Dietary Glutamine Supplementation Enhances Weaned Pigs Mitogen-Induced Lymphocyte Proliferation*

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ABSTRACT : Two experiments involving 92 crossbred, 21 day old weaned pigs were used to evaluate the effect of glutamine supplement in a dietary or culture medium on lymphocyte proliferation. In Exp. 1, 88 pigs were fed diets supplemented with 0, 0.5, 1.0, or 1.5% glutamine for 28 days. Lymphocytes were prepared from peripheral blood mononuclear cells (PBMC), ileal Peyer's patches (PP), the mesenteric lymph node (MLN), and the spleen in each dietary supplement group on days 7, 14, or 28 postweaning. Lymphocytes were cultured at 37°C for 72 h in a RPMI-1640 medium with or without mitogen-stimulated, and pulsed with 3H-thymidine for an additional 18 h. The stimulation index of PBMC proliferation in 1.0% dietary glutamine supplement group and both of the MLN and splenocytes proliferation in 1.5% dietary glutamine supplement group was significantly ($p<0.05$) increased at 14 days postweaning. In Exp. 2, four weaned pigs were fed a basal diet for 14 days. The 3H-thymidine incorporation of PBMC, PP, and MLN cells, incubated with 0.125 to 0.25 mM glutamine in culture medium were markedly enhanced with Con A-stimulated, however, the splenocyte proliferation was not affected in the addition of glutamine medium. These observations suggest that dietary glutamine supplement might enhance the lymphocyte proliferation of weaned pigs. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 8 : 1182-1187)***

Key Words : Glutamine, Weaned Pigs, Lymphocytes Proliferation

INTRODUCTION

The Peyer's patches (PP), mesenteric lymph node (MLN), and the spleen, located in intestinal surroundings known as the gut-associated lymphoid system, is the largest lymphoid organ in the body (Kuby, 1994). These lymphocytes concomitantly perform the local immune function of the intestine, maintaining the digestive tract integration, and keeping the animal healthy. Pigs weaned undergoing from maternal milk to solid feed seems to have underdeveloped gut-associated lymphoid system and this is associated with increased incidences of malnutrition and intestinal infection (Miller et al., 1986; Wilson et al., 1989). During this transition period, the protective immunoglobulins from maternal milk are no longer provided, and the integrity of gut-associated immune function must be protected to against invasion during the weaning procedure.

Glutamine is the predominant free amino acid in sow milk (Wu and Knabe, 1994), and could further be degraded to α-ketoglutarate and amide group after enterocyte uptake. These products can contribute for important biosynthesis pathways such as the formation of purines, pyrimidines, and glucosamine (Krebs, 1980; Wu et al., 1995), and consequently may be the benefit of intestinal lymphocytes meiosis. Glutamine is also an important fuel source for cultured cells including lymphocytes (Eagle, 1959; Wu et al., 1991). Supplementing rat lymphocytes with 0.2-1.0 mM of glutamine in culture mediums could increase the uptake of ^{3}H-thymidine (Ardawi and Newsholme, 1983). The proliferation rate of rat lymphocytes in culture increases with external glutamine concentration in a dose-dependent manner exceeding the fuel role as glucose (Calder, 1995; Dugan et al., 1994).

Dietary glutamine supplement enhances murine spleen T-lymphocyte response to mitogen stimulation (Kew et al., 1999), and normalizes the peripheral blood mononuclear cells (PBMC) function in infected early weaned pigs (Yoo et al., 1997). *In vitro* assay also found that glutamine supplemented to IEC-6 cell line enhances the synthesis of DNA, RNA and protein (Ko et al., 1993). Therefore, it has been recognized glutamine as a conditionally essential amino acid (Souba, 1991). Information on the effects of glutamine supplement on enteric immune cells in pigs is rare. The current study investigated the beneficial effect of glutamine supplement in both diet and culture medium on lymphocyte proliferation in weaned pigs.

MATERIALS AND METHODS

Animals and treatment

All pigs were the offspring of either Yorkshire or Landrace sows crossed with Duroc boar. Pigs were weaned

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at 21 days of age, and fed a basal diet containing corn, soybean meal, isolated soybean protein and whey, supplemented with vitamins and minerals. All nutrients met the standards of the National Research Council (NRC, 1998). Table 1 lists the diet formulation and nutrient composition. Feed and water were fed *ad libitum* for 28 days. The total content of glutamine and glutamate in the basal diet was 3.70% which was determined using the hydrolysis technique by amino acid automatic analyzer (System Gold, Beckman, USA).

In Exp. 1, 88 pigs from 12 litters were randomly allotted to dietary treatments based on sex and litter origin. Pigs were fed 0, 0.5, 1.0 or 1.5% glutamine supplement (Ajinomoto Inc., Tokyo, Japan) and housed in a nursery room with four pigs per pen. Temperature in the nursery room was maintained around 26°C with heating supply during the first 2 weeks. The blood samples in each treatment were obtained from the cervical vena at day 7 (n=4), 14 (n=18), and 28 (n=10), respectively. Four and eight pigs of each dietary treatment group were sacrificed at 7 and 14 days postweaning, respectively. After a citsol injection of 1 mg/kg body weight (i.v.) for pre-anesthesia, a mixture of 4% halothane and 95% oxygen for surgical anesthesia was administered by facemask. The abdomen was opened after anesthesia, and the ileal PP, MLN, and spleen were immediately dissected and washed with cold Hank's balanced buffer (Life Technologies Inc., USA). All tissues were ground for single cell preparation to do ^{3}H-thymidine incorporation.

Table 1. Formula and nutrient composition of basal diet

Ingredients (%)	
Corn	50.09
Soynbean meal	22.51
Soy protein (CP 65%)	12.00
Whey	10.00
DL- Methionine	0.03
Soybean oil	1.12
Glutamine-corn starch[a]	1.50
Dicalcium phosphate	1.27
Limestone, pulverized	0.88
Salt	0.30
Vitamin premix[b]	0.10
Trace mineral premix[c]	0.10
Antibiotic premix[d]	0.10
Calculated nutrient composition	
Metabolizable energy (kcal/kg)	3,265
Crude protein (%)	23.70
Ca (%)	0.80
P (%)	0.69
Analyzed nutrient composition	
Lys (%)	1.46
Met+Cys (%)	0.76
Glu+Gln (%)	3.70

[a]Glutamine substituted for corn starch. Glutamine levels were 0, 0.5, 1.0 or 1.5%.

[b] Supplied per kg diet: vitamin A, 6,000 IU; vitamin D, 900 IU; vitamin E, 30 IU; vitamin K_3, 3 mg; vitamin B_2, 6 mg; pantothenic acid, 18 mg; niacin, 60 mg; vitamin B_{12}, 30 μg, and choline-HCl, 525 mg.

[c] Supplied per kg diet: Cu, 20 mg; Zn, 100 mg; Fe, 140 mg; Mn, 4 mg; Se, 0.1 mg, and I, 0.2 mg.

[d] Supplied per kg diet: Lincomycin-HCl 44 mg and Spectinomycin sulfuate 44 mg.

In Exp. 2, four weaned pigs from the same litter were fed a basal diet without glutamine supplement for 14 days. The basal diet and the method to obtain lymphoid tissue samples were the same as in Exp. 1. The ileal PP, MLN, and spleen were immediately dissected out after anesthesia and the tissues were ground for single cell preparation to do ^{3}H-thymidine incorporation.

Preparation of lymphocytes

The lymphocytes prepared from the ileal PP, MLN, and the spleen were teased apart and cell suspensions were filtered through a 50-meshes stainless screen to remove debris. The cells and heparinized blood were centrifugally isolated on a cushion of Ficoll-Hypaques (Amersham Pharmacia Biotech Co., France). The lymphocytes were removed from the interface, and residual erythrocytes were lysed using NH_4Cl. Lymphocytes were then washed three times with Roswell Park Memorial Institute-1640 (RPMI-1640) medium and cultured at a concentration of 1×10^6 cells/ml in an RPMI-1640 culture medium. Cell viability was greater than 95% as assessed by trypan blue exclusion.

Measurement of ^{3}H-thymidine incorporation

Lymphocytes were cultured at 37°C in triplicate in 1×10^5cells/0.2 ml/well on a 96-well plate for 72 h in 5% CO_2 and 95% air. Culture media used was RPMI-1640 with glutamine medium (#23400, Life Technologies Inc.) or RPMI-1640 without glutamine medium (#21870, Life Technologies Inc.); both were supplemented with 10% fetal bovine serum and 100 U/ml penicillin in Exp. 1 and Exp. 2, respectively. In Exp. 2, 0 to 4.0 mM L-glutamine (Sigma Chemic Co., USA) was additionally added to the culture media. Actual glutamine concentrations in the basic culture medium were 2.05 and 0.27 mM in Exp. 1 and 2, respectively, using the enzyme measurement method (Lund, 1983). To determine the lymphocyte response to mitogen stimulation, the culture medium contained 10 μg/ml concanavalin A (Con A), 20 μg/ml phytohemagglutinin (PHA), or 10 μg/ml pokeweed mitogen (PWM) (Sigma Chemic Co.). The concentrations of mitogen used have been tested and shown optimal proliferation in a preliminary study (Lee et al., 2000). Lymphocyte proliferation was pulsed with 0.5 μCi/well ^{3}H-thymidine (Specific activity 80 Ci/mmol, Amersham Pharmacia Biotech.) for 18 h, and was terminated by harvesting cells onto glass fiber filters using an automatic cell harvester (Packard, USA). Filters were immersed in a cocktail after

drying, and radioactivity was measured using a microplate scintillation and luminescence counter (Packard). Stimulation index was calculated as counts per minute (cpm) of radioactivity in the presence of a mitogen/cpm of radioactivity in the absence of mitogen as a control treatment.

Statistical analysis

Experimental data was analyzed using the SAS (1999) statistical program. The general linear model was used to analyze variance, and Duncan's multiple range test was applied for comparing the differences between treatments.

RESULTS

PBMC proliferation

Table 2 shows the PBMC proliferation of dietary glutamine supplement in Exp. 1. A dosage of glutamine supplement greater than 1.0% could elevate ($p<0.05$) the PWM-stimulated PBMC proliferation at 14 days postweaning, but glutamine supplement did not influence the proliferation of PBMC at 7 and 28 days postweaning.

Gut-associated lymphocyte proliferation

The results of glutamine supplement on gut-associated lymphocyte proliferation in weaned pigs at 7 and 14 days is listed in Table 3. Dietary treatments did not affect lymphocyte proliferation after 7 days feeding. Following 14 days feeding increased in glutamine, MLN lymphocyte proliferation in both the basal culture medium and that co-culture with PHA were stimulated. The stimulation index was approximately 2.2 fold in the 1.5% glutamine supplement group as compared with the control group. A significant difference ($p<0.05$) of Con A-stimulated splenocytes proliferation among treatments was observed, the 1.5% glutamine supplement group being better than both 0.5 and 1.0% groups.

Lymphocytes incubated with glutamine

The proliferation of lymphocytes incubated with 0 to 4 mM of glutamine from Exp. 2, is shown in Figure 1. Results indicate that 0.25-1 mM glutamine supplement in medium could significantly increase the proliferation of PBMC after Con A-stimulation. When the glutamine level was increased to 2 mM, lymphocyte proliferation from the MLN and PP remained increased. Elevated to 4 mM high glutamine level did not keep the increasing trend in MLN and PP whereas the lymphocyte proliferation of splenocytes showed the increasing manner. In this lymphocyte proliferation system, MLN had the highest stimulation index, and splenocytes showed the lowest one.

DISCUSSION

Nutrients have a profound effect on the host immune defenses (Alexander, 1995), and glutamine seems particularly relevant because of its direct supply of requirements to the gastrointestinal epithelium and its associated lymph tissue (Kreb, 1980; Wu, 1996). However dietary glutamine supplement did not affect growth performance of pigs during 28 days postweaning (data not shown). The result of dietary glutamine supplement

Table 2. Effect of dietary glutamine supplement on ^{3}H-thymidine incorporation in cultured peripheral blood mononuclear cell lymphocytes (PBMC) of weaned pigs (Exp. 1)

Mitogen source	Glutamine, %				Pooled SEM
	0	0.5	1.0	1.5	
Day 7					
No. of pigs	4	4	4	4	
Control	1.00[†]	0.85	1.02	0.96	0.15
Con A	95.17	104.5	140.6	111.0	31.22
PHA	79.40	97.37	124.0	84.97	34.74
PWM	17.19	25.88	37.02	24.49	8.79
Day 14					
No. of pigs	18	18	18	18	
Control	1.00[†]	1.41	1.39	0.98	0.17
Con A	70.86	91.54	142.1	119.3	22.63
PHA	64.77	71.13	105.8	113.3	16.48
PWM*	14.79[a]	20.37[ab]	35.27[b]	34.86[b]	6.06
Day 28					
No. of pigs	10	10	10	10	
Control	1.00[†]	1.53	2.34	1.60	0.45
Con A	92.87	190.0	116.9	97.97	31.04
PHA	63.92	144.3	80.05	84.92	23.77
PWM	17.55	31.00	15.04	13.95	6.40

[†] The cpm value of control treatment non-mitogen stimulation was 90.75, 242.7, and 297.9 on days 7, 14, and 28, respectively. * Different letter represents the significant difference ($p<0.05$) between treatments.

enhanced PBMC proliferation is agreed with previous finding (Yoo et al., 1997). They found that PBMC proliferation from E. coli-infected pigs was significantly higher in pigs fed a diet containing 40 g glutamine/kg than that fed a basal diet. Studies of glutamine oxidation in pig intraepithelial lymphocytes have indicated a metabolic alteration from 8 days postweaning pigs to 35 days postweaning pigs (Dugan et al., 1994). This seems to be the case of the indifference in PBMC proliferation among dietary treatments at 28 days postweaning in this study. Additionally, the observation in which porcine lymphocyte proliferation increased after *in vitro* glutamine supplement was similar to findings in previous studies involving rats (Ardawi and Newsholme, 1983; Wu et al., 1992) and pigs (Wu, 1996). In the present study, the basal culture medium containing 0.266 mM glutamine plus 0.125-0.25 mM exogenous glutamine (making a total level of 0.39-0.52 mM glutamine) had the maximum proliferation. The total glutamine concentration of medium was close to the blood plasma level of weaned pigs (Wu et al., 1994). This glutamine level indeed meets the requirement of lymphocytes and maintains them in normal condition.

In Exp. 2, only PBMC, MLN and PP lymphocyte proliferation showed a glutamine dose-dependent manner after Con A-stimulated. However splenocytes did not present this effect. The stimulation index of PBMC, PP and MLN proliferation all were higher than splenocyte, and a higher glutamine dose-dependent pattern was evident *in vitro*. This difference may be associated with the lymphocyte configuration of different lymphoid organs (Solano-Aguilar et al., 2000, 2001). The PP possesses a higher proportion of B-cells. Increasing the dietary

Table 3. Effect of dietary glutamine supplement on ^{3}H-thymidine incorporation in cultured gut-associated lymphocytes of weaned pigs (Exp. 1)

Mitogen source	Glutamine, %				Pooled SEM
	0	0.5	1.0	1.5	
Day 7					
No. of pigs	4	4	4	4	
PP					
Control	1.00[†]	0.97	1.16	1.41	0.20
Con A	32.50	51.04	100.0	114.7	22.15
PHA	72.97	65.62	119.2	127.5	24.85
PWM	14.73	6.31	25.02	30.11	7.56
MLN					
Control	1.00[†]	1.03	0.91	0.99	0.07
Con A	168.1	245.8	137.0	149.0	51.48
PHA	175.3	263.5	185.3	186.2	43.59
PWM	76.69	103.3	68.14	64.06	29.44
Splenocytes					
Control	1.00[†]	1.09	1.03	0.98	0.17
Con A	34.66	43.98	69.19	49.76	15.54
PHA	30.03	38.80	60.04	47.90	10.70
PWM	7.78	13.06	17.60	14.74	4.02
Day 14					
No. of pigs	8	8	8	8	
PP					
Control	1.00[†]	1.01	1.07	1.07	0.14
Con A	73.72	69.36	114.0	76.31	31.71
PHA	91.67	97.65	165.8	108.6	42.67
PWM	25.52	29.56	43.62	34.42	11.48
MLN					
Control*	1.00[†a]	1.04[a]	1.12[a]	1.37[b]	0.09
Con A	312.0	340.5	379.2	615.4	108.7
PHA*	306.4[a]	342.0[a]	420.6[ab]	675.9[b]	89.16
PWM	132.4	151.5	185.6	232.7	28.38
Splenocytes					
Control	1.00[†]	1.30	1.12	1.46	0.24
Con A*	48.87[ab]	37.75[a]	33.22[a]	73.64[b]	9.91
PHA	48.41	37.36	34.83	51.92	5.90
PWM	14.87	11.05	12.31	17.48	3.38

[†] The cpm value of control treatment non-mitogen stimulation was PP: 41.00 and 66.50; MLN: 40.25 and 43.88; Splenocytes: 108.5 and 148.0 on days 7 and 14, respectively. * Different letter represents the significant difference ($p<0.05$) between treatments.

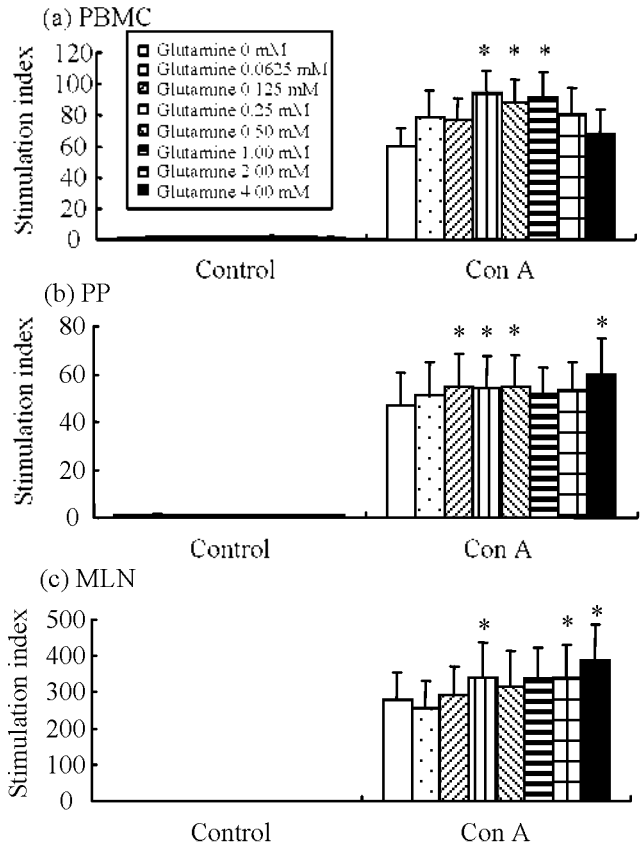

Figure 1. Effect of extracellular glutamine concentrations on stimulation index in cultured lymphocytes of weaned pigs using Con A-stimulation. The non-mitogen stimulation cpm value (mean ±SE) of glutamine concentration in 0 group was PBMC: 276.8±95.15; PP: 154.2±18.18; MLN: 60.37±12.07; and splenocytes: 362.0±92.64. An asterisk indicates a significant difference ($p<0.05$) between the group and the glutamine supplementation groups (Exp. 2).

glutamine content from 0.5 g/100 kcal to 3 g/100 kcal elevated the total lymphocyte and B-cell number in the PP of mice under health and infectious status (Manhart et al., 2001). However, there is no information in the literature on pig PP proliferation through dietary or culture medium glutamine supplement. Among these high T-cell percentages of lymphoid tissue (PBMC, MLN, and spleen), splenocytes have a particularly high proportion of cluster of differentiation antigen^{8+}(CD^{8+}) compared to PBMC or MLN (Boeker et al., 1999).

Glutamine performs a specific and unique role in the lymphocyte proliferation process. It is required for T cell activation, possibly due to it stimulating the expression of "late" activation markers (Hörig et al., 1993) and that other amino acids cannot replace glutamine in supporting lymphocyte proliferation (Ardawi and Newsholme, 1983). In the present study, MLN had the highest stimulation index in four kinds of lymphocytes, and that can stimulate over 600 fold by using different mitogens. Wu (1996) speculated that porcine MLN lymphocytes lack the ability to synthesis glutamine from glutamate and ammonia. The metabolic difference of glutamine in various organs may explain why is a glutamine-dependent of MLN lymphocyte proliferation in this study.

In conclusion, this study has shown that the lymphocyte proliferation is enhanced in weaned pigs with both dietary and medium glutamine supplement. The cell proliferations of PBMC, MLN, and spleen response to dietary glutamine supplement are a much higher extent than the PP. In contrast to in vivo result, splenocyte was poor response to glutamine in medium. Further studies on the effectiveness of dietary glutamine supplement in gut-associated immunity under bacterial challenges require to be done.

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