and various human tumors were studied in this project. Table 1 shows the principal characteristics of the specimens used. Bone marrow cells were aspirated from normal healthy volunteers whose peripheral blood counts were normal. The studies of melphalan with normal human bone marrow cells gave similar results in all individuals, and the results were therefore pooled. The proportion of tumor cells in each effusion studied was evaluated by cytological examination. Only one patient (C) had received chemotherapy prior to the tests in vitro.

Table 1. Origin and characteristic of human cells used in melphalan studies

Subjects	Type of sample	% Malignant cells in sample	Diagnosis	Chemo therapy
Healthy human subjects	Bone marrow (posterior iliac crest)	–	–	–
A	Pleural effusion	>90%	Breast cancer	No
B	Abdominal effusion	~50%	Adenocarcinoma (unknown etiology)	No
C	Pleural effusion	>80%	Breast cancer	Yes[a]
D	Abdominal effusion	>60%	Ovarian cancer	No
E	Pleural effusion	~20%	Breast cancer	No
F	Abdominal effusion	>50%	Adenocarcinoma (unknown etiology)	No

[a] Adriamycin, 5-fluorouracil, vinblastine, cyclophosphamide, methotrexate

The intracellular volume of human tumor cells was two to five times greater than that of bone marrow cells whose intracellular volume was approximately 0.18 ± 0.03 µl per 1×10^6 cells. The quantity of intracellular melphalan following incubation with 5.42 µ*M* melphalan at 35 min was 17.2 pmol per 1×10^6 bone marrow cells and between 8 and 71 pmol per 1×10^6 tumor cells.

In normal human bone marrow cells, 5.42 µ*M* melphalan uptake at 37 °C appears linear for approximately 3 min; attaining a cell: medium ratio (intracellular: extracellular concentration) of 17 at 35 min. In human tumor cells (patients A, B), melphalan uptake at 37 °C appeared linear for 3 min and resulted in a cell:medium ratio of 6- and 10-fold, respectively, at 35 min (Fig. 1). In the other human tumor cells (patients C, D, and F), the cell:medium ratios were, respectively, 12, 5, and 2 after 3 min of incubation. At 35 min, the corresponding ratios were 17, 16, and 7 respectively. Experiments at 4 °C indicate that melphalan uptake is strongly temperature-sensitive (data not shown). The K_m for melphalan uptake in tumor cells from patient B was 37 µ*M*, with a V_{max} of 45 pmol/intracellular µl/min for melphalan concentrations between 3 µ*M* and 75 µ*M* ($r = 0.9958$). In subsequent experiments we chose 3 min as a time point to look at uptake and 35 min for steady-state kinetics. While the 3-min observation may not be strictly a measure of unidirectional influx, it allows us to compare our results with previously published results that looked at uptake at 1.5–3 min [2, 17, 19].

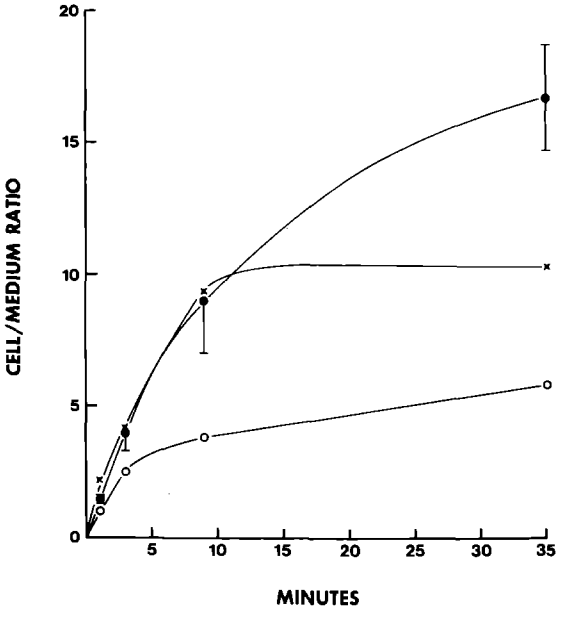

Fig. 1. Cell-to-medium ratios of 5.42 µ*M* melphalan transport into normal human bone marrow (●———●) and human tumor cells incubated in PAG medium without amino acids (means ±SE). The data represent results recorded in 10 individuals and in patient A (○———○) and patient B (×———×). Nonspecific adsorption of labeled melphalan was estimated by measuring drug binding at 0–4 °C following exposure of the cells to melphalan for periods up to 35 min. The determination of the amount of extracellular melphalan trapped in the cell pellet was made by incubating the cells with [carboxy-^{14}C]inulin. Corrections for these two factors were made. The intracellular water volume was estimated using tritiated water

In normal human bone marrow cells, after 3 min of incubation the uptake of melphalan was decreased in the presence of a 1 m*M* concentration of several amino acids in this order: tyrosine > glutamine > leucine > asparagine > methionine. No significant effect was observed with 1 m*M* glycine or alanine. (Table 2). After 35 min of incubation all amino acids tested, except glycine, cysteine, and glutamine, significantly decreased the intracellular concentration of melphalan.

In tumor cells from patient A (breast tumor), melphalan uptake was partially reduced after 3 min in the presence of 1 m*M* amino acids according to this sequence: leucine > tyrosine > methionine > cysteine. No significant effect was observed with glutamine, glycine, asparagine, or alanine at 1-m*M* concentrations. After 35 min of incubation, leucine, tyrosine, methionine, glutamine and asparagine significantly decreased the intracellular concentration of melphalan (Table 2).

In tumor cells from patient B (adenocarcinoma of unknown etiology), 1 m*M* glutamine, leucine, and tyrosine significantly decreased the uptake of melphalan after 3 min. Methionine, asparagine, alanine, and cysteine decreased melphalan uptake to a lesser, but not significant, extent. After 3 min, the intracellular melphalan concentration was reduced by glutamine, leucine, methionine, and asparagine. No significant effect was observed with alanine, tyrosine, glycine and cysteine at 35 min (Table 2).

In bone marrow cells, aminoisobutyuric acid (AIB), a specific substrate of the A (alanine-preferring) neutral

Table 2. Effect of several amino acids on the intracellular concentration of 5.42 μ*M* melphalan in human bone marrow cells and human tumor cells

	Human cells											
	3 min						35 min					
Amino acid (1 m*M*)	Bone marrow		Tumor A		Tumor B		Bone marrow		Tumor A		Tumor B	
Glutamine	10± 6***	(6)	71±12	(6)	19± 3**	(4)	59±18	(6)	45± 7***	(6)	24± 1*	(2)
Leucine	21± 6***	(10)	47± 4***	(6)	28± 5***	(6)	16± 2***	(9)	34± 7***	(6)	28± 8**	(6)
Tyrosine	8± 1***	(6)	57±13***	(6)	39±12***	(6)	42±13***	(8)	37±10***	(6)	74±23	(6)
Methionine	58±10***	(8)	61± 6***	(6)	50±24	(4)	15± 4***	(7)	39± 5**	(6)	45± 1***	(3)
Glycine	111±18	(2)	87±15	(6)	92± 1	(4)	127±26	(6)	110±38	(5)	131±15	(4)
Asparagine	34±22**	(6)	90± 3	(5)	56±11	(3)	31± 6***	(6)	78±13*	(6)	56±16***	(4)
Alanine	135± 1	(4)	100± 8	(6)	59±18	(4)	61± 6***	(4)	83± 3	(6)	84± 1	(4)
Cysteine	160±25**	(6)	65±16**	(6)	67± 7	(4)	123±31	(7)	71±17	(6)	84±41	(4)

[a] All values expressed as percentage of control ±SE; the number in parentheses represent the number of assays in eachcase
* P≤.05; ** P≤0.025; *** P≤0.01 (Student's *t*-test)

amino acid transport system, at 1–50 m*M* had no significant effect on the uptake of melphalan at 3 min (Fig. 2A). In human tumor cells, AIB at these concentrations had no significant effect in patients A, B or D at 3 min. However, AIB significantly reduced the uptake of melphalan in patient C.

The intracellular concentration of melphalan is significantly reduced after 35 min by at least one concentration of AIB in human bone marrow cells and in tumor cells of patients B and C (Fig. 2B).

BCH (2 m*M*), which is a specific substrate of the sodium-independent L system, significantly reduced the uptake of melphalan by bone marrow cells after 3 min of incubation. BCH at this concentration did not reduce melphalan uptake by the tumor cells except in those of patient C at 3 min (Fig. 3A). In particular, in the presence of

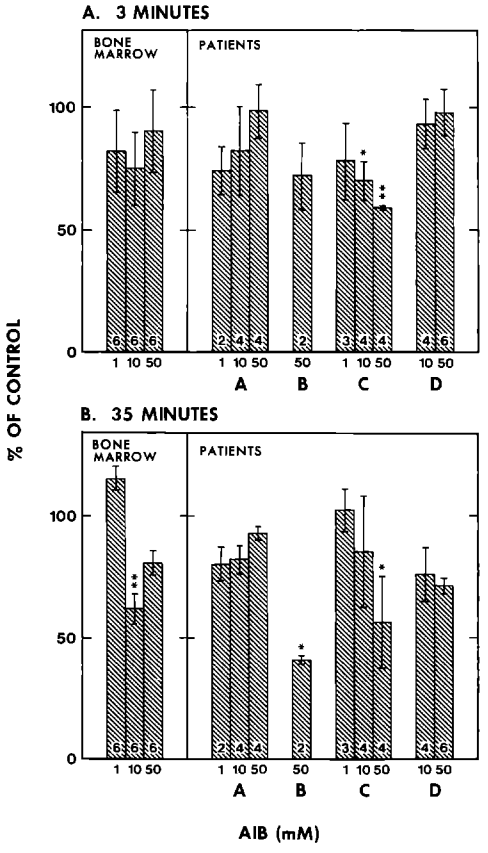

Fig. 2. Effect of AIB on the intracellular concentration of melphalan in human bone marrow cells and in human tumor cells incubated with 5.42 μ*M* melphalan at 37 °C. *Numbers* at base of each *bar* indicate number of experimental trials. * $P \leq 0.05$; ** $P \leq 0.01$

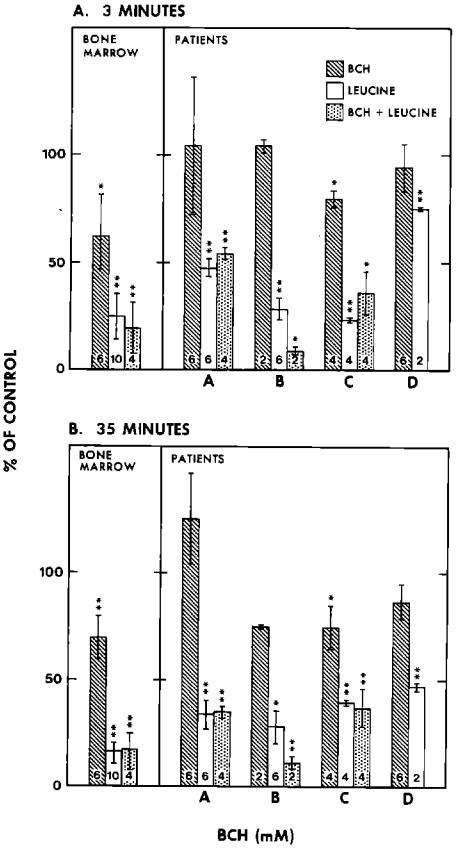

Fig. 3. Effect of BCH (2 m*M*), leucine (1 m*M*), and the combination BCH (2 m*M*) and leucine (1 m*M*) on the intracellular concentration of melphalan in human bone marrow cells and in human tumor cells incubated with 5.42 μ*M* melphalan at 37 °C. *Numbers* at base of each *bar* indicate the number of experimental trials. * $P \leq 0.05$; ** $P \leq 0.01$

Table 3. Effect of several amino acids on the cytotoxicity of melphalan in human bone marrow cells and one human breast tumor (tumor E) during a 35-min incubation at 37 °C

Malphalan (MLN)		Human bone marrow cell CFU-C Pecentage of Control ± SE	Human breast tumor (tumor E) HTSCA
1.2 μ*M*		4± 3	N.D.
6.1 μ*M*		N.D.	41±5
MLN + leucine	(0.1 m*M*)	53± 8	N.D.
	(1.0 m*M*)	80± 7	85±4
MLN + glutamine	(0.1 m*M*)	15±15	N.D.
	(1.0 m*M*)	43±19	57±5
MLN + tyrosine	(0.1 m*M*)	23±12	N.D.
	(1.0 m*M*)	45±19	N.D.
MLN + methionine	(0.1 m*M*)	11± 8	N.D.
	(1.0 m*M*)	38±23	N.D.
MLN + glycine	(0.1 m*M*)	4± 1	N.D.
	(1.0 m*M*)	5± 2	N.D.
MLN + asparagine	(0.1 m*M*)	10± 5	–
	(1.0 m*M*)	20± 4	38±6
MLN + alanine	(0.1 m*M*)	17± 8	N.D.
	(1.0 m*M*)	26±13	N.D.
MLN + cysteine	(0.1 m*M*)	5± 5	N.D.
	(1.0 m*M*)	4± 1	N.D.
MLN + BCH	(2 m*M*)	17± 4	55±2
MLN + AIB	(50 m*M*)	24± 3	66±4

2 m*M* BCH at 3 min melphalan uptake was 99% of control in tumor cells from patient F in which red blood cells were removed by centrifugation on Ficoll–Hypaque reagent. In addition, 1 m*M* leucine reduced melphalan uptake to 39% of control at 3 min in tumor cells from patient F. After 35 min of incubation of drug with human bone marrow cells the intracellular concentration of melphalan was approximately 70% that of the control in the presence of BCH. At 35 min 2 m*M* BCH significantly reduced the intracellular concentration of melphalan in tumor cells from patient C (Fig. 3 B) and patient F (52% of control). Melphalan (5.42 μ*M*) uptake into murine P388 cells separated from red blood cells by Ficoll–Hypaque reagent was linear up to 3 min. In the presence of 2 m*M* BCH melphalan uptake was approximately 55% of control. In the murine P388 leukemia cells in which red blood cells were removed by ammonium chloride lysis, the uptake of melphalan was also linear up to 3 min. In the presence of 2 m*M* BCH melphalan uptake was approximately 75% of control in murine P388 leukemia cells after ammonium chloride lysis.

Thin-layer chromatography suggests that melphalan is not metabolized after 35 min of incubation with human bone marrow or tumor cells from untreated patients in control samples. It should be noted, however, that this did not hold true for the one patient (C) who had already received multiple types of chemotherapy. In this case, it appears that melphalan was metabolized during this time. The separation between melphalan (R_f 0.9) and its metabolite (R_f 0.6) was not good however, but at least 50% of the radioactivity present was metabolized. A similar metabolite has been reported to be dihydroxymelphalan by Suzukake et al. [14].

In the CFU-C system, leucine, glutamine, tyrosine, methionine, alanine, and asparagine inhibit melphalan cytotoxicity. BCH (2 m*M*) slightly but significantly decreases melphalan cytotoxicity (Table 3). Linear regression analysis of the results of melphalan transport at 35 min in the presence or absence of each of ten amino acids, including 2 m*M* BCH and 50 m*M* AIB (y-axis), with corresponding cytotoxicity results (x-axis) demonstrated a correlation coefficient of 0.786 ($y = 103 - 1.35x$) which is significant ($p < 0.005$).

According to the HTSCA in patient E, 6.1 m*M* melphalan reduced the number of colonies to 40% of control (control plates had 473 ± 58 colonies). After a 35-min incubation, leucine, AIB, and to a lesser extent, glutamine and BCH, reduced the cytotoxicity of melphalan against this tumor (Table 3).

Discussion

In freshly obtained human cells, 5.42 μ*M* melphalan appears to be concentrated 6- to 17 fold in cells incubated in medium without amino acids. This observation correlates well with the findings of earlier studies by Goldenberg et al. [7, 8] and Vistica [17, 18, 20]. In human tumor cells (patients A and B), normal human bone marrow cells, and murine P388 leukemia cells, melphalan transport was approximately linear for at least 3 min. We chose 3 min as a time point to investigate influx and 35 min as a time point to investigate steady-state kinetics.

Amino acids reduced melphalan uptake in normal human bone marrow and tumor cells. Leucine, glutamine, tyrosine, and methionine gave the best reduction at 3 min, although there is some variation between the two tumor samples. After 35 min, several amino acids (such as methionine, leucine, tyrosine, and alanine) diminished the uptake and/or increased the efflux of melphalan as previously described [3]. Glycine generated neither of these effects in any of the human cells studied. No significant differences were noted between bone marrow and tumor cells with the amino acids used. The complexity and overlapping of the different amino acid transport systems complicates identification of the systems implicated in melphalan

transport. Leucine, glutamine, and alanine are transported to varying degrees by the L, A, and ASC systems, while methionine and tyrosine are transported by at least the L and A systems [4, 5].

The use of specific substrates yields interesting information. AIB generally had no effect on the uptake of melphalan at 3 min by either human bone marrow or tumor cells from previously untreated patients, suggesting that system A is not involved in melphalan uptake into these cells. In patient C, however, AIB either significantly altered melphalan uptake or altered its metabolism. Since chromatographic results are only available on control samples, it is not possible to clarify this situation.

BCH (2 m*M*) inhibited the uptake of melphalan to 63% of control in bone marrow cells at 3 min. In freshly obtained human tumor cells, however, it generally had no significant effect, except in patient C. Here again, either a change in the uptake of melphalan or an alteration in its metabolism might be responsible for this result. At 35 min, the BCH effect persisted in the bone marrow cells. In human tumor cells, BCH significantly decreased the intracellular concentration of melphalan in tumor cells from patients C and F at 35 min.

The dose-limiting toxicity of melphalan is leukopenia, probably as a result of damage to progenitor cells found in the bone marrow. These cells, which represent approximately 0.1% of all nucleated bone marrow cells, give rise to colonies in the CFU-C assay. The interaction of melphalan in the presence or absence of amino acids with the heterogenous population of nucleated bone marrow cells as determined by transport at 35 min appears to correlate well with cytotoxicity as measured by the CFU-C assay. For example, BCH diminished the cytotoxicity of melphalan in CFU-C to an extent that corresponded well with its ability to decrease transport of melphalan at 35 min in the entire heterogeneous population of bone marrow cells. This suggests that melphalan transport with and without amino acids at 35 min by the heterogenous nucleated bone marrow cell population is not significantly different from the drug's transport with or without amino acids at 35 min by the progenitor cells committed to form colonies in the CFU-C assay.

To correlate the transport results obtained with tumor cells and with the human tumor stem cells which can grow in culture, we used the human tumor stem cell assay. Insufficient growth in tumor cells from patient B, the presence of many clumps in tumor cells from patients A and C, plus too high a concentration of melphalan in tumor cells from patient D, preclude direct comparison of the results obtained in the transport studies with those obtained in the cytotoxicity studies. In tumor cells from patient E, leucine assures the best protection against melphalan toxicity, while BCH minimally alters melphalan cytotoxicity. Transport studies were not performed in tumor cells from patient E, since only 20% of the entire cell population were tumor cells. However, the cytotoxicity study with tumor cells from patient E correlates reasonably well with our transport studies of tumor cells from patients A, B, C, and D.

In comparison with murine tissue and human tumor cell lines, freshly isolated human bone marrow and tumor cells transport melphalan in a slightly different fashion. BCH (2 m*M*) had no effect on the cytotoxicity of melphalan in murine bone marrow cells and it decreased melphalan uptake to 50% of control in L1210 murine leukemia cells and MCF-7 human breast cancer cells in culture [2, 16]. In our study, BCH at this concentration slightly decreased melphalan uptake and cytotoxicity in human bone marrow cells, while BCH had no effect on drug uptake at 3 min into freshly isolated human tumor cells from patients who had not previously received chemotherapy. The presence of inflammatory or mesothelial cells in human effusions cannot explain this difference, because the percentage of tumor cells in patient A was greater than 90%. It is possible that ammonium chloride lysis selectively damaged these cells in such a fashion that only the BCH component of melphalan transport was altered. The results with murine P388 leukemia cells suggest that ammonium chloride lysis may diminish melphalan transport by a BCH component, but it does not abolish this component. Also, BCH had no effect on patient F's tumor cells, which were separated from red blood cells by Ficoll – Hypaque reagent. More recent studies show that the L system changes in activity during the initial 24–48 h in primary hepatocyte cultures [10, 11, 21]. Similar changes could also be the reason for the different effect of BCH in freshly isolated tumor cells compared with human tumor cell lines in culture. However, it will be necessary to verify that there is not a BCH component of melphalan uptake with more samples of freshly obtained human tumor cells not exposed to ammonium chloride lysis.

Results obtained from these studies with human cells suggest the existence of a minor BCH-sensitive component of melphalan transport and cytotoxicity in nucleated human bone marrow cells. In freshly obtained untreated human tumor cells from four patients there was not a BCH-sensitive component of melphalan uptake. These results are slightly different from those obtained by Vistica [16] with murine bone marrow and L1210 leukemia cells or by Begleiter et al. with human tumor cell lines [1, 2]. This suggests that there might be minor but potentially significant differences in melphalan transport amongst species and between freshly obtained human tumor cells and human tumor cell lines in continuous culture. Further studies will be necessary to determine whether the BCH-sensitive component of melphalan uptake is uniformly absent in freshly isolated human tumor specimens from previously untreated patients.

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