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Specificity of the suppression of metastatic phenotype by tyrosine and phenylalanine restriction

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Amino acid restriction modulates tumor growth, although effects on metastasis are poorly documented. We demonstrate that low levels of tyrosine (Tyr) and phenylalanine (Phe) suppress metastasis of B16-BL6 melanoma and that these effects are specific to these two amino acids. Weight loss and sustained low body weight in mice fed low Tyr and Phe diet do not contribute to the antimetastatic effects. Furthermore, methionine (Met) restriction, which decreased survival of mice inoculated i.p. with B16 melanoma, only slightly inhibited spontaneous metastasis compared to the dramatic inhibition during Tyr and Phe restriction. Tyr and Phe restriction inhibited spontaneous metastasis by impairing the ability of tumor cells to establish metastatic foci and not via differential tumor cell removal from the blood. Spontaneous metastasis is blocked by Tyr and Phe intervention even in mice with established lymph node tumors. Tumors isolated from mice fed low Tyr and Phe diet reinoculated into mice fed normal diet exhibited lower experimental metastatic potential, reflected by decreased formation of lung tumor colonies and increased survival of inoculated mice. This decrease in metastatic potential is not associated with tumor chemosensitivity. These findings indicate that Tyr and Phe restriction could become an important adjuvant to effective melanoma treatment.

Introduction

Nutritional factors play a role in cancer treatment [16, 65]. Manipulation or intervention, typically via nutrient restriction or supplementation, can alter tumor growth [43], morphology [67], membrane characteristics [46, 67], metastatic potential [10, 11], and host immune responses [8, 9, 30]. Perturbations in the nutrient availability of carbohydrates [16, 54], fats [4, 7, 33, 59, 62], vitamins [22, 29, 44], and other trace elements [42, 53, 65] affect a wide range of tumor systems. Additionally, some tumors have specific requirements for amino acids that are different from those of normal cells [43, 46, 61]. This differential requirement for particular amino acids has been exploited to preferentially inhibit these tumors during dietary amino acid deprivation [12, 43], via enzymatic destruction [6] or analog substitution [32, 35, 61, 66]. The clinical development of L-asparaginase has resulted in an important drug for treatment of leukemia [6, 55, 58].

Our research has focused on the role of two amino acids, tyrosine (Tyr) and phenylalanine (Phe), as an adjunct to enhance treatment of melanoma. Tyr and Phe restriction is effective not only against melanoma [17, 46] but also against several other murine and human tumor systems [1, 36, 50, 51, 64]. Pigmented melanomas

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have a dual requirement for these amino acids in both protein and melanin synthesis. We previously showed that Tyr and Phe restriction via diet [2, 40] or enzyme degradation [18, 19] inhibits melanoma growth [18, 37, 39–41], increases survival of tumor-bearing mice [40, 41, 48], enhances the therapeutic effectiveness of levodopa methylester [41, 48], and inhibits spontaneous metastasis [2]. Administration of tyrosine phenol-lyase and phenylalanine ammonia-lyase, specific Tyr- and Phe-degrading enzymes, decreases plasma levels of these amino acids in mice and inhibits growth of established subcutaneous melanoma tumors without compromising body weight [18, 19, 39]. These observations allude to the specificity of this amino acid restriction. Restriction of Tyr and Phe in the diet from 0·3 per cent Tyr and 0·6 per cent Phe to 0·04 per cent and 0·08 per cent, respectively, moderately increases median survival time of mice bearing i.p. B16 melanoma up to 42 per cent [40, 41]. Furthermore, we found this restriction to enhance the antitumor activity of levodopa methylester [41, 48, 49] and, in combination with sodium ascorbate supplementation, to more than double the survival of mice bearing s.c. B16 melanoma tumors [48].

Clinically, the more pertinent question is whether Tyr and Phe restriction has an effect on metastasis, the major cause of lethality in most cancers [34, 47]. Our studies have shown that Tyr and Phe restriction dramatically inhibits spontaneous metastasis of the highly invasive and metastatic BL6 variant of B16 melanoma and that this inhibitory effect is applicable to other tumor systems including Lewis lung carcinoma in mice and RT74bs hepatocarcinoma in rats [2]. Tyr and Phe restriction also inhibits brain metastasis of L1210 leukemia [51]. In this paper we demonstrate that these antimetastatic effects are specific to Tyr and Phe restriction, and that they are due to impaired ability of the tumor cells to establish metastatic foci. Tumor cells selected from mice restricted in Tyr and Phe, and also exhibiting a reduced metastatic phenotype, did not show differences in sensitivity to various chemotherapeutic agents.

Methods and materials

Animals and diets

Specific-pathogen-free, female, B6D2F$_1$ mice were purchased from Jackson Laboratories, Bar Harbor, ME, at 6 weeks of age. C57BL/6 mice were bred locally at Washington State University from brother–sister mating. Male and female, C57BL/6 mice were used in some experiments. All mice were housed in the Wegner Hall Vivarium at Washington State University, which is accredited by the American Association for Accreditation of Laboratory Animal Care. Mice were housed in polycarbonate cages with hardwood shavings. Mice at least 3 months old were used for experiments involving deficient diets. Unless otherwise noted, mice were fed the test diets 2 weeks before tumor cells were inoculated. When food consumption was determined, mice were housed singly.

Mice were fed one of three crystalline amino acid diets (BioServ, Inc., Frenchtown, NJ). The composition of the nutritionally complete diet (normal diet) is fully characterized elsewhere [41]. The three diets were different only in their amino acid content. All diets were isonitrogenous, provide 4 Cal/g, and consist of 15·7 per cent amino acids, 10 per cent fat, 61·9 per cent carbohydrate, and 5 per cent fiber. Normal diet contained 0·3 per cent Tyr, 0·6 per cent Phe, 0·4 per cent Met, 0·2 per cent cystine and 1·04 per cent serine. The Tyr- and Phe-restricted diet (low Tyr

and Phe diet) contained 0·04 per cent Tyr and 0·08 per cent Phe. The Met-restricted diet (low Met diet) contained 0·1 per cent Met and no cystine or serine. The levels of glycine and glutamic acid were altered to make the diets isonitrogenous. Major constituents and Tyr or Met content, as appropriate, were assayed by BioServ and did not vary by more than 5–10 per cent. Body weights and food consumptions were determined daily. The accuracy of food consumption determination was ±0·5 g [41].

Tumors and culture conditions

The well-characterized B16 murine melanoma and its highly invasive and metastatic B16-BL6 variant [24], originally obtained from the Mason Research Institute, Worcester, MA, were grown *in vitro* in Dulbecco's modified Eagle's complete minimal essential medium supplemented with 10 per cent heat-inactivated fetal bovine serum, sodium pyruvate, nonessential amino acids, 2-fold vitamin solution (Gibco Laboratories, Grand Island, NY), L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (DME). Cultures were incubated as monolayers on tissue culture plastic in a humidified atmosphere of 5 per cent CO_2/95 per cent air at 37°C and harvested with 4 mM EGTA to avoid the membrane-altering effects of trypsin.

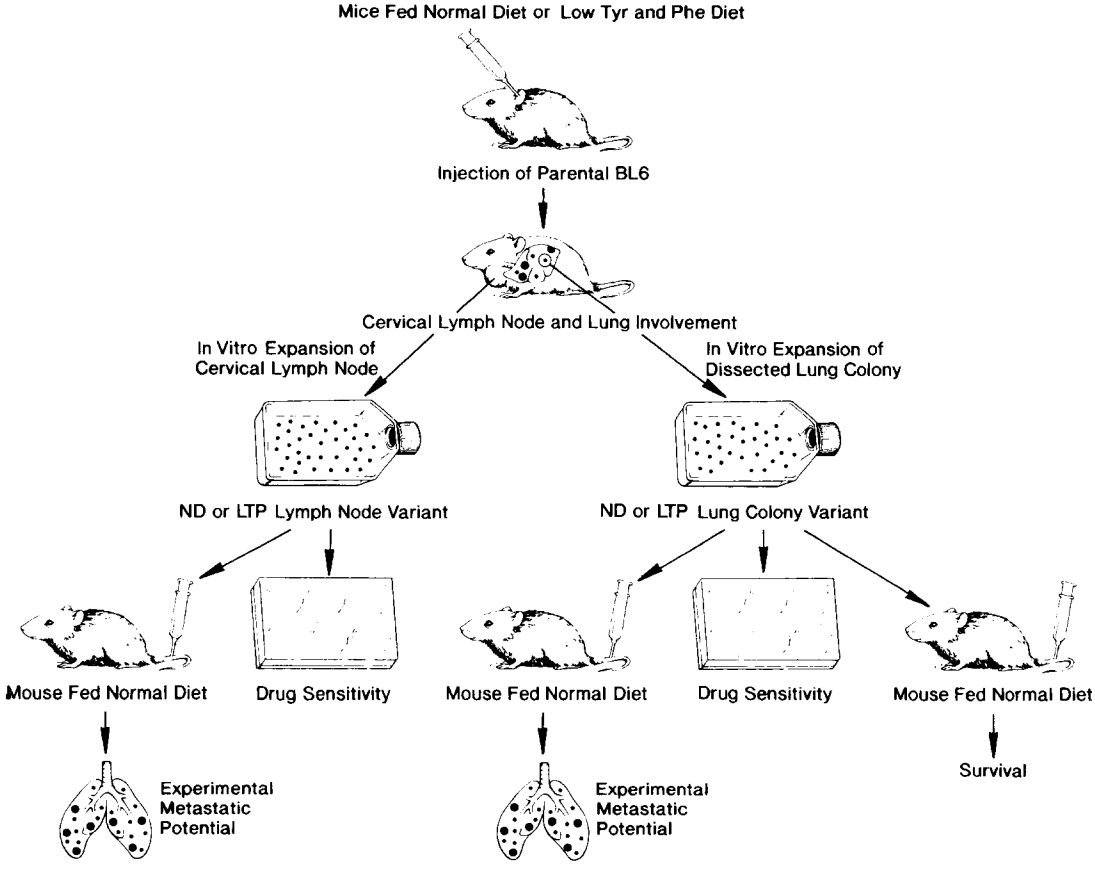


Figure 1. Flow diagram describing the isolation and characterization of ND and LTP dietary lymph node and lung colony variants.

Figure 1 shows the isolation of the tumors (variants) from female, B6D2F$_1$ mice and the experimental protocol to evaluate the effects of Tyr and Phe restriction on tumor heterogeneity and metastatic phenotype. B16-BL6 served as the parental source of the isolated tumors. *In vitro* passages 2–9 of B16-BL6, after *in vivo* passage as a s.c. tumor, were used for experimentation. No significant differences in lung colonizing potential and drug sensitivity were noted among these passages. Forty dietary-modulated variants of the B16-BL6 melanoma parent were isolated from spontaneous cervical lymph node metastases and individual spontaneous lung metastases. For purposes of isolation, mice were fed either normal or low Tyr and Phe diet for 2 weeks and subsequently inoculated subcutaneously into the ventral surface of the pinna of the ear with 5×10^4 viable B16-BL6 cells in 0·02 ml Ca^{2+} and Mg^{2+}-free balanced salt solution (CMF). Tumor-bearing ears were amputated under chloral hydrate anesthesia when the primary tumor reached 0·5 cm in diameter. Individual lymph node metastases from 10 mice per dietary group were excised, separated from extraneous tissue, minced, and repeatedly digested in 0·1 per cent collagenase. Contaminating host cells were removed from the tumor cell suspension by centrifugation on a continuous metrizamide gradient [60]. Ten individual, isolated lung tumor colonies of approximately the same small size range were dissected under a microscope from nine mice fed normal diet and from five mice fed low Tyr and Phe diet. Variants isolated from mice fed the normal diet are designated as 'ND' and variants isolated from mice fed the low Tyr and Phe diet are designated as 'LTP'. ND lymph node and lung colony variants were isolated 27–75 days postinjection. LTP variants were isolated 36–85 days after inoculation of B16-BL6 into the ear. Both groups of dietary variants showed heterogeneity for pigmentation. All isolated variants were expanded *in vitro* and stored in liquid nitrogen, which does not alter metastatic characteristics of tumor cells [52]. Variants were always initiated from freezer stock and never maintained *in vitro*. Culture conditions were identical to those of B16 and B16-BL6.

Effect of dietary Met restriction on primary tumor growth and survival

B16 melanoma was used for experiments involving primary tumor growth and survival. Female, B6D2F$_1$ mice were fed low Met diet 14 days before i.p. inoculation of 1×10^6 B16 melanoma cells.

Assessment of spontaneous metastasis

Anesthetized mice were injected into the ventral surface of the pinna of the ear with viable B16-BL6 tumor cells in a volume of 0·02 ml of CMF. Ears of anesthetized mice were amputated by electrocautery surgery when the primary tumor was approximately 0·5 cm in diameter. Mice were sacrificed when moribund. Upon necropsy, cervical lymph node tumors were weighed and lungs were collected and fixed in Bouin's fixative to facilitate visualization of surface lung tumor colonies. Tumor colonies were counted with the aid of a dissecting microscope.

Effect of food restriction on spontaneous metastasis. To determine the effect on metastasis of the weight loss induced by the low Tyr and Phe diet, female, B6D2F$_1$ mice were singly housed in polycarbonate cages and divided into three groups: (1) mice fed normal diet *ad libitum* (control group), (2) mice fed low Tyr and Phe diet *ad libitum*, and (3) mice pair-fed normal diet at a level that induced a weight loss

equivalent to that of the low Tyr and Phe diet *ad libitum* group and that maintained the lower weight after the transient weight loss period (normal diet food-restricted group). Mice were inoculated with 1×10^5 B16-BL6 melanoma cells.

Effect of Tyr and Phe intervention subsequent to lymph node involvement. To examine the therapeutic effects of low Tyr and Phe diet on spontaneous metastasis in mice with established lymph node tumors, male or female, C57BL/6 mice were fed normal diet for 2 weeks and inoculated with 5×10^4 B16-BL6 tumor cells. Cervical lymph node tumor involvement was estimated at the time of primary tumor removal by measuring the involved area with calipers in two perpendicular planes. The diameter values were averaged, and volume was calculated using the formula $V = \pi r^3$. One group of mice (switch group) was taken off normal diet and fed the low Tyr and Phe diet at the time of ear amputation to evaluate the effectiveness of Tyr and Phe restriction to block further metastasis after establishment of lymph node tumors. Control groups were fed either normal or low Tyr and Phe diets throughout the study.

Presence of pulmonary micrometastases

Female $B6D2F_1$ mice were fed either normal or low Tyr and Phe diet and inoculated into the pinna of the ear with 5×10^4 viable B16-BL6 tumor cells. After lymph node involvement in these mice (donor mice), lungs were excised and separated into five lobes. Individual lobes were implanted s.c. into the dorsal hips of a second group of anesthetized, non-tumor-bearing mice fed normal diet (recipient mice). All lobes were implanted into recipient mice 33 days after tumor inoculation into the ears of donor mice. Host survival, tumor pigmentation characteristics, and the presence of residual lung tissue at the time of necropsy were noted.

Clearance of ^{125}I-labeled B16-BL6 and Tyr/Phe-modulated cells [13, 15, 21, 31]

Cell cultures were labeled with 0·5 μCi [^{125}I]5-iodo-2′-deoxyuridine/ml of DME for 24 h. Labeled cells were harvested and injected intravenously into the tail veins of mice fed normal diet. Three days prior to injection the drinking water of the mice was supplemented with 0·1 per cent sodium iodide to prevent thyroidal accumulation of radioiodine. Mice received 2×10^4 viable cells in a volume of 0·2 ml CMF, averaging approximately 20 000 CPM per inoculation. Six mice were sacrificed by cervical dislocation at intervals ranging from 15 min to 48 h. Lung, liver, spleen, and kidneys were collected from each animal at each time point. Organs were extracted in FAA fixative (10 ml formaldehyde, 90 ml 80 per cent ethanol, 5 ml glacial acetic acid) for 3 days to rinse away free isotope and precipitate bound activity. Fixative was changed daily. Cell suspensions and organs were counted on a gamma scintillation counter; counts were corrected for radioactive decay.

Injected mice were divided into three treatment groups. Control mice were injected with either viable B16-BL6 melanoma cells or cells killed by heating at 70°C for 10 min (HK-BL6). A third group of mice was injected with B16-BL6 tumor cells which were passaged *in vivo* as a s.c. tumor in the dorsal hip of mice fed low Tyr and Phe diet (LTP1) and which demonstrated a significant ($P < 0{\cdot}05$) decrease in lung colonizing potential after i.v. inoculation (manuscript in preparation).

Experimental metastasis of tumor cell variants

As described [2, 37], unanesthetized mice, fed the normal diet, were inoculated i.v. with 2×10^4 viable B16-BL6, ND variants, or LTP variants in 0·2 ml CMF into the lateral tail vein into female, $B6D2F_1$ mice. (See section entitled 'Tumors and culture conditions' for isolation of ND and LTP variants.) The animals were killed 3 weeks after inoculation and examined for pulmonary and extrapulmonary metastases. Lungs were removed, weighed, and fixed in Bouin's solution to facilitate visualization of superficial tumor colonies. The numbers of tumor nodules on each of the five lobes were counted twice under a dissecting microscope.

Survival of mice inoculated with tumor cell variants

Female, $B6D2F_1$ mice were fed normal diet and inoculated with parental B16-BL6 and selected ND and LTP lung colony variants which demonstrated representative experimental metastatic potentials. Ten mice per treatment group were inoculated i.v. into the lateral tail vein with 2×10^4 viable tumor cells in a volume of 0·2 ml CMF. Survival of mice was determined from the date of tumor injection to the date of sacrifice of moribund mice.

In vitro *assay for drug sensitivity of tumor cell variants*

Drug sensitivities of the tumor cells were assayed in liquid cell culture DME (Whittaker M.A. Bioproducts, Walkersville, MD) in six-well tissue culture plates according to the method of Talmadge *et al.* [63]. Three hundred single, viable tumor cells were exposed to the following drugs: adriamycin (ADR) at 0, 2, 4, 6, 8, 10 ng/ml; dihydroxyphenylalanine methyl ester (DOPA) at 0, 50, 100, 150, 200, 250 μg/ml; bleomycin (BLEO) at 0, 500, 1000, 1500, 2000, 2500 ng/ml; and vincristine (VCR) at 0, 20, 30, 40, 50, 60 ng/ml. Drug concentration ranges were determined in preliminary experiments. Following a 12-h incubation in the presence of drug, plates were rinsed twice with CMF to remove residual drug, supplemented with 4 ml of fresh DME, and incubated an additional 8–10 days to allow maximum outgrowth of surviving cells. Resulting colonies were rinsed, fixed in absolute methanol, stained with 2 per cent methylene blue, and colonies containing at least 50 cells were enumerated with the aid of an automated colony counter (NBS Model C111, New Brunswick Scientific Co, Inc., Edison, NJ). Drug concentrations which inhibited colony formation by 50 per cent (IC_{50}) values were calculated. Assays were run in triplicate and IC_{50} values were averaged. For randomly selected variants, assays were repeatable (data not shown).

Statistical analyses

To determine the effectiveness of Tyr and Phe restriction after establishment of lymph node metastases, the Kruskal–Wallis test after non-parametric one-way analysis of variance was used to confirm differences between experimental groups. The Wilcoxon rank-sum or Mann–Whitney U test was used to compare differences in the numbers of lung colonies between two experimental groups. Analyses were considered significant at $P < 0{\cdot}05$. Linear regression analysis was used to correlate the numbers of pulmonary tumor colonies and the volume of lymph node tumors at the time of ear amputation and the weight of lymph node tumors at necropsy. The analysis revealed a lack of correlation in these parameters. Comparisons of lung colonizing potentials and ADR drug resistance between the 40 isolated dietary variants and the B16-BL6 parent were analyzed with the Wilcoxon two-sample test.

For comparisons of the two dietary groups an unpaired t-test was applied to the transformed data. Because of unequal variances in the data among the variants, the data were transformed with either a log (y) transformation, for lymph node variants, or a log ($y+1$) transformation, for lung colony variants. Differences in survival and drug sensitivity were respectively determined by the Kruskal–Wallis test and the Wilcoxon two-sample test. Levels of significance were set at $P<0{\cdot}05$. Differences in body weights, food consumptions, tumor weights, and distributions of ^{125}I-labeled cells were analyzed with an unpaired Student's t-test and were considered significant at $P<0{\cdot}05$.

Results

Experiments were designed to address three main questions: (1) the specificity of Tyr and Phe restriction for modifying metastasis of B16-BL6 murine melanoma, (2) the ability of Tyr and Phe restriction to inhibit metastasis after tumor establishment, and (3) the mechanism underlying the ability of Tyr and Phe restriction to inhibit metastasis.

Specificity of Tyr and Phe restriction for modifying metastasis of B16-BL6 murine melanoma

The role of the lower body weight of mice fed low Tyr and Phe diet was examined by inducing weight loss through food restriction of mice fed normal diet. To address the selectivity of Tyr and Phe restriction for modifying metastasis, we compared the effect of Met restriction on primary B16 melanoma tumor growth, host survival, and B16-BL6 spontaneous metastasis. This amino acid was selected because melanoma cells exhibit Met dependence and because growth is inhibited in Met-depleted culture media or in media where Met is replaced by L-homocysteine (unpublished observations [27, 28]). This dependence has been exploited to inhibit a variety of tumors, including melanoma [12, 32, 35, 61, 66].

Effect of body weight loss and low Tyr and Phe diet on metastasis. Body weights and food consumptions before and after tumor inoculation are indicated in table 1. At the time of tumor inoculation, mice were 21 weeks of age. Initial body weights of mice in all three groups were not different ($P>0{\cdot}05$) and averaged approximately 23 g. Mice fed normal diet *ad libitum* maintained a fairly constant body weight of 22·5 g throughout the study. Mice fed the low Tyr and Phe diet initially lost weight during the first 10 days, after which body weight stabilized at approximately 90 per cent of control body weight. After tumor inoculation, body weight averaged 20 g. Body weights of the normal diet food-restricted group paralleled those of mice fed low Tyr and Phe diet *ad libitum*. To allow identical weight losses in group three, food was frequently restricted to 2 g/day of normal diet in this group. Except when mice were moribund, no visible physical signs of malnourishment (emaciation, greasy appearance or matted fur, tremors or humped behavior, inactivity, watery eyes) were noted in the normal diet food-restricted group, indicating general health status of the mice was not compromised.

Nutritional parameters of the mice fed the low Tyr and Phe diet were well within the ranges that have been observed in our other studies [2, 37, 38, 40, 41, 48]. Dietary restriction of Tyr and Phe is accompanied by respective 32 per cent and 11 per cent reductions in plasma concentrations of Tyr and Phe [40]. Plasma levels ($\pm$SD) decreased from $66\pm2\,\mu$M to $45\pm3\,\mu$M for Tyr ($P<0{\cdot}05$, Student's t-test) and

Table 1. Average body weights and food consumptions of female B6D2F$_1$ mice before and after B16-BL6 melanoma inoculation.

	Body weight (g ± SD)		Food consumption (g ± SD)	
Treatment group	Preinoculation	Postinoculation	Preinoculation	Postinoculation
Normal diet *ad libitum*	22·47 ± 0·17	22·61 ± 0·25	2·92 ± 0·08	2·80 ± 0·18
Low Tyr and Phe diet *ad libitum*	20·71 ± 0·62[a]	20·12 ± 0·16[a]	3·33 ± 0·58[a]	3·00 ± 0·32
Normal diet food-restricted	20·68 ± 0·74[a]	20·20 ± 0·65[a]	2·34 ± 0·40[a,b]	2·03 ± 0·44[a,b]

The preinoculation period encompasses the 18 days prior to tumor inoculation (days 0–18), the postinoculation period involves a 22-day interval from days 19 to 40. Mice were fed normal diet or low Tyr and Phe diet, as indicated. Each treatment group consisted of 30 mice.

[a] Significantly different ($P < 0·05$) from normal diet *ad libitum* group by Student's *t*-test.

[b] Normal diet food-restricted group significantly different ($P < 0·05$) from low Tyr and Phe *ad libitum* group by Student's *t*-test.

61 ± 1 μM to 51 ± 3 μM for Phe ($P < 0.05$, Student's *t*-test) when mice were switched from normal diet to low Tyr and Phe diet.

At necropsy, cervical lymph node tumors from mice fed low Tyr and Phe diet *ad libitum* were smaller ($P < 0.05$) than those excised from normal diet and normal diet food-restricted mice (table 2). Pulmonary colonization in low Tyr and Phe *ad libitum* mice was significantly different ($P < 0.0001$) from metastasis in the normal diet or the pair-fed, normal diet food-restricted groups (table 2).

Modulation of tumor growth, survival, and metastasis by a low Met diet. Mice were fed low Met diet to determine the effect on primary and metastatic melanoma growth of another amino acid restriction that also induced weight loss. Mice were given the low Met diet 14 days before tumor inoculation and throughout the remainder of the experiment. No differences were observed in food consumption compared to mice fed normal diet (data not shown). The mean plasma Met level ($\pm$SD) was reduced from 56 ± 4 μM to 28 ± 1 μM ($P < 0.05$, Student's *t*-test) at 14 days after initiation of the low Met diet.

Mice fed low Met diet lost 23 per cent of their original starting weight by day 14 and an additional 3 per cent after tumor inoculation. Thus, mice were relatively weight-stable at the time of tumor inoculation. Body weight loss was actually greater in mice fed the low Met diet than in mice fed the low Tyr and Phe diet. Mice lost a total of 26 per cent of their body weight when fed the low Met diet compared to 10 per cent weight loss in mice fed low Tyr and Phe diet (table 1). Met restriction, however, did not increase, but rather decreased, survival of i.p. B16 melanoma-bearing mice in spite of its pronounced inhibitory effect on primary tumor growth. Median survival decreased from 21 days (range 16–24 days) to 17 days (range 11–23), $P < 0.05$. The average tumor weight in mice fed the low Met diet was 1.7 ± 1.1 g compared to 6.4 ± 2.3 g in mice fed the normal diet, $P < 0.05$.

Although the low Met diet inhibited spontaneous metastasis of B16-BL6 melanoma (table 3), the effect was far less pronounced than the inhibitory effect of Tyr and Phe limitation on spontaneous metastasis (tables 2 and 5, ref. 2). This was

Table 2. Effect of weight loss on spontaneous metastasis of B16-BL6 melanoma and on weight of cervical lymph tumors at time of necropsy.

Treatment group	N[a]	Average weight of lymph node tumors at necropsy (g $\pm$ SD)	Number of lung colonies	
			Median	Range
Normal diet *ad libitum*	19 (30)	4.2 ± 1.7	>300	73>300
Low Tyr and Phe diet *ad libitum*	23 (30)	2.9 ± 0.8[b,c]	24[d]	5–189
Normal diet food-restricted	22 (30)	4.7 ± 1.1[b]	283	96>300

[a] N = number of mice that developed lymph node tumors. Number in parentheses is the number of mice inoculated with B16-BL6.

[b] Significantly different ($P < 0.05$) from normal diet *ad libitum* by the Student's *t*-test.

[c] Significantly different ($P < 0.05$) from normal diet food-restricted group by the Student's *t*-test.

[d] Significantly different ($P < 0.001$) from normal diet *ad libitum* and normal diet food-restricted groups by the Wilcoxon two-sample test.

also true even though the low Met diet also had a greater inhibitory effect on lymph node growth (see tables 2 and 3). Interestingly, lung tumor colonies in mice fed the low Met diet were well developed and as large or larger than the lung tumors in mice fed normal diet.

Ability of Tyr and Phe restriction to inhibit metastasis after tumor establishment

In this experiment we tested whether Tyr and Phe restriction could influence spontaneous metastasis in mice with established lymph node tumor involvement. As shown in table 4, the switch group exhibited an intermediate number of lung tumor colonies. Neither the volume of lymph node at primary tumor amputation nor the weight of lymph node tumor at death correlated with the number of lung tumor colonies formed ($R \leqslant 0{\cdot}4670$ for all experiments).

Table 3. Effect of low Met diet on spontaneous metastasis of B16-BL6 melanoma.

Group	No. of animals developing lymph node metastases	Weight of lymph node metastases at autopsy (g ± SD)	Number of lung metastases	
			Median	Range
Normal diet	10 (33)[a]	5·6 ± 2·6	>300	80>300
Low Met diet	12 (28)	1·7 ± 1·0[b]	234[c]	70>300

[a] Values in parentheses indicate number of inoculated animals developing a primary tumor in the pinna of the ear.

[b] Significantly different ($P<0{\cdot}05$) from mice fed normal diet by Student's *t*-test.

[c] Significantly different ($P<0{\cdot}05$) from mice fed normal diet by Mann–Whitney U-test.

Table 4. Effectiveness of Tyr and Phe limitation after B16-BL6 melanoma tumor establishment.

Dietary group	N[a]	Number of lung tumor colonies		Volume of lymph node at primary tumor amputation (cm^3 ± SD)	Weight of lymph node tumor at death (g ± SD)
		Median	Range		
Experiment 1 (females)					
Normal	14(14)	277	33>300	0·79 ± 0·72	6·48 ± 2·47
Switch	13(15)	95[b]	9>300	1·07 ± 0·71	4·04 ± 1·08[b]
Low Tyr and Phe	11(15)	9[c]	0–77	0·30 ± 0·23[b]	2·13 ± 0·93[c]
Experiment 2 (males)					
Normal	12(15)	156	53>300	0·32 ± 0·54	7·63 ± 2·29
Switch	11(13)	58[b]	4–288	0·22 ± 0·23	4·33 ± 1·38[b]
Low Tyr and Phe	8(10)	8[c]	0–93	0·26 ± 0·34	2·62 ± 1·50[c]

[a] N = number of mice that developed lymph node metastasis. The number in parentheses is the number of mice inoculated with B16-BL6.

[b] Significantly different ($P<0{\cdot}05$) from normal diet group in experiment.

[c] Significantly different ($P<0{\cdot}05$) from all groups in experiment.

Mechanism underlying the ability of Tyr and Phe restriction to inhibit metastasis
Presence of micrometastases in lungs from mice fed low Tyr and Phe diet. This experiment was designed to determine if the observed antimetastatic effects of Tyr and Phe restriction were caused by an impaired ability of B16-BL6 melanoma cells to establish secondary metastatic foci. The results are presented in table 5. Lung lobes, excised from lungs of mice fed low Tyr and Phe diet (LTP lobes), visually exhibited numbers of tumor colonies ranging from 0 to 3; whereas visible tumor involvement was greater in lungs excised from mice fed normal diet (ND lobes), 26–81 per lobe. Subcutaneous tumors resulted after implantation of all lobes; however, recipient mice implanted with LTP lobes lived 31 per cent longer compared to mice receiving ND lobe implants (mean = 58·0 vs 40·8 days, respectively). Subcutaneous tumors arising from implanted LTP lobes were generally gray in color and residual lung tissue was present. On the other hand, tumors arising from implanted ND lobes were heavily pigmented with no evidence of residual lung tissue. Final tumor weights were not significantly different.

Distribution of ^{125}I-labeled tumor cells. Experiments were designed to determine differential tumor cell fate and retention *in vivo* as possible mechanisms to explain the antimetastatic activity of dietary Tyr and Phe restriction. The dissemination and arrest after i.v. injection of tumor cells labeled with [^{125}I]UDR, which is not

Table 5. Presence of micrometastases of B16-BL6 melanoma embedded in the lung.

Number of visible tumor colonies on implanted lobes from donor mice	Recipient mice fed normal diet	
	Survival (days)	Final tumor weight (g)
Donor mouse fed low Tyr and Phe diet		
0	31[a]	0[a]
0	48	5·0
1	67	12·2
2	52	9·9
3	69	16·6
Mean	58·0[b]	10·9
SD	10·6	4·8
Donor mouse fed normal diet		
26	43	10·5
32	41	7·3
58	45	11·8
70	43	7·8
81	32	7·6
Mean	40·8	9·0
SD	5·1	2·0

[a] Mouse became ill and was sacrificed. Survival was not included in the calculation of the mean and standard deviation.

[b] Significantly different ($P < 0·05$) from mice receiving lung from mice fed normal diet.

Table 6. Clearance of ^{125}I-labeled control B16-BL6 and Tyr and Phe modulated cells from lung, liver, spleen, and kidney after i.v. inoculation.

Organ	Cell line	Sampling time (h)							
		0·25	1	2	4	6	12	24	48
Lung	B16-BL6	67·4	35·2	30·8	22·0	23·6	6·0	2·8	1·0
	LTP1	36·7	37·1	21·6	21·1	19·6	8·3	1·7	2
	HK-BL6	43·7	36·6	17·7	12·1	5·1[a]	0·8[a]	0·2[a]	0[a]
Liver	B16-BL6	1·1	3·3	1·3	0·6	0·9	0·2	0·1	0·1
	LTP1	1·5	2·1	1·4	1·1	1·0	0·4	0·1	0
	HK-BL6	0·6	1·4	1·2	1·1	0·6	0	0·3	0
Spleen	B16-BL6	0	0·2	0·2	0·1	0·2	0·1	0·1	0
	LTP1	0	0·1	0·7	0·2	0·2	0·1	0·1	0
	HK-BL6	0	0	0·1	0·1	0·2	0·1	0	0
Kidney	B16-BL6	0	0·5	0·1	0·1	0·1	0	0	0
	LTP1	0	0·1	0·1	0·1	0·1	0	0	0
	HK-BL6	0	0	0	0·1	0·1	0	0	0

Results are expressed as percentage of original cell suspension
[a] Significantly different from B16-BL6 control ($p < 0·05$, Student's t-test).

reutilized after cell death, in various organs through time is shown in table 6. Within 15 min most tumor cells were arrested in the lung. Comparable observations have been reported elsewhere [13, 15, 21, 31]. Roughly 40–70 per cent of the original cell suspensions were detected in the lung within this time interval. Few (< 2 per cent) cells were detected in the liver, spleen, or kidney. Tumor cell death or loss to systemic distribution, as evidenced by the loss of labeled cells, in the lung began within 2 h. Less than 10 per cent of the cells survived after 12 h. Only 1 per cent (approximately 200 cells) of the original cell suspension of B16-BL6 was retained in the lung after the last sampling time (48 h). These surviving cells produce approximately 35–55 lung metastases (refer to figure 2), indicating that very few cells injected i.v. into the bloodstream are responsible for the formation of metastases and the majority of cells do not survive to establish lung metastases [21]. Clearance of the Tyr- and Phe-modulated LTP1 cells did not differ from that of viable B16-BL6 cells. HK-BL6 cells were more rapidly eliminated from the lung. Less than 13 per cent of the injected tumor cells were retained in the lung after 4 h, and most of the dead HK-BL6 cells were eliminated within 12 h. Fidler [21] reported a similar loss of radioactivity within 8 h. Corresponding, delayed fluxes of labeled cells in the other organs were not obvious.

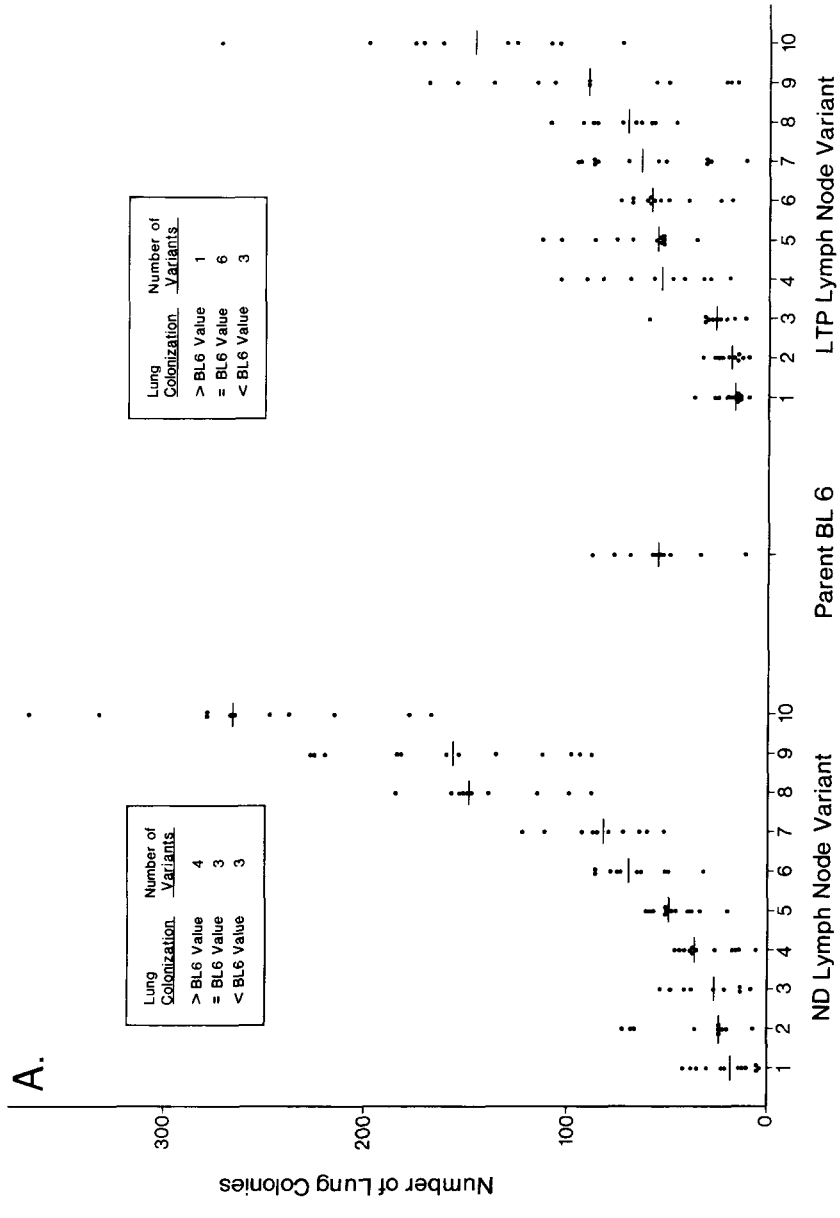
Lung colonizing potentials of the B16-BL6 melanoma parent and isolated lymph node and lung colony variants. B16-BL6 melanoma is a heterogeneous tumor and its metastatic potential is influenced by the *in vivo* host environment. To evaluate the degree to which Tyr and Phe limitation influences tumor heterogeneity and malignant phenotype we isolated and characterized 40 spontaneous cervical lymph node and individual lung metastases from mice fed normal diet or low Tyr and Phe diet, and inoculated with B16-BL6 melanoma. Lung colonizing abilities of the

isolated lymph node and lung colony variants and the parent B16-BL6 controls are characterized in figure 2. ND and LTP lymph node variants show variation in experimental metastatic potential when inoculated into mice fed normal diet (figure 2A), excluding a direct influence of the low Tyr and Phe diet on lung colonization. A trend ($P=0{\cdot}105$) toward suppressed metastatic phenotype is apparent when the LTP lymph node group is compared to the ND lymph node group. Additionally, while only three ND lymph node variants have metastatic potentials equal to that of B16-BL6, six out of 10 LTP lymph node tumors have the same metastatic potential as that of the B16–BL6 parent. Four out of 10 ND lymph node variants have metastatic potentials greater than the B16-BL6 parent ($P<0{\cdot}05$) compared to only one LTP variant. These data suggest less variation in the LTP variant group relative to the ND variant group.

The LTP lung colony variants demonstrate even less variation in experimental metastatic potential after inoculation into mice fed normal diet (figure 2B). In addition, these variants are less lung colonizing than either the ND lung colony variants or the B16-BL6 parent. The lung colonizing ability of the LTP lung colony group is markedly different ($P<0{\cdot}001$) from that of the ND lung colony group. Furthermore, eight of these LTP variants form significantly fewer lung colonies than the B16-BL6 parent after i.v. inoculation ($P<0{\cdot}05$); two are not significantly different from the parental cells. This is in contrast to ND lung colony variants which exhibit variability similar to the ND lymph node tumor variants. These results demonstrate that prior exposure of B16-BL6 melanoma to the low Tyr and Phe diet changes the tumor so that it has a reduced or delayed ability to form lung colonies.

Survival of mice inoculated with representative lung colony variants. Median survival time of mice inoculated i.v. with LTP lung colony variants was significantly greater ($P<0{\cdot}05$) than that of mice inoculated with ND lung colony variants (table 7). Mice inoculated with LTP variants 1, 3, 5, and 7, having low lung colonizing potentials, survived 13–65 per cent longer than mice inoculated with the B16-BL6 parent. Three mice inoculated with LTP variant 1 and four mice inoculated with LTP variant 7 were long-term survivors and were sacrificed on day 170; no evidence of tumor involvement was apparent upon necropsy. Survival of mice inoculated with LTP lung colony variant 10, which has a lung colonizing ability comparable to that of B16-BL6 (figure 2B), was not different from BL6-recipient mice. In mice inoculated with ND variants, lung colonizing ability was not associated with survival. Even though some differences were noted in median survival time of the ND variants relative to the B16-BL6 parent, these differences were less than 12 per cent. There were no long-term ND survivors. The increased survival of mice inoculated with the LTP variants supports the lung colonization results in figure 2 and advances the hypothesis that a low Tyr and Phe diet suppresses tumor cell colonization and/or outgrowth in the lung.

Drug sensitivities of the B16-BL6 melanoma parent and variant lines. We have previously shown that low Tyr and Phe diet increases sensitivity of B16 melanoma to DOPA *in vivo* [37, 41, 48, 49] and our preliminary studies indicate that B16-BL6 is significantly more sensitive to DOPA when cultured *in vitro* in media restricted in Tyr and Phe (unpublished observations). Overall, however, the LTP variants were not more sensitive to DOPA or to the other selected chemotherapeutic agents *in*


A.
Number of Lung Colonies
300
200
100
0
Lung Colonization
Number of Variants
> BL6 Value 4
= BL6 Value 3
< BL6 Value 3
Lung Colonization
Number of Variants
> BL6 Value 1
= BL6 Value 6
< BL6 Value 3
1 2 3 4 5 6 7 8 9 10
ND Lymph Node Variant
Parent BL 6
1 2 3 4 5 6 7 8 9 10
LTP Lymph Node Variant

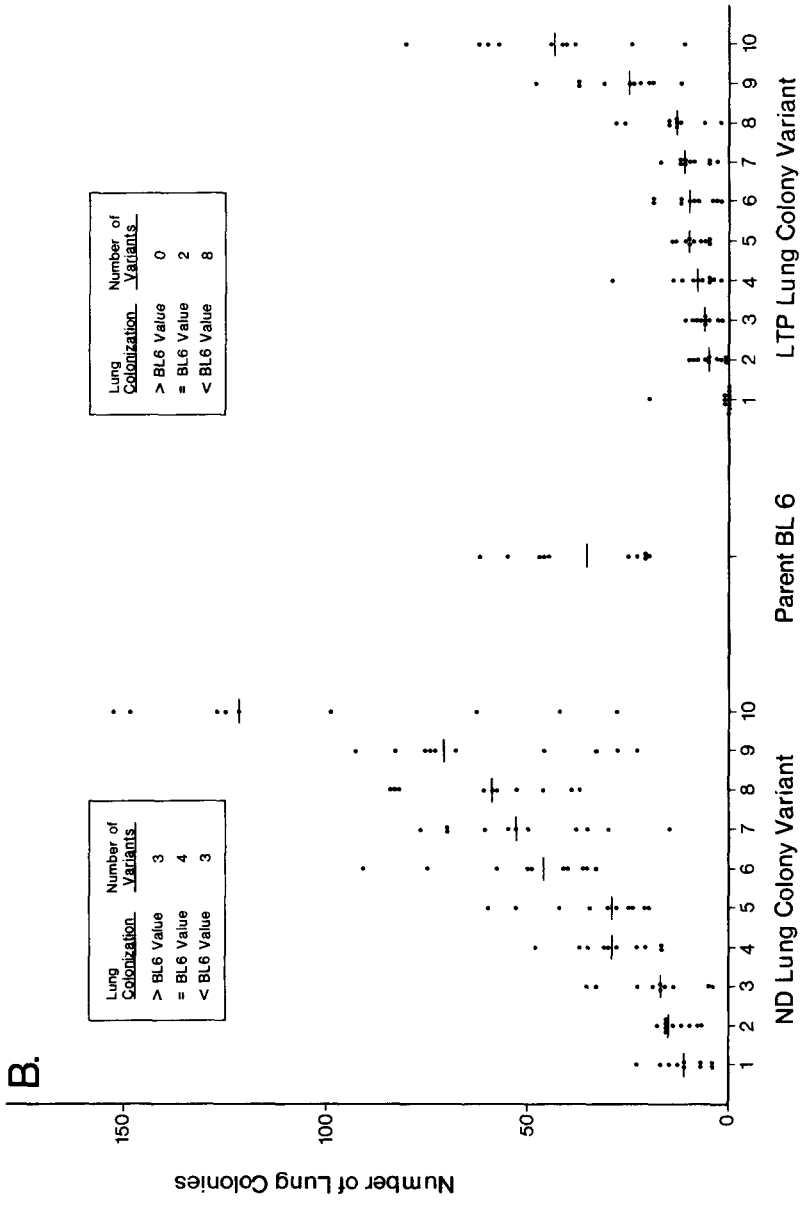


Figure 2. Lung colonizing potentials of ND and LTP lymph node (**A**) and lung colony (**B**) variants and parent B16-BL6 controls. Each dot represents the number of pulmonary metastases in one mouse fed normal diet and inoculated i.v. with 2×10^4 viable tumor cell variants. Heavy lines represent the median number of lung colonies from 10–12 mice for each variant. Comparisons of the number of lung colonies from the dietary variants relative to the number from the B16-BL6 parent are depicted in the panel inserts. Differences in lung colonization compared to parental B16-BL6 (inserts) were statistically significant by the Wilcoxon two-sample test ($P < 0{\cdot}05$).

Table 7. Survival of mice inoculated with parental B16-BL6 and selected ND and LTP lung colony variants demonstrating representative experimental metastatic potentials.

Cell line (variant)	Survival (days) Median (range)	Percentage increase/decrease in survival[a]
B16-BL6 Parent	34·5 (33–38)	
ND variant		
1	35·5 (31–39)	+2·9
3	32·5 (33–38)	−5·8
5	32·5 (28–37)[b]	−5·8
7	38·5 (33–85)	+11·6
10	38·0 (36–45)[b]	+10·0
LTP variant[c]		
1	57·0 (48–170)[b,d]	+65·0
3	39·0 (31–45)	+13·0
5	46·0 (39–99)[b]	+33·0
7	43·5 (30–170)[b,d]	+26·0
10	33·5 (33–39)	−2·9

[a] Percentage increase/decrease in survival =

$$\frac{\text{Median survival of variant—Median survival of B16–BL6 parent}}{\text{Median survival of B16–BL6 parent}} \times 100$$

[b] Median significantly different from B16-BL6 parent by Kruskal–Wallis test ($P<0·05$).
[c] LTP variant group significantly different ($P<0·05$) from ND variant group by unpaired *t*-Test.
[d] Long-term survivors were sacrificed 170 days after i.v. inoculation.

vitro in nutritionally complete DME (tables 8 a and 8 b). Of interest is that LTP lung colony variants were more sensitive to ADR compared to the B16-BL6 parent ($P<0·05$). Moreover, metastatic potential was divorced from chemosensitivity, as shown by the heterogeneous response given by all variants to drug exposure.

Discussion

We have previously shown that Tyr and Phe restriction inhibits primary tumor growth and metastasis [2, 39–41, 48], enhances the antineoplastic effectiveness of DOPA against murine melanoma [41, 48, 49], and prolongs host survival [40, 41, 48]. Even though food and caloric intake are not different in mice fed the low Tyr and Phe diet compared to mice fed the normal diet, mice fed the low Tyr and Phe diet transiently lose weight during the first 10–14 days after initiation of the diet [2, 37, 38, 40, 41, 48]. To eliminate weight loss as a variable during tumor establishment, in most experiments tumor cells were inoculated 14 days after initiation of the low Tyr and Phe diet. The initial weight loss results in smaller mice, which may behave differently immunologically and metabolically than do mice of normal weight and, consequently, may respond differently to tumor cells. Experiments conducted without the 2-week prefeeding regimen, however, still demonstrate the antimetastatic activity of the diet (ref. 37, and table 4).

Table 8a. Sensitivity of lymph node variants and B16-BL6 melanoma to ADR, DOPA, BLEO, and VCR.

Tumor variant	IC_{50}^{a}			
	ADR	DOPA	BLEO	VCR
Parent B16-BL6				
Mean	13·0	129·6	1764·8	72·5
SD	2·9	32·5	214·3	44·0
ND Lymph node variant[b]				
1	27·7	113·7	1162·4	39·7
2	26·9	110·7	1464·5	65·8
3	14·3	129·0	1547·3	120·0
4	16·9	118·8	1894·4	26·5
5	24·5	141·9	939·2	48·1
6	45·8	—[c]	1793·9	52·7
7	9·1	140·9	1080·6	65·3
8	318·5	201·7	4014·7	122·4
9	11·5	190·0	2005·9	14·0
10	29·2	140·7	2211·0	173·9
Mean	52·4	143·0	1811·4	72·8
SD	94·1	32·3	879·7	50·2
LTP Lymph node variant[b]				
1	15·9	233·4	1664·5	33·6
2	39·1	83·5	1425·5	40·3
3	66·1	238·0	2577·0	110·0
4	42·8	116·7	1569·3	33·6
5	8·9	107·3	1510·7	39·7
6	41·5	173·6	1856·0	168·8
7	31·9	115·1	1487·9	32·1
8	36·5	243·9	1416·7	—[c]
9	75·7	168·2	1041·7	546·8
10	7·2	193·8	1902·3	100·1
Mean	36·6	167·4	1645·2	122·8
SD	22·5	59·5	407·2	166·0

[a] Each value represents the average of triplicate determinations. For parental B16-BL6, the values represent the mean of two to four triplicate determinations.

[b] All dietary variants isolated from separate mice.

[c] Data not collected.

Table 8b. Sensitivity of lung colony variants and B16-BL6 melanoma to ADR, DOPA, BLEO, and VCR.

	IC_{50}^{a}			
Tumor variant	ADR	DOPA	BLEO	VCR
Parent B16-BL6				
Mean	13·0	129·6	1764·8	72·5
SD	2·9	32·5	214·3	44·0
ND Lung colony variant[b]				
1	4·6	160·1	1620·8	125·2
2	19·9	307·4	2030·1	44·3
3	13·1	172·9	1058·7	49·3
4	6·0	411·8	1963·9	98·3
5	10·1	191·1	2317·1	34·3
6	30·3	128·6	987·9	162·6
7	4·9	69·2	1261·5	55·5
8	7·0	166·9	1534·6	68·6
9	5·0	271·9	1876·4	—[c]
10	8·5	132·5	2226·5	50·4
Mean	10·9	201·2	1687·8	76·5
SD	8·3	101·0	472·6	43·4
LTP Lung colony variant[b]				
1	6·3	143·9	2102·6	135·9
2	8·1	154·9	1494·4	109·9
3	9·8	128·0	2022·7	144·6
4	7·6	265·1	1527·6	57·3
5	4·4	341·6	860·1	122·6
6	9·6	203·9	1474·5	56·7
7	7·2	272·0	1672·2	—[c]
8	20·2	104·2	971·3	200·4
9	7·6	232·7	2010·4	47·7
10	6·6	110·9	933·9	158·9
Mean	8·7[d]	195·7	1507·0	114·9
SD	4·3	80·1	463·9	52·3

[a] Each value represents the average of triplicate determinations. For parental B16-BL6, the values represent the mean of two to four triplicate determinations.

[b] Normal diet variants isolated from nine different mice. Low Tyr and Phe diet variants isolated from five different mice.

[c] Data not collected.

[d] Different from parent B16-BL6 ($P<0·05$).

Others have reported that calorie (food) restriction, which causes weight loss, inhibits tumor growth [5, 20, 56, 57]. In this study we demonstrate that while weight losses in the low Tyr and Phe *ad libitum* and normal diet food-restricted mice are identical, spontaneous metastasis of B16-BL6 in the low Tyr and Phe diet *ad libitum* group is significantly inhibited; whereas metastasis is not influenced by food restriction (tables 1 and 2). Numbers of metastatic lung colonies formed after s.c. injection of B16-BL6 in the normal diet food-restricted group are not significantly different from those in the normal diet control group which experienced no weight loss.

Thus these data strongly suggest that the antitumor effects of the Tyr and Phe restriction are specific and not a result of the initial weight loss or sustained low body weight. Further evidence indicating that the effects of Tyr and Phe limitation are specific and not associated with general malnutrition or weight loss include: (*a*) inhibition of growth of established subcutaneous melanomas via specific Tyr and/or Phe degrading enzymes, which do not alter body weight (18, 19, 39); (b) increase in survival of mice fed higher levels of Tyr and Phe that do not cause weight loss [40]; (c) failure of food (calorie)-restricted mice to exhibit increased survival [41]; and (d) increase in lung colonization of B16-F10 after i.v. inoculation in calorie-restricted mice, even though calorie restriction decreased primary growth of the B16-F1 melanoma [20]. It is conceivable that mice, with time, would adapt to the changes induced during dietary Tyr and Phe restriction, and that different results might be obtained after a longer feeding interval before tumor inoculation. However, mice inoculated with B16-BL6 melanoma after 30 days of feeding the low Tyr and Phe diet still showed decreased metastasis (unpublished observations).

Specificity of Tyr and Phe restriction is also indicated when the effects of Met restriction are compared to those of Tyr and Phe. Melanoma cells, as well as many other tumor systems, are Met-dependent [12, 27, 28, 32, 35, 61] and the low Met diet was expected to dramatically inhibit growth and metastasis of these cells. Although we found that tumor growth is inhibited during restriction of this amino acid, survival is significantly decreased. The effect on spontaneous metastasis is not dramatic compared to effects of Tyr and Phe restriction. Additionally, it is important that Tyr and Phe restriction blocked further tumor outgrowth in mice with established lymph node metastasis, supporting the use of this intervention as an adjuvant to clinical treatment of cancer. Responsiveness was evident in both female and male mice.

Although spontaneous metastasis is dramatically inhibited in mice fed low Tyr and Phe diet, it is possible that metastatic tumor cells were able to colonize the lung but that outgrowth was inhibited, and that tumor colonies remained embedded in the lung but were not visible. These micrometastases would therefore not be counted, thus underestimating metastatic potential. Our experiment in which individual lung lobes from mice fed low Tyr and Phe diet were implanted in mice fed normal diet (table 5) suggested that the low Tyr and Phe diet either slows tumor progression (reduces the actual numbers of tumor cells that metastasize) or impacts the metastatic process at the secondary (lung) site by influencing outgrowth, which may be related to decreased invasive capability of the tumor. The fact that residual lung tissue was evident only in mice implanted with LTP lobes supports these hypotheses.

Additional experiments indicated that the low Tyr and Phe diet modifies tumor cell characteristics and selects for tumor cells with low metastatic potential. Low

dietary Tyr and Phe levels altered the highly invasive and metastatic B16-BL6 melanoma so that it exhibited a reduced or delayed ability to form lung colonies when inoculated i.v. into mice. The surface tumor colonies that grew out after i.v. inoculation of the LTP lymph node or lung colony variants were well developed and of comparable size to those of parental B16-BL6 and of the ND lymph node and lung colony variants. This indicates that colonization and/or growth were affected. This hypothesis is also supported by the fact that survival was increased in mice after i.v. inoculation of LTP variants. Pigmentation characteristics of all variant and parent B16-BL6 experimental metastases after i.v. inoculation were similar, including both pigmented and mixed-pigmented characteristics.

The alteration in tumor cell subpopulations is a dynamic process which is modulated by environmental circumstances [26], such as the nutritional status of the host and dietary deprivation of certain nutritional factors. Whether or not the changes induced in the B16-BL6 melanoma in mice fed the low Tyr and Phe diet are caused by a direct effect of the amino acid restriction on the tumor or by an indirect modulation of some host response is presently unknown. Modulation of immune responses has been attributed to Tyr and Phe, as well as to other amino acid restrictions [8, 9, 30]. Bounous and Kongshavn [8] found that limiting Tyr and Phe in the diet to 0·05 and 0·10 per cent, respectively, which are similar to the amounts in our diet, did not affect plaque-forming cell responses and hemagglutinin antibody titers in mice immunized with sheep red blood cells. Jose and Good [30] observed impairment in cytotoxic T cell-mediated immunity. Additionally, Pine [51], restricting only Phe in the diet to 0·08 per cent, observed a uniform decrease in complement independent cell-mediated cytotoxicity against L1210 tumors in mice. Yet this restriction suppressed brain metastasis after i.p. implantation of L1210 tumor cells. We have found that dietary restriction of Tyr and Phe decreases natural killer cell activity in normal and nude mice [3, 38]; however, metastasis is still inhibited even when B16-BL6 melanoma is inoculated into nude mice (unpublished observations). Thus, host immune responses appear not to play a significant role in the antimetastatic activity of dietary Tyr and Phe restriction. This does not exclude the involvement of other host factors in modulating the phenotype of B16-BL6 melanoma or in contributing to the overall antimetastatic activity of the diet. Whether directly or indirectly, the characteristics of the B16-BL6 melanoma tumor are altered after passage in mice fed the low Tyr and Phe diet, as evidenced by the decrease in experimental metastatic potential of the variants.

Attachment of tumor cells in the lung may not be affected since preferential colonization and differential dissemination patterns of ^{125}I-labeled tumor cells were not observed (table 6). These data also suggest that hematogenous spread of tumor cells is not decreased by Tyr and Phe restriction, and that the dietary modulated variants are not more sensitive to removal or clearance from the blood, possibly by natural killer cells. In fact the numbers of tumor colonies are increased in mice prefed low Tyr and Phe diet and inoculated i.v. with B16-BL6 melanoma [2, 37, 38]. This probably results from decreased natural killer cell activity in mice fed the low Tyr and Phe diet, since the numbers of tumor colonies are not increased if mice are fed low Tyr and Phe diet after tumor inoculation [3, 38].

Because metastatic tumor cells are thought to be more resistant to chemotherapy [14, 23, 25, 26, 45], we had expected dietary Tyr and Phe restriction to modulate tumor cell chemosensitivity, as well as lung colonizing ability. Overall, Tyr and Phe restriction, with the exception of sensitivity of the LTP lung colony variants to

ADR, did not enhance the *in vitro* susceptibility of B16-BL6 to selected chemotherapeutic agents. Furthermore, the metastatic potential of a tumor cell variant did not indicate susceptibility to chemotherapeutic agents, as evidenced by the heterogeneous response given by all variants to drug exposure.

Even though there was little correlation between metastatic potential and susceptibility to chemotherapeutic drugs when examined in Tyr and Phe complete medium, the variants may respond differently under reduced levels of Tyr and Phe during exposure to these chemotherapeutic agents, as our preliminary *in vitro* studies indicate for DOPA (unpublished observations). We have also shown that DOPA-resistant tumors are sensitized *in vivo* to the drug during Tyr and Phe dietary restriction [37].

Further studies on the mechanisms responsible for our results are under investigation, and we are continuing to assess the specificity associated with the Tyr and Phe restriction. Several explanations for our results are possible. Tyr and Phe limitation may specifically suppress the phenotypic emergence of highly metastatic clones within the heterogeneous B16-BL6 tumor, thus selecting for and enriching in subpopulations which are tumorigenic but exhibit a low metastatic phenotype. Tumor cell invasion and outgrowth may be impacted. Host factors may also contribute to this selection. Whatever the mechanisms, our results dramatically show that the malignant phenotype of B16-BL6 melanoma can be suppressed by dietary Tyr and Phe restriction, which can also block the formation of new lung colonies in mice with established metastatic lesions. The consequences for cancer therapy are important, and as the mechanisms of this dietary influence of Tyr and Phe on metastasis are elucidated, the relevance of this dietary limitation as an adjunct to the control of cancer will increase.

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