Effect of glutamine supplementation on lymphocyte subsets in children with acute diarrhea

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To study the effect of glutamine supplementation on lymphocyte subpopulation counts in children with acute diarrhea, children aged 6-24 months were enrolled in a double-blind randomized study. Cases had received either 0.3 g/kg/day of glutamine or placebo orally for seven days. The counts of blood leukocytes, lymphocytes and lymphocyte subpopulations (CD3+, CD4+, CD8+, CD19+, CD16+CD56+) were determined both on admission and seven days later using a flow cytometry. When adjusting for sex, current breastfeeding status, dehydration, and nutritional status of children, lymphocyte subpopulations did not differ significantly between the glutamine- and placebo-supplemented groups on the 7th day of intervention.

Key words: lymphocyte subset, acute diarrhea, glutamine, children, supplementation.

Previous studies have indicated that glutamine (glu) was an important fuel and/or substrate for lymphocytes and was critical for lymphocyte differentiation in cell culture[1,2]. Studies have also reported that, even in a healthy state, the addition of glu to the diet improved measures of cell-mediated immunity - an increase in the proportion of various T-cell subsets and in the ability of T-lymphocytes to respond to mitogenic stimulation in murine studies[3,4]. Enteral glu supplementation increased the blood lymphocyte CD4+/CD8+ ratio in patients in intensive care[5], and parenteral glu increased mitogen-stimulated proliferation of blood lymphocytes from patients after colorectal surgery[6]. Bone marrow transplant patients receiving total parenteral nutrition (TPN) with glu also exhibited higher total lymphocyte, T-lymphocyte, CD4+, and CD8+ lymphocyte counts than did patients receiving glu-free TPN[7]. Moreover, a blind, randomized controlled study of enterally glu-supplemented premature formula in 68 very low birth weight neonates between days 3 and 30 of life demonstrated that the glu recipients had improved markers of T-cell function[8].

Although glu plays important roles in the gut integrity and the immunologic responses[1,9,10], there are limited studies clarifying the role of glu supplementation during acute diarrhea[11]. As one of the primary nutrients promoting growth, function and maturation of the intestinal tract and immune system[9,10], glu may aid in the control of gastroenteritis. In our previous study, a reduction in the duration and severity of diarrhea as a result of glu supplementation was detected in children with acute diarrhea[11]. As a result, there has been some interest in the role of glu supplementation in modulating immune response in children with acute diarrhea. However, the mechanism of dietary glu action in these patients remains to be determined. Therefore, we aimed in the current study to investigate the effects of oral glu supplementation upon lymphocyte subsets in cases with acute diarrhea.

Material and Methods

Children aged 6-24 months admitted to the Diarrheal Diseases Training and Treatment Center with the complaint of acute diarrhea were enrolled in a prospective, placebo-controlled, double-blind randomized study. Infants with chronic illness, immunologic disorders, severe malnutrition (weight <60%

of weight standard for age according to the National Center for Health Statistics [NCHS])[12], associated infectious diseases (urinary tract infections, pneumonia), history of prior antibiotic or antidiarrheal drug use, or stool smear containing leukocytes (more than 5 cells in 1 high power field) were not taken into the study. The design of the study was given previously and done as a part of a previous study[11]. The study was approved by the Ethical Committee for Medical, Surgery and Drug Research at Hacettepe University Faculty of Medicine, Ankara, Turkey (29/7/1999; TBK 99/2-10). Informed consent was obtained from parents of all the children studied.

At the initial appointment, infants were examined physically, and dehydration was assessed and corrected according to World Health Organization (WHO) guidelines by using glucose-oral rehydration solution[13]. Nutritional status (weight for age) was expressed as a percent of the median NCHS standard[12]. A predesigned, pretested file including age, weight and length, birth order, birth weight, age and educational level of parents, diarrheal duration, frequency of diarrhea and vomiting, and presence of fever (axillary temperature ≥ 38°C) was completed on admission.

Cases had received either 0.3 g/kg/day of glu or placebo (plb) orally for seven days[11]. In cases who admitted in the morning on admission, peripheral venous blood samples (2 ml) were obtained in collection tubes containing ethyl enediaminetetraacetic acid both on admission and on the 7th day of follow-up to observe any change in lymphocyte subsets. Blood samples were stored at room temperature for no longer than four hours and assayed for complete blood cell count and lymphocyte subset profiles. Of 159 cases taken in the previous study[11], 71 cases admitted in the morning and were also enrolled for the current study. In two cases, appropriate blood samples were not taken on day 7. Overall, 33 cases in the glu-supplemented group and 36 in the plb group completed the study. Blood samples were taken again seven days later.

A complete blood cell count was performed with Coulter STKR counter (Counter®, Hialeah, FL). Absolute lymphocyte counts were calculated as the product of the white blood count and lymphocyte differential percentage. Two-color flow cytometric immunophenotyping of lymphocytes was performed (Becton Dickinson FACScalibur, Immunocytometry Systems, E2810, CA, USA) using matched combinations of monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (IO Test® Conjugate Antibodies, Immunotech, Coulter Company, Marseille, France) at the Flow Cytometry Laboratory, Unit of Hematology, Department of Pediatrics, Hacettepe University Faculty of Medicine. The lymphocyte staining was performed in complete blood samples by using erythrocyte lysis. Absolute lymphocyte subset counts were calculated as the percentage of positive cells times the number of total lymphocytes. Circulating CD3+ (total T-lymphocyte); CD4+ (helper T-cell); CD8+ (suppressor T-cell); CD16+CD56+ (total natural killer cell); and CD19+ (total B-lymphocyte) cell numbers were analyzed. In all cases, the sum of lymphocyte lineage percentages (CD3+, CD19+, CD16+) was 100% + 5%. The parallel determinations of CD3 (CD3+/CD19+ and CD3+/CD16+CD56+) permitted internal control check.

All analyses were compared with SPSS for Windows (SPSS Inc., Chicago, IL, USA). Statistical methods used were t-test for comparing means, χ^2 for comparing proportions on admission and paired samples t test for comparing changes during the follow-up period. The effects of supplementation (glu vs. plb) on the changes in diarrheal recovery time, lymphocytes and lymphocyte subset counts from baseline to the seventh day of examination were analyzed by repeated measure analysis of ANOVA when adjusting for sex, current breastfeeding status, dehydration status (normal vs. mild-moderate dehydration), and nutritional status (normal vs. malnourished) of children.

Results

The admission characteristics in terms of age, sex, weight, duration and frequency of diarrhea, frequency of vomiting, breastfeeding status, presence of mild malnutrition (weight for age <90% of standard), presence of mild-moderate dehydration, birth order, birthweight including mother's and father's age and education, and frequency of anemia were comparable between the groups (Table I).

Table I. Baseline Characteristics of Glutamine and Placebo Groups

	Glutamine group	Placebo group
N	33	36
Age (mos)*	13.8±5.2	12.8±5.0
Male**	16 (48.5)	21 (58.3)
Birthweight (kg)*	3.36±0.55	3.27±0.43
Diarrheal duration on admission (day)*	4.27±2.80	3.67±2.18
Frequency of diarrhea / day*	6.30±3.42	7.08±3.49
Frequency of vomiting / day*	1.42±2.11	2.22±2.59
Body weight on admission (kg)*	9.68±1.66	9.29±1.63
Breastfed infants**	12 (36.4)	11 (30.6)
Mild-moderate dehydrated cases**	18 (54.5)	21 (58.3)
Malnourished infant**	9 (27.3)	14 (38.9)
Anemia (hemoglobin value <11 g/dl)**	11 (33.3)	12 (33.3)

*Mean ± SD, ** n(%) p>0.05 for comparison between glu and plb groups.

The duration (mean ± SD) of diarrhea after the intervention in the glu group was significantly shorter than in the plb group (3.58±1.98 days, 4.61±2.10 days, respectively; p=0.040). Further, the same relation was detected in diarrheal recovery time when analysis was repeated after controlling for sex, current breastfeeding status, dehydration status (normal vs. mild-moderate dehydration), and nutritional status (normal vs. malnourished) of children (p=0.043).

No differences were found between groups in the absolute count of leukocytes and lymphocytes on admission. The absolute counts of CD4+, CD8+, CD3+, CD16+CD56+, and CD19+ cells were similar in the glu and plb groups on admission (Table II).

When adjusting for sex, current breastfeeding status, dehydration status (normal vs. mild-moderate dehydration), nutritional status (normal vs. malnourished) of children, the changes in absolute counts of leukocytes, lymphocytes, CD3+, CD4+, CD8+, CD16+CD56+, and CD19+ cells and CD4+/CD8+ ratio from day 0 to day 7 were similar in the glu- and plb- supplemented groups (Table II).

Table II. Absolute Counts of Leukocytes, Lymphocytes, Lymphocyte Subsets ($10^3/\mu l$) According to Time and Supplementation Group When Adjusting for Sex, Dehydration, Nutritional Status and Current Breastfeeding Status (Repeated Measures for ANOVA, mean±SEM)

Cells ($10^3/\mu l$)	Time	Glutamine group	Placebo group
Leukocytes	Day 0	12273±856	12383±819
	Day 7	12787±593	11512±567
Lymphocytes	Day 0	6120±514	6409±492
	Day 7	7013±437	6600±418
CD3+	Day 0	3805±339	4036±324
	Day 7	4625±288	4203±276
CD4+	Day 0	2284±223	2597±213
	Day 7	2745±193	2755±185
CD8+	Day 0	1862±158	1745±151
	Day 7	2270±156	1982±149
CD4+/CD8+	Day 0	1.32±0.09	1.56±0.08
	Day 7	1.26±0.07	1.50±0.07
CD19+	Day 0	1616±154	1633±147
	Day 7	1711±137	1705±131
CD16+CD56+	Day 0	628±79	715±76
	Day 7	680±82	656±78

p>0.05 for comparison between glu and plb groups.

Discussion

The results of the current study showed that the distributions of CD3+, CD4+, CD8+, CD19+, and CD16+CD56+ cells in blood did not differ with supplementation. As shown in the previous study, glu supplementation had a beneficial effect on diarrheal recovery in children with acute diarrhea. Glu (0.3 g/kg/d) administered in water seems to be orally and metabolically tolerated in children with acute diarrhea; however, glu supplementation did not affect the systemic interleukin (IL)-8 and secretory IgA responses[11]. In addition, in the current study, glu supplementation did not seem to enhance the blood lymphocyte subpopulation in children with acute diarrhea. It is possible that the absence of the effect of dietary glu on the lymphocyte subpopulation in the systemic circulation might reflect the use of glu in the gut, and oral glu might exert effects principally at the level of the gut-associated immune system. Enteral glu might be taken by rapidly dividing cells (enterocytes), could not appear, or could appear in a lesser amount in the systemic circulation, which in turn may be the reason for the absence of systemic immune response[14]. Similarly, glu supplementation was shown to improve the nitrogen balance and enhance lymphocyte subpopulation activity at the site of injury[3,15]. Lai et al.[15] demonstrated that preventive use of glu in rats with cecal ligation and puncture promoted proliferation of total lymphocytes in gut-associated lymphoid tissue and maintained T-lymphocyte populations in Peyer's patches. Gismondo et al.[3] also showed that orally administered glu to nude mice significantly increased intestinal CD3+, CD4+ and CD8+ lymphocytes when compared with the group without glu supplementation. Lopez-Pedrosa et al.[16] reported that healthy pigs had a total number of lymphocytes, B-cells (CD21+) and helper (CD4+) T-cells in Peyer's patches similar to the malnourished group supplemented with *N*-acetyl-glu; however, they were significantly higher than in other malnourished pigs that received calcium caseinate and glu as supplements. However, Yeh et al.[17] reported that oral glu supplementation also maintained total T-lymphocytes, and a glu-enriched diet before cecal ligation and puncture preserved blood CD4+ cells in rats with gut-derived sepsis. These controversial results might be due to the differences in the severity of the catabolic state[18]. Glu as a conditionally essential amino acid has been shown to enhance immune function in stressed, human and animal models. Acute mild-moderate diarrhea may have no immunosuppressive effect. The association of T-lymphocyte subsets and persistent diarrhea has been known[19]; in further studies, the immunomodulatory effect of glu supplementation can be studied in persistent diarrhea in which the catabolic state is much higher. Contrary to this hypothesis, Luo et al.[20] showed that alanyl-glu administration for eight days by enteral or parenteral routes did not appear to affect T-lymphocyte subset (CD3, CD4, CD8) number or gut barrier function compared to unsupplemented critically ill patients requiring enteral tube feeding in a double-blind, pilot clinical trial. As suggested by Luo et al.[20], the second blood analysis (day 7) might be done somewhat early, as adaptive immune functions such as changes in lymphocyte number likely take several weeks to develop. Furthermore, the observed effect of glu might be not mediated through a lymphocyte-associated mechanism. As a limitation, we only studied the number of blood lymphocyte subpopulations; however, lymphocyte functions could be analyzed in further studies. Whether or not glu supplementation enhances T-lymphocyte subset requires further investigation with a long follow-up period including local response and peripheral blood analysis.

In this study, patients with stool smear containing leukocytes (more than 5 cells in 1 high power field) and those with positive stool culture for salmonella or shigella were not included. As a limitation of this study, other diarrheal pathogens were not studied. However, we intended to detect the overall effect of glu in cases with acute diarrhea with a limited sample size (n=69). Additional studies might be done to detect the effect of glu supplementation in different diarrheal pathogens with a larger sample size.

In conclusion, glu supplementation did not show any effect on lymphocyte subpopulations in cases with acute diarrhea on the seventh day of glu supplementation. Further studies are necessary regarding gut-associated immunity in cases with acute diarrhea.

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