Effects of Glutamine Supplementation on Macrophage Phagocytic Activity and Inflammatory-related Cytokines in Mice with Gut-origin Sepsis

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Running title: Effect of glutamine on phagocytosis in sepsis

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Abstract

This study examined the effect of administering glutamine-supplemented diets before sepsis on the phagocytic activity of peritoneal macrophages and inflammatory-related cytokines in mice with gut-origin sepsis. Male BALB/c mice were assigned to 2 groups. One group was fed a control diet (control) in which all amino acids were provided by casein. The other group was fed a diet containing glutamine (Gln) in which Gln replaced 25% of the total nitrogen in casein. After feeding the experimental diets to mice for 4 weeks, sepsis was induced by cecal ligation and puncture (CLP). All mice were sacrificed 24 h after CLP to examine their immune responses. The results showed that anaerobic bacteria colony counts in peritoneal lavage fluid (PLF) were significantly reduced, and the phagocytic activity of peritoneal macrophages was enhanced in the Gln group compared to those of the control group. The concentrations of interleukin-1β and interleukin-6 in plasma and tumor necrosis factor-α levels in PLF were significantly lower in the Gln group than in the control group. These findings suggest that Gln supplementation before sepsis significantly enhanced peritoneal macrophage phagocytic activity and reduced total bacteria counts in PLF in septic mice. The production of inflammatory mediators systemically and at the site of injury was reduced after Gln administration, indicating that the prefeeding of a Gln-enriched diet may be beneficial in attenuating

the inflammatory reaction after sepsis.

Keywords: gut-origin sepsis; glutamine; phagocytic activity; cytokines

1. Introduction

Sepsis is a common clinical problem with extremely high mortality rates. When bacterial toxins insult the body, inflammatory mediators are oversecreted, which may result in an imbalance in metabolic pathways and consequently lead to multiple organ failure [1,2]. Numerous studies have focused on attenuation of the inflammtory reaction and bacterial insult after sepsis. In recent years, glutamine (Gln) has elicited great attention for its therapeutic use in treating diseases, because it has been demonstrated to have several desirable biological properties. Gln is the most abundant free amino acid in plasma and the tissue pool [3]. It has traditionally been thought of as a nonessential amino acid, but laboratory and clinical data suggest that it may be essential during certain inflammatory conditions, such as infection and injury [4,5]. A number of studies have demonstrated the beneficial effects of supplying Gln for metabolic-stressed conditions; these effects include increasing nitrogen retention, preserving the integrity of the intestinal mucosa and intestinal permeability, maintaining immunologic function, and reducing infections [4-8]. Gianotti et al. [9] demonstrated that oral Gln decreases bacterial translocation and improves survival in gut-origin sepsis. Parry-Billings et al. [5] reported that the depressed Gln concentrations were associated with reduced proliferation of lymphocytes from healthy volunteers and depressed phagocytosis by peritoneal macrophages from normal mice. Ogle et al. [10] also reported that Gln improved the bactericidal ability of abnormal neutrophils from pediatric patients after burns. An in vitro study by Wallace and Keast [11] showed that Gln is required for phagocytosis of opsonized sheep erythrocytes in macrophage culture. Furukawa et al. [12] revealed that supplemental Gln enhances phagocytosis by neutrophils from postoperative patients in vitro. Although Parry-Billings et al. [5] and Ogle et al. [10] suggested the efficacy of Gln supplementation, they did not supply Gln to their patients. In in vitro studies Gln was added to the culture medium, so the beneficial effect of Gln on phagocytosis observed in those studies may not reflect in vivo situations. To our knowledge, there has been no study, so far, investigating the effect of dietary Gln supplementation on macrophage phagocytic activity in sepsis. Therefore, the aim of this research was to study the effect of prefeeding a Gln-supplemented diet on peritoneal macrophage phagocytic activity and in vivo cytokine production in septic mice. In this study, we used cecal ligation and puncture (CLP) as a sepsis model, because CLP is very clinically relevant and is considered to be a simple and reproducible model of gut-origin sepsis in rodents [13].

2. Materials and Methods

2.1. Animals

BALB/c mice (4 weeks of age) weighing 10-15 g were used in this study. All mice were housed in temperature- and humidity-controlled rooms and were allowed free access to standard chow for 3 days prior to the experiment. The care of the animals used in this study followed standard experimental animal care procedures.

2.2. Study protocol

Thirty-five mice were randomly assigned to 2 groups, 17-18 mice to a group. One group was fed a control diet (control) in which all amino acids were provided by casein. The other group was fed a diet with glutamine (Gln) in which Gln replaced 25% of the total nitrogen in casein (Table 1). After feeding the experimental diets for 4 weeks, sepsis was induced by CLP according to the method of Wichterman et al. [13]. Briefly, mice were anesthetized with intraperitoneal pentobarbital (0.71 ug/g body weight), 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 a 25-gauge needle and was replaced back into the abdomen. The abdominal wound was closed in 2 layers.

2.3. Measurements and analytical procedures

Twenty-four hours after CLP, all mice were weighed and anesthetized. A middle abdominal incision was made, and 2 ml of phosphate-buffered saline (PBS) was intraperitoneally injected to elute the peritoneal cells. After harvesting the peritoneal lavage fluid (PLF), the mice were sacrificed by heart puncture. Fresh PLF was used for bacteriological analysis and macrophage phagocytic activity, and the remainder of the sample was stored at –80 ℃ until being assayed for cytokines. Blood samples were collected in tubes containing heparin and were centrifuged immediately. Plasma was stored at –80 ℃ until cytokine analysis.

2.3.1. Technique for quantitative bacteriologic culture

A total aerobic bacterial count was made by spreading 50 μl of whole blood or 10-fold serially diluted PLF on TSA blood agar plates (BBL® media, Becton Dickinson, Sparks, NJ) and incubating them at 37 ℃ overnight. A total anaerobic bacteria count was made by spreading 50 μl of the above samples on CDC blood agar plates (BBL® media) and incubating them under anaerobic conditions (GasPak System, Becton Dickinson Microbiology Systems) at 37 ℃ for 2 days. The total number of colonies formed on each plate was counted. The results were expressed as colony forming units per milliliter (CFU/mL) of blood or PLF.

2.3.2. Phagocytosis assay of peritoneal macrophages

A Vybrant ™ Phagocytosis Assay kit (Molecular Probes) was used to evaluate the phagocytic activity of peritoneal macrophages. After washing the peritoneal macrophages 3 times with HBSS, the cell concentration was counted, and the cell number was adjusted to 10^6 cells/ml with RPMI-1640 supplemented with 5% fetal bovine serum and an adequate amount of antibiotics. After distributing 100 μl of diluted solutions into each well on a 96-well microplate, it was transferred to a 37 ℃ CO_2 incubator for 1 h to allow the cells to adhere to the microplate surface. The RPMI solution was removed from all microplate wells by vacuum aspiration, and then 100 μl of prepared FITC-labeled *E. coli* was added to each well for 2 h. Labeled bacteria were removed by vacuum aspiration, and 100 μl of trypan blue suspension was added to all wells within 1 min. The excess trypan blue was immediately aspirated, and the experimental and control wells (without peritoneal macrophages) were read in a fluorescence plate reader using ~480 nm for excitation and ~520 nm for emission.

2.3.3. Measurement of cytokine concentrations

Concentrations of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in plasma and PLF were determined with a commercially available enzyme-linked immunosorbent assay in microtiter plates. Antibodies specific for mouse IL-1β, IL-6, and TNF-α were coated onto the wells of the microtiter strips provided (Amersham Pharmacia Biotech, Bukinghamshire, UK).

2.4. Statistical Analysis

Data are expressed as the mean ± SD. Differences between the 2 groups were analyzed by *t*-test. A p value < 0.05 was considered statistically significant.

3. Results

There were no differences in initial body weights (control 15.6 ±2.6 g vs. Gln 15.1 ±2.0 g, $p > 0.05$) or body weights after feeding the diets for 4 weeks (control 20.3 ±2.2 g vs. Gln 20.5 ±1.7 g, $p > 0.05$) between the experimental groups.

No significant difference in the cell numbers of peritoneal macrophages was observed between the 2 groups (control [6.25 ±2.44] x 10^6/mL vs. Gln [5.16 ±1.80] x 10^6/mL, $p > 0.05$). The numbers of anaerobic bacteria in PLF of the Gln group was significantly less than that of the control group. There was no significant difference in aerobic bacteria counts in PLF between the 2 groups (Table 2). The phagocytic activity was significantly higher in the Gln group than the control group (Fig 1).

Plasma concentrations of TNF-α were not detectable, whereas IL-1β, and IL-6 levels in the Gln group were significantly lower than those of the control group. There were no significant differences in PLF concentrations of IL-1β, and IL-6 between the 2 groups. However, TNF-α levels in PLF were significantly lower in the Gln group than in the control group (Table 3).

4. Discussion

In this study, 25% of total nitrogen in the diet was supplied by Gln. This amount of Gln was found to enhance the immune response in rodents [14,15]. We provided oral Gln supplementation before sepsis induction. This model mimics the septic complications in critically ill patients, in whom preventive use of a Gln-supplemented enteral diet may be recommended.

Previous reports have shown that parenterally or enterally administered Gln lowered the incidence of infection in patients with bone marrow transplantation [16] and multiple trauma [17]. Supplemental Gln improved the survival in experimentally *Escherichia coli*-induced peritonitis in rodents [9,18]. Nevertheless, the mechanisms underlying the enhancing effect of Gln on bactericidal capacity have not been fully elucidated. Gln is an important fuel for immune cells [5]. Macrophages use Gln at a very high rate [19]. In this study we found that the phagocytic activity of peritoneal macrophages was much higher, and anaerobic bacteria counts in PLF were significantly reduced in the Gln group compared to the control group. CLP causes peritoneal contamination with mixed bacteria flora, mostly anaerobic bacteria originating from enteric organisms. These findings indicate that Gln supplementation enhances the activity of peritoneal macrophages, and may consequently affect bacterial killing at the site of injury, possibly by enhancing the production of reactive oxygen intermediates within phagocytic vacuoles [12].

Surgical injury and infection stimulate the production of a variety of endogenous mediators. TNF-α, IL-1β, and IL-6 are major proinflammatory mediators present in septic conditions [20]. Although these cytokines have beneficial effects following injury, exaggerated or prolonged secretion of these proteins, however, is detrimental to the host [21]. Studies had shown that TNF-α is instrumental in triggering the cytokine response to severe bacteremia, and high plasma concentrations of IL-1 and TNF-α are associated with increased severity of inflammatory diseases [22]. Also, previous studies revealed that IL-6 was strongly associated with adverse events and outcomes after catabolic conditions, and is thought to be the most consistently identified cytokine mediator of postinjury complications [23]. In this study, TNF-α concentrations in plasma were not detectable at the time we took measurements. However, plasma IL-1β and IL-6 levels were significantly lower in the Gln group than in the control group. Also, the TNF-α concentrations in PLF were significantly lower after CLP in the Gln group than in the control group. This result may indicate that the inflammatory-related cytokines in systemic circulation and at the location of injurious stimulus can be reduced when Gln is administered before sepsis. An in vitro study by Rohde et al. [24] showed that Gln had no effect on the production of IL-1β, IL-6, or TNF-α. Also, O'Riordain et al. [25] revealed that Gln supplementation did not influence IL-2, IL-6, or TNF-α production in peripheral blood mononuclear cells of surgical patients. Our findings are inconsistent with those of the reports mentioned above. There are 2 possible explanations for this: 1) the result of in vitro studies may not actually reflect the in vivo situations; and 2) samples used for evaluating the effect of Gln on cytokine production were derived from healthy volunteers or patients with different disease conditions. This may differ from the stressed metabolic condition observed in this study, and may consequently lead to different immune responses.

In summary, this study shows that Gln supplementation before sepsis significantly enhances peritoneal macrophage phagocytic activity and bacterial clearance in mice with gut-origin sepsis. Also, the production of inflammatory-related mediators were reduced systemically and at the site of injury, indicating that prefeeding a Gln-enriched diet may be beneficial in attenuating the inflammatory reaction after sepsis.

Acknowledgements

This study was supported by research grant NSC 90-2815-C-038-001-B from the National Science Council, ROC.

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Figure legend

Fig. 1. Phagocytic activity of peritoneal macrophages. The phagocytic activity of the Gln group was significant higher than that of the control group ($p < 0.05$).

Table 1. Composition of the experimental diet (g/kg)

Ingredient	GLN group	Control group
Casein	150	200
Protein N	22.8	30.4
Glutamine	40	0
Total N	30.5	30.4
Corn starch	661	651
Soybean oil	35	35
DL-Methionine	3	3
Methyl cellulose	60	60
Mineral mix[a]	40	40
Vitamin mix[b]	10	10
Choline chloride	1	1

[a] The salt mixture contains the following (mg/g): calcium phosphate diabasic 500, sodium chloride 74, potassium sulphate 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 sulphate 0.55.

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

Table 2. Number of aerobic and anaerobic bacteria colonies in peritoneal lavage fluid after 100- and 1000-fold dilutions between the 2 groups.

	Aerobic		Anaerobic	
Dilution times	10^2	10^3	10^2	10^3
Gln	7.0 ± 8.6	2.0 ± 2.5	9.8 ± 12.4*	2.6 ± 5.1*
Control	16.5 ± 19.3	1.2 ± 2.2	243.0 ± 131.2	14.0 ± 6.5

Values are expressed as the mean ± SD.

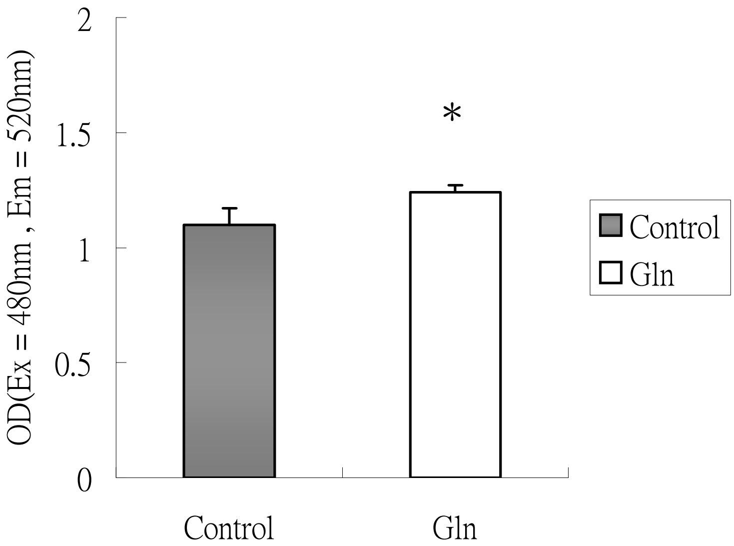
*Significantly different from the control group at $p < 0.05$.

Table 3. Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α concentrations in plasma and peritoneal lavage fluid (PLF) of the 2 groups

	GLN group	Control group
	(pg/mL)	
Plasma		
IL-1β	18.3 ±11.9*	28.2 ±20.5
IL-6	555.7 ±287.3*	945.8 ±535.7
TNF-α	ND	ND
PLF		
IL-1β	109.5 ±88.9	142.0 ±77.1
IL-6	2331.2 ±581.7	2301.6 ±501.7
TNF-α	18.5 ±11.0*	39.6 ±33.2

Values are the mean ±SD. ND: not detectable.

*Significantly different from the control group at $p < 0.05$


2
1.5
1
0.5
0
OD(Ex = 480nm , Em = 520nm)
*
Control
Gln
Control
Gln