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Arginine-Supplemented Diets Improve Survival in Gut-Derived Sepsis and Peritonitis by Modulating Bacterial Clearance

The Role of Nitric Oxide

Luca Gianotti, M.D., J. Wesley Alexander, M.D., Sc.D.,
Tonyia Pyles, B.A., and Ryoji Fukushima, M.D.

From the Transplantation Division of the Department of Surgery, University of Cincinatti College of Medicine, Cincinnati, and the Shriners Burn Institute, Cincinnati, Ohio

Objective
The effect of arginine on survival rates and host defense mechanisms was studied using two clinically relevant models of infection that included transfusion-induced immunosuppression.

Summary Background Data
Dietary arginine will improve resistance to infection but its role in transfusion-induced immunosuppression and bacterial translocation (gut-derived sepsis) has not been defined.

Methods
Balb/c mice were fed for 10 days with either a defined AIN-76A diet, an AIN-76A diet supplemented with 2% arginine, an AIN-76A diet supplemented with 4% glycine, or standard laboratory chow. In most experiments, the mice were then transfused with allogeneic blood and allowed to feed for an additional 5 days before undergoing either cecal ligation and puncture (CLP) or gavage with 10^{10} *Escherichia coli* and a 20% burn injury. Additional animals fed with the arginine supplemented diet were treated with the nitric oxide inhibitor Nω-Nitro-L-arginine (NNA) before gavage and burn. The effect of these diets and NNA on the degree of translocation of ^{14}C-radiolabeled *E. coli* from the intestine and the ability of the host to kill translocated organisms was also investigated. Mice were fed and received transfusion, gavage, and burn as above. Mesenteric lymph nodes (MLN), liver and spleen were harvested 4 hours postburn.

Results
Survival after CLP was 56% in the arginine-supplemented group versus 28% in the AIN-76A group and 20% in the chow group ($p < 0.02$). After gavage and burn, survival was 100% in the arginine-supplemented group versus 50% in both the glycine-supplemented and chow groups and 35% in the AIN-76A group ($p < 0.01$). In animals receiving the arginine-supplemented diet, treatment with NNA decreased survival from 95% to 30.5% ($p < 0.0001$). Greater translocation, as measured by radionuclide counts, was observed to the MLN of the AIN-76A group. However, there was no difference in translocation to the liver and spleen related to dietary group. Quantitative colony counts and the calculated percentage of remaining viable bacteria showed that the ability to kill translocated organisms was significantly enhanced in animals receiving arginine. Treatment with NNA reversed the beneficial effects of arginine on immune defense.

Conclusions

The benefit of arginine appears to be mediated by improved bactericidal mechanisms via the arginine-nitric oxide pathway.

Recent experimental[1,2] and clinical studies[3,4] have strongly suggested that microorganisms, endotoxin, and other toxic compounds may escape from the gut lumen by crossing the intestinal mucosal barrier and eventually spread to systemic compartments via lymphatics and blood vessels or by traversing the bowel wall to enter the peritoneal cavity. This process, defined as microbial translocation, occurs with microorganisms passing directly through rather than between intact mucosal cells.[5] It has been postulated that translocation plays a central role in the development of devastating sequelae such as endotoxemia,[6] systemic infections,[7] the septic syndrome,[8] hypermetabolic responses,[9] and multiple-system organ failure,[10] conditions often complicating critical illnesses and trauma.

Two major factors determine the occurrence and the severity of infections associated with microbial translocation from the gut: the magnitude of the bacterial load and the ability of the host defense to kill the invading organisms. An ideal therapeutic modality should decrease the burden of microbes and microbial products crossing the mucosal barrier and stimulate the immune bactericidal mechanisms to increase clearance. We have demonstrated previously that decreasing the load of organisms translocating from the gut with prostaglandin E analogs significantly improves both extent and length of survival,[11] even though these drugs possess mild immunosuppressive properties and slightly reduce the clearance of translocated bacteria.[12]

Arginine has been shown to have potent effects on immune defense, particularly after trauma, and this effect may be mediated primarily by enhancing T-cell mediated responses.[13] Recent *in vitro* experiments[14] have shown that the key role of arginine on macrophage and lymphocyte-mediated toxicity toward tumor and/or infected cells is largely via by the production and release of nitric oxide (NO). NO is the intermediate metabolite generated during the biochemical transformation of arginine to citrulline.[15] The effects of arginine on gut barrier function have not been studied previously.

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Address reprint requests to J. Wesley Alexander, M.D., Sc.D., Department of Surgery, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0558.

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The following experiments were designed to investigate the effects of diets enriched with arginine on the resistance of animals to bacterial peritonitis after cecal ligation and puncture (CLP) and on the susceptibility of burned animals to gut-origin sepsis. The influence of arginine on intestinal translocation of enteric bacteria and on the ability of the host to kill translocated organisms that invade systemic organs was also determined. An arginine derivative that inhibits the production of NO was used to study the role of the NO pathway.

MATERIALS AND METHODS

Animals and Animal Care

Adult female Balb/c mice (H-2^d) (Charles River, Wilmington, MA) and adult female C3H/HeJ mice (H-2^k) (Jackson Laboratory, Barhrbor, ME) weighing between 19–22 grams were used for all experiments. The animals were quarantined for 1 week to allow adaptation to the environmental conditions and to exclude animals with preexisting diseases. During this period, the animals were provided food (Rodent Laboratory Chow 5001, Purina Mills Inc., St. Louis, MO) and water *ad libitum* until the start of the experiments. The experimental protocols were approved by the University of Cincinnati Animal Care and Use Committee, and the animals were maintained in an AAALAC-approved facility. In conducting the described research, the investigators adhered to the Guide for the Care and Use of Laboratory Animals as set forth by the Committee on the Care and Use of Laboratory Animals, National Research Council, and United States Department of Health and Human Service, National Institutes of Health. The animals were killed using an overdose of methoxyflurane and cervical dislocation or by carbon dioxide inhalation according to the Guide for the Care and Use of Laboratory Animals.

Diet Preparation and Feeding Protocols

In these experiments, Balb/c mice were fed with four different pelleted diets. Two of these were commercially available: AIN-76A semipurified diet (ICN Biomedicals Inc., Costa Mesa, CA) or natural chow (Rodent laboratory chow 5001, Purina Mills, Inc., St. Louis, MO). The other two experimental diets were prepared in our laboratory by modification of the basic composition of the

AIN-76A diet. The first was enriched with 2% arginine (weight/weight) and the second with 4% glycine (weight/weight). Four percent glycine (nitrogen content 18.7%) was used to balance the nitrogen content of the 2% arginine diet (32.1%) so that the two diets were near isonitrogenous. The AIN-76A diet contains 0.7% arginine and 0.35% glycine, while Purina chow 5001 contains 0.5% arginine and 0.3% glycine (all percentages are expressed as weight/weight). Detailed compositions of the 2% arginine and 4% glycine diets are described in Table 1.

Pair feeding was felt to be unnecessary since preliminary experiments showed that during 15 days of feeding the amount of diet intake was similar among individual animals receiving different diets (range 2.8–3.2 g; mean 3.07 ± 0.03 SEM).

Blood Harvesting and Transfusion Procedures

Under methoxyflurane inhalation anesthesia, blood was obtained from C3H/HeJ mice by cardiac puncture using aseptic technique. The blood was mixed with citrate phosphate dextrose adenine anticoagulant (Fenwal Laboratories, Deerfield, IL) at a 7:1 ratio and stored at 4°C overnight. Groups of Balb/c mice were transfused via a tail vein with 0.2 ml of allogeneic blood after 10 days of feeding.

Preparation of Nω-Nitro-L-Arginine (NNA)

NNA (Sigma Chemical Co., St. Louis, MO) was diluted in phosphate-buffered saline (PBS, pH 7.4) to obtain a desired concentration of 20 mg/ml. Sodium hydroxide (1.0 normal) was added until the pH of the suspension reached 11 to allow the solubilization of NNA. Then the pH was brought to 7.4 with hydrochloric acid (1.0 normal). NNA was administered subcutaneously within 10 minutes because this compound precipitates about 15 minutes after preparation.

Table 1. COMPOSITION OF THE PELLETED DIETS

	2% Arginine	4% Glycine
L-Arginine (free base)	24 g	0 g
Glycine	0 g	40 g
Corn oil	50 ml	50 ml
Butylate hydroxytoluene	1 ml	1 ml
Lecithin (refined soybean)	1 ml	1 ml
Casein-protein	200 g	200 g
Corn starch	142 g	142 g
AIN 76A vitamin mixture	10 g	10 g
AIN mineral mix 76	35 g	35 g
DL-methionine-free grade	3 g	3 g
Choline bitartrate	2 g	2 g
D-(+)-sucrose	476 g	476 g
Alphacel fiber (cellulose)	50 g	50 g
Water	76 ml	76 ml

The AIN-76A diet contained all components except the added arginine or glycine. All components were obtained from ICN Biomedicals Inc., Costa Mesa, California.

Preparation of ^{14}C-Radiolabeled *Escherichia Coli*

Escherichia coli (stock #53104; University of Minnesota, Minneapolis, MN) was inoculated into a glucose deficient-minimal nutrient broth and 500 μCi of ^{14}C glucose (New England Nuclear/DuPont, Boston, MA) were added at the same time. The culture was incubated for 18 hours at 37°C in a shaking incubator. The culture was then centrifuged at 4000 revolutions per minute for 10 minutes and the pellet was washed twice in sterile saline to remove the unincorporated isotope. The final pellet was resuspended in sterile saline at a concentration of 1×10^{10} organisms/0.2 ml using a standard Klett densitometer (Klett Mfg. Co., New York, NY). The viability of the organisms was confirmed by plating 10-fold dilutions of the bacterial suspension on brain heart infusion agar plates. Radionuclide counts of the gavaged bacterial suspension were 6.31×10^7 dpm/0.2 ml. This corresponded to 158 organisms/dpm. The pellet of bacteria before suspension had 1.31×10^9 dpm, and the supernatant after two washes had 3.66×10^4 dpm/ml. Thus, the pellet had approximately 36,000 fold more dpm than the supernatant suggesting stable incorporation of the isotope in the bacteria.

The same stock of *E. coli* was grown in brain heart infusion broth for 18 hours at 37°C without addition of the isotope. The culture was processed as above to obtain a final concentration of 1×10^{10} organisms/0.2 ml.

Cecal Ligation and Puncture (CLP) Procedure

Using sterile technique under methoxyflurane anesthesia, Balb/c mice underwent a minimal midline laparotomy (0.7 cm) to expose the cecum, which was ligated with a silk suture and punctured with a 25 G needle according to a previously described technique.[16] CLP was performed after 15 days of feeding.

Burn Model and Gavage Procedure

After 14 days of feeding, all animals had hair removed from their entire back and flanks by clipping. The follow-

ing morning all mice, while awake, were gavaged with 0.2 ml containing 1×10^{10} ^{14}C labeled or unlabeled *E. coli* according to the experimental design. The animals were then anesthetized by methoxyflurane inhalation and immediately subjected to a 20% total body surface area, full thickness flame burn[17] and resuscitated with intraperitoneal injection of 20 ml/kg of saline. The burn wound was left exposed throughout the remainder of the experiments.

Experimental Design

All the following experiments were repeated two or three times and the results presented herein represent data pooled from replicate studies.

Experiment 1

Balb/c mice (n = 15/group) were fed with either the arginine enriched diet, the AIN-76A diet or laboratory chow for 15 days. Then the animals were subjected to CLP and afterward allowed to feed their designated diets *ad libitum*. All animals were observed for survival for 10 days.

Experiment 2

Three groups of Balb/c mice were fed either the arginine diet (n = 25), the AIN-76A diet (n = 25), or laboratory chow (n = 20) for 10 days. Then all animals were transfused with allogeneic blood (from C3H/HeJ mice) and allowed to continue feeding with their designated diets for an additional 5 days. CLP was performed 5 days post-transfusion and the animals were observed for survival for 10 days.

Experiment 3

Four groups of Balb/c mice were fed either the arginine-enriched diet (n = 30), glycine-enriched diet (n = 20), AIN-76A diet (n = 20), or laboratory chow (n = 20) for 10 days. Then all animals were transfused will allogeneic blood (from C3H/HeJ mice) and allowed to ingest their assigned diets for the remainder of the experiment. Five days after transfusion, the mice were gavaged with 1×10^{10} *E. coli* followed immediately by a 20% thermal injury. Survival was observed for 10 days postburn.

Experiment 4

Additional groups of mice (n = 14/group) underwent the same procedures described in experiment 3, but they were gavaged with 1×10^{10} ^{14}C-labeled *E. coli* in 0.2 ml and killed 4 hours after burn. The mesenteric lymph nodes complex (MLN), liver, and spleen were excised using sterile techniques. These were placed in sterile Petri dishes, weighed, and homogenized in 1.0 ml of saline. One hundred μl of each homogenate were plated on eosin methylene blue plates (Becton Dickinson Microbiology System, Cockeyville, MD), a selective media for aerobic gram-negative bacteria, incubated aerobically at 37°C for 24 hours and the number of colony-forming units (CFU) were counted. To ensure that the *E. coli* grown from the tissues was the same species as the gavaged *E. coli*, the Sceptor System Test® #80401 (Becton Dickinson, Towson, MD) was used. This system uses a combination of antimicrobial susceptibility tests, biochemical identification, and a beta-lactamase test to identify specific species of enteric bacteria. The entire set of data obtained from the gavaged *E. coli* matched the data from the *E. coli* recovered from the culture plates.

The remainder of the tissue homogenates were lyophilized overnight and decolorized as previously described.[1] The radionuclide counts (as measured by degradation per minutes; dpm) were determined using a liquid scintillation counter (Beckman Model LS 3133, Beckman Instruments, Inc., Fullerton, CA). The CFU and dpm counts were adjusted to be expressed as per gram of tissue and the percentage of translocated bacteria that remained viable was calculated as a ratio between CFU/g and dpm/g using a formula described previously.[1] In some animals, the small bowel was excised and the lumen irrigated with 10 ml of saline. The effluent had 3×10^7 CFU/0.2 ml and 2.41×10^5 dpm/0.2 ml. This corresponded to 121 organisms/dpm. This value was similar to the original suspension of gavaged bacteria suggesting that 4 hours after burn the *E. coli* retained all of the radiolabel and remained viable.

Experiment 5

Balb/c mice were fed with the arginine-enriched diet for 10 days, transfused with allogeneic blood, and allowed to feed for an additional 5 days. They were then randomized to two groups on day 14 of feeding; one group (n = 23) was injected subcutaneously each day for 2 days with 2 mg NNA/mouse (100 mg/kg/day) in 0.1 ml of PBS while the second group (control, n = 20) received 0.1 ml of PBS each day. After the second treatment, all animals were gavaged with 1×10^{10} ^{14}C-*E. coli* and received a 20% burn injury. Survival was observed for 10 days postburn.

Experiment 6

In this experiment, the animals (n = 15/group) were treated as in experiment 5, but they were gavaged with 1×10^{10} ^{14}C-labeled *E. coli* and killed 4 hours postburn. The MLN, liver, and spleen were harvested aseptically

and processed as described in experiment 4 to determine radionuclide counts, CFU, and percentage of bacteria alive.

Statistical Analysis

Since the data obtained were not normally distributed, the differences among the means of the continuous variables (dpm, CFU, and percentage alive) were analyzed using a nonparametric analysis of variance (Kruskal-Wallis ANOVA). P values < 0.05 were considered significant. The survival rates of the five groups were compared using the chi-square test of independence.

RESULTS

Experiment 1: Survival After CLP

The survival rate of the animals fed the arginine-supplemented diet was 46.6% (7/15) versus 26.6% (4/15) in both AIN-76A and chow groups (Fig. 1). Although the arginine group showed a 45% improvement in survival this difference was not statistically significant ($p = 0.4$).

Experiment 2: Survival After Transfusion and CLP

Survival of the animals fed with the arginine-supplemented diet was 56% (14/25) versus 28% (7/25) in the group fed the AIN-76A diet without added arginine and 20% (4/20) in the group fed chow (Fig. 2). The arginine supplemented group showed a significant improvement in survival compared with AIN-76A and chow groups ($chi^2 = 7.28$; $p = 0.02$ and $chi^2 = 6.0$; $p = 0.01$, respectively).

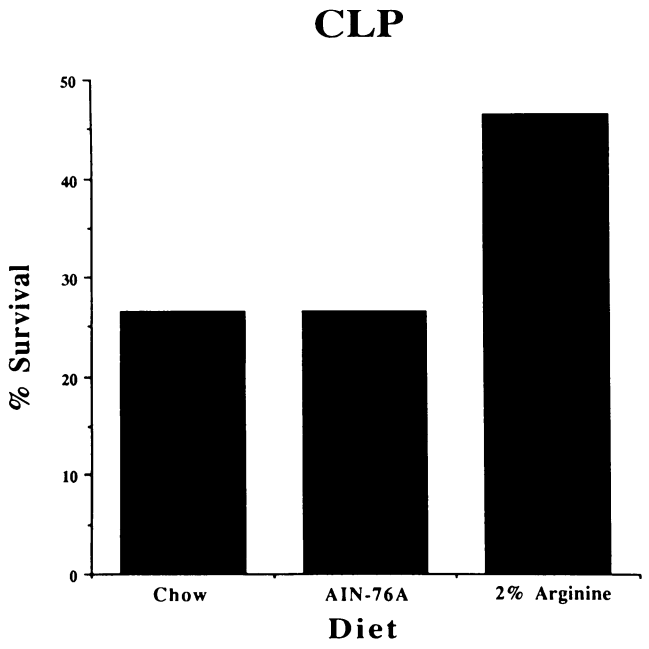

Figure 1. Survival rates of animals fed with either laboratory chow, AIN-76A semipurified diet, or 2% arginine- supplemented diet undergoing cecal ligation and puncture (CLP). $p = 0.4$

Experiment 3: Survival After Transfusion, Bacterial Gavage and Burn Injury

In this model, the survival rate of the animals fed with the arginine-supplemented diet was 100% (30/30) compared with 50% (10/20) in the animals fed with either the glycine-supplemented diet or chow ($chi^2 = 9.35$; $p = 0.01$) and 35% (7/20) in the AIN-76A group ($chi^2 = 2.0$; $p = 0.001$) (Fig. 3).

Experiment 4: Study of Translocation and Bacterial Survival

The magnitude of translocation of ^{14}C-*E. coli*, as measured by dpm/g of tissue, for the four groups is shown in Table 2. Animals fed with AIN-76A diet had a greater amount of translocation to the MLN compared with all other groups ($p < 0.05$). No difference was observed when the other three groups were compared with each other. The AIN-76A group had also higher translocation to the liver but this difference was not significant.

The analysis of the number of viable *E. coli* (as measured by CFU/g of tissue) recovered from the tissues showed that the animals fed with arginine supplementation had significantly fewer bacteria in the MLN and spleen compared with all other groups ($p < 0.05$) and fewer viable organisms in the liver compared with the AIN-76A and glycine-supplemented groups ($p < 0.05$) (Table 3).

The calculated percentage of translocated bacteria that remained alive showed that the diet enriched with arginine improved the ability of the host to kill translocated organisms in the MLN and spleen compared with AIN-76A, glycine, and chow diets ($p < 0.05$). A similar trend was observed for the liver. A significant difference was reached when the arginine diet was compared with AIN-76A and glycine-enriched diets ($p < 0.05$) but not versus chow (Table 4).

Experiment 5: Survival Study After Treatment with NNA

Transfused mice fed with the arginine diet and subsequently receiving bacterial gavage and burn injury had a survival of 95% (19/20) while only 7 of 23 (30.5%) animals with similar treatment but also given the arginine inhibitor NNA survived ($chi^2 = 21.90$; $p < 0.0001$) (Fig. 4).

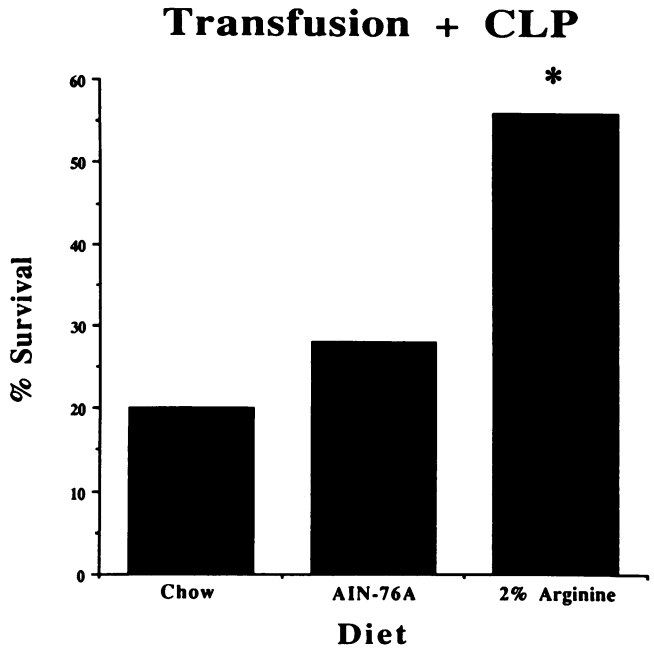

Figure 2. Survival rates of animals fed with either laboratory chow AIN-76A semipurified diet or 2% arginine supplemented diet undergoing blood transfusion and cecal ligation and puncture (CLP). *p < 0.02 versus all other diets.

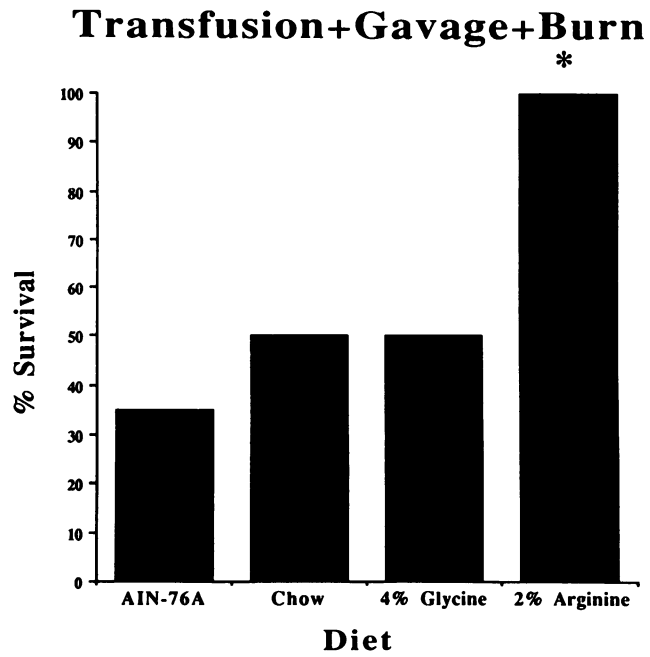

Figure 3. Survival rates of animals fed with either laboratory chow, AIN-76A semipurified diet, 4% glycine-supplemented diet, or 2% arginine-supplemented diet undergoing blood transfusions, bacterial gavage (10^{10}), and a 20% burn injury. *p < 0.01 versus all other diets.

Table 2. DEGREE OF TRANSLOCATION OF ^{14}C-LABELED *E. COLI* AS MEASURED BY DPM/G OF TISSUE

Diet	Mesenteric Lymph Nodes	Liver	Spleen
Arginine	2.784,262 ± 1,309,706	25,624 ± 4,824	30,755 ± 6,002
Glycine	1,031,898 ± 420,677	24,958 ± 6,925	53,233 ± 19,730
Chow	1,715,068 ± 547,382	33,223 ± 4,908	32,458 ± 3,860
AIN-76A	11,769,923 ± 4,664,100	52,976 ± 30,699	49,776 ± 16,335

* $p < 0.05$ vs. all other diets.
Values are expressed as mean ± SEM.

Experiment 6: Effect of NNA of Translocation and Bacteria Survival

Radionuclide counts (dpm/g of tissue) showed that the administration of NNA did not affect the degree of translocation of ^{14}C-*E. coli* to any of the tested tissues. In contrast, in all organs the number of viable *E. coli* was significantly higher in the NNA treated group compared with controls ($p < 0.05$). As a consequence, the calculated percentage of translocated bacteria that remained alive was significantly higher in all tissues of the animals treated with arginine plus NNA versus control mice receiving only the arginine-supplemented diet ($p < 0.05$) (Table 5).

DISCUSSION

It has been known since 1930 that normal young rats grow more rapidly and maintain a positive nitrogen balance when receiving dietary arginine.[18] In 1978, Seifter et al. showed that postoperative weight loss was significantly reduced in rats fed with arginine while wound healing was markedly improved.[19] Furthermore, patients undergoing moderate surgical trauma had a reduction of nitrogen excretion when fed arginine compared with controls.[20] The important metabolic role of argi-

Table 3. NUMBER OF VIABLE *E. COLI* AS MEASURED BY CFU/G OF TISSUE

Diet	Mesenteric Lymph Nodes	Liver	Spleen
Arginine	0*	483 ± 137†	162 ± 108*
Glycine	4,240 ± 2,649	3,913 ± 1,362	2,338 ± 1,591
Chow	1,434 ± 251	2,622 ± 776	1,625 ± 435
AIN-76A	15,216 ± 10,899	5,762 ± 1,186	13,857 ± 10,468

* $p < 0.05$ vs. all other diets.
† $p < 0.05$ vs. glycine and AIN-76A.
Values are expressed as mean ± SEM.

Table 4. CALCULATED PERCENTAGE OF TRANSLOCATED BACTERIA THAT REMAINED ALIVE IN THE TISSUES

Diet	Mesenteric Lymph Nodes	Liver	Spleen
Arginine	0*	0.02 ± 0.004†	0.007 ± 0.006*
Glycine	0.010 ± 0.008	0.36 ± 0.18	0.080 ± 0.06
Chow	0.002 ± 0.001	0.08 ± 0.02	0.040 ± 0.01
AIN-76A	0.008 ± 0.004	0.22 ± 0.04	0.016 ± 0.07

* $p < 0.05$ vs. all other diets.
† $p < 0.05$ vs. glycine and AIN-76A.
Values are expressed as mean ± SEM.

nine was studied further by Barbul et al.[21,22] using rats subjected to a bilateral closed femoral fracture. Supplementation of the diet with arginine-reduced nitrogen loss, improved the healing of wounds, increased thymic weight and lymphocyte content of the thymus and enhanced the T-cell blastogenic responses to different mitogens. This thymotrophic effect of arginine was also observed in nontraumatized and less severely injured animals.[23] Furthermore, arginine could increase extrathymic T-cell maturation and function in athymic mice.[24]

The potent immunomodulatory effect of arginine has been demonstrated in a variety of models that have focused primarily on T-cell mediated immune mechanisms. Animal studies showed that cytotoxic T-lymphocyte development and activation, and natural killer cell activity and expression of interleukin-2 receptors were significantly improved after dietary supplementation with arginine.[25] In tumor-bearing mice, arginine appeared to represent an essential substrate for optimal generation of lymphokine-activated killer cells after interleukin-2 administration.[26] As a result, tumor growth and engraftment were reduced in animals fed with arginine compared with glycine, and the duration of host survival was significantly prolonged. Similar improvement in the immune response was observed in cancer patients undergoing major surgery. They had increased T-lymphocyte response and a higher concentration of CD4-positive cells.[27] Moreover, arginine was shown to be a safe nutritional stimulator of lymphocyte immune reactivity in healthy humans.[28]

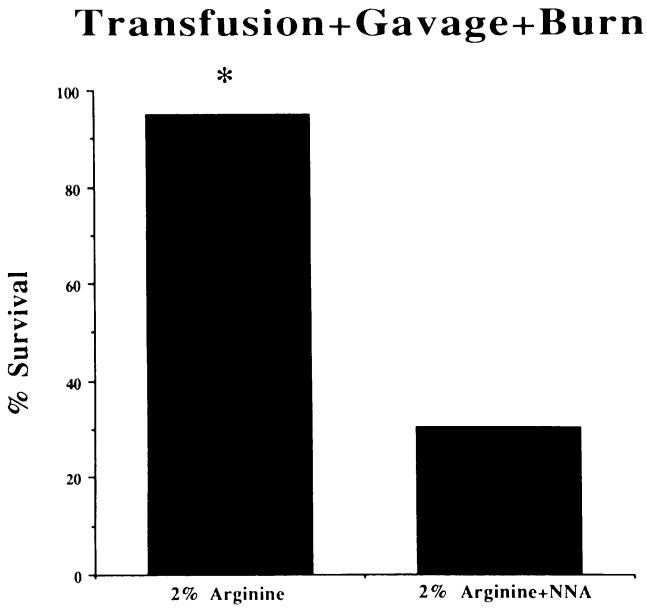

Figure 4. Survival rates of animals fed with 2% arginine-supplemented diet or 2%-arginine supplemented diet plus N-nitro-L-arginine (NNA) and receiving blood transfusion, bacterial gavage, (10^{10}) and a 20% burn injury. *$p < 0.0001$ versus arginine plus NNA.

Madden et al. showed that arginine supplementation given orally 3 days before inducing peritonitis by CLP or started intravenously after established peritonitis significantly increased animal survival.[29] This effect was not observed when arginine was given by gavage after CLP possibly because arginine absorption via the intestine was markedly impaired during peritonitis. Our results with CLP are consistent with these findings. Saito et al. showed that guinea pigs fed enterally with a 2% arginine diet after inflicting a 30% burn injury, had improved cell-mediated immunity, better clearance of *Staphylococcus aureus* from skin injection sites and improved survival compared with animals fed with 4% arginine or no arginine.[30] In a recent prospective randomized clinical trial, Daly showed that surgical patients receiving enteral nutritional support with a diet containing supplemental arginine, RNA, and omega-3 fatty acids had significantly fewer infectious complications and a shorter postoperative stay.[31]

Part of the positive effect of arginine supplementation on immune function is thought to be mediated by the potent secretogogue effect of arginine on several endocrine glands. Specifically, arginine given either intravenously or orally may increase the secretion of pituitary growth hormone and prolactin[32,33] and cause an increased release of pancreatic insulin, somatostatin, and pancreatic polypeptide.[34] These hormones may have a trophic effect on the intestinal mucosa[35] which is believed to play an important role in preventing the translocation of enteric bacteria.[36] Additionally, arginine is a precursor of the polyamines putrescine, spermine, and spermidine, which are considered important mediators of cell growth and differentiation.[37] Polyamines are able to stimulate the normal rate of growth of the intestinal mucosa and prevent the inhibition of epithelial cell growth induced by difluromethylornithine.[38] We have previously reported that the combined administration of basic fibroblast growth factor and sucralfate could signifi-

Table 5. DEGREE OF TRANSLOCATION OF ^{14}C *E. COLI* (DPM/G OF TISSUE), NUMBER OF VIABLE *E. COLI* (CFU/G OF TISSUE) AND CALCULATED PERCENTAGE OF TRANSLOCATED BACTERIA REMAINING ALIVE IN GROUPS FED DIETS SUPPLEMENTED WITH ARGININE AND TREATED WITH NNA OR A PLACEBO

	dpm/g	CFU/g	% Alive
Mesenteric lymph nodes			
Arginine	3,325,970 ± 1,302,020	31 ± 21*	0.00001 ± 0.00001*
Arginine + NNA	1,779,536 ± 373,814	1,298 ± 274	0.00152 ± 0.00050
Liver			
Arginine	54,299 ± 19,535	360 ± 76*	0.018 ± 0.007*
Arginine + NNA	59,323 ± 15,336	3,585 ± 572	0.148 ± 0.045
Spleen			
Arginine	48,083 ± 7,207	224 ± 57*	0.005 ± 0.002*
Arginine + NNA	65,237 ± 13,734	738 ± 100	0.019 ± 0.005

* $p < 0.05$ vs. arginine + NNA.
Values are expressed as mean ± SEM.

cantly reduce the magnitude of translocation in burned animals, and the improvement in gut barrier function was correlated with the maintenance of normal gut morphology.[39] Other growth factors have been shown to decrease the rate of translocation in animal models.[40] Whatever the mechanism might be by which arginine enhances barrier function of the mucosa and decreases translocation, our results clearly showed that this effect was weak and limited to the mesenteric lymph nodes. The magnitude of translocation to systemic organs was similar among all groups studied. Therefore, the improved outcome observed in the arginine fed animals was more related to the influence of arginine on immune function.

Surprisingly, few studies have addressed the importance of dietary arginine on macrophage function after trauma, infection, or sepsis. Macrophages are involved in the early and nonspecific host-defense responses. They exhibit microbicidal and tumoricidal properties and function as accessory cells in T-cell activation. Tachibana and colleagues reported that arginine-enriched solutions suppressed subcutaneous tumor growth and prevented metastasis to systemic organs when given to rats. After infusion with arginine, it was possible to demonstrate an enhanced phagocytic activity of alveolar macrophages.[41]

To clarify further the mechanisms of arginine in improved resistance to bacterial infections, we used an animal model that can measure *in vivo* the host's ability to kill viable organisms that translocate from the gut after thermal injury.[1] This model combines burn injury, which is responsible for increased permeability of the gut to enteric bacteria, and allogeneic blood transfusion that impairs macrophage activity[42] and causes a deficient killing of organisms that translocate without affecting the gut barrier function.[2] The synergistic negative effect of these two insults results in a high mortality from sepsis.[2,11] The analysis of the percentage of translocated microorganisms that remained alive suggested that the ability of animals fed with arginine to kill bacteria was strikingly enhanced and correlated well with lower mortality. Activated macrophages exhibit high arginase activity.[43] Therefore, the immune properties of arginine may be mediated through the metabolism of arginine within the macrophages.

Hibbs et al. demonstrated that L-arginine is required *in vitro* for the cytotoxic effector mechanisms of macrophages and that the production of NO via the conversion of arginine to citrulline is controlled by the arginine deaminase pathway.[44] The L-arginine-NO pathway has been proposed to be the primary defense mechanism for killing intracellular organisms and to be the main mechanism of macrophage toxicity for target cells.[45] This is consistent with our results showing that arginine strongly affects bacterial killing. The macrophage NO-mediated toxicity appears to represent the primary immune mechanism involved in the clearance of bacteria since pretreatment with the NO inhibitor NNA completely reversed the beneficial effects of arginine on bacterial clearance. Moreover, the ablation of the arginine effect on the host antibacterial defense was demonstrable by a significant decrease in survival in the animals treated with the NO inhibitor. Previous observations showing increased mortality in endotoxic rodents[46] and induction of severe hepatic damage in rats[47] challenged with endotoxin and treated with NO inhibitors, also support the present results.

Somewhat in contrast with these findings are the reports that indicate NO is a significant contributor to the pathogenesis of the severe blood pressure alterations ob-

served during therapy with interleukin-2 and septic shock in both animals and humans.[48] The proposed explanation behind this observation is the demonstration that NO synthesis is induced by the principal mediators of the detrimental sequelae of septic shock, such as interleukin-1 and tumor necrosis factor.[49] Preliminary studies in patients with severe septic shock[50] or undergoing interleukin-2 therapy[51] reported a successful restoration of blood pressure and favorable outcome after administration of NO inhibitors. The negative effect of the overproduction of NO from arginine in the setting of established sepsis was also shown indirectly by Gonce et al. who reported adverse outcomes in guinea pigs when high doses of arginine were administered 3 days after the onset of peritonitis.[52]

In conclusion, the present studies show that diets supplemented with arginine affect mortality when given before different types of infection. The increased survival observed in the animals fed with arginine appears to be mediated by improved macrophage bactericidal killing mechanisms mediated by the arginine-NO pathway.

References

1. Alexander JW, Gianotti L, Pyles T, Carey MA, Babcock GF. Distribution and survival of Escherichia coli translocating from the intestine following thermal injury. Ann Surg 1991; 213:558–566.
2. Gianotti L, Pyles T, Alexander JW, Carey M, Babcock GF. Impact of blood transfusion and burn injury on microbial translocation and bacterial survival. Transfusion 1992; 32(4):312–317.
3. Border JR, Hassett J, LaDuca J, et al. The gut origin sepsis in blunt multiple trauma (ISS = 40) in the ICU. Ann Surg 1987; 206:427–448.
4. Deitch EA. Simple intestinal obstruction causes bacterial translocation. Arch Surg 1989; 124:699–701.
5. Alexander JW, Boyce ST, Babcock GF, et al. The process of microbial translocation. Ann Surg 1990; 212:496–512.
6. Schoeffel U, Windfuhr M, Freudenberg KH, et al. The role of intestinal endotoxin in experimental peritonitis. Circ Shock 1989; 27:83–91.
7. Rush BF, Redan JA, Flanagan JJ, et al. Does bacteremia observed in hemorrhagic shock have clinical significance? A study in germ-free animals. Ann Surg 1989; 210:342–347.
8. Offenbartl K, Bengmark S. Intraabdominal infections and gut origin sepsis. Word J Surg 1990; 14:191–195.
9. Mochizuki H, Trocki O, Dominioni L, et al. Mechanism of prevention of postburn hypermetabolism and catabolism by early enteral feeding. Ann Surg 1984; 200:297–310.
10. Carrico CJ, Meakins JL, Marschall JC, et al. Multiple-organ-failure syndrome. Arch Surg 1986; 121:196–208.
11. Fukushima R, Gianotti L, Alexander JW, Pyles T. The degree of bacterial translocation is a determinant factor for mortality after burn injury and is improved by prostaglandin analogs. Ann Surg 1992; 216:438–445.
12. Gianotti L, Alexander JW, Pyles T, Fukushima R, Babcock GF. Prostaglandin E1 analogues, misoprostol and enisoprost decrease microbial translocation in thermally injured mice. Circ Shock (In press).
13. Barbul A. Arginine: biochemistry, physiology and therapeutic implications. JPEN 1986; 10:227–238.
14. Hibbs JB, Vavrin Z, Taintor RR. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. J Immunol 1987; 138:550–565.
15. Marletta MA, Yoon PS, Iyengar R, Leaf CD, Wishnok JS. Macrophage oxidation of L-arginine to nitrite and nitrate: nitric oxide is an intermediate. Biochemistry 1988; 27:8706–8711.
16. Galandiuk S, George CD, Pietsch JD, et al. An experimental assessment of the effect of blood transfusion on the susceptibility to bacterial infection. Surgery 1990; 108:567–571.
17. Stieritz DD, Holder IA. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa:* description of a burned mouse model. J Infect Dis 1975; 131:688–691.
18. Scull CW, Rose WC. Arginine metabolism: I. the relation of the arginine content of the diet to the increments in tissue arginine during growth. J Biol Chem 1930; 39:109–114.
19. Seifter E, Rettura G, Barbul A, Levenson SM. Arginine: an essential amino acid for injured rats. Surgery, 1978; 84:224–230.
20. Elsair J, Poey J, Issad H, et al. Effect of arginine chlorhydrate on nitrogen balance during the three days following routine surgery in man. Biomed Express 1978; 29:312–317.
21. Barbul A, Wasserkrug HL, Yoshimura N, Tao R, Efron G. High arginine levels in intravenous hyperalimentation abrogate post-injury immune suppression. J Surg Res 1984; 36:620–624.
22. Barbul A, Sisto DA, Wasserkrug HL, Yoshimura NN, Efron G. Metabolic and immune effect of arginine in post injury hyperalimentation. J Trauma 1981; 21:970–974.
23. Barbul A, Wasserkrug HL, Seifter E, et al. Immunostimulatory effects of arginine in normal and injured rats. J Surg Res 1980; 29:228–235.
24. Kirk SJ, Regan NC, Wasserkrug HL, Sodeyama M, Barbul A. Arginine enhances T-cell responses in athymic nude mice. JPEN 1992; 16:429–432.
25. Reynolds JV, Daly JM, Zhang S, et al. Immunomodulatory mechanisms of arginine. Surgery 1988; 104:142–151.
26. Lieberman MD, Nishioka K, Redmond P, Daly JM. Enhancement of interleukin-2 immunotherapy with L-arginine. Ann Surg 1992; 215:157–165.
27. Daly JM, Reynolds J, Thom A, et al. Immune and metabolic effects of arginine in surgical patients. Ann Surg 1988; 208:512–523.
28. Barbul A, Sisto DA, Wasserkrug HL, Efron G. Arginine stimulates lymphocyte immune response in healthy human beings. Surgery 1981; 90:244–251.
29. Madden HP, Breslin RJ, Wasserkrug HL, Efron G, Barbul A. Stimulation of T-cell immunity by arginine enhances survival in peritonitis. J Surg Res 1988; 44:658–663.
30. Saito H, Trocki O, Wang SL, et al. Metabolic and immune effects of dietary arginine supplementation after burn. Arch Surg 1987; 122:784–789.
31. Daly JM, Lieberman MD, Goldfine J, et al. Enteral nutrition with supplemental arginine, RNA and Omega-3 fatty acids in patients after operation: Immunologic, metabolic, and clinical outcome. Surgery 1992; 112:56–67.
32. Merimee TJ, Rabinowitz D, Riggs L. Plasma growth hormones after arginine infusion: Clinical experiences. N Engl J Med 1967; 276:434–439.
33. Isidori A, LoMonaco A, Cappa M. Study of growth hormone release in man after oral administration of amino acids. Curr Med Res Opin 1981; 7:475–481.
34. Utsumi M, Makimura H, Ishihara K, et al. Determination of immunoreactive somatostatin in rat plasma and responses to arginine, glucose and glucagon infusion. Diabetologia 1979; 17:319–323.

35. Williamson RCN, Bauer FLR, Ross JS. Contribution of bile and pancreatic juice to cell proliferation in ileal mucosa. Surgery 1978, 83:570–576.
36. Ma L, Ma J-W, Deitch EA, Specian RD, Berg RD. Genetic susceptibility to mucosal damage leads to bacterial translocation in a murine burn model. J Trauma 1989; 29:1245–1251.
37. Kay JE, Lindsay VJ. Polyamine synthesis during lymphocyte activation. Exp Cell Res 1973; 77:428–436.
38. Wang JY, McCormack SA, Viar MJ, Johnson LR. Stimulation of proximal small intestinal mucosal growth by luminal polyamines. Am J Physiol 1991; 261:G504–G511.
39. Gianotti L, Alexander JW, Fukushima R, Pyles T. Reduction of bacterial translocation with oral fibroblast growth factor and sulcralfate. Am J Surg 1993; 165:195–201.
40. Huang KT, Chung DH, Herndon DN. Insulin-like growth factor reduces gut atrophy and bacterial translocation after severe burn injury. 12th Annual Meeting Surgical Infection Society 1992; 51 (abstract).
41. Tachibana K, Mukai K, Hiraoka I, et al. Evaluation of the effect of arginine-enriched amino acid solution on tumor growth. JPEN 1985; 9:428–434.
42. Waymack JP, Rapien J, Garnett D, Tweddell JS, Alexander JW. Effect of transfusion on immune function in a traumatized animal model. Arch Surg 1986; 121:50–55.
43. Currie GA, Gyurel L, Cifuentes L. Macroenvironmental arginine depletion by macrophages *in vivo*. Br J Cancer 1979; 39:613–620.
44. Hibbs JB, Taintor RR, Vavrin Z. Macrophage cytotoxicity: role for L-arginine deiminase and imino nitrogen oxidation to nitrate. Science 1987; 235:473–476.
45. Moncada S, Higgs EA. Endogenous nitric oxide: physiology, pathology and clinical relevance. Eur J Clin Invest 1991; 21:361–374.
46. Nava E, Palmer RM, Moncada S. Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? Lancet 1991; 338:1555–1557.
47. Billiar TR, Curran RD, Harbrecht BG, et al. Modulation of nitrogen oxide synthesis *in vivo:* N^G-monothyl-L-arginine inhibits endotoxin-induced nitrate/nitrite biosynthesis while promoting hepatic damage. J Leukoc Biol 1990; 48:565–569.
48. Kilbourn RG, Griffith OW. Overproduction of nitric oxide in cytokine—mediated and septic shock. J Nat Cancer Inst 1992; 84:827–831.
49. Kilbourn RG, Belloni P. Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with necrosis factor, interleukin-1 or endotoxin. J Natl Cancer Inst 1990; 82:772–776.
50. Petros A, Bennett D, Vallance P. Effect of nitric oxide synthase inhibitors on hypotension in patients with septic shock. Lancet 1991; 338:1557–1558.
51. Hibbs JB, Westenfelder C, Taintor R, et al. Evidence for cytokine inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. J Clin Invest 1992; 89:867–877.
52. Gonce SJ, Peck MD, Alexander JW, Miskell PW. Arginine supplementation and its effect on established peritonitis in guinea pigs. JPEN 1990; 14:237–244.

Discussion

DR. EDWIN A. DEITCH (Shreveport, Louisiana): The concept that the composition of dietary administration can affect the incidence of infection and almost certainly survival seems clear from numerous experimental and an increasing number of clinical studies. However, the mechanisms by which dietary modulation affects survival and the composition of the optimal diet remain to be fully elucidated. The current study by Alexander and his coworkers tackles both issues. First, they document that by supplementing a complete diet with arginine they could increase survival in both a peritonitis and a burn model. Secondly, they documented that by blocking nitric oxide production from arginine with the inhibitor NNA, they blocked the protective effect of arginine on injury-induced mortality and increased the survival of translocating bacteria. Since arginine has immuno-potentiating effects and the percent of translocating bacteria that survived in the arginine-supplemented mice was less, the authors concluded that the benefits of arginine appear to be mediated by improved bacteriocidal mechanisms through the arginine nitric oxide pathways. Although there are a number of important areas that could be addressed, including methodologic questions about stability of the label on the bacteria, I would like to focus on what I think is the most important concept; that is, the potential relationship between bacterial translocation, survival, and nitric oxide in the macrophage. To begin with, although my bias fits their bias that loss of intestinal barrier function and the translocation of bacteria and endotoxin can adversely affect the host, what the authors have shown is an association between an impaired inability of the host to kill translocating bacteria and survival. Thus, my first question is, have you carried out any experiments to directly test the causal role of bacteria in the death of these animals? This is a critical issue since it is possible it is not the bacteria that are directly killing the host but instead the overproduction of host-derived factors such as cytokines or the products of activated inflammatory cells and humoral systems. In this regard, we recently presented data documenting that it is possible to dissociate bacterial translocation from mortality in a model of systemic inflammation by depleting the rodents of macrophages. That is, in the absence of macrophages, more bacterial translocation occurred, but the animals did not get as sick and mortality was significantly less. We concluded that gut-derived bacteria or endotoxin may be the triggers, but the macrophage products and the inflammatory response appear to be the bullets. Thus my final two questions. Since arginine has multiple effects and many components of the immune system modulates wound healing and cell proliferation, could it be that arginine's protective effect is multi-factorial and not just due to improving host bacteriocidal activity? Secondly, since nitric oxide through its vasodilatory properties is important in maintaining organ blood flow and inhibition of nitric oxide production potentiates organ injury and death in many models including endotoxemia, have you examined the possibility that nitric oxide is exerting its protective effect by maintaining organ blood flow rather than or in addition to its bacteriocidal activity.

DR. ACHILLES DEMETRIOU (Los Angeles, California): The authors demonstrated a significant beneficial effect and presented data on mechanisms that suggest that the beneficial arginine effect described here is due to enhanced bacterial killing by macrophages rather than decreased bacterial translocation across the gut wall. They also demonstrated that the macrophage effect is mediated through a nitric oxide mechanism. What is the minimum amount of arginine needed per day to provide a protective effect? Is the 2% supplementation that was