

Dietary Arginine Influences Rous Sarcoma Growth in a Major Histocompatibility B Complex Progressor Genotype (43325)

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Abstract. L-Arginine (L-Arg) can serve as a substrate for the production of reactive nitrogen intermediates. One of these metabolites, nitric oxide, has been shown to possess significant antitumor properties *in vitro*. To investigate the importance of this system *in vivo*, we have examined the dietary L-Arg host tumor interaction in the chicken. Since chickens are incapable of *de novo* L-Arg synthesis, concentration of this amino acid is readily controlled by diet. Line UNH 105 New Hampshire chickens having the major histocompatibility complex genotype, B²⁴/B²⁴, were used to study *in vivo* effects of dietary L-Arg on Rous sarcoma growth. After 5 weeks on a standard diet, 119 chicks were fed either a basal (0.92% L-Arg) diet or a high arginine (2.40% L-Arg) diet. One week later, chicks were wing-web inoculated with subgroup A Rous sarcoma virus. Tumor growth was monitored weekly for 12 weeks after inoculation. Plasma L-Arg levels and body weights from birds on each dietary treatment were analyzed. Neither body weight gains nor latent period for tumor development was affected by diet. However, plasma L-Arg levels were significantly different between dietary treatments (basal, 0.245 ± 0.01 μmol/ml; high, 0.738 ± 0.03 μmol/ml). In addition, mean tumor size scores were significantly (*P* < 0.05) lower over time in chickens fed the high L-Arg diet. The results suggest that dietary L-Arg in excess of the amount required for growth reduces tumor load.

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Selected dietary factors are known to be important in modulating immune function and enhancing protection from diseases, including cancer. Most investigations have focused on certain micronutrients, including such factors as vitamin E (1, 2) and selenium (3, 4). Others have concentrated on dietary fatty acids as important modulators of immune protection (5), while total dietary protein intake has also been an important consideration in cancer (6). Specific amino acids, as a dietary category, have received less attention as modulators of tumor immunity.

Recently, one amino acid, L-arginine (L-Arg), was shown to serve as a substrate for the production of reactive nitrogen intermediates (RNI), including nitric

oxide, by activated macrophages (7). Production of RNI is dependent both on the availability of L-Arg and the activation status of macrophages (8). Using *in vitro* models, the conversion of L-Arg to nitric oxide has been demonstrated to be important in the antimicrobial (9), antiparasite (10), and antitumor (11, 12) actions of macrophages. Arginine was recently reported to influence the growth of a murine neuroblastoma *in vivo* (13).

The chicken represents a potentially useful model to study the effects of L-Arg on tumor growth. The production of RNI by chicken macrophages is exquisitely sensitive to L-Arg availability and activation with lipopolysaccharide (14). Furthermore, because the chicken is uricotelic and cannot produce endogenous L-Arg (15), the availability of this important substrate can be effectively controlled by dietary administration (16–18). The present report describes the influence of dietary L-Arg on the growth of virally induced sarcomas. The results provide a connection between prior *in vitro* tumor cell studies (11, 12) and the utility of employing dietary strategies for optimizing tumor immunity.

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Materials and Methods

Stock. Line UNH 105 New Hampshire chickens having the major histocompatibility (*B*) complex genotype B^{24}/B^{24} were used. This *B* genotype progresses Rous sarcoma virus-induced tumors (19–22). Progeny were produced by mating B^{24}/B^{24} parents previously classified by blood typing using a panel of alloantisera (23). The chicks were immunized against Marek's disease upon hatching and Newcastle bronchitis at 10 days of age.

Experimental Diets. Two trials were conducted. All chicks were housed in heated brooders and fed a commercial chick starter mash that contained the recommended levels of all nutrients for the first 5 weeks. At 5 weeks of age, all birds were weighed, placed in grower cages, and then assigned to one of three replicates for either a basal (0.92% L-Arg) diet or a high L-Arg (2.40% L-Arg) diet. Both experimental diets contained 16.4% crude protein and were isocaloric (1337 kcal/kg). Additional body weights were taken after 3 and 7 weeks on the experimental diets.

Virus Inoculation. A highly purified pseudotype of Bryan high-titer Rous sarcoma virus, subgroup A was used at a 1/4000 dilution containing approximately 50 pock-forming units. Six-week-old chickens were inoculated in the left wing web with 0.1 ml of the diluted virus.

Assessment of Antitumor Response. For the first 2 weeks after inoculation, tumor growth was monitored in each bird daily to determine the latent period for tumor development. Thereafter, tumors were scored weekly until 12 weeks after inoculation, which corresponded to 18 weeks of age. Scoring was based on the scale listed in Table I (24). This scoring system has been highly correlated with tumor volume (24). Birds that died during the study received subsequent tumor scores of 7. All mortality was due to Rous sarcomas and total mortality was determined for each dietary treatment.

Arginine Analysis. Blood was collected in heparin from 10 birds per dietary treatment after 3 and 7 weeks on the diets. Plasma was separated by centrifugation and stored at -20°C . Prior to analysis, plasma samples

were deproteinized in 1.6% sulfosalicylic acid. Triplicate 1-gram samples of each diet were hydrolyzed for 20 hr in sealed tubes in 50 ml of 6 *N* HCl under N_2 at 15 psi. After filtration through Whatman No. 41 filter paper and dilution to 100 ml with water, samples were evaporated using a rotary vacuum evaporator and dissolved in 0.05 *M* lithium citrate buffer (pH 2.0). L-Arg in plasma and dietary samples was determined by ion exchange chromatography using a model TSM amino acid analyzer (Technicon Instruments Corp., Tarrytown, NY).

Statistical Analysis. Mean weekly tumor scores of 119 chickens (61 basal diet and 58 high L-Arg diet) were evaluated by least squares analysis of variance using a repeated-measures split-plot design. Initial body weight, weight gain at 3 and 7 weeks, latent period, and plasma L-Arg levels were analyzed by least-squares analysis of variance. Significant means were separated by Fisher's Protected LSD at $P < 0.05$. Mortality in the two dietary treatments was tested by chi-square.

Results

Chickens fed two discrete levels of dietary L-Arg had plasma L-Arg levels that were significantly different (Fig. 1). After 3 weeks, chickens fed the basal diet had $0.184 \pm 0.01 \mu\text{mol/ml}$ of plasma L-Arg compared with $0.784 \pm 0.03 \mu\text{mol/ml}$ of plasma L-Arg for chickens on the high L-Arg diet. Similar results were found in samples taken after 7 weeks on the diets. The basal group had $0.245 \pm 0.01 \mu\text{mol/ml}$ of plasma L-Arg, while the high L-Arg group had $0.734 \pm 0.03 \mu\text{mol/ml}$ of plasma L-Arg (Fig. 1).

Despite the significant differences in plasma L-Arg, growth was not affected. Initial body weights when the experimental diets were begun were not significantly different (data not shown). Furthermore, weight gains were not significantly different between dietary treat-

Table I. Criteria for Tumor Size Scores of Rous Sarcomas in Line UNH 105 B^{24}/B^{24} New Hampshire Chickens^a

Score	Criterion
0	No palpable tumor
1	Small tumor (up to 0.5 cm in diameter)
2	Tumor (>0.5 up to 1.2 cm in diameter)
3	Tumor (>1.2 cm up to 1/2 wingweb area)
4	Tumor (>1/2 wingweb area, but < entire wingweb)
5	Tumor (filled entire wingweb)
6	Tumor (massive; extended beyond wingweb)

^a See Ref. 24.

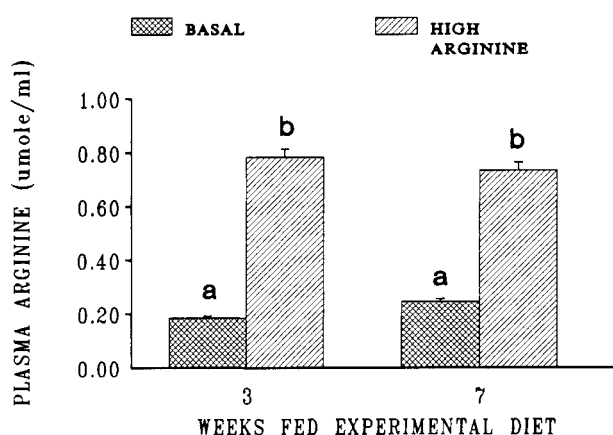


Figure 1. Mean plasma arginine concentration in line UNH 105 B^{24}/B^{24} New Hampshire chickens fed either basal (0.92% L-Arg) or high L-Arg (2.40% L-Arg) diet. Bars indicate the standard error. Dietary groups within each time period having unlike superscripts differ significantly ($P < 0.05$).

ments. Chickens fed the basal diet gained 425.4 ± 10.5 g and 908.4 ± 31.4 after 3 and 7 weeks, respectively. The high arginine diet group gained 423.4 ± 9.6 g and 919.6 ± 33.2 g after 3 and 7 weeks, respectively.

The latent period for tumor development following Rous sarcoma virus inoculation was similar in both groups. Chickens fed the basal L-Arg diet developed tumors in 10.1 days, while the high L-Arg group developed tumors in 10.5 days. These values did not differ significantly.

Analysis of the tumor growth revealed that the high L-Arg diet group had significantly lower tumor growth over time than did the basal L-Arg diet group (Fig. 2). Lower mean tumor size scores were observed beginning at the 10-week scoring period and continued to the end of the experiment. Mortality was not significantly different between dietary treatments: 16.4% in the basal diet and 8.6% in the high L-Arg diet.

Discussion

Recent evidence suggests that the availability of L-Arg is a crucial factor in the ability of activated macrophages to produce nitric oxide in mammalian (8) and avian (14) species. Nitric oxide secretion is reported to represent a major macrophage antitumor effector function, at least *in vitro* (11). Therefore, the *in vivo* status of L-Arg availability to macrophages should represent an important factor in host tumor protection. However, the relevance of the production of nitric oxide from L-Arg to *in vivo* tumor protection has received little attention.

In the present study, a solid tumor model, Rous sarcoma, was employed. This virally induced tumor model has the advantage of possessing a known early tumor growth phase that is usually unaffected by immune response, followed by a secondary phase that results either in a progressive or a regressive tumor (25). This latter phase is the period in which a genetically

determined immune response attributable to the major histocompatibility complex is known to influence the tumor growth profile (24, 26). For these studies, chickens were employed that possessed a B complex haplotype (B^{24}) known to result in a progressive sarcoma. When different levels of dietary L-Arg were provided to these chickens during the period of tumor production, a significant influence on tumor growth was observed (Fig. 2). It is important to note that the modulation of tumor growth occurred in the absence of significant overall growth differences among the chickens. Furthermore, the latency period required for tumor induction was unaffected by the dietary difference between groups. Differential dietary administration of L-Arg did result in differential arginine availability *in vivo*, as reflected by the plasma concentrations of L-Arg.

The plasma concentrations of arginine in the basal group ($0.184\text{--}0.245$ $\mu\text{mol/ml}$) were similar to the levels reported by others in chicks receiving adequate dietary arginine (16–18) and are well below the concentration (<0.4 $\mu\text{mol/ml}$) which results in maximum RNI production by activated chicken macrophages *in vitro* (14). Conversely, the plasma arginine concentrations ($0.734\text{--}0.784$ $\mu\text{mol/ml}$) in the chickens receiving supplemental L-Arg are well above the arginine concentration that maximizes RNI production. Studies of mouse macrophages *in vitro* also indicate that approximately 0.4 $\mu\text{mol/ml}$ of L-Arg is required for maximal production of RNI (9).

Reynolds *et al.* (13) investigated the effect of dietary arginine on the tumor growth in mice that had been induced by injecting two related neuroblastoma cell lines into the flanks of 6-week-old mice. Supplementation of the diet with 1.0% arginine increased the serum arginine concentration from 0.108 $\mu\text{mol/ml}$ to 0.173 $\mu\text{mol/ml}$. Tumors arising from both neuroblastoma lines were significantly decreased by arginine during 28 days of nutritional treatment. It is clear from both the chicken and mouse studies that dietary arginine levels that would be expected to increase RNI production by activated macrophages suppress the growth of tumors. Whether this effect on tumor growth is due to RNI or to other effects of arginine remains to be determined.

Clark *et al.* (27) studied dietary effects on the growth of Rous sarcomas. Inbred chickens possessing the B complex genotypes B^2/B^2 (regressor) and B^5/B^5 (progressor) had protein and calories restricted to 60% of full-fed controls. In both regressor and progressor B genotypes, the restricted group had significantly fewer tumors and a significantly longer latent period for tumor development compared with full-fed animals. Furthermore, feed restriction significantly reduced mean tumor size for the first 4 weeks after inoculation, but not during subsequent time periods through 10 weeks after infection.

This dietary manipulation employed by Clark *et*

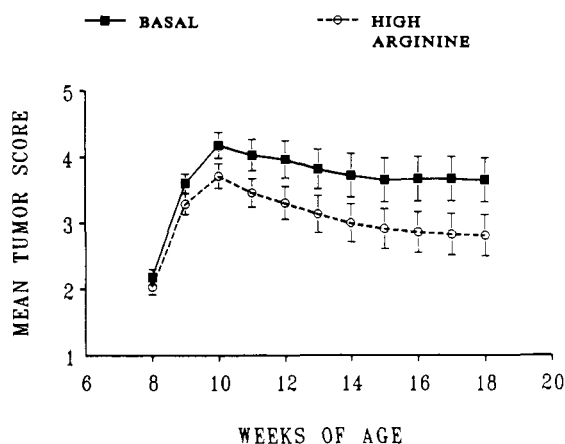


Figure 2. Mean tumor size scores for Rous sarcomas in line UNH 105 B^{24}/B^{24} New Hampshire chickens fed either basal (0.92% L-Arg) or high L-Arg (2.40% L-Arg) diet. Bars indicate the standard error. Dietary treatments differ significantly ($P < 0.05$) over time.

al. (27) was not limited to a single amino acid. While arginine would have been lower in the feed-restricted animals, other essential amino acids and calories would have been lower also. The restriction of total protein and calories reduced body growth as well as tumor growth. Differences in early tumor growth may have been caused by insufficient viral replication due, in part, to the dietary restriction.

The current study found that the feeding of different arginine levels did not affect body growth, tumor susceptibility, or the tumor latency. Yet, differential L-Arg concentrations *in vivo* were found to be associated with different tumor growth profiles. Therefore, we hypothesize that L-Arg may modulate tumor immunity through increased production of nitric oxide by macrophages. Additional studies concerning host immune effector functions and L-Arg-dependent tumor protection are warranted.

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