Dietary Glutamine Supplementation Increases the Activity of Peritoneal Macrophages and Hemopoiesis in Early-Weaned Mice Inoculated with Mycobacterium bovis Bacillus Calmette-Gue´rin1,2

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Abstract

Infants who are breast-fed have been shown to have a lower incidence of certain infectious diseases compared with formula-fed infants. Glutamine is one of the most abundant amino acids found in maternal milk and it is essential for the function of immune system cells such as macrophages. The purpose of this study was to investigate the effect of glutamine supplementation on the function of peritoneal macrophages and on hemopoiesis in early-weaned mice inoculated with Mycobacterium bovis bacillus Calmette-Gue´rin (BCG). Mice were weaned at 14 d of age and distributed to 2 groups and fed either a glutamine-free diet (n = 16) or a glutamine-supplemented diet (+Gln) (n = 16). Both diets were isonitrogenous (with addition of a mixture of nonessential amino acids) and isocaloric. At d 21, 2 subgroups of mice (n = 16) were intraperitoneally injected with BCG and all mice were killed at d 28. Plasma, muscle and liver glutamine concentrations and muscle glutamine synthetase activity were not affected by diet or inoculation with BCG. The +Gln diet led to increased leukocyte and lymphocyte counts in the peripheral blood (P < 0.05) and granulocyte and lymphocyte counts in the bone marrow and spleen (P < 0.05). The +Gln diet increased spreading and adhesion capacities, hydrogen peroxide, nitric oxide, and tumor necrosis factor-α (TNF α) syntheses and the phagocytic and fungicidal activity of peritoneal macrophages (P < 0.05). The interaction between the +Gln diet and BCG inoculation increased the area under the curve of interleukin (IL)-1β and TNF α syntheses (P < 0.05). In conclusion, the intake of glutamine increases the function of peritoneal macrophages and hemopoiesis in early-weaned and BCG-inoculated mice. These data have important implications for the design of breast milk substitutes for human infants. J. Nutr. 138: 1343–1348, 2008.

Introduction

Immaturity of the neonatal immune system predisposes infants to the development of infection during the first year of life. Infants who are breast-fed have been shown to have a lower incidence of certain infectious diseases compared with formula-fed infants (1–5). Some mechanisms that might explain the active stimulation of the infant’s immune system with breastfeeding involve the bioactive factors in maternal milk, such as hormones, growth factors, and colony-stimulating factors, as well as specific nutrients, like glutamine (6,7). Glutamine and glutamate are the most abundant amino acids in proteins and in the free form in human milk, as well as in rat and mouse milk (8,9). Furthermore, the concentrations of free glutamine and glutamate in maternal milk increase ~20 and 2.5 times, respectively, with the progression of lactation in humans and represent >50% of the total free amino acids in human milk by mo 3 of lactation (10). In contrast to infants fed with maternal milk, early-weaned infants are deprived of this source of glutamine, which renders them exclusively dependent on endogenous synthesis of this amino acid or supplementation by means of commercially available formulas for babies (10).

Glutamine, traditionally considered a nonessential amino acid, now appears to be a conditionally essential nutrient during stress, injury, or illness (11,12). This amino acid is utilized at high rates by isolated cells of the immune system, such as macrophages, which are involved in innate immunity and whose...
abilities to phagocytose pathogens, kill fungi, and produce nitric oxide (NO), interleukin (IL)6, IL-6, tumor necrosis factor-α (TNFα), and reactive oxygen species are dependent on the extracellular concentration of glutamine (13–19). Furthermore, glutamine plays a key role in hematopoiesis, because it acts in the proliferation and differentiation of bone marrow cells (20–22). The objective of this study was to investigate the effect of glutamine supplementation on the function of peritoneal macrophages and on hemopoiesis in early-weaned mice inoculated with Mycobacterium bovis bacillus Calmette-Guérin (BCG).

Methods

Mice and treatments. Male Swiss Webster mice obtained from the Animal Laboratory of the Faculty of Pharmaceutical Sciences at the University of São Paulo were weaned at 14 d of age. The mice were kept in a room at an ambient temperature of 22 ± 2°C and a relative humidity of 55 ± 10% under a 12-h-light/12-h-dark cycle (lights on at 0700). The mice were weighed daily and we recorded their final mass before they were killed.

All mice were generated by mating 2 2-mo-old primiparous females with a single male. After pregnancy was confirmed, the females (n = 8) were maintained isolated in individual cages throughout gestation. After the birth of their offspring, 8 males were chosen and then maintained with their mother during the next 14 d. At the moment of weaning (at the age of 14 d), 32 infant mice, 4 from each female, were distributed into 2 groups: 16 of them were fed a glutamine-free diet (−Gln), while the other 16 were fed a glutamine-supplemented diet (+Gln). Mice of the −Gln (n = 16) and +Gln (n = 16) groups consumed water and a diet made specifically for infant mice (AIN-93G) (23) ad libitum from d 14–28 (Table 1). To ensure that the diet was glutamine free, casein was substituted with a mixture of amino acids (Ajinomoto Interamericana Indústria e Comércio) in similar quantities to those found in casein, but lacking glutamine (Table 2). The amount of nitrogen corresponding to the withdrawal of glutamine (3.33 g nitrogen/kg diet) was substituted with dispensable amino acids (alanine, aspartic acid, glycine, proline, and serine), which were added to produce equivalent quantities of nitrogen. Mice from the +Gln group were also fed a casein-free diet, which was supplemented with the addition of each one of the amino acids present in the casein, plus additional glutamine (40 g glutamine/kg diet).

On d 21 of age, 8 mice from the −Gln group and 8 mice from the +Gln group were intraperitoneally inoculated with 10⁷ viable units of BCG, Mycobacterium bovis cepa Moreau, provided by the Butantan Institute (São Paulo, SP, Brazil). Thus, the study was performed with 4 experimental groups: −Gln/−BCG (n = 8), −Gln/+BCG (n = 8), +Gln/−BCG (n = 8), and +Gln/+BCG (n = 8).

On d 28, the mice from the 4 groups were intraperitoneally anesthetized with ketamine hydrochloride (100 mg/kg body mass) associated with xylazine hydrochloride (50 mg/kg body mass) and then killed by cervical dislocation method. Blood, spleen, femur, liver, muscle tissue from the gastrocnemius, and peritoneal macrophages were collected for analyses. All of the mice were killed between 0800 and 1200. All procedures carried out on the mice were approved by the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences, University of São Paulo, according to the guidelines of the Brazilian College on Animal Experimentation.

Biochemical analyses. We measured the serum corticosterone concentration was measured with a commercially available radioimmunoassay kit (DSL, Diagnostic Systems Laboratories). The plasma glutamine concentration was measured according to the method described by Lund (24). Muscle glutamine was extracted as described by Sahlin et al. (25) and glutamine concentration was determined as described by Lund (24). The maximal activity of the enzyme glutamine synthetase in the gastrocnemius muscle was determined according to Minet et al. (26).

### Table 1 Composition of the diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>−Gln diet</th>
<th>+Gln diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constarch</td>
<td>562.4</td>
<td>562.1</td>
</tr>
<tr>
<td>Amino acid mixture</td>
<td>190.1</td>
<td>170.4</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>70.0</td>
<td>70.0</td>
</tr>
<tr>
<td>Fiber source (cellulose)</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Choline bitartrate (41.1% choline)</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>tert-Butylhydroquinone</td>
<td>0.014</td>
<td>0.014</td>
</tr>
</tbody>
</table>

1 Based on AIN-93G (23).
2 Both diets (−Gln and +Gln) are isocaloric (1674 kJ/100 g) and isonitrogenous (2.45 g nitrogen/100 g).
3 Due to the removal of casein from the formulation of the diet, the mineral mix was enriched with 180.68 g potassium phosphate/kg mix, which results in the supply of 3 g phosphorous/kg diet.
4 AIN-93G (23) mineral mixture.
5 AIN-93G (23) vitamin mixture.

Hematological parameters. Whole blood samples with EDTA (1 g/L, Sigma Chemical) were obtained via cardiac puncture. Total cells were quantified in a hemocytometer. Differential leukocyte counts were performed on blood smears stained by the standard May-Grünwald and Giemsa solutions (Sigma Chemical). The hematocrit levels and the concentrations of hemoglobin were determined according to Dacie and Lewis (27). Bone marrow cells were obtained by flushing the femoral cavity with McCoy’s 5A medium (Sigma Chemical). The spleen was removed, placed in petri dishes containing McCoy’s 5A medium with EDTA (1 g/L), and dissociated gently using needles and tweezers. Total cells were quantified using a Neubauer chamber and cytocentrifuge smears were stained with the standard May-Grünwald-Giemsa solutions. Differential cell counts were performed considering 500 cells per slide per mouse.

### Table 2 Amino acid concentrations of the diets

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>−Gln diet</th>
<th>+Gln diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>26.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Serine</td>
<td>14.7</td>
<td>3.1</td>
</tr>
<tr>
<td>NEAA-N²</td>
<td>11.52</td>
<td>11.52</td>
</tr>
<tr>
<td>Essential</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>Cysteine</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Leucine</td>
<td>15.4</td>
<td>15.4</td>
</tr>
<tr>
<td>Lysine</td>
<td>16.2</td>
<td>16.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>9.3</td>
<td>9.3</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Valine</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>NEAA-N:NEAA-N ratio</td>
<td>12.94</td>
<td>12.94</td>
</tr>
</tbody>
</table>

1 Based on the composition of amino acids present in the AIN-93G diet (23).
2 NEAA-N, Nonessential amino acid nitrogen; EAA-N, essential amino acid nitrogen.

6 Abbreviations used: BCG, bacillus Calmette-Guérin; −Gln, glutamine-free diet; +Gln, glutamine-supplemented diet; IL, interleukin; TNFα, tumor necrosis factor-α.
Bone marrow, n x 10^6/femur

Total cell count 240 ± 16 154 ± 14 263 ± 18 0.001
Granulocytes 18 ± 3 32 ± 4 12 ± 2 <0.001
Erythroblasts 12 ± 2 0.001
Lymphocytes 4 ± 0.3 0.001

Spleen, n x 10^8/spleen

Total cell count 74 ± 1 4.1 ± 0.3 0.001
Granulocytes 20 ± 1 0.001
Erythroblasts 12 ± 2 0.001
Lymphocytes 0.3 ± 0.01 0.001

1 Values are means ± SEM, n = 8. Means in a row with superscripts without a common letter differ, P < 0.05.

Statistical analysis. Initial body weight was expressed as means ± SEM and analyzed by 1-way ANOVA after checking the homogeneity of variances using a Bartlett test. Tukey's honestly significant difference test was applied to compare means. The effects of glutamine supplementation (diet) and inoculation with BCG were expressed as means followed by SEM and analyzed by factorial ANOVA (2^2) followed by Tukey honestly significant difference test. When the effects of 2 factors were taken throughout time, results were submitted to repeated-measures factorial ANOVA (diet x infection x time). Differences of P < 0.05 were considered significant. Calculations and graphics were performed using Statistica version 7.1 (StatSoft).

Results

Diet intake, body weight, and blood and tissue parameters. Daily diet intakes (3.2 ± 0.5 g) did not differ among groups. Despite the fact that the initial body weight (8.2 ± 0.7 g) did not differ among groups, BCG inoculation reduced (P < 0.001) final body weight (–BCG = 18.6 ± 0.4 g; +BCG = 16.0 ± 0.6 g). Serum corticosterone concentration; plasma, muscle, and liver glutamine concentrations; and muscle glutamine synthetase activity were not affected by either diet or BCG inoculation. The plasma glutamine concentration tended to be greater (P = 0.054) in the +Gln groups than in the –Gln groups but was not affected by BCG.

Increase in hemopoiesis induced by glutamine supplementation. The leucogram showed that the glutamine-supplemented diet increased leukocyte, lymphocyte, and neutrophil counts in peripheral blood, whereas BCG inoculation led to decreased
H$_2$O$_2$ and NO syntheses and spreading and adhesion capacities of peritoneal macrophage of 28-d-old mice weaned to −Gln or +Gln diets at 14 d of age and with (+BCG) or without (−BCG) BCG inoculation at 21 d.$^1$

<table>
<thead>
<tr>
<th>Variable</th>
<th>−Gln −BCG</th>
<th>−Gln + BCG</th>
<th>+Gln −BCG</th>
<th>+Gln + BCG</th>
<th>Diet</th>
<th>Infection</th>
<th>Diet × infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O$_2$ (basal), nmol H$_2$O$_2$-2 × 10$^6$ cells$^{-1}·$h$^{-1}$</td>
<td>3.3 ± 0.4</td>
<td>35.2 ± 2.0</td>
<td>4.4 ± 0.5</td>
<td>38.9 ± 1.8</td>
<td>0.005</td>
<td>&lt;0.001</td>
<td>0.076</td>
</tr>
<tr>
<td>H$_2$O$_2$ (PMA), nmol H$_2$O$_2$-2 × 10$^6$ cells$^{-1}·$h$^{-1}$</td>
<td>4.4 ± 0.4</td>
<td>53.8 ± 2.8</td>
<td>7.8 ± 1.0</td>
<td>82.0 ± 1.6</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.116</td>
</tr>
<tr>
<td>NO, nmol N0$_2$/mg protein</td>
<td>683 ± 82</td>
<td>2978 ± 269</td>
<td>778 ± 79</td>
<td>4062 ± 381</td>
<td>0.023</td>
<td>&lt;0.001</td>
<td>0.053</td>
</tr>
<tr>
<td>Spreading, %</td>
<td>34 ± 2</td>
<td>56 ± 3</td>
<td>42 ± 2</td>
<td>69 ± 3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.305</td>
</tr>
<tr>
<td>Adhesion, cells/mm$^2$</td>
<td>106 ± 5</td>
<td>92 ± 4</td>
<td>143 ± 15</td>
<td>134 ± 8</td>
<td>0.001</td>
<td>0.160</td>
<td>0.194</td>
</tr>
</tbody>
</table>

$^1$Values are means ± SEM, n = 8.

The BCG inoculation increased H$_2$O$_2$ and NO synthesis in the group (Table 3). The glutamine-supplemented diet as well as glutamine supplementation. The Increase in peritoneal macrophage function induced by group. The peritoneal macrophage count was higher peritoneal macrophage count than the group. The +Gln+/BCG group had increased granulocyte counts in the spleen compared with the −Gln+/BCG group, while the +Gln−/BCG group had higher granulocyte counts in the spleen than the −Gln−/BCG group.

**Increase in peritoneal macrophage function induced by glutamine supplementation.** The +Gln+/BCG group had a higher peritoneal macrophage count than the −Gln+/BCG group (Table 3). The glutamine-supplemented diet as well as BCG inoculation increased H$_2$O$_2$ and NO synthesis in the peritoneal macrophage culture (Table 4). The glutamine-supplemented diet increased spreading and adhesion capacities, as well as phagocytic (Fig. 1A) and fungicidal (Fig. 1B) activities of peritoneal macrophages. Glutamine supplementation had no effect on cytokine kinetics at each assessed time point (Fig. 2). The BCG inoculation decreased IL-6 and IL-10 synthesis, while increasing IL-1β and TNFα synthesis of lipopolysaccharide-stimulated peritoneal macrophages. The glutamine-supplemented diet caused an increase in the area under the curve of TNFα synthesis of peritoneal macrophages (P < 0.05). The area under the curves of IL-1α (P = 0.001) and TNFα (P = 0.032) synthesis were higher in the +Gln+/BCG group than in the −Gln+/BCG group.

**Discussion**

The period of growth and development between birth and weaning is crucial for the long term welfare of the body (33). Weaning, which is the transition period between the substitution of mother’s milk for other food, represents, for infants, the period with the highest risk of malnutrition, infections, and growth faltering (7,34). Glutamine, one of the most abundant amino acids in human milk (8–10), is also an essential nutrient for hemopoiesis (20–22) and macrophage function (13–19). In the present study, oral glutamine supplementation caused an increase in the function of peritoneal macrophages and hemoipoiesis in early-weaned mice inoculated with BCG. However, the effects of a glutamine-supplemented diet were not followed by significant changes in glutamine metabolism-related parameters, because plasma, muscle, and liver glutamine concentra-tions and maximal activity of the enzyme glutamine synthetase in the gastrocnemius muscle were not influenced by diet or BCG inoculation.

The plasma glutamine concentration tended to be greater (P = 0.054) in the +Gln groups than in the −Gln groups but was not affected by BCG. Enterally administered glutamine is
entirely metabolized in the splanchnic compartment and does not appear in the systemic circulation. Therefore, enterally administered glutamine will primarily affect the metabolism of the splanchnic tissue, in particular the gastrointestinal tract, its barrier function, and mucosal immunity (35). This fact may explain the possible effect of glutamine-supplemented diet on the plasma concentration of glutamine in the present study, because the presence of glutamine in the intestinal lumen causes reduced uptake of glutamine from the bloodstream by the enterocyte (36), which consequently favors an increased plasma concentration of this amino acid. This effect of glutamine supplementation by the diet may have important implications in studies involving neonates and children during acute stress and illness (35,37), given that in such situations there is a reduction in the plasma concentration of glutamine (38).

The glutamine-supplemented diet increased total nucleated cell, granulocyte, and lymphocyte counts in the bone marrow and spleen, a fact that influenced the total and differential leukocyte counts in the peripheral blood, which indicates that glutamine intake plays a key role in hematopoietic tissues of early-weaned mice. This result may be related to the fact that glutamine is essential to cell division processes, including hemopoiesis in the bone marrow, because that amino acid acts as a nitrogen donor for the biosynthesis of pyrimidines and purines (20,39,40).

In this study, the comparison between BCG-inoculated groups with regard to the functionality of peritoneal macrophages showed that the +Gln/+BCG group exhibited increased adhesion and spreading capacities, fungicidal activity, and NO, H2O2, TNFα, and IL-1β syntheses compared with the −Gln/+BCG group. These effects of glutamine supplementation are associated with metabolic alterations resulting from the BCG-induced macrophage activation process, which leads to increased glutamine metabolism, such as increased maximum glutaminase enzyme activity and glutamine consumption (41). Thus, the increased consumption of glutamine by BCG-activated macrophages suggests that the intake of glutamine through diet facilitated the activation of glutaminolysis in macrophages and, consequently, increased macrophage functional capacity in the +Gln/+BCG group compared with the −Gln/+BCG group.

Similar to the results obtained in the present study concerning cytokine synthesis, other studies have demonstrated that in vivo dietary supplementation with glutamine can increase TNFα and IL-1 synthesis by thioglycollate-elicited peritoneal macrophages from mice (42) or reverse the diminished TNFα synthesis by macrophages obtained from rats fed a low-protein diet (43).

Despite the absence of significant differences in the ability to phagocytize Candida albicans, peritoneal macrophages from the +Gln/+BCG group exhibited higher fungicidal activity toward this pathogen than those from the −Gln/+BCG group. That result may be explained by the increased TNFα, H2O2, and NO syntheses from peritoneal macrophages from the +Gln/+BCG group compared with those from the −Gln/+BCG group, because these factors are crucial for killing the intracellular pathogens (44,45). The increased NO synthesis from peritoneal macrophages induced by the intake of glutamine through diet could be related to 2 mechanisms. First, stimulated macrophages would have increased arginase secretion, thus decreasing the extracellular concentration of arginine and increasing the demand for intracellular glutamine. Thus, glutamine would have acted as a precursor of arginine, because macrophages possess enzymes for the conversion of glutamine to citrulline and citrulline to arginine (46). Second, the activity of NO synthase in macrophages is dependent on NADPH and the metabolism of glutamine might have supplied NADPH by means of a catabolic pathway involving NADP+-dependent malate dehydrogenase (glutamine → glutamate → α-ketoglutarate → malate → pyruvate) (19).

Glutamine, traditionally considered a nonessential amino acid, now appears to be a conditionally essential nutrient during stress, injury, or illness (12). These situations are associated with an increased susceptibility to infections and it has been suggested that this may be due in part to a diminished supply of glutamine to immunocompetent cells, such as macrophages (11,19,45). As a result, there is great interest in supplying glutamine to subjects in stress situations. Within this context, the present study showed that the intake of glutamine through diet increases the function of peritoneal macrophages in early-weaned and BCG-inoculated mice, which suggests that this amino acid may also be
conditionally indispensable in the first months of life of early-weaned children with a clinical picture of infectious diseases.

In summary, this study has shown that the function of macrophages and hemopoiesis is increased by the intake of a glutamine-supplemented diet in early-weaned and BCG-inoculated mice. Thus, other studies are necessary to characterize the possible benefit of glutamine in feeding formulas for early-weaned infants to increase hematopoietic and immune responses during this phase of life.

**Literature Cited**


