Glutamine Supplementation Enhances Mucosal Immunity in Rats With Gut-Derived Sepsis

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OBJECTIVE: Supplemental glutamine (Gln) has been demonstrated to improve the immunologic response and reduce mortality in rodents with sepsis. However, the effects of Gln on gut-associated lymphoid tissue function after infection and sepsis are not clear. We investigated the effects of Gln-supplemented diets before sepsis, Gln-enriched total parenteral nutrition (TPN) after sepsis, or both on the intestinal immunity in rats with gut-derived sepsis.

METHODS: Male Wistar rats were assigned to control and four experimental groups. The control and experimental groups 1 and 2 were fed a semi-purified diet; in experimental groups 3 and 4, part of the casein in the diets was replaced with Gln. After feeding rats the respective diets for 10 d, sepsis was induced by cecal ligation and puncture (CLP) in the experimental groups, whereas the control group underwent a sham operation; at the same time, the internal jugular vein of all rats was cannulated. All rats were maintained on TPN for 3 d. The control group and groups 1 and 3 were infused with conventional TPN, and groups 2 and 4 were given a TPN solution supplemented with Gln, which provided 25% of total amino acid nitrogen. All rats were killed 3 d after the sham operation or CLP. Intestinal immunoglobulin A levels, total lymphocyte yields, and lymphocyte subpopulations in Peyer’s patches were analyzed.

RESULTS: Total Peyer’s patch lymphocyte numbers were significantly higher in the Gln-supplemented groups than in the control group. Distributions of CD3⁺ and CD4⁺ in group 1 were significantly lower than those in the control group, whereas no differences were observed among the control and Gln-supplemented groups. Plasma immunoglobulin A levels were higher in the Gln-supplemented groups than the control group and group 1. Intestinal immunoglobulin A levels were significantly higher in groups 2 and 4 than in the control group and group 1.

CONCLUSIONS: Preventive use of a Gln-supplemented enteral diet before CLP or intravenous Gln supplementation after CLP have similar effects in promoting proliferation of total lymphocyte in gut-associated lymphoid tissue, enhancing IgA secretion, and maintaining T-lymphocyte populations in Peyer’s patches. Gln administered before and after CLP did not seem to have a synergistic effect on enhancing mucosal immunity in rats with gut-derived sepsis. Nutrition 2004;20:286–291. ©Elsevier Inc. 2004

KEY WORDS: glutamine, Peyer’s patches, immunoglobulin A, gut-derived sepsis

INTRODUCTION

Sepsis is a frequent clinical problem in patients undergoing major surgery. Under a condition of sepsis, bacterial toxins insult the body, and profound tissue breakdown and alterations in substrate metabolism may occur. The development of total parenteral nutrition (TPN) has afforded the means to provide adequate calories and protein to patients whose gastrointestinal tract is unusable. However, TPN has been shown to cause atrophy of the gut and impairment of gut-associated lymphoid tissue (GALT). Small intestinal GALT is a major contributor to primary immunologic protection at all mucosal sites. Peyer’s patches have attracted special attention, because the genesis of generalized mucosal immunity resides within Peyer’s patches in the GALT that process intraluminal antigens and stimulate naive B and T cells to those antigens. Studies have shown that B- and T-cell populations in Peyer’s patches are significantly reduced when TPN is administered. Also, intestinal interleukin (IL)–4 and IL-10 accompanied by immunoglobulin (Ig) A levels decreased in parenterally fed animals. Clinically, enteral nutrition is considered a better delivery route than parenteral feeding for critically ill patients. However, sepsis has been shown to reduce mesenteric blood flow and adversely affect the barrier and metabolic functions of the small intestine. Gardiner et al. suggested that under the condition of gut-derived sepsis, the parenteral rather than the enteral route has benefits for improving survival in rats.

Glutamine (Gln) is a semi-essential amino acid that has been shown to possess numerous useful physiologic properties. Inoue et al. found that bowel weight was greater and disaccharidase activity of the intestinal mucosa was higher in an intravenous Gln-treated group than in the control group in a peritonitis model. Nakamura et al. examined the effect of an intravenous Gln dipeptide...
on septic mortality and found that mortality was significantly lower in the Gln-TPN group than in animals receiving conventional TPN. The beneficial effects of Gln were thought to result from its protective effect on increasing intestinal blood flow and in maintaining a functioning bowel barrier. Human studies also have reported that the Gln-treated group experienced fewer clinical infections and shorter hospital stays. Some investigators have suggested that Gln may be useful in the treatment of established infections and shorter hospital stays. Some investigators have suggested that Gln may be useful in the treatment of established infections and shorter hospital stays. Some investigators have suggested that Gln may be useful in the treatment of established infections and shorter hospital stays. Some investigators have suggested that Gln may be useful in the treatment of established infections and shorter hospital stays. Some investigators have suggested that Gln may be useful in the treatment of established infections and shorter hospital stays.

**MATERIALS AND METHODS**

**Animals**

Male Wistar rats weighing 230 to 250 g were housed in stainless steel cages maintained in a temperature- and humidity-controlled room with a 12-h light, 12-h dark cycle. Animals were allowed free access to standard rat chow for 3 d before the experiment. All procedures conducted in this study were approved by the National Taiwan University Animal Care Committee.

**Surgical Procedure and Grouping**

Rats were divided into a control and four experimental groups. The control group and experimental groups 1 and 2 were fed a common semi-purified diet. Rats in experimental groups 3 and 4 were fed an identical diet except that part of the casein was replaced by Gln, which provided 25% of the total amino acid nitrogen (Table I).

### Table I.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>With Gln</th>
<th>Without Gln</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>165</td>
<td>220</td>
</tr>
<tr>
<td>Gln</td>
<td>45</td>
<td>—</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>34.4</td>
<td>34.4</td>
</tr>
<tr>
<td>Corn starch</td>
<td>667</td>
<td>657</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Vitamin*</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Salt mixture†</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Methyl-cellulose</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

* The vitamin mix contained the following (mg/g): thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; b-vitamin, 0.02; cyanocobalamin, 0.001; retinyl palmitate, 1.6; DL-α-tocopherol acetate, 20; cholecalciferol, 0.25; and menaquinone, 0.005.

† The salt mixture contained the following (mg/g): calcium phosphate diabasic, 500; sodium chloride, 74; potassium sulfate, 52; potassium citrate monohydrate, 220; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; and chromium potassium sulfate, 0.55.

Gln, glutamine

After feeding rats the respective diets for 10 d, sepsis in the experimental groups was induced by CLP, whereas sepsis in the control group was induced by a sham operation. CLP was performed according to the method of Wichterman et al. Briefly, rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and the abdomen was opened through a midline incision. The cecum was isolated, and a 3-0 silk ligature was placed around it, ligating the cecum just below the ileocecal valve. The cecum was then punctured twice with an 18-gauge needle and replaced back into the abdomen. The abdominal wound was closed in two layers. Immediately after the sham operation or CLP, all rats underwent placement of a catheter for TPN infusion. A silicon catheter (Dow Corning, Midland, MI, USA) was inserted into the right internal jugular vein. The distal end of the catheter was tunneled subcutaneously to the back of the neck and exited through a coiled spring, which was attached to a swivel allowing free mobility of animals inside their individual metabolic cages. Two milliliters per hour of TPN was administered on the first day. Full-strength TPN was given thereafter. The infusion speed was controlled by a Terufusion pump (model STC-503, Terumo, Tokyo, Japan). The TPN solution without fat was prepared in a laminar flow hood. Sterilized fat emulsions were added to the TPN solution daily just before use. The TPN solution was infused for the entire day at room temperature. All rats were maintained on TPN for 3 d. No enteral nutrition was administered during the period of TPN. The control group and experimental groups 1 and 3 were infused with conventional TPN. Experimental groups 2 and 4 were supplemented with Gln, which provided 25% of the total amino acid nitrogen in the TPN solution. TPN provided 280 kcal/kg of body weight, and the kilocalorie:nitrogen ratio was 120:1. The TPN solutions were isonitrogenous and identical in nutrient composition except for the difference in the amino acid content (Table II). There were five groups of rats in this study: control group, Gln not supplemented before or after the sham operation; group 1, Gln was not supplemented before or after CLP; group 2, a semi-purified diet was given before and Gln-containing TPN was given after CLP.
To determine the phenotypes of lymphocytes isolated from Peyer’s patches, 10^5 cells were suspended in 100 μL of Hank’s balanced salt solution containing fluorescein-conjugated mouse anti-rat CD3 (Serotec, Oxford, UK) and phycoerythrin-conjugated mouse anti-rat CD45Ra (Serotec, Oxford, UK) to distinguish T cells and B cells, respectively. After staining for 15 min, 1 mL of red blood cell-lysing buffer (Serotec) was added to lyse the red blood cells and to fix the stained lymphocytes. Fluorescence data were collected for 5 × 10^4 viable cells and analyzed by flow cytometry (Coulter, Miami, FL, USA).

**Lymphocyte Isolation From Peyer’s Patches**

On day 3 after the sham operation or CLP, all surviving rats were anesthetized and then killed by drawing arterial blood from the aorta of the abdomen. Blood samples were collected in tubes containing heparin and immediately centrifuged. Plasma amino acids were analyzed by standard ninhydrin technology (model 6300, Beckman Instruments, Palo Alto, CA, USA) after deproteination of the plasma with 5% salicylic acid.23

**Antibody Quantitative Analysis**

IgA was measured in plasma and intestinal washings. Collection of intestinal washings was modified from a previously described procedure.25 After excising Peyer’s patches, a 12-cm, small intestinal segment proximal to the cecum was removed; the intestinal fragment was washed intraluminally using 5 mL of phosphate buffered saline with 1% protease inhibitor (Sigma Chemical Co.). Debris was removed by centrifugation for 10 min at 3000 rpm to harvest the supernatant for analysis of intestinal IgA after an appropriate dilution. The plasma and intestinal IgA levels were determined by sandwich-type enzyme-linked immunosorbent assay, in which the anti-rat IgA capture antibody (clone A93-3; PharMingen, San Diego, CA, USA) was coated on the plate, and it was detected with the peroxidase-labeled antihuman IgA detecting antibody (clone A93-2; PharMingen). IgA in the intestinal washing was expressed as milligrams per milliliter.

**RESULTS**

**Body Weight and Plasma Gln Levels**

There were no differences in initial body weights, body weights after feeding the experimental diets for 10 d, or after TPN administration for 3 d among the five groups (data not shown). The Gln-supplemented groups (groups 2, 3, and 4) had significantly higher plasma Gln levels than did group 1, but showed no differences from groups 2 (−/+−) and 3 (+/−−; Figure 2A). The total Peyer’s patch lymphocyte yields were significantly higher in the Gln-supplemented groups than in the control group, whereas no difference was observed between the control group and group 1 (Figure 2B).

**Total Peyer’s Patch Lymphocyte Yields**

Total numbers of Peyer’s patches on the small intestine were significantly higher in group 4 (+/+/+) than in the control and group 1 (−/−−), and there were no differences from groups 2 (−/+−) and 3 (+/−−; Figure 2A). The total Peyer’s patch lymphocyte yields were significantly higher in the Gln-supplemented groups than in the control group, whereas no difference was observed between the control group and group 1 (Figure 2B).
There were no differences in the distributions of CD45Ra+/H11001 Lymphocyte Subpopulations

There were no differences in the distributions of CD45Ra+ and CD8+ cells in Peyer’s patches among the five groups. The distributions of CD3+ and CD4+ in group 1 (−−−) were significantly lower than those in the control group, whereas no differences were observed among the control and Gln-supplemented groups (Figure 3).

Plasma and Intestinal IgA Levels

Plasma IgA levels were significantly higher in the Gln-supplemented groups than in the control group and group 1. IgA levels in intestinal lavage fluid were significantly higher in groups 2 (−−−−) and 4 (−−−+) than in the control group and group 1 (−−−−; Figure 4).

In Vitro Cytokine Production

The IL-10 secreted by Peyer’s patch lymphocytes after stimulation were significantly lower in the experimental groups than the control group. IFN-γ levels did not differ across groups, whereas IL-2 and IL-4 levels were not detectable (Figure 5A,B).

DISCUSSION

Gln supplementation has been demonstrated to improve the immunologic response in studies done in vivo and in vitro. The clinical relevance of these effects has been documented in several animal and human experiments.26–30 In this study, 25% of total amino acid nitrogen was supplied by Gln, because this amount of Gln was found to enhance murine immune responses.29,30 We administered TPN for 3 d after CLP because, in a preliminary study, we found that the total numbers of Peyer’s patches on the serosal side of the intestine were much greater on day 3 than on any other day after CLP. In this study, we did not observe a survival benefit with Gln supplementation after CLP. Because survival was noted for only 3 d, determining whether Gln supplementation may improve survival over a longer period requires further investigation.

Peyer’s patches are specialized lymphoid aggregates within the gastrointestinal tract.6 Naive lymphocytes are sensitized within Peyer’s patches, proliferate within the mesenteric lymph nodes, and migrate via the thoracic duct to the lamina propria of various mucosal sites, where they produce secretory IgA.6 In this study, we found that the total Peyer’s patch lymphocyte yields were higher in Gln-supplemented groups than in the control group, whereas no difference was observed between the control group and group 1. This result indicates that Gln supplementation enhanced the proliferation of total lymphocyte number in GALT after CLP. To understand the effect of Gln on the distribution of total B lymphocytes (CD45Ra+), total T cells (CD3+), Th cells (CD4+), and cytotoxic T cells (CD8+) within Peyer’s patches, the subpopulations of lymphocytes were evaluated. The results showed that the distributions of B and cytotoxic T cells in Peyer’s patches did not differ across groups. However, the distributions of CD3+ and CD4+ in group 1 (−−−) were significantly lower than those in the control group, whereas no differences were observed between the
control and Gln-supplemented groups. This finding indicates that Gln supplementation before and/or after CLP helped maintain total T and Th lymphocyte populations in Peyer’s patches in septic condition. This result is similar to that of a report by Alverdy et al., in which addition of Gln to the standard TPN solution helped maintain levels of CD4+ cells in gut lamina propria similar to those of chow-fed animals. Secretory IgA is the principal effector of specific immunity against invading pathogens. In this study, we found that plasma and intestinal IgA levels were significantly higher in groups 2 (−/+ ), and 4 (+/+ ) than in the groups without Gln. Also, plasma IgA concentrations were higher in group 3 (+/+ ) than in the control group and group 1. These findings indicate that Gln supplementation before and/or after CLP enhances the secretion of IgA. Because there were no differences in Peyer’s patch lymphocyte yields, lymphocyte distribution, and IgA production among the Gln-supplemented groups, Gln administered before and after CLP seemed to have no synergistic effect on enhancing mucosal immunity.

Cytokines are peptides produced by cells of the immune system that act as mediators of the immune response and the response of tissues to injury. To understand the possible roles of cytokine secretions on intestinal immunity, IL-2, IFN-γ, IL-4, and IL-10 productions in mitogen-stimulated Peyer’s patch lymphocyte cultures were analyzed. IL-2 and IFN-γ are produced by Th1 lymphocytes. Th1 cytokines enhance cell-mediated immunity. A predominant Th1 effect results in activation of T lymphocytes. Th2 cytokines, including IL-4 and IL-10, enhance humoral immunity. A predominant Th2 effect results in activation of B lymphocytes and upregulation of antibody production. The effects of Th1 or Th2 lymphocytes are counter-regulatory.

that Gln-enriched TPN had a normalizing effect on intestinal IL-4 and IL-10 production, which as a consequence may preserve intestinal IgA levels. We analyzed in vitro cytokine production by Peyer’s patch lymphocytes and found that IL-2, IL-4, and IFN-γ were undetectable or that there were no differences among the five groups after mitogen stimulation. IL-10 production was significantly lower in the four experimental groups than in the control group, and no difference was observed across the experimental groups. In this study, we did not observe comparable changes between intestinal IgA and in vitro Peyer’s patch IL-4 or IL-10 production. It is possible that the peak time of action of IL-4 or IL-10 preceded the time we took the measurement, or perhaps these cytokines are bioactive at levels well below the range of detectability by current immunoassays. Quantitation of cytokine mRNA expression in the spleen and Peyer’s patches is now under investigation in our laboratory.

In conclusion, the results of this study have shown that preventive use of a Gln-supplemented enteral diet and Gln begun intravenously after CLP have similar effects in promoting proliferation of total lymphocyte in GALT, enhancing IgA secretion, and maintaining Peyer’s patch T-lymphocyte populations and plasma Gln levels in septic rats. Gln administered before and after CLP did not seem to have a synergistic effect on enhancing mucosal immunity in rats with gut-derived sepsis.

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