High doses of dietary arginine during repletion impair weight gain and increase infectious mortality in protein-malnourished mice

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There is considerable evidence for the beneficial effects of dietary arginine, a conditionally-essential amino acid that enhances anabolism and T-cell function. However, the safety and efficacy of higher doses of arginine supplementation following infection have not been investigated completely. These issues were explored therefore, in a murine model of malnutrition and infection. Severe protein malnutrition was induced by feeding mice for 6 weeks on an isoenergetic diet containing only 10 g protein/kg. Mice were then allowed to consume diets with normal amounts of protein (200 g/kg) with 50 g/kg provided as amino acid mixtures of glycine and arginine in which the arginine content ranged from 0 to 50 g/kg. During the repletion period a significant weight gain was noted in the groups fed on diets with either 10 or 20 g arginine/kg, but not in the group fed on the diet with 50 g arginine/kg, compared with the diet with 0 g arginine/kg. Mortality rates after infection with *Salmonella typhimurium* were not decreased by the addition of 10 or 20 g arginine/kg to the diet, and were in fact worsened by supplementation with 50 g arginine/kg. The results of the present study showed that not only are the beneficial effects of arginine supplementation after infection lost when high doses are administered, but also that these high doses become toxic. Mice fed on higher doses showed significant impairment of weight gain and an increase in mortality rates.

Arginine: Malnutrition: Infection

Arginine has been described as a non-essential dietary amino acid. Biological functions requiring arginine in adult humans can occur in the absence of dietary arginine because arginine can be synthesized from ornithine, a urea cycle intermediate. Under certain conditions, however, the endogenous synthesis of arginine is apparently inadequate, and dietary supplements are needed. For example, Borman *et al.* (1946) showed that dietary arginine is necessary for normal growth in immature rats. Subsequently, Barbul *et al.* (1980*a*, 1984) demonstrated that arginine supplementation improves weight gain in rats recovering from trauma, that it leads to increased weight and cellularity of the thymus, and that lymphocytes from animals fed on the arginine-supplemented diets show an enhanced proliferative response when stimulated by lectins.

Several studies, both in man and in rodents, have confirmed the thymotropic effects of arginine (Barbul *et al.* 1980*b*, 1981, 1984; Daly *et al.* 1988). In addition, three animal studies have produced results suggesting that dietary arginine supplementation improves outcome from infection. First, Saito *et al.* (1987) noted that arginine supplementation after injury is associated with improved cell-mediated immunity and survival in burned guinea-pigs. Second, Madden *et al.* (1988) observed that survival from caecal ligation and puncture (CLP) is improved in rats given either oral supplementation before or intravenous arginine supplementation after the onset of infection. Finally, Gianotti *et al.* (1993) noted that mice fed on supplemental dietary arginine for 10 d before CLP have higher survival rates than controls.

Some evidence, however, suggests that high doses of arginine carry some risk. For example, in the study of burned guinea-pigs by Saito *et al.* (1987) the beneficial effects of arginine supplementation were lost when the level of supplementation was increased to 4% total energy (en %). In addition, Gonce *et al.* (1990) found that arginine supplementation at 2 and 4 en % was not associated with higher survival rates in septic guinea-pigs, and that supplementation at a higher level (6 en %) was associated with decreased N retention and increased mortality.

The work reported in the present paper describes our attempts to define the safety and efficacy of arginine supplementation during dietary repletion of malnourished mice. Several doses of arginine supplementation were used in an attempt to investigate the effects on weight gain and rates of survival after *Salmonella typhimurium* infection over a range of doses. In addition, *in vitro* testing of lymphocyte proliferation and assays of the endproducts of reactive (arginine) N metabolism were performed.

METHODS

All mice were purchased from Charles River Suppliers (Cambridge, MA, USA), and allowed free access to natural laboratory chow (Wayne Rodent Blox®; Libertyville, IN, USA) and water for 1 week. Female C3H/HeN mice weighing 18–20 g were used. All procedures were conducted with the previous approval of the University of Cincinnati Animal Care and Use Committee according the guidelines established in the *Guide for the Care and Use of Laboratory Animals* (National Research Council Committee on the Care and Use of Laboratory Animals, 1985).

Experimental diets

Using food-grade ingredients from ICN Biochemical (Cleveland, OH, USA), diets were prepared in our laboratory. The basic diet formulation used was the purified diet AIN-76 recommended by the American Institute of Nutrition (1977) for the support of growth and reproduction in rodents. In this purified diet the protein source was 200 g vitamin-free casein/kg, the fat source was 50 g maize oil/kg, and the carbohydrates used were 150 g maize starch/kg and 500 g sucrose/kg. AIN-76 vitamin and mineral mixes were used.

The experimental diets were based on the AIN-76 formulation, except that they were made with a fixed amount of casein (150 g/kg) and with varying amounts of supplemental arginine and glycine. Glycine was used to balance the amount of free amino acid in the diet, so that the total weight of additional free amino acids remained constant. However, because arginine has a higher N content than glycine, the total amount of supplementary N increased with increasing amounts of arginine. As the amount of supplementary arginine was increased there was a corresponding decrease in the amount of glycine. Mice were fed on 3 g chow/d, which was a slight food restriction but did ensure that all chow was consumed each day.

The diet used to induce protein malnutrition was also based on the AIN-76 formulation. However, the casein content was reduced to 10 g/kg, and there was a corresponding increase in carbohydrates. No other components were changed. This diet has been used to induce severe malnutrition over a 6-week period in C3H/HeN mice, which then have an increased susceptibility to infection with *S. typhimurium* (Peck & Alexander, 1992).

Infection with Salmonella typhimurium *by intraperitoneal injection in malnourished mice*

In this model we tested the hypothesis that enteral feeding of arginine would improve survival from a subsequent parenteral challenge of live bacteria in protein-malnourished mice. *S. typhimurium* is an intracellular pathogen to which sensitivity is increased by malnutrition.

The six groups may be summarized as follows: group 1, female C3H/HeN mice (*n* 64) were fed on the standard purified diet (AIN-76) containing casein 200 g/kg for 59 d (42 d while the other groups were protein-malnourished, 3 d while the other groups were repleted, and 14 d during the 2-week observation period following *S. typhimurium* infection). Their nutritional status and body weight remained normal; group 2, mice (*n* 60) were fed on the low-protein (casein 10 g/kg) diet for 6 weeks to induce protein malnutrition, infected with *S. typhimurium* (strain 1344; 10^4 colony-forming units by intraperitoneal injection) and continued on the low-protein diet until death from *S. typhimurium* infection ensued; group 3, mice (*n* 61) were fed on the low-protein diet for 6 weeks, and then repleted for 3 d with diets containing (g/kg): casein 150, arginine 0, glycine 50. They were then infected with *S. typhimurium* and continued on this experimental diet. Mice that did not succumb to infection were killed 14 d after the infectious challenge; group 4, mice (*n* 61) were fed on the low-protein diet for 6 weeks, and then repleted for 3 d with diets containing (g/kg): casein 150, arginine 10, glycine 40. They were then infected with *S. typhimurium* and continued on this experimental diet; group 5, mice (*n* 59) were fed on the low-protein diet for 6 weeks, and then repleted for 3 d with diets containing (g/kg) casein 150, arginine 20, glycine 30. They were then infected with *S. typhimurium* and continued on this experimental diet; group 6, mice (*n* 59) were fed on the low-protein diet for 6 weeks, and then repleted for 3 d with diets containing (g/kg) casein 150, arginine 50, glycine 0. They were then infected with *S. typhimurium* and continued on this experimental diet. The mice were weighed on day 0, on day 42 after 6 weeks of protein underfeeding, and on day 45 after 3 d of repletion.

Another five groups of mice (*n* 47) were fed as described previously but were not infected with *S. typhimurium*. After 7 d of refeeding the animals were weighed and killed by cervical dislocation, and the spleens were removed aseptically for assays of lymphocyte blastogenesis. Serum samples were taken by intracardiac puncture just before death. These serum samples were assayed for total nitrogen oxides, both nitrates and nitrites (NO_2^-/NO_3^-).

Lymphocyte proliferation

Splenic lymphocytes were isolated from freshly killed animals. At the time of death the spleen was aseptically removed and placed in Hanks' balanced salt solution (HBSS). After aseptic removal the spleens were minced with forceps and a scalpel and filtered through glass wool to remove debris. Erythrocytes were lysed hypotonically and residual leucocytes were washed twice in HBSS and suspended in M199 medium containing 10 mg streptomycin + 100 mg penicillin + 100 ml autologous serum/l. The cells were then added to tissue culture plates and incubated for 1 h to allow adherence of the macrophages, and the non-adherent cells were removed. Viability of the cells was ascertained by trypan blue exclusion.

This mixture was centrifuged at 400 *g*, for 10 min at 4°, decanted and resuspended in HBSS. The wash procedure was repeated. The pellet was resuspended in HBSS to a 1:10 (v/v) dilution and counted with gentian violet dye. Responder lymphocytes were resuspended to a final concentration comprising 1×10^9/l in RPMI 1640 with L-glutamine, 100 ml autologous plasma/l, 1 mM-sodium pyruvate 5×10^5 mM-2-mercaptoethanol, 100 mg streptomycin/l and 100 mg penicillin/l.

The mononuclear cells were stimulated with 1 mg lipopolysaccharide (LPS)/l which selectively stimulates B cells. Stimulation was done in tissue culture plates in RPMI 1640 (Gibco, Grand Island, NY, USA) medium supplemented with 10 mM-Hepes, 10^5 units penicillin/l, 100 mg streptomycin sulphate/l, 5×10^{-5} M-2-mercaptoethanol, and 100 ml pooled mouse serum/l from each experimental group in tissue culture plates. Following incubation for 24–48 h at 37° the cells (2×10^5/200 μl) were pulsed for the final 24 h of culture with [^{3}H]TdR (1·0 μCi/2×10^5 cells). The amount of [^{3}H]TdR incorporation was measured in a liquid-scintillation counter (Model LS 3155T; Beckman® Instruments, Irvine, CA, USA). The stimulation index (SI) was calculated as:

$$\frac{\text{splenocytes} + \text{mitogens}}{\text{splenocytes} + \text{medium}}.$$

Assays of total nitrogen oxides

Plasma NO_2^-/NO_3^- levels were measured using a modification of an HPLC assay, based on the Griess reaction. In brief, plasma samples were collected in heparinized tubes and stored at −70° until assayed. A 200 μl portion of sample was made alkaline with 400 μl 0·5 M-NaOH. The sample was then deproteinized with 400 μl $ZnSO_4$ (100 g/l), and centrifuged at 10000 rev./min for 5 min. The supernatant fraction was then collected and used immediately. Using an HPLC system, NO_3^- was reduced to NO_2^- with the use of a Cu-coated Cd column. With this technique, recovery of NO_2^-/NO_3^- has been over 95% (Wagner *et al.* 1983; Stuehr & Marletta, 1985).

Statistics

Results were analysed statistically using a Macintosh SE™ computer (Apple Computer, Inc., Cupertino, CA, USA) with Statview 512™ software (BrainPower, Inc., Calabasas, CA, USA). Repeated-measures ANOVA was used to compare the change in weight among groups with time. One-way ANOVA was used to compare the SI calculated from lymphocyte proliferation studies and the levels of nitrates and nitrites among groups. Scheffé's *F* procedure was used for multiple two-group comparisons. Since the SI and the levels of nitrates and nitrites were not normally distributed, the values were $\log_e$-transformed before analysis. Mortality rates were compared using the chi-square test of independence. Length of survival was compared using the Kruskal-Wallis test. Simple linear regression was done using the method of least squares to test the presence of a dose–response effect of the amount of dietary arginine on either B cell blastogenesis or on total nitrogen oxides. A probability level of 0·05 was accepted as significant.

RESULTS

Survival after infection

The survival rate after infection was 0% (0/60) in group 2, the malnourished mice which were not repleted. Survival (%) was 57 (35/61) in group 3, 62 (38/61) in group 4, 68 (40/59) in group 5, and 39 (23/59) in group 6. Although there was a trend toward a higher survival rate in groups 4 and 5 compared with group 3, this difference was not statistically

Table 1. *Weight change (g) of malnourished C3H/HeN mice after a period of refeeding with supplementary arginine*

(Mean values with their standard errors)

Dietary group*...	1		3		4		5		6	
Arginine supplement (g/kg)...	Control		0		10		20		50	
n...	64		61		61		54		59	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Day 0	18·9	0·15	18·0	0·18	18·2	0·12	18·3	0·13	18·4	0·15
Day 42	23·7	0·21	13·5	0·21	13·5	0·21	13·3	0·19	13·4	0·18
Day 45	24·5	0·19	15·7	0·20	16·8	0·16	16·7	0·17	14·8	0·18
Statistical significance of difference (by Scheffé's *F* procedure): *P* =										
Group 1	—		—		—		—		—	
Group 3	0·0001		—		—		—		—	
Group 4	0·0001		0·2233		—		—		—	
Group 5	0·0001		0·4823		0·9950		—		—	
Group 6	0·0001		0·7787		0·0093		0·0453		—	

* Group 1 was fed the AIN-76 diet (American Institute of Nutrition, 1977) for 45 d, groups 3–4 were fed on low-protein diets for 42 d, and then were repleted on experimental diets for 3 d before infection (days 43–45). For details of diets and procedures, see pp. 788–790.

significant. However, the survival rate in group 6 was significantly lower than that in groups 3, 4 and 5 (χ^2: $P = 0{\cdot}01$). Similarly, the median length of survival was shorter in group 6 (10 d) compared with groups 3, 4, and 5 (15 d each).

Weight change during repletion of malnourished mice

After 6 weeks of protein malnutrition the mice were weighed and found to have lost a mean of 28·2 (SEM 0·6) % of initial body weight. The mice were weighed after 3 d of refeeding, just before infection with *S. typhimurium* (Table 1). Weight gain from days 42 to 45 was significantly higher in groups 3, 4, 5, and 6 compared with group 1 (repeated-measures ANOVA: df 4, F 560, $P = 0{\cdot}0001$), as the malnourished animals began to replete body mass during the refeeding period. Among the malnourished mice undergoing body mass repletion, those animals from groups 4 and 5 had higher weight gain than those from groups 3 and 6, but not to a degree that was statistically significant. Animals from group 6, on the other hand, had a significantly lower weight gain than those from groups 4 and 5 during the refeeding period ($P = 0{\cdot}0093$ and 0·0453 respectively, by Scheffé's procedure).

Assays of lymphocyte responsiveness

Response to the mitogen LPS was higher in group 1 than in group 3 (Table 2). Response to LPS was also higher in groups 4, 5, and 6 compared with group 3, but not to a degree that was statistically significant. Stimulation of B cells by LPS was associated with the amount of arginine in the diet ($P = 0{\cdot}03$, r 0·378), suggesting a dose–response effect.

Assays of total nitrogen oxides (nitrites and nitrates)

Serum NO_2^-/NO_3^- levels were higher in groups 1 and 6 compared with groups 3 and 4, but not to a degree that was statistically significant (Table 2). Serum levels of NO_2^-/NO_3^- did not correlate with lymphoproliferation, but was related to the amount of arginine in the diet ($P = 0{\cdot}0106$, r 0·452), which suggested a dose–response effect.

Table 2. *Lymphocyte proliferation and nitrate/nitrite levels in malnourished C3H/HeN mice after a period of refeeding with supplementary arginine*

(Mean values with their standard errors before $\log_e$ transformation for statistical analysis)

Dietary group...	1		2		3		4		5		6	
Arginine supplement (g/kg)...	Control		Control, malnourished		0		10		20		50	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
n...	9		7		7		8		8		8	
LPS (SI)†	8·8	1·2	3·9	0·41	3·1*	0·54	6·7	1·35	6·2	1·32	7·6	1·15
n...	8		0‡		7		8		8		8	
NO_2^-/NO_3^- (mol/l)§	20·9	4·30			14·1	3·16	13·7	2·52	18·9	2·84	25·8	4·11

LPS, lipopolysaccharide; SI, stimulation index $\left(\frac{\text{splenocytes + mitogens}}{\text{splenocytes + medium}}\right)$.

* Mean value for group 3 was significantly lower than that for group 1 (paired comparisons by Scheffé's *F* procedure): $P = 0{\cdot}0137$.

† Statistical significance of difference by one-way ANOVA on $\log_e$-transformed data: df 5, F 4·427, $P = 0{\cdot}026$.

‡ Reactive nitrogen intermediates were not measured in group 2; because of severe malnutrition we were unable to obtain enough serum to do the assays.

§ Statistical significance of difference by one-way ANOVA on $\log_e$-transformed data: df 5, F 2·796, $P = 0{\cdot}0419$.

DISCUSSION

The effect of dietary arginine supplementation on survival

Malnourished mice were repleted with diets containing varying amounts of supplementary arginine before challenge with *S. typhimurium*. Although there was a trend towards higher survival rates with lower levels of arginine supplementation (10 and 20 g/kg), these differences were not statistically significant. Similarly, one recent study performed in this laboratory has also shown no beneficial effect of supplemental arginine at lower levels (1 and 2 en%) in guinea-pigs made septic with *Staphylococcus aureus* and *Escherichia coli* infused into the peritoneal cavity from osmotic pumps (Gonce *et al.* 1990). Other studies that showed improved survival with arginine supplementation were different in that they were performed in rats challenged with CLP (Madden *et al.* 1988; Gianotti *et al.* 1993), in which tissue necrosis of the caecum leads to a polymicrobial peritoneal infection, with peritonitis and abscess formation. Differences in results may be due to differences in the models.

Of concern in this current report is the evidence that with high levels of dietary arginine supplementation much of the survival advantage conferred by this amino acid is lost. We have previously reported that septic guinea-pigs suffered a higher mortality rate when fed on diets with 6 en% as supplementary arginine (Gonce *et al.* 1990). Similarly, in our current experiments, malnourished mice fed on diets with 50 g supplementary arginine/kg (equivalent to 5 en%) had a higher mortality rate when challenged with *S. typhimurium* than similarly treated mice fed on diets with lower amounts of arginine. Others have noted previously that the beneficial effects of supplementary arginine were lost when higher levels were used (Saito *et al.* 1987).

The mechanisms responsible for the loss of survival benefit observed with high levels of dietary arginine are not understood. It is known that arginine plays a role in the urea cycle, in polyamine synthesis, and in the production of reactive nitrogen intermediates, especially NO. For example, these reactive nitrogen intermediates are involved in macrophage

cytotoxicity against target tumour cells, and are capable of mediating the effect of Kupffer cells on hepatocyte protein synthesis (Hibbs *et al.* 1988; Billiar *et al.* 1989). Furthermore, byproducts of the reactive nitrogen intermediates, i.e. nitrates and nitrites, occur in higher levels in the plasma of septic humans (Ochoa *et al.* 1991), suggesting that NO, as endothelium-derived relaxing factor, diminishes vascular tone in sepsis (Palmer, 1993). In the present study we found elevated serum levels of NO_2^-/NO_3^- which paralleled the increase of arginine in the diets.

Arginine may also affect outcome by changing the hormonal milieu. For example, rats administered parenteral arginine (5 mg/kg) subsequent to the intravenous injection of endotoxin had significantly higher mortality than controls (Yelich & Filkins, 1983). This phenomenon was associated with hyperinsulinaemia and hypoglycaemia, suggesting a mechanism for the increased susceptibility to endotoxin. In another study, plasma glucose levels from burned guinea-pigs were elevated during feeding with diets containing arginine at 4 en% (Saito *et al.* 1987).

Enhancement of B-cell function by arginine supplementation may also alter the response of mice to bacterial infection. Biozzi *et al.* (1979) observed that mice can be selectively bred for either low or high antibody responsiveness to Salmonella antigens. Both natural and induced resistance to infection with *S. typhimurium* is stronger in low antibody responders. It is possible that arginine increases antibody production and, thus, lowers resistance to infection in this model.

The effects of dietary arginine supplementation on weight gain during repletion following malnutrition

Lower levels of arginine supplementation in diets fed to malnourished mice during the repletion period improved weight gain, but this effect was lost when high levels of arginine were fed.

In the present study severe protein malnutrition was induced by feeding mice for 6 weeks with diets containing 10 g casein/kg. We had noted already that this protocol results in severe weight loss and wasting, and an increased susceptibility to *S. typhimurium* (Peck & Alexander, 1992). The current experiments allowed protein-malnourished mice to consume diets with normal amounts of protein and free amino acids (150 g casein/kg and 50–60 g amino acids/kg) during which time a significant weight gain was noted in the groups fed on diets with either 10 or 20 g arginine/kg. This is consistent with previous reports describing the benefit of dietary arginine supplementation on repletion of body mass in malnourished rats (Kari *et al.* 1981; Iyamu & Adamson, 1982; Mulloy *et al.* 1982).

Higher levels of arginine supplementation were associated with loss of this beneficial effect. That is, malnourished mice repleted on diets containing 50 g arginine/kg did not manifest the same rate of weight gain as did their counterparts in the 10 and 20 g arginine/kg groups. Previous reports have shown that as the amount of arginine in the diet is increased there is a loss of beneficial effect on N balance. Both burned and septic guinea-pigs exhibited increasing N losses when supplementary dietary arginine was elevated to 4 and 6 en% respectively (Saito *et al.* 1987; Gonce *et al.* 1990).

The effects observed in group 6 may also be due to the relative imbalance in amino acids which occurred when glycine was totally eliminated from the supplementary amino acid mixture. Stucki & Harper (1962) found that diets which contained relatively large excesses of either indispensable or dispensable amino acids were inferior to diets with more balanced proportions of amino acids when growth, food consumption, and final body composition were measured in rats. The more balanced amino acid mixtures in groups 4 and 5 had the highest survival rates in our study. Nonetheless, of the two relatively-imbalanced amino

acid supplements which we used, arginine had a more pronounced deleterious effect on survival than glycine.

The effect of dietary arginine supplementation on lymphocyte proliferation

Splenocytes from malnourished mice repleted with diets containing supplementary arginine had an increased response to the mitogen LPS, although not to a degree that was statistically significant. There was a weak dose–response relationship to the amount of arginine in the diet. Previous studies have shown that arginine supplementation improves the mitogenic response of lymphocytes, specifically T cells. In general, lymphocyte responsiveness to both concanavalin A and phytohaemagglutinin is increased by arginine supplementation in both normal and injured rats (Barbul *et al.* 1980, 1984). Our results also show that increasing amounts of supplementary arginine are associated with increases in the lymphoblastogenic response of B cells.

The effect of dietary arginine supplementation on total nitrogen oxides

Serum levels of NO metabolites (NO_2^- and NO_3^-) increased with the amount of supplementary dietary arginine in repleted malnourished mice which were not infected. However, the serum levels of NO_2^-/NO_3^- were not correlated with lymphocyte mitogenic response.

Conclusions

The effect of dietary arginine supplementation during repletion of malnourished mice on the outcome from bacterial challenge is beneficial to restoration of body mass, but only at moderate levels (i.e. 10 or 20 g/kg). Dietary arginine supplementation also stimulates the lymphocyte response to mitogen stimulation in a dose-dependent manner. These findings are consistent with those from other experiments in which recovery from trauma, burns, or malnutrition have been studied in animals.

The results also show that arginine supplementation during repletion following protein malnutrition has a marginally beneficial effect on survival rates in moderate doses which is lost at high doses. Dietary arginine supplementation at 50 g/kg nullified the improvement in weight gain seen at moderate doses and worsened mortality rates from *S. typhimurium* infection. NO production contributes to the complex cellular interactions in the septic animal, and excessive NO production associated with higher levels of arginine supplementation may contribute to the loss of the beneficial effects which are seen at lower levels. However, this hypothesis, despite its attractiveness, remains unproven.

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REFERENCES

American Institute of Nutrition (1977). Report of the American Institute of Nutrition *Ad Hoc* Committee on Standards for Nutritional Studies. *Journal of Nutrition* **107**, 1340–1348.

Barbul, A., Fishel, R. S., Shimazu, S., Wasserkrug, H. L., Yoshimura, N. N., Tao, R. C. & Efron, G. (1985). Intravenous hyperalimentation with high arginine levels improves wound healing and immune function. *Journal of Surgical Research* **38**, 328–334.

Barbul, A., Sisto, D. A., Waserkrug, H. L. & Efron, G. (1981). Arginine stimulates lymphocyte immune response in healthy human beings. *Surgery* **90**, 244–251.

Barbul, A., Wasserkrug, H. L., Seifter, E., Rettura, G., Levenson, S. M. & Efron, G. (1980*a*). Immunostimulatory effects of arginine in normal and injured rats. *Journal of Surgical Research* **29**, 228–235.

Barbul, A., Wasserkrug, H. L., Sisto, D. A., Seifter, E., Rettura, G., Levenson, S. M. & Efron, G. (1980*b*). Thymic stimulatory actions of arginine. *Journal of Parenteral and Enteral Nutrition* **4**, 446–449.

Barbul, A., Wasserkrug, H. L., Yoshimura, N. N., Tao, R. & Efron, G. (1984). High arginine levels in intravenous hyperalimentation abrogate post-traumatic immune suppression. *Journal of Surgical Research* **36**, 620–624.

Billiar, T. R., Curran, R. D., Stuehr, D. J., West, M. A., Bentz, B. G. & Simmons, R. L. (1989). An L-arginine-dependent mechanism mediates Kupffer cell inhibition of hepatocyte protein synthesis in vitro. *Journal of Experimental Medicine* **169**, 1467–1472.

Biozzi, G., Mouton, D., Sant'Anna, O. A., Passos, H. C., Gennari, M., Reis, M. H., Ferreira, V. C., Heumann, A. M., Bouthillier, Y., Ibanez, O. M., Stiffel, C. & Siqueira, M. (1979). Genetics of immunoresponsiveness to natural antigens in the mouse. *Current Topics in Microbiology and Immunology* **85**, 31–98.

Borman, A., Wood, T. R., Black, H. C., Anderson, E. G., Oesterling, M. J., Womack, M. & Rose, W. C. (1946). The role of arginine in growth with some observations on the effects of arginic acid. *Journal of Biological Chemistry* **166**, 585.

Daly, J. M., Reynolds, J., Thom, A., Kinsley, L., Dietrick-Gallagher, M., Shou, J. & Ruggieri, B. (1988). Immune and metabolic effects of arginine in the surgical patient. *Annals of Surgery* **208**, 512–516.

Gianotti, L., Alexander, J. W., Pyles, T. & Fukushima, R. (1993). Arginine-supplemented diets improve survival in gut-derived sepsis and peritonitis by modulating bacterial clearance: The role of nitric oxide. *Annals of Surgery* **217**, 644–654.

Gonce, S. J., Peck, M. D., Alexander, J. W. & Miskell, P. W. (1990). The effect of supplemental arginine on recovery from peritonitis in guinea pigs. *Journal of Parenteral and Enteral Nutrition* **14**, 237–244.

Hibbs, J. B., Taintor, R. R., Vavrin, Z. & Rachlin, E. M. (1988). Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochemical and Biophysical Research Communications* **157**, 87–94.

Iyamu, J. O. & Adamson, I. (1982). Effect of supplemental arginine on rehabilitation of the starved rat. *Nutrition Reports International* **26**, 335–345.

Kari, F. W., Ulman, E. A., Mulloy, A. L. & Visek, W. J. (1981). Arginine requirement of mature protein-malnourished rats for maximal rate of repletion. *Journal of Nutrition* **111**, 1489–1493.

Madden, H. P., Breslin, R. J., Wasserkrug, H. L., Efron, G. & Barbul, A. (1988). Stimulation of T cell immunity by arginine enhances survival in peritonitis. *Journal of Surgical Research* **44**, 658–663.

Mulloy, A. L., Kari, F. W. & Visek, W. J. (1982). Dietary arginine, insulin secretion, glucose tolerance and liver lipids during repletion of protein-depleted rats. *Hormonal and Metabolic Research* **14**, 471–475.

National Research Council Committee on the Care and Use of Laboratory Animals (1985). *Guide to the Care and Use of Laboratory Animals. National Institutes of Health Publication* no. 86-23. Bethesda, Md: National Institutes of Health.

Ochoa, J. B., Udekwu, A. O., Billiar, T. R., Curran, R. D., Cerra, F. B., Simmons, R. L. & Peitzman, A. B. (1991). Nitrogen oxide levels in patients following trauma and during sepsis. *Annals of Surgery* **214**, 621–626.

Palmer, R. M. J. (1993). The discovery of nitric oxide in the vessel wall: A unifying concept in the pathogenesis of sepsis. *Archives of Surgery* **128**, 396–401.

Peck, M. D. & Alexander, J. W. (1992). The interaction of protein and zinc malnutrition with the murine response to infection. *Journal of Parenteral and Enteral Nutrition* **16**, 232–235.

Peitzman, A. B. (1991). Nitrogen oxide levels in patients following trauma and during sepsis. *Annals of Surgery* **214**, 621–626.

Saito, H., Trocki, O., Wang, S., Gonce, S. J., Joffe, S. N. & Alexander, J. W. (1987). Metabolic and immune effects of dietary arginine supplementation after burn. *Archives of Surgery* **122**, 784–789.

Stucki, W. P. & Harper, A. E. (1962). Effects of altering the ratio of indispensable to dispensable amino acids in diets for rats. *Journal of Nutrition* **78**, 278–286.

Stuehr, D. J. & Marletta, M. A. (1985). Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proceedings of National Academy of Sciences USA* **82**, 7738.

Wagner, D. A., Young, V. R. & Tannenbaum, S. R. (1983). Mammalian nitrate biosynthesis: Incorporation of $^{15}NH_3$ into nitrate is enhanced by endotoxin treatment. *Proceedings of National Academy of Sciences USA* **80**, 4518.

Yelich, M. R. & Filkins, J. P. (1983). Insulin hypersecretion and potentiation of endotoxin shock in the rat. *Circulatory Shock* **9**, 589–603.

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