Amino Acids (1991) 1: 7–16

Amino
Acids

Effect of alanylglutamine-enriched infusion on tumor growth and cellular immune function in rats

M. N. Kweon[1], S. Moriguchi[1], K. Mukai[2], and Y. Kishino[1],

[1] Department of Nutrition, School of Medicine, The University of Tokushima, Tokushima, Japan
[2] Otsuka Pharmaceutical Factory, Inc., Naruto, Tokushima, Japan

Summary. The effects of total parenteral nutrition containing alanylglutamine (Ala-Gln) on tumor growth and cellular immune response were examined in rats inoculated Yoshida sarcoma cells subcutaneously. Rats given Ala-Gln-enriched solution intravenously showed a positive nitrogen balance and the increased function of alveolar macrophages. Studies on in vitro effect of Ala-Gln on immune cells in control rats showed a significant increase in phagocytic activity of alveolar macrophages and in blastogenic response of splenocytes, respectively. In vitro experiment of Gln or Ala-Gln addition to culture medium showed a remarkable enhancement of incorporation of ^{3}H-thymidine into Yoshida sarcoma cells, but in vivo administration of Ala-Gln containing solution did not accelerate the growth of the same tumor as measured by changes in the weight and volume. These results suggest that Ala-Gln infusion does not stimulate tumor growth due to maintenance of some immune-enhancing effect by Gln liberated from Ala-Gln in tumor-bearing hosts.

Keywords: Ala-Gln – Tumor growth – Alveolar macrophage

Introduction

The prolonged use of total parenteral nutrition provokes mucosal atrophy of the small intestine [1] and it may be related to the lack of glutamine (Gln) in standard currently available parenteral solutions. Because Gln is poorly soluble and instable, it has been not generally used as one of amino acids in parenteral nutrition. However, Gln is a nutrient necessary for the intestinal mucosal metabolism as a major oxidative fuel. Up to date, great attention has been paid to prevent the atrophy of the intestinal mucosa in patients with parenteral nutrition

[2]. Recently, Fürst et al. [3] indicated a safe and efficient utilization of Gln-containing dipeptide, Ala-Gln, as a source of free Gln in parenteral nutrient solutions. On the other hand, although free Gln is highly consumed by rapidly proliferating tumor cells [4], it is not clearly known whether tumor growth rate may be increased by intravenous supply of Ala-Gln. This problem is especially important to consider in treating tumor-bearing patients with Gln-enriched solution to prevent mucosal atrophy of the small intestine and to maintain good nutritional status of them. In addition, Gln is also preferentially used for the provision of fuels in proliferating lymphocytes [5] and macrophages [6]. In an attempt to determine the influence of Gln in tumor growth, the present study was undertaken and evaluated the changes of tumor volume and weight, and cellular immune response following the administration of Ala-Gln enriched solution to provide Gln.

Materials and methods

Experimental animals

Male Donryu rats of 5 wk old, weighing 140 g, were used. The animals were obtained from Shizuoka Animal Facility Center, Shizuoka, Japan. They were acclimatized for 1 wk to our laboratory conditions. During this acclimatization period, they were given a stock diet (Oriental Yeast Co., Chiba, Japan) and some of them were subcutaneously inoculated Yoshida sarcoma cells (2×10^6) in the abdominal wall. This cell line was supplied from Sasaki Kenkyusho, Tokyo, Japan. After the inoculated tumors became a visual size, the rats were anesthetized with Nembutal and a silicon rubber catheter (Dow Corning Corp., Midland, MI) was inserted into the right atrium of the heart through the right jugular vein, and an exit from the back of the neck was sutured subcutaneously and fixed onto the back of rats. The catheter of rat was connected to continuous infusion pump (Tokyo Rikakikai Co., Tokyo, Japan). During the experimental period, they could move freely but were infused continuously via the catheter. At first day they were infused a half of daily volume, and then they received infusion solution at a rate of 270 ml/kg body weight per day for 8 days. The rats were divided into two groups, which were infused with standard solution and Ala-Gln enriched solution, respectively. The control group was fed an isoenergetically controlled amount of a stock diet compared with that of the infused groups.

Composition of infusion solutions

As shown in Table 1, both standard and Ala-Gln-enriched solutions used in this experiment were nearly isoenergetic and isonitrogenous. Ala-Gln containing solution was prepared at the ratio of 30% to total amino acids as Gln.

Measurement

The size of subcutaneous tumors was measured with calipers. Tumor volumes were calculated from the formula $L \times (S)^2 \times 1/2$, where L and S represent the long and short diameters of the tumor, respectively. Urine samples were collected daily and their nitrogen contents were measured by total nitrogen analyzer (TN-02 type, Mitsubishi Co., LTD., Tokyo, Japan). Nitrogen balance was calculated by subtracting the amount of urinary nitrogen from the amount of nitrogen infused. Fecal nitrogen was neglected in the calculation of nitrogen balance, because there was no significant fecal output during the infusion period. At the end

Table 1. Composition of infusion solution

Ingredient	Standard (ml)	Ala-Gln rich (ml)
Amiparen[1]	127	0
Ala-Gln suppl. A.A. sol.	0	77
41.7% Glucose and electrolyte sol.[2]	140	140
Vitamin[3]	0.2	0.2
Water	3.0	53
Total volume (ml)	270	270
Total energy (kcal)	283	278
Gln/Total A.A. (%)	0	30
Total A.A. (g/270 ml)	12.7	11.6
Nitrogen (g/270 ml)	2.0	2.0
E.A.A./N.E.A.A.	1.44	0.65

[1] Amino acid content of final infusion solution (g/270 ml): L-Arg, 1.33; L-His, 0.64; L-Ile, 1.02; L-Leu, 1.78; L-Lys, 1.88; L-Met, 0.50; L-Phe, 0.89; L-Thr, 0.72; L-Trp, 0.25; L-Val, 1.02; L-Cys, 0.13; L-Tyr, 0.19; L-Ala, 1.02; L-Pro, 0.64; L-Ser, 0.38; L-Asp, 0.27; L-Glu, 0.27.
[2] GE-3, Otsuka Pharmaceutical Factory, Inc.
[3] Otsuka MV, Otsuka Pharmaceutical Co., Ltd.

of the infusion period, rats were anesthetized with Nembutal and 60 min after stopping infusion venous blood was taken from the inferior vena cava. Main organs were removed and weighed. Amino acid concentrations in the plasma and muscle were measured by an automated amino acid analyzer (Hitachi-835, Tokyo, Japan).

Preparation and culture of alveolar macrophages

Alveolar macrophages (AMø) were obtained aseptically by tracheopulmonary lavage of rats after bled by cutting both renal arteries [7]. The total number and viability of nuclear cells were assessed by the trypan blue staining. According to positive reaction for non-specific esterase, more than 95% of the lavaged cells were AMø. AMø collected by centrifugation of the lavage fluid were resuspended in 2 ml of RPMI 1640 (Nissui Seiyaku, Tokyo, Japan) with 5% FBS and plated in Multiwell plates (Falcon Plastics, Oxnard, CA). Nonadherent cells including neutrophils were removed by washing the plates with medium after plating for 1 hr. More than 99% of the adherent cells were mononuclear and used for the following phagocytosis assay.

Phagocytosis assay

Sheep red blood cells (SRBC, Nihon Biotest, Tokyo) were maintained in Alserver's solution (Grand Island Biological, Grand Island, NY) at 4°C. Opsonization was accomplished by incubating 10 ml of a 0.2% v/v SRBC suspension with 0.2 ml of rat anti-SRBC antiserum (heat-inactivated) for 1 hr at 37°C. The antiserum was prepared by injecting intraperitoneally 1.0 ml washed SRBC into rats at weekly intervals (7). Radioactive labeling of the opsonized SRBC was accomplished by incubation with 200 μCi of sodium chromate ($Na_2{}^{51}CrO_4$;

specific activity 128 μCi/mg, New England Nuclear) for 1 hr at 37°C. The opsonized SRBC labeled with ^{51}Cr were added. After 2 hr incubation at 37°C, the cultures were rinsed once with distilled water to lyse nonphagocytosed SRBC and then washed twice with 0.1 M phosphate buffer, pH 7.2. All remaining cells were lysed with 0.1 M NaOH, and the radioactivity of the lysate was measured in a gamma counter (ARC-361, Aloka, Tokyo). Phagocytic index was calculated by assigning a value of 1 to the average of phagocytic activity per rat of stock diet group.

In vitro effect of Gln and Ala-Gln on tumor growth

In vitro effect of Gln and Ala-Gln on tumor growth was investigated on Yoshida sarcoma cells. Tumor cells (1×10^6 cells/ml) were cultured with various concentrations of Gln and Ala-Gln, concomitantly pulsed with ^3H-thymidine (1 μCi/well) and incubated for 24 hr. Then tumor cells were harvested by an automated cell harvester (Flow Lab., Rockville, MD) and their radioactivities were counted by a liquid scintillation counter (LSC-703 Aloka Corp., Tokyo).

In vitro effect of Ala-Gln on AMø phagocytosis

AMø prepared from rats fed control stock diet were incubated at 37°C in 0.5 ml of medium alone and medium containing 0.1–10 μmol/ml of Ala-Gln. Six hr later, AMø were washed thoroughly with medium before addition of radiolabelled SRBC and then the phagocytosis was assessed by the method described above.

In vitro effect of Ala-Gln on blastogenesis of splenocytes

Spleen from rats fed control stock diet was minced with scissors and passed through a stainless steel sieve. Numbers of splenocytes were counted microscopically. Splenic T- and B-lymphocyte responses to mitogens such as phytohemagglutinin (PHA; 20 μg/ml), concanavalin A (Con A; 20 μg/ml) and lipopolysaccharide (LPS; 40 μg/ml) were determined as described previously (8).

Statistical analyses

The data were analyzed by Student's t-test and differences associated with p values <0.05 were regarded as statistical significance.

Results

Body, organ and tumor weights

As shown in Table 2, there was no significant difference in the body weight gain for 8 days between two infusion groups of tumor-bearing rats. The weight of organs including the intestine, spleen and thymus per final body weight was not different between both infusion groups. Tumor weights were also not different significantly. The rats of both infusion groups showed a positive nitrogen balance during the infusion period (data not shown).

Table 2. Weight of body, organ and tumor

	Standard ($n = 6$)	Ala-Gln rich ($n = 9$)
Body weight gain (g)	19.38 ± 8.51	19.28 ± 5.22
Intestine*	1.49 ± 0.05	1.50 ± 0.10
Spleen*	0.44 ± 0.15	0.46 ± 0.08
Thymus*	0.28 ± 0.08	0.29 ± 0.12
Tumor weight/ Carcass weight (%)	0.85 ± 0.47	0.62 ± 0.97

Data are mean ± SD.
* g/100 g body weight.

Tumor growth

The volume of tumors in rats infused Ala-Gln-enriched solution was smaller than that of standard parenteral nutrition, but was not statistically significant (Fig. 1).

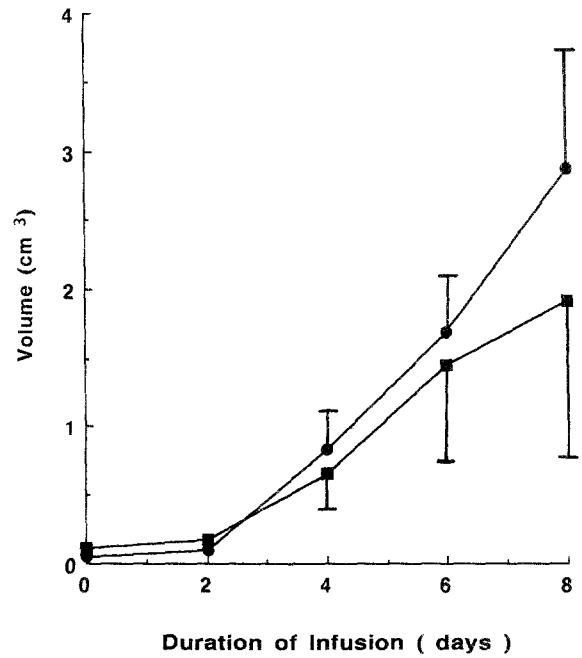

Fig. 1. Changes of tumor volume in rats infused with standard parenteral nutrition (–●–) and Ala-Gln containing parenteral nutrition (–■–). Bars indicate SD

Composition of amino acids in plasma and skeletal muscle (Fig. 2A and B)

As shown in Fig. 2, there was no significant difference in the concentration of Gln in plasma among standard parenteral nutrition group, Ala-Gln-enriched

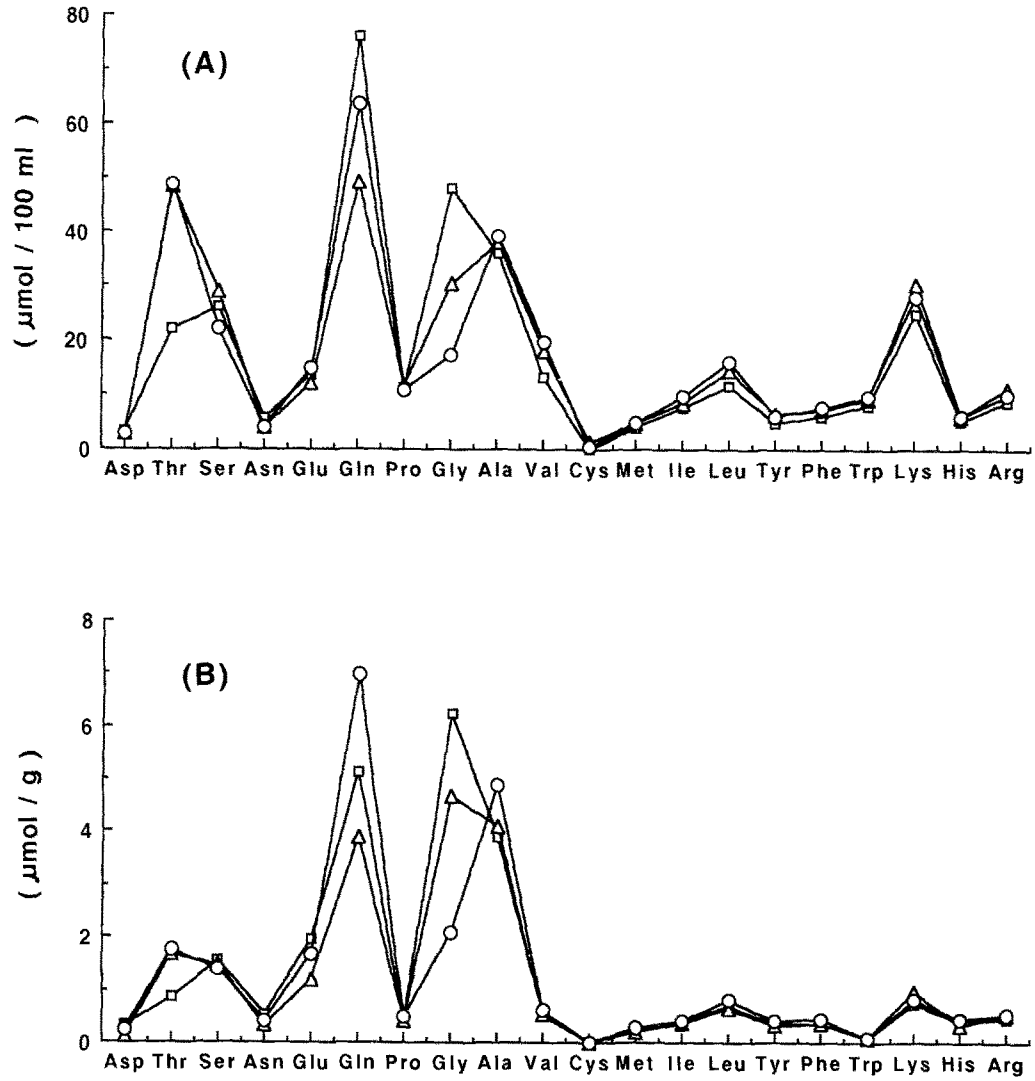

Fig. 2. Changes of amino acid concentration in plasma (**A**) and muscle (**B**). —□— control rats fed stock diet, —△— tumor-bearing rats infused with standard parenteral solution, —○— tumor-bearing rats infused with parenteral solution containing Ala-Gln

group and control stock diet group. In muscle, Ala-Gln-enriched solution induced a significant increase of Gln. The level of Thr in skeletal muscle of both parenteral nutrition groups was higher than that in the stock diet group. Conversely, Gly level was decreased in plasma and skeletal muscle of both infusion groups.

In vivo and in vitro phagocytosis of opsonized SRBC by AMø

As shown in Fig. 3, phagocytosis of AMø from tumor-bearing rats infused with the solution containing Ala-Gln was significantly higher than that of the rats infused with standard solution. Studies on in vitro activation of AMø from

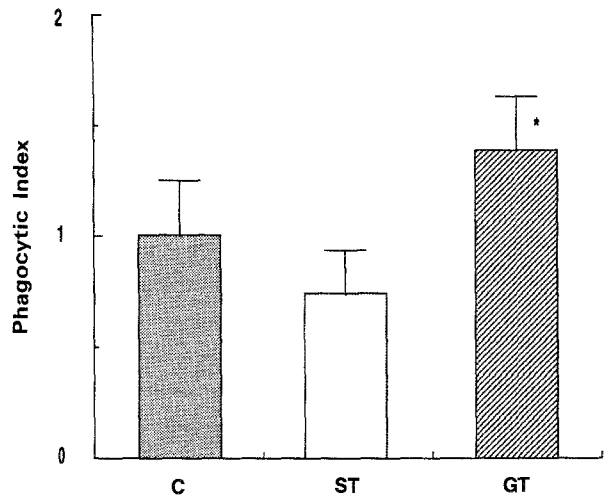

Fig. 3. Phagocytosis of ^{51}Cr-labeled opsonized SRBC by AMø of tumor-bearing rats infused with standard parenteral solution (*ST*) and parenteral solution containing Ala-Gln (*GT*) compared to that of control stock diet (*C*). Significantly different vs ST groups. Bars indicate SD. $^{*}P < 0.05$

control rats by addition of Ala-Gln showed a significant increase in phagocytic activity at 0.1–1.0 μmol/ml and 10.0 μmol/ml of Ala-Gln (Fig. 4).

In vitro blastogenesis of splenocytes by Ala-Gln (Fig. 5)

The blastogenic response of splenocytes incubated with Con A, in particular, for 3 or 6 hr was significantly increased in addition of Ala-Gln and it reached a maximum in the concentration of 0.5 μmol/ml of Ala-Gln. The response of splenocytes to PHA and LPS was not so large compared to those with Con A.

In vitro effect of Gln and Ala-Gln on the proliferation of tumor cells (Fig. 6A and B)

Incorporation of ^{3}H-thymidine into Yoshida sarcoma cells increased in both groups adding Gln (A) or Ala-Gln (B) at the levels more than 0.1 μmol/ml in the culture medium.

Discussion

Ala-Gln is readily hydrolyzed after its intravenous administration and efficient as a source of free Gln in total parenteral nutrition [3]. The results of our study also showed that infusion of the solution containing Ala-Gln to the tumor-bearing rats had a beneficial effect on muscle Gln and nitrogen balance. Hwang et al. [9] and Klimberg et al. [10] described the preservation of intestinal mucosal weight, DNA content and villous height in healthy animals following Gln-enriched parenteral nutrition compared to those of standard Gln-depleted

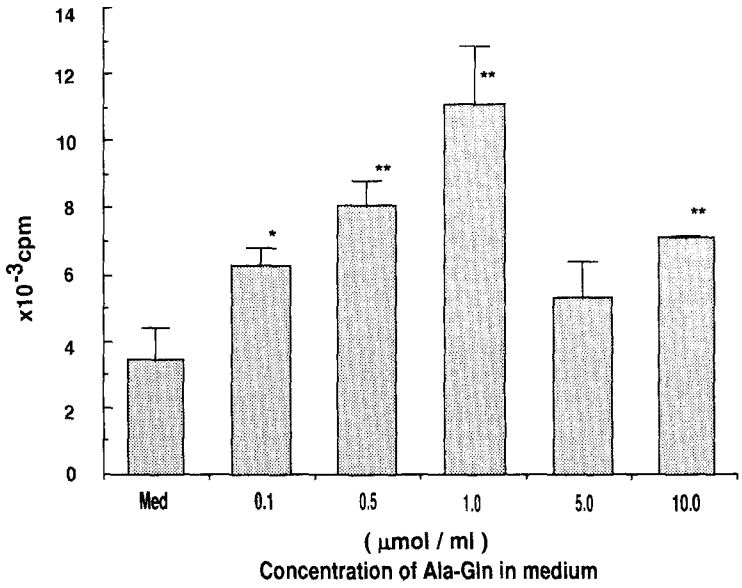

Fig. 4. In vitro effect of Ala-Gln on phagocytic activity of AMø in rats fed stock diet. Significantly different vs culture with RPMI 1640 medium without Ala-Gln. Bars indicate SD of three separate experiments. $*P < 0.05$, $**P < 0.01$

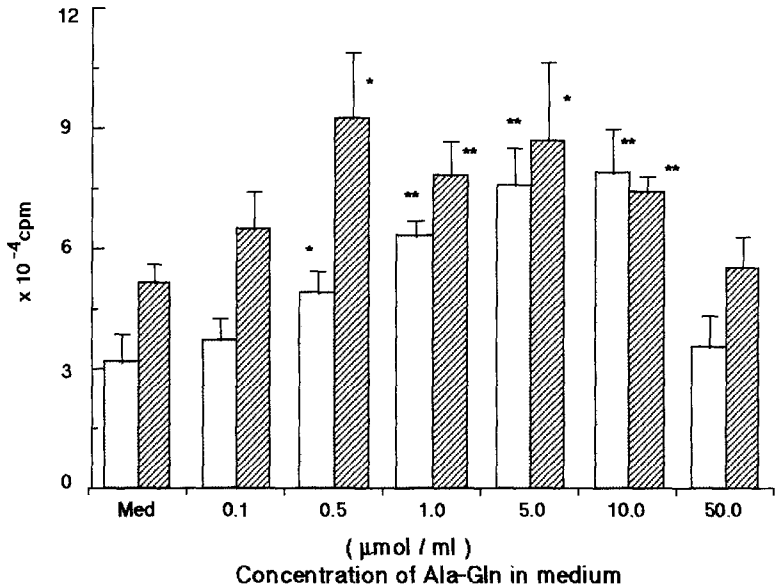

Fig. 5. In vitro effect of Ala-Gln on blastogenesis of splenocytes from rats fed stock diet. Bars indicate SD of three seperate experiments. Significant different vs culture with medium alone. □ 3 hr ▨ 6 hr incubation. $*P < 0.05$, $**P < 0.01$

nutrition. In vitro experiment in the present study showed that the supplement of Gln or Ala-Gln increased significantly in the incorporation of ^{3}H-thymidine to Yoshida sarcoma cells. At the same time, it devotes our attention of Gln by

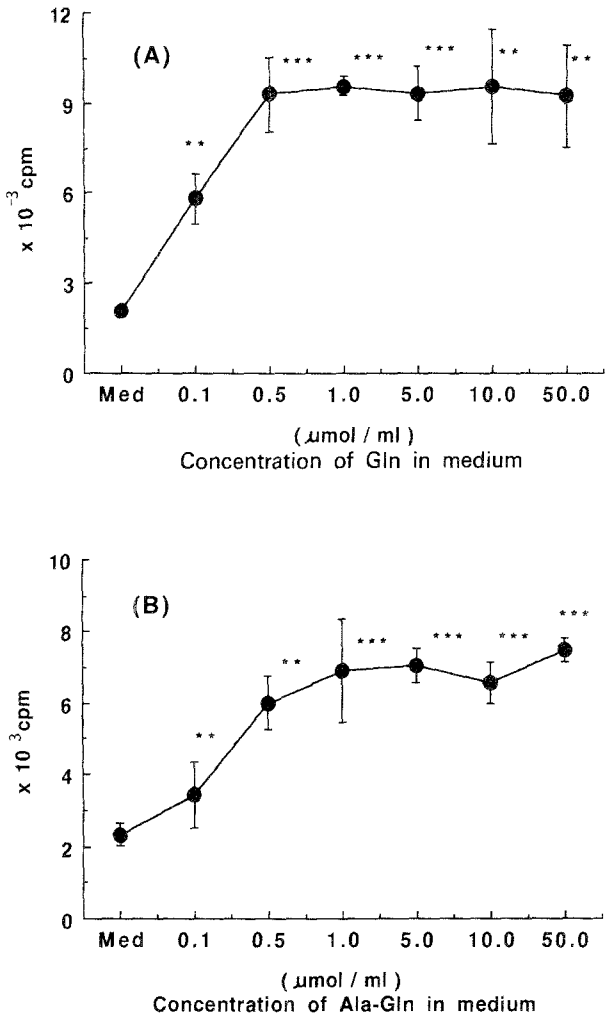

Fig. 6. In vitro effect of Gln (**A**) and Ala-Gln (**B**) on the incorporation of ^{3}H-thymidine to Yoshida sarcoma cells. Significantly different vs culture with RPMI medium without Gln or Ala-Gln. Bars indicate SD. $^{**}P < 0.01$, $^{***}P < 0.001$

rapidly growing tumors as preffered energy and nucleotides, and then may accelerate tumor growth in vivo. However, in the present experiment in vivo using Yoshida sarcoma cells, we have not observed the detectable acceleration of tumor growth subcutaneously inoculated in rats after treated with Ala-Gln infusion. Recently Klimberg et al. [11] also reported that Gln-enriched elemental diet improved nutritional status without increase in the rate of fibrosarcoma growth. On the other hand, a relationship between Gln and cells of the immune system has been postulated by Newsholme and his co-workers. Ardawi and Newsholme [12] and O'Rourke [13] reported that Gln is important in not only provision of energy, but also precursor for proliferation of the splenic and thymic lymphocytes in respect to DNA synthesis. Gln utilization of mitogen-activated thymocytes was 8-fold increased as compared to the resting ones [14]. When Gln

was added to cultured lymphocytes from lymphnodes, remarkable incorporation of ^{3}H-thymidine into Con A-activated lymphocytes occurred [12]. Newsholme and Newsholme [6] observed the enhancement of glucose utilization of macrophages by addition of Gln to culture medium and suggested that macrophages have a high capacity to take up Gln, which may act as an intracellular store for both energy formation and provision of precursors for purine and pyrimidine biosynthesis. Alverdy [15] and Burke et al. [16] demonstrated that Gln-enriched parenteral nutrition protects against bacterial translocation from the intestinal walls by maintaining secretory IgA and Wells et al. [17] also suggested that Gln may directly affect macrophage-mediated bactericidal activity in mesenterial lymphnodes. As shown in our experiment, in vivo and in vitro addition of Ala-Gln increased in phagocytosis of AMø and blastogenesis of splenocytes in rats. The depletion of Gln in rats was found to be immunosuppressive but the immunosuppression was reversed by normal concentration of Gln [18] and then it was indicated that Gln is an important cellular immune system modulator.

In summary, our present study showed that Ala-Gln as a 30% Gln in amino acids maintained tumor-bearing rats in an adequate nutritional state and did not accelerate tumor growth by increased host-resistance through maintaining lymphocyte and macrophage functions at normal levels.

References

1. Grant JP, Synder PJ (1988) J Surg Res 44: 506–513
2. Souba WW, Strehel FR, Bull JM, Copeland EM, Teagtmeyer H, Cleary K (1988) J Surg Res 44: 720–726
3. Fürst P, Albers S, Stehle P (1989) Metabolism 38 [Suppl]: 67–72
4. Fischer JE, Chance WT (1990) J Parenter Enteral Nutr 14 [Suppl]: 86S–89S
5. Ardawi MSM, Newsholme EA (1982) Biochem J 201: 743–748
6. Newsholme P, Newsholme EA (1989) Biochem J 261: 211–218
7. Moriguchi S, Sone S, Kishino Y (1983) J Nutr 113: 40–46
8. Moriguchi S, Kobayashi N, Kishino Y (1990) J Nutr 120: 1096–1102
9. Hwang TL, O'Dwyer ST, Smith RJ, Wilmore DW (1987) Surg Forum 38: 56–58
10. Klimberg VS, Souba WW, Sitren H, Plumley DA, Salloum RM, Hautamaki D, Bland Kl, Copeland EM (1989) Surg Forum 40: 175–177
11. Klimberg VS, Souba WW, Salloum RM, Plumley DA, Cohen FS, Dolson DJ, Bland Kl, Copeland EM (1990) J Surg Res 48: 319–323
12. Ardawi MSM, Newsholme EA (1983) Biochem J 212: 835–842
13. O'Rourke AM, Rider CC (1989) Biochem Biophys Acta 1010: 342–345
14. Brand K, Leibold W, Luppa P, Schoerner C, Schulz A (1986) Immunobiology 173: 23–34
15. Alverdy JC (1990) J Parenter Enteral Nutr 14: 109S–113S
16. Burke DJ, Alverdy JC, Aoys E, Moss GS (1989) Arch Surg 124: 1396–1399
17. Wells CL, Maddacs MA, Simmons RL (1987) Arch Surg 122: 48–53
18. Kafkewitz D, Bendich A (1983) Am J Clin Nutr 37: 1025–1030

Authors' address: Dr. Y. Kishino, Department of Nutrition, School of Medicine, The University of Tokushima, Kuramoto-cho, Tokushima 770, Japan.