Cancer Chemother Pharmacol (1986) 16: 229–236

Cancer Chemotherapy and Pharmacology

Interaction between specific dietary factors and experimental chemotherapy of metastatic melanoma*

Gary G. Meadows[1], Rokia M. Abdallah[1], and Jean R. Starkey[2]

[1] College of Pharmacy, Washington State University, Pullman, WA 99164-6510, USA
[2] Department of Microbiology, Montana State University, Bozeman, MT 59717, USA

Summary. The single and combined effects of (a) dietary restriction of phenylalanine and tyrosine, (b) levodopa methylester chemotherapy, and (c) megadose sodium ascorbate supplementation on experimental metastasis was determined in B16–BL6 melanoma. Dietary restriction and levodopa methylester therapy inhibited tumor outgrowth, whereas ascorbate alone was inactive. In combination, however, the effect of dietary restriction and levodopa methylester chemotherapy was augmented by sodium ascorbate. Tumor cells surviving this combination therapy (treated population) were isolated from the lungs of treated mice, and proved to be tumorigenic when inoculated SC into the back of naive mice. The resulting tumors grew more slowly than those produced by inoculation of similarly isolated control cells (control population), irrespective of whether the diet was adequate or deficient in phenylalanine and tyrosine. Failure of the treated tumor cell population to exhibit reduced sensitivity to the combination chemotherapy or, unlike the control population, to exhibit variation in pigmentation levels, suggests that the restriction of phenylalanine and tyrosine during drug therapy alters the tumor response to reduce heterogeneity and perhaps interferes with the emergence of drug resistance.

Introduction

Disseminated malignant melanoma presents a major therapeutic problem, because metastasis frequently occurs early in the course of the disease and is characteristically resistant to conventional therapies. New approaches to the treatment of this disease are needed. Levodopa methylester chemotherapy combined with dietary restriction of phenylalanine and tyrosine and megadose supplementation with sodium ascorbate more than doubles the survival time of mice bearing primary subcutaneous B16 melanoma tumors [21] and may, therefore, be useful in the treatment of human patients with melanoma. Although treatment of primary tumors has been used for many years in preclinical screens for potential antitumor agents, it has become increasingly apparent that primary and metastatic tumors can respond differently to drug therapy [9, 25, 28, 32, 33] and that the response of metastatic cells to chemotherapy is dependent on the local environment as well as on various other host factors [5, 26]. Differences in drug susceptibility are related to the heterogenous nature of malignant neoplasms, and in murine melanoma drug resistance is reported to occur more frequently in the metastatic B16 cell lines than in their parent tumors [2, 17, 33]. Therefore, assays using tumor cell subpopulations with defined metastatic capabilities have a higher predictive value in identifying the activity of potential agents for treatment of disseminated disease [6]. We report here the effects of (a) dietary restriction of phenylalanine and tyrosine; (b) levodopa methylester chemotherapy; and (c) ascorbate supplementation, on the establishment and growth of experimental metastasis of the bladder-6 (BL6) cell line of B16 melanoma, a tumor line originally selected for increased invasive and metastatic capabilities [23].

Materials and methods

Tumor cell line. The highly invasive and metastatic B16-BL6 (bladder-6) melanoma variant isolated by Dr Ian Hart [23] was used in all experiments. This cell line was obtained from the Mason Research Institute, Mass, and grown in T-75 flasks in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Sterile Systems, Inc.), 100 IU/ml penicillin and 100 μg/ml streptomycin (complete medium). For subculture, cell monolayers are incubated at room temperature in Ca^{2+}- and Mg^{2+}-free Tyrode's balanced saline for 10 min, then exposed to 0.05% trypsin, 0.02% EDTA for 2 min followed by addition of complete medium to inhibit further enzyme action. The cells are subcultured weekly at a split ratio of 1:4 and incubated at 37 °C in a humidified atmosphere of 7% CO_2 in air.

Animals and diets. Female, $B6D2F_1$ mice 23 weeks of age and ranging in weight from 22 to 25 g were used in these studies. All mice were purchased from The Jackson Laboratories, Bar Harbor, Me, and 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 on experiment were housed three per cage in polycarbonate cages containing hardwood shavings and fed one of two synthetic crystalline amino acid diets. The composition of these diets has

* The work described in this paper was supported in part by funds provided to Washington State University through the NIH Biomedical Research Support grant and by funds provided by the College of Pharmacy

Offprint requests to: G. G. Meadows

been previously reported [19]. Both diets are equivalent to an 11.8% casein diet, and provide approximately 4.0 kcal/g. The basal diet contains 0.6% L-phenylalanine and 0.3% L-tyrosine, and the deficient diet, which is isonitrogenous to the basal diet, contains 0.08% L-phenylalanine and 0.04% L-tyrosine. Both diets were purchased from BioServ, Inc., Frenchtown, NJ and were assayed for tyrosine content within a range of analytical variability and content of 10%–15%.

Experimental metastasis (lung colony assay) and antitumor therapy. Mice were preconditioned on either the basal or deficient diets for 2 weeks prior to inoculation of B16-BL6 melanoma cells, to allow for equilibration of body weight and stabilization of diet and fluid intake, thus eliminating these experimental variables. Initially mice fed the deficient diet lose weight, but they become weight-stable between the 10th and 14th day after initiation of the diet [19]. During the conditioning period and throughout the experimental period mice were given either distilled-deionized water or a solution of 30 mg/ml sodium ascorbate dissolved in distilled-deionized water. The fluids and diets were offered ad libitum, and fresh ascorcabte solution was provided daily. Body weight changes and fluid and diet intake were similar to those reported in previous studies [19, 21]. At the end of the 2-week conditioning period each mouse was inoculated via the lateral tail vein with 1.25×10^4 B16-BL6 melanoma cells or selected B16-BL6 cell populations (see below) in a total volume of 0.2 ml. Cells for inoculation were prepared from subconfluent cultures and harvested using EGTA to obtain single cell suspensions. Cell monolayers were washed twice with Ca^{2+}- and Mg^{2+}-free Tyrode's balanced salt solution (CMF) and then incubated at room temperature with a small volume of 10^{-4} *M* EGTA in Ca^{2+}- and Mg^{2+}-free Puck's saline G. Incubation was continued until the cell sheet loosened (several minutes), and then the flasks were tapped sharply to dislodge the monolayer. Cells were pelleted, washed three times with CMF, resuspended, and counted. Suspensions for inoculation were greater than 95% viable as judged by trypan exclusion.

Drug treatment groups were given levodopa methylester (LDME) in the presence of an aromatic amino acid decarboxylase inhibitor, benserazide (to prevent dopamine toxicity). These groups received daily injections of benserazide (100 mg/kg) 90 min before injection of LDME (1000 mg/kg) IP for 12 days, beginning 24 h after tumor inoculation. Benserazide by itself lacks anti-B16 melanoma activity (Meadows, unpublished data). Levodopa methylester was purchased from Sigma Chemical Co., St. Louis, Mo, and Benserazide was a gift from Hoffman-La Roche, Inc., Nutley, NJ. Both drugs were prepared in sterile saline and injected IP in a volume equal to 1% of mouse body weight. All other groups were given equivalent injections of sterile saline. Mice were killed 19–21 days after tumor inoculation and examined for pulmonary and extrapulmonary metastases. Their lungs were removed, washed in saline, and then fixed in Bouin's solution. The numbers and size of pulmonary colonies were counted twice and the results were averaged. When tumor growth was excessive and the pulmonary tumor colonies could not be counted accurately, the weight of the tumor-bearing lungs was used to assess tumor burden.

Isolation of pulmonary tumor populations. Two B16-BL6 tumor cell populations were isolated from whole lungs of mice previously inoculated IV via the lateral tail vein with 1.25×10^4 BL6 cells using a method similar to that described by Suzuki [29]. The first (treated population) was isolated from lungs of five mice fed the deficient diet, given sodium ascorbate in the drinking water, and treated with LDME for 12 days as described in the "Experimental Metastasis (Lung Colony Assay) and Antitumor Therapy" section under *Methods.* A second B16-BL6 tumor cell population (control population) was isolated from whole lungs of four untreated mice fed the basal diet. On day 13 after inoculation of tumor, the mice were killed and the lungs aseptically transferred into sterile petri dishes containing 20 ml complete medium and washed twice with complete medium. The lungs were minced and then serially digested with sterile collagenase isolated from *Clostridium histolyticum* (type IV, Sigma Chemical Co., St. Louis, Mo). A 0.5% solution of collagenase dissolved in phosphate buffered saline (20 ml) was added to the minced tissue and incubated at 37 °C for 30 min with periodic agitation. The collagenase solution was removed and centrifuged at 200 *g* for 5 min at 4 °C, and the pellet washed twice with 10 ml complete medium. After the third resuspension of the pellet in complete medium the contents were transferred to a 100-mm tissue culture dish. The collagenase digestion was repeated four times and each digest was plated separately. Five additional digests were then completed, using 0.05% trypsin with agitation, until the lungs were fully decomposed. The resultant cells were centrifuged, washed twice in complete medium, resuspended in complete medium, and transferred to a 100×20 mm tissue culture dish. These were incubated at 37 °C in a humidified atmosphere of 7% CO_2 in air. The cultures were fed every 5 days starting on day 2. On day 16, representative dishes from each digest were fixed in methanol and stained with hematoxylin, after which the tumor colonies were enumerated. By the next passage the tumor cells had overgrown the normal lung cells, so that essentially only B16 cells were evident in the confluent culture. At this point, combined aliquots of each tumor cell population were frozen in liquid nitrogen for use later.

Subcutaneous growth of B16-BL6 lung tumor cell populations. Mice were preconditioned for 2 weeks on one of two dietary regimens: (a) the basal diet with distilled-deionized water or (b) the deficient diet with sodium ascorbate (30 mg/ml) in the drinking water. At the end of the conditioning period, 10^5 cells of the control and treated BL6 tumor lung populations in a volume of 0.2 ml were inoculated into the left back. The tumor latent period and the tumor growth rate were determined. Tumor volume was calculated from the mean of three individual caliper measurements of tumor diameter using the formula for a hemiellipsoid ($\pi/6 \times$ mean diameter^3).

Statistics. Statistical analyses were performed using SAS computer programs. Chi-square analysis was used to assess the degree of significance in tumor colony size distribution between two groups using actual colony numbers. The percentages of colonies distributed into small, medium and large groups are presented in Tables 1 and 2. The Kruskal-Wallis test after NParlWay ANOVA was used to confirm that the numbers of pulmonary colonies were different. The Wilcoxon rank-sum test was used to compare differences between two groups (Tables 1 and 2). All pair-wise comparisons of lung weight and subcutaneous tumor volume with-

in and among the experimental groups involving the control and treated BL6 cell populations were accomplished using Duncan's multiple range test (Table 3 and Fig. 4). The difference in the average latent period was determined using students *t*-test. Differences revealed by analyses using these tests were considered significant at $P<0.05$.

Results

Effect of dietary restriction of phenylalanine and tyrosine, LDME chemotherapy, and ascorbate supplementation on experimental metastasis

The results of dietary restriction of phenylalanine and tyrosine, LDME chemotherapy and ascorbate supplementation on lung colonization and tumor lung colony growth of B16-BL6 melanoma are presented in Figs. 1 and 2 and Tables 1 and 2. A pronounced effect on tumor colony outgrowth was observed in mice fed the deficient diet, treated with LDME, and given ascorbate supplementation, where over 60% of the tumor lung colonies were small, as against only 9%–12% small colonies in untreated mice fed the basal diet (Tables 1 and 2). Combination therapy with LDME and ascorbate supplementation also reduced the tumor colony size in mice fed the basal diet, but the effect was not as pronounced as that observed in mice fed the deficient diet.

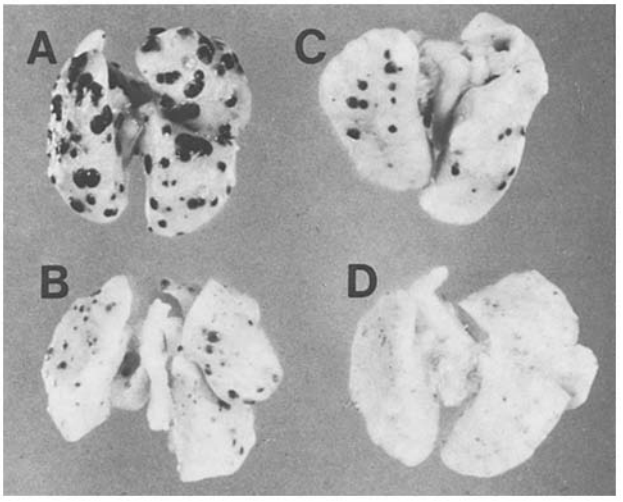

Fig. 1 A–D. Representative lungs from mice 21 days after inoculation of 1.25×10^4 cells of B16-BL6 melanoma: **A** from untreated mouse fed the basal diet; **B** from untreated mouse fed the deficient diet; **C** from mouse fed the basal diet, given supplemental ascorbate, and treated for 12 days with LDME; **D** from mouse fed the deficient diet and given supplemental ascorbate and treated 12 days with LDME

In experiment 2 (Table 2), the individual effects of LDME chemotherapy and ascorbate supplementation on tumor lung colony size were studied. For both dietary groups, the addition of ascorbate supplementation to LDME chemotherapy led to some increased inhibition of tumor lung colony growth. On the other hand, ascorbate supplementation alone had no significant effect on tumor lung colony growth in mice fed either the amino acid-deficient diet or the basal diet. Thus, the beneficial effects of ascorbate supplementation are only evident in combination with LDME chemotherapy.

Neither LDME treatment nor ascorbate supplementation significantly affected the numbers of lung colonies produced after IV inoculation of B16-BL6 melanoma (Tables 1 and 2). The net effect of dietary phenylalanine and tyrosine deficiency was to increase the number of resultant lung colonies, but there was wide variation in the numbers. This effect was most evident in experiment 2 (Table 2). From the data on groups A, E, and I in Table 2 it appears that this increase may be related to modulation of host mice during the dietary preconditioning period, because initiation of the deficiency 3 days after inoculation did not increase the number of tumor colonies formed (group I). The effect on colony size in group I closely resembled that of mice treated with LDME and fed the deficient diet throughout the experimental period (group F, Table 2). Importantly, these data indicate that the deficient diet can exert its influence on tumor growth 3 days after tumor inoculation, presumably when tumor extravasation and implantation have already occurred.

The majority of tumor lung colonies in mice receiving LDME chemotherapy and ascorbate supplementation and also fed the amino acid-deficient diet presented grossly as small, flat, irregularly shaped patches of pigmentation on the surface of the lung. In contrast, the prevalent tumor lung colonies in similarly treated mice fed the basal diet were large or medium in size, raised, regularly shaped, and

Table 1. Effect of diet and combination chemotherapy on the size and number of pulmonary colonies in a lung colony assay (exp 1)

Diet-treatment	*N*	Colony size (%)[a]			No. of lung colonies[b]
		Small	Medium	Large	
Basal					
Untreated	12	12.30	56.15	31.55	58.9 ± 36.4
LDME + ascorbate	10	37.04	50.00	12.96[c]	36.4 ± 34.5
Deficient					
Untreated	12	19.70	60.10	20.20[d]	91.9 ± 55.8
LDME + ascorbate	9	64.68	33.46	1.86[c, e]	109.2 ± 33.1[f]

[a] Small < 1.3 mm; medium, > 1.3 < 6.5 mm; large, ≥ 6.5 mm in diameter
[b] Mean ± SD
[c] Colony size distribution is different from that in untreated mice within same dietary group
[d] Colony size distribution is different from that in treated and untreated mice fed basal diet, and treated mice fed deficient diet
[e] Colony size distribution is different from that in all other groups
[f] Different from that in untreated and treated mice fed the basal diet

Table 2. Effect of diet, ascorbate, and LDME singly and in combination on the size and number of pulmonary tumors in a lung colony assay (exp 2)

Group	Diet-treatment	*N*	Colony size (%)[a]			No. of lung colonies[b]
			Small	Medium	Large	
	Basal[c]					
A	Untreated	12	8.94	25.20	65.85	44.3 ± 23.6
B	LDME[f]	11	17.90	38.27	43.83	49.2 ± 22.7
C	Ascorbate[g]	10	11.50	34.50	54.00	61.3 ± 61.9
D	LDME + ascorbate[h]	10	27.80	38.27	33.94	54.5 ± 25.7
	Deficient[d]					
E	Untreated	12	27.83	48.28	23.89	112.8 ± 92.1
F	LDME[i]	13	51.38	43.32	5.30	135.2 ± 103.0
G	Ascorbate[j]	9	22.08	53.25	24.68	137.4 ± 54.6
H	LDME + ascorbate[k]	9	61.89	33.55	4.56	108.0 ± 39.3
I	Basal-Deficient[e]					
	LDME + ascorbate	11	50.94	37.74	11.32	48.4 ± 28.3

[a] Small < 1.3 mm; medium, > 1.3 < 6.5 mm; large, ≥ 6.5 mm in diameter

[b] Mean ± SD

[c] Colony size distribution in all groups within diet (A – D) are different from that in groups E – I. Within diet the numbers of pulmonary colonies are not significantly different, nor are they different from group I. The numbers are different from groups E – H except that group C and D are not significantly different from groups E and H, and group D is not different from group E

[d] Colony size distribution of all treatment groups within diet (E – H) are different from groups A – D, and group I. Within the diet the numbers of pulmonary colonies are not significantly different from each other

[e] Mice were given the basal diet and ascorbate during the pretumor conditioning period and during the first 3 days of treatment with LDME. On the 4 th day of treatment, mice were switched to the deficient diet for the remainder of the experiment. Colony size distribution in this group is different from that in all other groups. The number of pulmonary colonies were not significantly different from groups A – D, but were different from groups E – H

[f] Colony size distribution within diet is different from that in group A and group D, but not group C

[g] Colony size distribution within diet is different from that in group D, but not group A or B

[h] Colony size distribution within diet is different from that in groups A, B, and C

[i] Colony size distribution within diet is different from that in groups E, G, and H

[j] Colony size distribution within diet is different from that in group G and H, but not group E

[k] Colony size distribution within diet is different from that in groups E, F, and H

well demarcated from the surrounding lung tissue and other tumor colonies (Fig. 2). Conceivably, the pigmented areas observed on the surface of lungs from treated mice fed the deficient diet could merely have represented melanin within lung macrophages; however, histopathological examination of the lungs indicated that viable tumor cells were present in these areas.

In vitro and SC growth characteristics of isolated treated and control tumor cell populations

To examine the viability and tumorigenicity of these small, flat colonies further, we repeated the therapeutic protocol, but killed the mice after the 12-day drug treatment period and immediately isolated the tumor cells from the lungs. Fi-

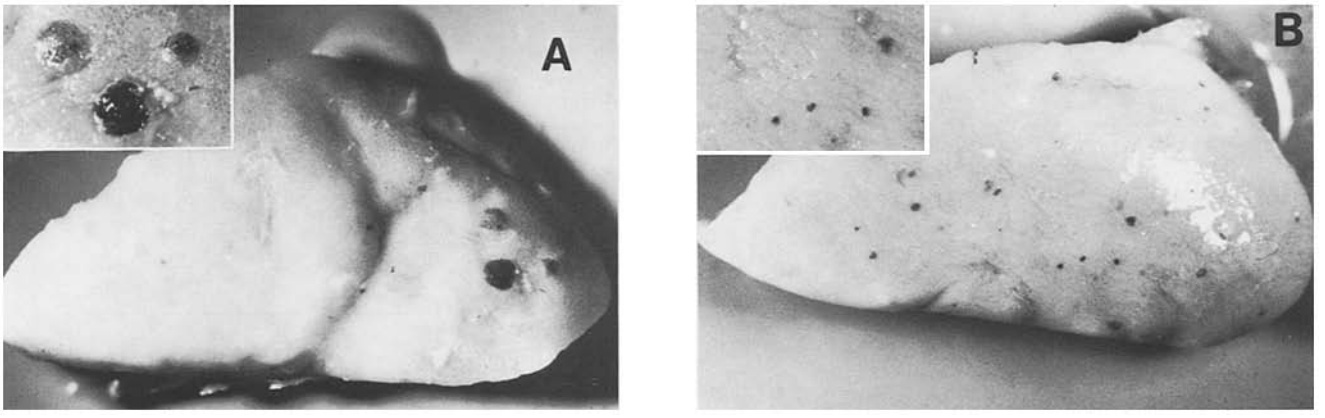

Fig. 2 A, B. Enlargement of right pulmonary lobes, showing the influence of diet on size of tumor colonies in mice given supplemental ascorbate and treated for 12 days with LDME. **A** basal diet; **B** deficient diet. (Lobes ×3; *Insert* ×6)

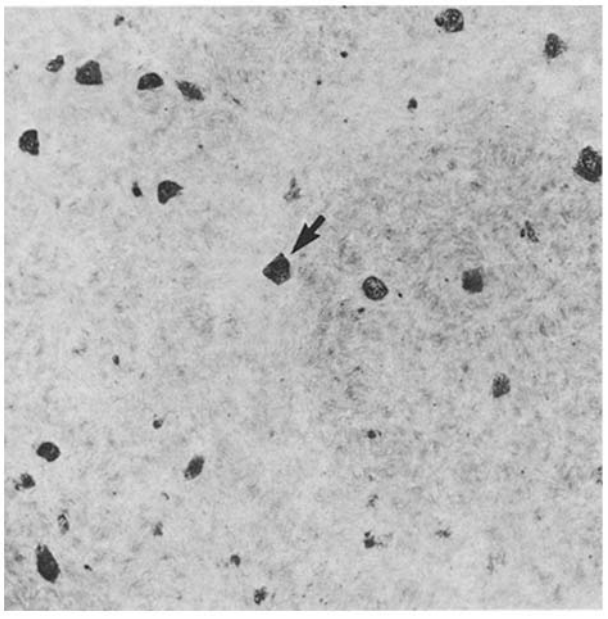

Fig. 3. Hematoxylin stain of methanol-fixed culture plate, indicating B16-BL6 tumor colonies isolated from the lungs of mice fed the deficient diet, given ascorbate, and treated with LDME for 12 days. The *arrow* indicates a darker stained dense tumor colony (× 5.5)

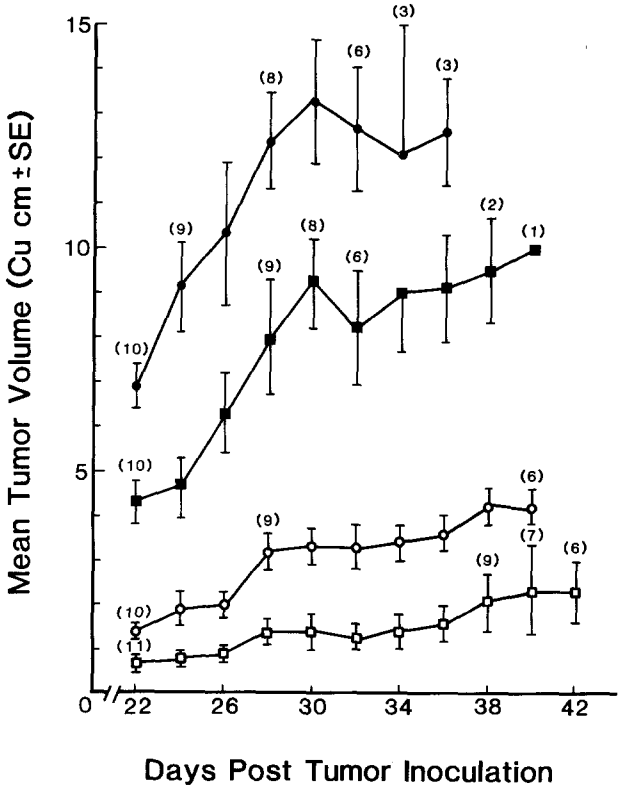

Fig. 4. Growth curve of control and treated cell populations of B16-BL6 inoculated SC into the left flank and fed either basal diet or deficient diet and ascorbate. ●———●, control cell population, basal diet; ■———■, treated cell population, basal diet; ○———○, control cell population, deficient diet and ascorbate; □———□, treated cell population, deficient diet and ascorbate. Each group is significantly different from all others ($P < 0.05$)

gure 3 shows the in vitro growth of tumor colonies resulting from disaggregation of lungs from treated mice fed the deficient diet. About 56% fewer tumor colonies developed in vitro from this group than from in vitro culture of disaggregated lungs from untreated mice fed the basal diet, confirming that the combined drug and diet therapy had significantly reduced tumor burden.

The two cell lines obtained after proteolytic enzymatic disaggregation were designated *treated cell population* (isolated from the lungs of mice fed the deficient diet and treated with LDME and ascorbate) and *control cell population* (isolated from untreated mice fed the basal diet). Both cell lines, when inoculated SC, proved to be tumorigenic, and both retained their sensitivity to growth inhibition by phenylalanine and tyrosine restriction in combination with ascorbate supplementation, as reported previously [21]. All mice inoculated with either of the cell populations developed tumors. The latent periods of control and treated populations within each dietary group were the same, but amino acid deficiency did slightly increase latency by 4 days. The average latent period for all mice fed the basal diet was 10.3 ± 1.5 (SD) days, and that in mice fed the deficient diet and given ascorbate supplementation was 15.8 ± 3.1 days ($P < 0.05$).

Tumors resulting from inoculation of the treated cell population grew at a slower rate than those resulting from inoculation of the control cells, whether mice were fed the basal diet alone or the amino acid-deficient diet supplemented with ascorbate (Fig. 4). Neither cell line metastasized from this implantation site as was characteristic of our previous study with the B16 melanoma parent tumor [21]. This could be related to the anatomical placement of the tumor, and regional differences in placement of SC tumors are known to affect metastasis of primary tumors [20]. Alternatively, the mice may have died of primary tumor involvement before evidence of gross pulmonary metastasis could be detected. In our hands B16-BL6 will metastasize from this SC implantation site when mice are inoculated with a lower number of cells (1×10^4 cells per mouse).

Tumors from mice inoculated with the control cell population were heterogeneously pigmented, consisting of white, gray, and black areas, but tumors from mice inoculated with the treated cell population were homogeneously black, like tumors resulting from inoculation of unselected B16-BL6 melanoma cells. These pigmentation patterns were consistently observed in all tumors, irrespective of the diets fed.

Experimental metastasis of isolated treated and control tumor cell populations

All mice developed tumor lung colonies, and tumor growth was excessive in those fed the basal diet. The numbers of tumor lung colonies could not be counted; therefore the weights of tumor-bearing lungs are presented (Table 3). Combined LDME and ascorbate therapy decreased tumor burden in mice inoculated with the treated, but not with the control, cell population. Dietary deficiency of phenylalanine and tyrosine reduced tumor burden similarly in mice inoculated IV with either control or treated cells and had a greater effect than the combined therapy in mice inoculated with the treated cell line. The deficient diet enhanced the effect of LDME and ascorbate therapy, reducing the tumor burden by about 76% compared with that in untreated mice fed the basal diet irrespective of the cell population that was

Table 3. Response of control and treated B16-BL6 cell populations to treatment with LDME, ascorbate, and dietary deficiency of tyrosine and phenylalanine

Diet	Chemotherapy regimen	Tumor-bearing lung weight (g)[a]			
		N	Control cell population	*N*	Treated cell population
Basal	Untreated	14	0.86 ± 0.31	13	0.77 ± 0.28
Basal	LDME + ascorbate	11	0.96 ± 0.22	11	0.57 ± 0.17[b,c]
Deficient	Untreated	11	0.37 ± 0.12[b]	10	0.42 ± 0.10[b]
Deficient	LDME + ascorbate	9	0.19 ± 0.04[d]	9	0.19 ± 0.03[d]
Deficient-basal[e]	LDME + ascorbate	7	0.29 ± 0.04[b]	10	0.31 ± 0.07[b]

[a] Mean ± SD. Lung weight for tumor-free mice fed the basal diet was 0.23 ± 0.04 ($N = 12$) and that for mice fed the deficient diet was 0.21 ± 0.03 ($N = 12$)

[b] Different from untreated mice fed basal diet

[c] Different from control cell population group fed basal diet and given LDME + ascorbate

[d] Different from untreated mice fed basal or deficient diet

[e] Mice were fed the deficient diet before tumor inoculation and during treatment, but switched to the basal diet after the chemotherapy was stopped

inoculated, thus breaking the resistance of the control population to this therapy. Both cell lines began growing when the diet was switched from deficient to basal after LDME treatment was stopped, but growth remained inhibited if the amino acid deficient diet was continued (Table 3 and Fig. 5). Similar results were also obtained in mice inoculated with nonselected B16-BL6 cells (data not shown). These data, obtained with the experimental metastasis protocol, indicate that the phenylalanine and tyrosine dietary deficiency and LDME therapy effects are primarily related to blockade of tumor outgrowth in the lung.

Discussion

A major finding in this report is that dietary restriction of phenylalanine and tyrosine exerts its antitumor activity in a lung colony assay by inhibiting outgrowth of the melanoma cells. In addition, it enhances the cytostatic activity of levodopa methylester chemotherapy against this neoplasm, an effect which is further enhanced by oral supplementation with sodium ascorbate. Neither the LDME nor the ascorbate supplementation influences lung colonization, but pre-existing dietary deficiency increases the number of pulmonary colonies. Since the properties of the BL6 tumor cell line are consistent, the increase probably results from modulation of the host by phenylalanine and tyrosine deficiency to allow for greater tumor cell survival in the early stages after IV inoculation [7]. Our observation that no increase in number occurred when the deficient diet was started 3 days after tumor inoculation is consistent with this explanation. The increase in the number of tumor lung colonies in mice preconditioned with the deficient diet could be related to lowered natural killer cell activity than in mice fed the basal diet [1]. Lower natural killer cell activity is known to increase and high levels are known to decrease the number of pulmonary tumor colonies developing after IV tumor inoculation [11–15, 30]. Factors such as changes in the interaction between the tumor cells and host lymphocytes [8] or platelets [10], in the size of the lumen or blood flow in vessels, or in other host immune responses [7], however, can also affect the number of tumor cells capable of colonizing the lung, and cannot be disregarded.

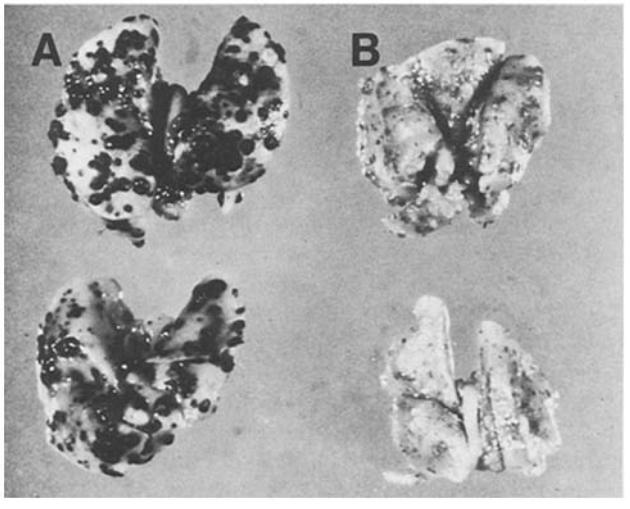

Fig. 5A, B. Dietary inhibition of B16-BL6 outgrowth. **A** Lungs from mice inoculated with treated cell population, fed the deficient diet and ascorbate, treated for 12 days with LDME, and switched to the basal diet for 9 days after chemotherapy was stopped; **B** lungs from mice treated identically except that mice continued on the deficient diet for 9 days after chemotherapy was stopped

The relative effects of the diet and drug or vitamin treatments on tumor outgrowth and colony size generally parallel the effects previously observed on tumor volume in mice bearing SC B16 melanoma tumors [21]. In both cases dietary restriction of phenylalanine and tyrosine exhibited a pronounced effect on tumor growth rate, with levodopa methylester exhibiting a lesser effect. Ascorbate supplementation alone showed little or no activity on either SC tumor growth of B16 or on pulmonary colony outgrowth of B16-BL6 melanoma. This lack of direct antimetastatic activity is in accord with available studies indicating no activity on experimental pulmonary metastases of B16 melanoma [35] and lack of effect on the numbers and size of metastases formed after SC implantation of CA-51 colon carcinoma and 6C3HED lymphosarcoma [27]. Although ascorbate lacks direct antitumor activity, it does

somewhat augment the growth-inhibitory effects of phenylalanine and tyrosine deficiency in combination with levodopa methylester treatment. We observed this in the present study of experimental metastasis and previously in primary SC B16 melanoma, where ascorbate supplementation significantly prolonged survival relative to mice that did not receive supplementation [21].

The mechanism underlying the effects of dietary restriction of phenylalanine and tyrosine on tumor outgrowth is unknown, but it is generally assumed that restriction starves the tumor, thereby decreasing growth. It is unlikely that amino acid starvation completely explains the effect of phenylalanine and tyrosine restriction on tumor outgrowth, because only a limited reduction in the plasma levels of these amino acids (33% and 21%, respectively) is necessary to achieve a full antitumor response [19]. Furthermore, moderate dietary restrictions that similarly alter plasma phenylalanine and tyrosine levels but do not result in weight loss of the host increase survival of B16-bearing mice [18]. Perhaps other host immune responses known to be altered by phenylalanine and/or tyrosine restriction play a role in blocking tumor outgrowth [3, 4, 16].

Tumor cells isolated from the lungs of mice fed the deficient diet after levodopa methylester and ascorbate therapy exhibited an alteration in growth rate when subsequently implanted SC, and the resulting tumors were heavily pigmented compared with the mixed pigmentation of tumors derived from the control cell population. This was a surprising finding, because levodopa inhibits pigmented melanoma cells but not apigmented melanoma cells [34]. Therefore, we had expected an apigmented cell population to become the dominant resistant cell population and not a highly pigmented one.

Because heterogeneity for pigmentation did not occur and because resistance to neither the nutritional nor the drug therapy developed, it seems possible that phenylalanine and tyrosine deficiency may modify the expression of intralesional heterogeneity of B16-BL6 melanoma [22, 24, 31]. Any strategy capable of reducing or delaying the emergence of tumor heterogeneity, which is accompanied by emergence of drug resistance, would lead to an improved therapeutic effectiveness and increased long-term remission rates of patients with cancer. We would emphasize that the experiments described here cannot properly evaluate the heterogeneity question, since the numbers of tumor cell generations are not quantitated. We are currently studying the possible modulation of tumor heterogeneity by dietary phenylalanine and tyrosine restriction. Other important findings from this and other studies are that dietary restriction not only inhibits growth of primary SC B16 melanoma tumors but also the outgrowth of metastatic B16-BL6 melanoma tumors in the lung, and that restriction enhances the antimelanoma activity of combined supplemental ascorbate and LDME chemotherapy against these tumors.

Acknowledgements. The authors thank Dr R. B. Bendel, Statistical Services, for aiding the statistical analysis of data.

References

1. Abdallah RM, Starkey JR, Meadows GG (1983) Alcohol and related dietary effects on mouse natural killer-cell activity. Immunology 50: 131
2. Biorklund A, Hakansson L, Stenstamm B, Trope C, Akerman M (1980) Heterogeneity of non-Hodgkin's lymphomas as regards sensitivity to cytostatic drugs. An in vitro study. Eur J Cancer 16: 647
3. Bounous G, Kongshavn PAL (1978) The effect of dietary amino acids on immune reactivity. Immunology 35: 257
4. Bounous G, Sadarangani C, Pang KC, Kongshavn PAL (1981) Effect of dietary amino acids on tumor growth and cell mediated immune responses. Clin Invest Med 4: 109
5. Donnelli MG, Colombo T, Broggini M, Garattini S (1977) Differential distribution of antitumor agents in primary and secondary tumors. Cancer Treat Rep 61: 1319
6. Fidler IJ (1984) Recent concepts of cancer metastasis and their implications for therapy. Cancer Treat Rep 68: 193
7. Fidler IJ, Gersten DM, Hart IR (1978) The biology of cancer metastasis. Adv Cancer Res 28: 149
8. Fidler IJ, Gersten DM, Kripke ML (1979) Influence of immune status on the metastasis of three murine fibrosarcomas of different immunogenicities. Cancer Res 39: 3816
9. Fugmann RA, Anderson JC, Stolfi RL, Martin DS (1977) Comparison of adjuvant chemotherapeutic activity against primary and metastatic spontaneous murine tumors. Cancer Res 37: 496
10. Gasic GJ, Gasic TB, Galanti N, Johnson T, Murphy S (1973) Platelet-tumor cell interaction in mice. The role of platelets in the spread of malignant disease. Int J Cancer 11: 704
11. Gorelik E, Wiltrout RH, Okumura K, Habu S, Herberman RB (1982) Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. Int J Cancer 30: 107
12. Hanna N (1980) Expression of metastatic potential of tumor cells in young nude mice is correlated with low levels of natural killer cell-mediated cytotoxicity. Int J Cancer 26: 675
13. Hanna N (1982) Inhibition of experimental tumor metastasis by selective activation of natural killer cells. Cancer Res 42: 1337
14. Hanna N, Fidler IJ (1980) Role of natural killer cells in the destruction of circulating tumor emboli. JNCI 65: 801
15. Hanna N, Fidler IJ (1981) Relationship between metastatic potential and resistance to natural killer cell-mediated cytotoxicity in three murine tumor systems. JNCI 66: 1183
16. Jose DG, Good RA (1973) Quantitative effects of nutritional essential amino acid deficiency upon immune responses to tumors in mice. J Exp Med 137: 1
17. Lotan R, Nicolson G (1979) Heterogeneity in growth inhibition by β-*trans*-retinoic acid of metastatic B16 melanoma clones and in vivo selected cell variant lines. Cancer Res 39: 4767
18. Meadows GG, Oeser DE (1983) Response of B16 melanoma-bearing mice to varying dietary levels of phenylalanine and tyrosine. Nutr Rep Int 28: 1073
19. Meadows GG, Pierson HF, Abdallah RM, Desai PR (1982) Dietary influence of tyrosine and phenylalanine on the response of B16 melanoma to carbidopa-levodopa methylester chemotherapy. Cancer Res 42: 3056
20. Meyvisch C (1983) Influence of implantation site on formation of metastasis. Cancer Met Rev 2: 295
21. Pierson HF, Meadows GG (1983) Sodium ascorbate enhancement of carbidopa-levodopa methylester antitumor activity against pigmented B16 melanoma. Cancer Res 43: 2047
22. Poste G (1982) Experimental systems for analysis of the malignant phenotype. Cancer Res 1: 141
23. Poste G, Doll J, Hart IR, Fidler IJ (1980) In vitro selection of murine B16 melanoma variants with enhanced tissue-invasive properties. Cancer Res 40: 1636
24. Poste G, Doll J, Fidler IJ (1981) Interactions among clonal subpopulations affect stability of the metastatic phenotype in polyclonal populations of B16 melanoma cells. Proc Natl Acad Sci 78: 6226
25. Schabel FM Jr (1975) Concepts for systemic treatment of micrometastasis. Cancer 35: 15
26. Shami J, Wolf W (1977) A model for prediction of chemotherapy response to 5-fluorouracil based on the differential distrib-

ution of 5' ^{18}F fluorouracil in sensitive vs resistant lymphocytic leukemia in mice. Cancer Res 37: 2306
27. Silverman J, Rivenson A, Reddy B (1983) Effect of sodium ascorbate on transplantable murine tumors. Nutr Cancer 4: 192
28. Slack NH, Bross IDJ (1978) The influence of site of metastasis on tumor growth and response to chemotherapy. Br J Cancer 32: 78
29. Suzuki N (1983) Variant selection and blood-borne "clonogenic" tumor cells in metastasis of FSA cell clones. Br J Cancer 48: 827
30. Talmadge JR, Meyers KM, Prieur DJ, Starkey JR (1980) Role of natural killer cells in tumor growth and metastasis: C57BL/6 normal and beige mice. JNCI 65: 929
31. Talmadge JE, Benedict K, Madsen J, Fidler IJ (1984) Development of biological diversity and susceptibility to chemotherapy in murine cancer metastases. Cancer Res 44: 3801
32. Trope C (1975) Different sensitivity of cytostatic drugs of primary tumor and metastasis of Lewis carcinoma. Neoplasma 22: 171
33. Tsuruo T, Fidler IJ (1981) Differences in drug sensitivity among tumor cells from parental tumors, selected variants and spontaneous metastasis. Cancer Res 41: 3058
34. Wick MM, Beyers L, Frei E (1977, L-Dopa: selective toxicity for melanoma cells in vitro. Science 197: 468
35. Zbytntewski Z, Kanclerz A (1980) Effect of ascorbic acid on experimental pulmonary metastases of B-16 melanotic melanoma in C57BL/6J mice. Cesk Dermatol 55: 251

Received March 4, 1985/Accepted August 30, 1985