Glutamine decreases lipopolysaccharide-induced intestinal inflammation in infant rats

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Submitted 21 November 2003; accepted in final form 8 January 2004

Glutamine decreases lipopolysaccharide-induced intestinal inflammation in infant rats. Am J Physiol Gastrointest Liver Physiol 286: G914–G921, 2004. First published January 15, 2004; 10.1152/ajpgi.00493.2003.—Using a gastrostomy-fed (GF) rat infant “pup-in-a-cup” model, the effects of protein deprivation and supplemental glutamine (Gln) and glutamate (Glu) were examined to test the hypothesis that Gln decreases the proinflammatory response induced by LPS in the developing infant rat small intestine. Four groups of 6- to 7-day-old pups were fed a rat milk substitute (RMS), one providing 100% and three providing 25% normal protein intake for another 6 days. Two of the 25% protein-fed groups received supplemental Gln or Glu. GF and LPS treatment blunted body growth and intestinal villus height and increased intestinal cytokine-induced neutrophil chemoattractant (CINC) mRNA in the protein-deprived, non-Gln-treated group compared with mother-fed pups (P < 0.05). Gln blunted intestinal CINC mRNA (P < 0.05), but Glu did not. Intestinal CINC peptide in the LPS-treated pups provided 100 and 25% protein was elevated ~13-fold compared with the mother-reared pups (P < 0.001). Gln and Glu decreased intestinal CINC peptide by 73 and 80%, respectively. GF, LPS-treated pups also had a higher level of plasma CINC peptide (P < 0.05). Gln but not Glu decreased plasma CINC peptide (P < 0.05). An approximate sixfold elevation of intestinal MPO activity in the GF, LPS-treated rats was decreased by Gln and Glu by 92% (P < 0.001) and 54% (P < 0.05), respectively. Intestinal and plasma TNF-α were increased in GF, LPS-treated pups (P < 0.01), and Gln and Glu both blunted this increase (P < 0.05) in the intestine but not in the plasma. The results indicate that Gln decreases the LPS-induced inflammatory response in infant rat intestine under different conditions of protein intake. nutrition; proinflammatory response

METHODS

Animal model. The University of Florida Institutional Animal Care and Use Committee approved the following study.

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OVER THE PAST DECADE, several studies in critically ill infants and adults have demonstrated efficacy and safety of glutamine (Gln) supplementation (16, 23, 28, 30, 35, 36, 54, 56). In adult trauma patients, Gln supplementation resulted in decreased pneumonia and sepsis and prevented the elevation of proinflammatory cytokines involved in the systemic inflammatory response syndrome (23). In sick premature infants, there is a strong theoretical rationale for supplementation with Gln. When born prematurely, they are suddenly deprived of the abundant supply of Gln derived in utero from their mothers and the placenta (4). Furthermore, these infants are highly stressed and thus are likely to have an increased utilization of Gln during their first several weeks of life. Intravenous solutions used for infants do not supply Gln, and the clinician, because of feeding intolerance and concerns about necrotizing enterocolitis, generally limits enteral feedings that contain Gln as a component of the protein matrix.

The proinflammatory response in the form of a cytokine cascade is recognized as a critical component of several pathological entities including sepsis syndrome, lung injury, necrotizing enterocolitis, premature labor, inflammatory bowel disease, and central nervous system damage (7, 10–13, 29, 32, 33, 44, 49, 51, 55). A critical component of this process, the migration of polymorphonuclear leukocytes into tissues containing high concentrations of chemoattractant chemokines and cytokines, is a hallmark of these inflammatory conditions. IL-8 is a chemokine that recruits polymorphonuclear leukocytes, which, in turn, mediate tissue destruction via MPO. This process has been implicated in the pathogenesis of various forms of tissue injury (2, 8). In the rodent, cytokine-induced neutrophil chemoattractant (CINC), which has a similar function to human IL-8 (18), released after NF-κB pathway activation, has been suggested as a major early signal in the enterotoxic cascade and is implicated in the pathogenesis of distal organ damage originating from the intestine (26). The interaction of inflammatory mediators and their signaling pathways with immunonutrients such as Gln is poorly defined in developing animals. Studies using intestinal cell cultures support that Gln deprivation can severely exacerbate the intestinal inflammatory response in the form of increased IL-8 production in human intestinal epithelial cells (25). Studies in human volunteers have shown that Gln decreases IL-6 and -8 in the intestine (8, 9). The likely combination of Gln fortifying the intestinal mucosa barrier and preventing propagation of the proinflammatory injury to target organs (intestine, lung, liver, and brain) forms the foundation for the proposed hypothesis: Gln supplementation via the enteral route can downregulate proinflammatory cytokine production by the small intestine, thereby decreasing intestinal and distal organ injury. Here, we used a rat infant feeding model provided with different protein intakes to evaluate whether Gln can ameliorate the effects of LPS-induced inflammation on intestinal morphology, enzymes involved with absorption of nutrients and differentiation, the proinflammatory mediators CINC and TNF-α in the intestine, and plasma and MPO activity in the small intestine.

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In two separate experiments, Sprague-Dawley (Taconic, Germantown, NY) infant rats were randomly assigned to four gastrostomy feeding groups with five rats per group. Three mother-reared rats of the same age were used as reference controls. Gastrostomy feeding using the rat infant “pup-in-the-cup” model began on day 7 of life through feeding tubes constructed from 14-cm sections of polyethylene tubing that were inserted into the stomach, as previously described (17, 39). This is a commonly used model in studies of developmental nutrition when it is important to manipulate nutritional composition in the absence of maternal feedings. The gastrostomy placement was done under isoflurane anesthesia. Timer-controlled syringe pumps were connected to the feeding tubes and were set to feed the rats for the first 20 min of every hour at a weight-dependent flow rate. After a 1- to 2-day acclimation period during which they were fed with rat milk substitute (RMS) (3), the protein component was decreased to 25% of the usual quantity provided (30–40 g kg⁻¹ day⁻¹), the quantity required for normal growth) (3). One of the protein-deprived groups was given a supplement of Gln at 3.5 g kg⁻¹ day⁻¹, and another group was given 3.5 g kg⁻¹ day⁻¹ of glutamate (Glu). This dose of Gln was chosen because Gln comprises ~10% of the milk protein matrix and would be approximately the amount of Gln the animal would be receiving with a full protein diet. All the gastrostomy-fed groups received the same quantity of fat and carbohydrates. The protein-deprived rats were partially deprived of mimetic nutritional conditions in the Neonatal Intensive Care Unit, where very low body weight (VLBW) infants are frequently not enterally fed for days to weeks and have their total parenteral nutrition (TPN) amino acids protocol slowly advanced over the first 1–2 wk of life. LPS from Escherichia coli 0127:B8 (LPS; Sigma, St. Louis, MO) was dissolved in water by vortexing at a concentration of 2 μg/ml. All of these rats were given 0.25 mg/kg day⁻¹ of LPS via the gastrostomy tube starting 2 days after the initiation of artificial feeding. This dose was determined in our pilot studies to result in occasional shivering, piloerection, and poor weight gain but was not associated with a significant increase in mortality over a 6-day period. At the end of the 6-day treatment period, the rat pups were euthanized with an overdose of pentobarbital sodium. The small intestine samples were homogenized on ice in 0.01 M KH₂PO₄ buffer. After centrifugation at 10,000 rpm for 20 min at 4°C, the pellets were resuspended by sonication in cetyltrimethylammonium bromide buffer (13.7 mM CTAB, 50 mM KH₂PO₄, and 50 mM acetic acid, pH 6.0). The supernatant was kept for ELISA analysis. The suspension was centrifuged again at 10,000 rpm for 15 min. The supernatant was incubated in a 60°C water bath for 2 h. MPO concentration of the supernatant was measured by the H₂O₂-dependent oxidation of tetramethylbenzidine. Absorbance was determined at 650 nm, and concentration was calculated using the equation derived from a linear standard curve.

**RESULTS**

**Growth.** To compare the growth rate among the dietary groups, pups’ body weights were measured every day in the morning. Growth curves were made as percentage increases from the beginning of the study.

**Intestinal morphology.** We focused the microscopy studies on the ileum because this is a region that is most highly susceptible to certain pathology in VLBW infants (e.g., necrotizing enterocolitis and non-necrotizing enterocolitis-related perforations). Furthermore, pilot evaluations of the duodenum and jejunum did not suggest major differences. Formalin-fixed ileum samples were embedded in paraffin; 6-μm sections were cut using a 2030 Reichert-Jung paraffin microtome. The sections were then stained with a routine hematoxylin and eosin (H&E) stain. Villus height and width were measured using a Nikon microscope (Universal Imaging, Westchester, PA) with an ocular micrometer without the examiner knowing the group assignment. Villus height was calculated by first measuring from the tip of the villus to the base of the tunica muscularis. Next, measurements were taken from the base of the villus to the base of the tunica muscularis on both sides of the villus; these values were then averaged. The averaged value from the bottom of the villus to the base of the tunica muscularis was then subtracted from the first measurement, therefore giving the true villus height. Approximately 5–10 villi were measured for each stained H&E section; each rat had three to four stained sections (15–40 villi/rat). Villus height and corresponding villus width data were combined to analyze villus height-to-width ratios.

**Dissaccharidase activity assays.** Harvested distal small intestinal tissues were homogenized in homogenate buffer (0.01 M NaH₂PO₄/Na₂HPO₄, pH 6.0, and 0.002% Triton X-100). Maltase, lactase, and sucrase activities were determined at 37°C in 250 μl reaction volume in 50 mM sodium phosphate buffer (pH 6) using 0.0156 M maltose, 0.188 M lactose, or 0.04 M sucrose as the substrates, respectively. Specific activity was expressed as total enzyme activity per milligram of protein per hour. Protein was measured using the BioRad DC Protein Assay (BioRad, Hercules, CA).

**RNA isolation and RT-PCR for CINC.** Total RNA small intestine was isolated using the TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer’s recommendation. Total RNA was reversed into cDNA using a SuperScript First-Strand Synthesis System kit (Invitrogen Life Technologies). PCR amplification was performed in a total volume of 50 μl including Taq DNA polymerase and specific primers. After initial incubation at 94°C for 5 min, PCR was performed with 30 cycles consisting of denaturation (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 1.5 min), followed by an extension at 72°C, 10 min (using DNA Thermal Cycle 480). The oligonucleotide primers used were as follows (24) (from GenoMechanix, Gainesville, FL): CINC forward: 5'-CTC CAG CAC TCC AAC AGA-3', CINC reverse: 5'-CAC CCT AAC ACA AAA AAC CAC GAT-3' (670 bp); and β-actin forward: 5'-ATG CCA TCC TTC GTC TGG ACC TGG C-3', β-actin reverse: 5'-AGC ATT TGC CCT GCA CGATGG AGG G-3' (607 bp). β-actin was used as an internal control.

**ELISA for CINC and TNF-α.** Small intestine and plasma CINC and TNF-α were determined by TiterZyme Enzyme Immunoassay kits for rat growth-related oncogene/CINC and TNF-α (Assay Designs, Ann Arbor, MI), respectively. Absorbance was determined at 450 nm, and concentration was calculated using the equation derived from a linear standard curve.

**MPO assay.** Intestinal total MPO activity, a measure of neutrophil accumulation and a marker of tissue injury, was determined by a standard enzymatic procedure as previously described (45). Briefly, intestine samples were homogenized on ice in 0.01 M KH₂PO₄ buffer. After centrifugation at 10,000 g for 20 min at 4°C, the pellets were resuspended by sonication in cetyltrimethylammonium bromide buffer (13.7 mM CTAB, 50 mM KH₂PO₄, and 50 mM acetic acid, pH 6.0). The supernatant was kept for ELISA analysis. The suspension was centrifuged again at 10,000 g for 15 min. The supernatant was then incubated in a 60°C water bath for 2 h. MPO concentration of the supernatant was measured by the H₂O₂-dependent oxidation of tetramethylbenzidine. Absorbance was determined at 650 nm and compared with a linear standard curve. Protein was measured using the BioRad DC Protein Assay (BioRad).

**Statistical analysis.** Sigmatostat statistical software (SPSS, Chicago, IL) was used to analyze body weight, villus measurements, enzyme activities, MPO, ELISA for CINC, and TNF-α and densitometry results for RT-PCR. All data were reported as means ± SD. A one-way ANOVA was used to determine whether a significant difference was present among all treatment groups. Additionally, Bonferroni t-tests were performed for pairwise comparisons when the ANOVA was significant at P < 0.05.
protein over the same time period, but no LPS, showed comparable growth with that of mother-reared rats (data not shown). This demonstrated that LPS at the dose used in these experiments causes growth failure in infant rats that cannot be reversed by supplementation with Gln or Glu.

**Intestinal morphology.** To compare intestinal morphology among the dietary groups, small intestinal villus morphology was evaluated using light microscopy (Fig. 2). The proximal, middle, and distal small intestinal villus lengths, widths, area, intervillus distances, and crypt depths did not differ significantly within the gastrostomy-fed groups. Villus lengths from the all gastrostomy-fed groups were, however, ~20% smaller than mother-reared controls killed at the same age (data not shown). Comparisons of ileal morphology in Fig. 2 suggest that the groups fed 25% protein had greater distortion of overall villus architecture than the other groups.

**Intestinal enzymes.** To further determine the effects of diet on enzymes involved with absorption of nutrients and to make standard baseline comparisons of differentiation status between the mother-reared and gastrostomy-fed rats, lactase, maltase, and sucrase activity assays were performed on the distal region of the small intestine. As shown in Fig. 3, lactase and maltase activities did not differ in the small intestine (Fig. 3, A and B). However, sucrase activity was barely detectable in the mother-fed groups and was significantly greater in the artificially reared animals receiving LPS (Fig. 3C; \( P < 0.01 \) vs. 100% protein group).

**CINC and MPO.** To clarify the effects of diets on the CINC mRNA in small intestine, RT-PCR was performed. Figure 4A shows that protein deprivation and LPS (25% protein-only group) increased CINC mRNA expression. Meanwhile, Gln supplementation decreased CINC mRNA to a level similar to mother-fed animals. Densitometry analysis using β-actin as an internal control shows that the 25% protein-fed rat intestine had a significantly higher level of CINC mRNA compared with mother-fed pups (\( P < 0.05 \)), and Gln reversed this effect (\( P < 0.01 \)).
However, Glu did not show the same effect as Gln.

To further investigate the effects of the diets on CINC peptide, CINC production was evaluated by ELISA in both the small intestine and plasma. As shown in Fig. 5, intestinal CINC levels in pups fed 100 and 25% protein increased by 13-fold compared with mother-reared pups (both \( P < 0.001 \)). Gln and Glu decreased intestinal CINC significantly (Fig. 5A) compared with the other two gastrostomy-fed groups. Despite the Gln- and Glu-mediated decreases in intestinal and plasma CINC, neither Gln nor Glu supplementation caused a decrease in plasma CINC (Fig. 5B).

**DISCUSSION**

These results show a blunting of both somatic and intestinal growth in gastrostomy-fed infant rat pups treated with LPS compared with mother-reared controls. The group provided 100% of the protein they would normally receive from their mothers also had very poor growth, and their intestinal morphometrics were similar to those of the LPS-treated animals that were administered the lower quantity of protein with or without the Gln or Glu. Other studies in our laboratory have demonstrated that intestinal morphometrics in gastrostomy-fed rat pups not treated with LPS are comparable with those of mother-reared pups at comparable ages. Thus LPS at the dose used in this study results in marked growth retardation, and growth LPS-mediated retardation could not be rescued by using higher quantities of protein or supplementing protein-deprived rats with Gln or Glu. Intestinal morphology was subjectively most distorted at the lowest protein intake despite MPO activity in 100 and 25% protein groups that also received LPS (\( P < 0.001 \)). Gln and Glu supplementation decreased intestinal MPO activities by 92% (\( P < 0.001 \)) and 54% (\( P < 0.05 \)) in these groups, respectively.

**Intestinal and plasma TNF-\(\alpha\) peptide.** The dietary effects on the intestinal and plasma TNF-\(\alpha\) peptide concentrations were determined by ELISA. As shown in Fig. 7, intestinal TNF-\(\alpha\) increased in gastrostomy-fed, LPS-treated pups (\( P < 0.01 \)). Gln and Glu decreased intestinal TNF-\(\alpha\) significantly (Fig. 7A) compared with the other two gastrostomy-fed groups. Despite the Gln- and Glu-mediated decreases in intestinal and plasma TNF-\(\alpha\) in gastrostomy-fed, LPS-treated pups (\( P < 0.05 \)), neither Gln nor Glu supplementation caused a decrease in plasma TNF-\(\alpha\) (Fig. 7B).
not showing difference in morphometrics among the gastrostomy-fed, LPS-treated groups.

Protein intake is critical for intestinal mucosal growth. Burrin et al. (6) have shown that at least 40% of total calories need to be taken by the enteral route to demonstrate increased mucosal growth. However, smaller amounts have been found to have beneficial effects on the intestine, including a modulation of inflammation that can affect distal organs (27). Of interest in the current study is the fact that neither Gln nor Glu provided an improvement in growth or intestinal mucosal growth. In this study, standard techniques to determine intestinal morphology were employed. Newer stereological methods are becoming available that might offer increased sensitivity of measurement. Numerous previous studies (15, 46, 47) in stressed animals undergoing chemotherapy, radiotherapy, or TPN have demonstrated that Gln supplementation results in increased intestinal growth, adaptation, or at least the prevention of atrophy. A previous study from our laboratory (39), wherein an amino acid formulation not containing Gln or Glu was the only source of amino acids and wherein endogenous Gln synthesis was also inhibited with methionine sulfoximine, supplementation with Gln resulted in improved growth of the small intestinal villi. It is possible that the gastrostomy-fed, LPS-treated rat pups used in the current studies were so catabolic secondary to the LPS treatment that providing the "100%" protein diet was not enough to counteract the catabolic effect of the LPS-induced stress.

Disaccharidases are commonly used as markers of intestinal epithelial cell differentiation (19–22). In this study, small intestinal disaccharidases were measured to have a commonly measured comparative reference between mother-reared and gastrostomy-fed infant rats, whereas they are undergoing a rapid period of differentiation. Sucrase activity was higher in all the gastrostomy-fed rat pups compared with the mother-reared controls, but none of the enzymes demonstrated different activities in the four groups of gastrostomy-fed rats. Intestinal explants from mouse embryos have been demonstrated to respond to Gln by increasing formation of villi, initiating absorptive cell differentiation, and increasing DNA synthesis and the number of epithelial cells (5). Our previous studies in cell cultures have demonstrated that Gln deprivation inhibits differentiation (52). Here, we see no dependency of these differentiation markers on protein, Gln, or Glu intake. The sucrase activity was higher in the gastrostomy-fed animals that were also given LPS compared with the mother-reared controls ($P < 0.001$). We speculate that this probably reflects stress, which is known to increase sucrase activity in preweaned rat pups.
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animals, in which the activity would be almost negligible under baseline mother-reared conditions.

LPS had a marked effect on CINC mRNA and peptide in the intestine, and this was also reflected in the plasma (Figs. 3 and 4). CINC was markedly decreased with Gln supplementation to a level comparable with that seen in comparable aged mother-reared rat pups. The blunting of intestinal MPO activity with Gln and Glu correlated with the blunting of CINC peptide. Both Gln and Glu supplementation in the intestine altered TNF-α, but this was not reflected in the plasma.

In this study, we did not evaluate the relative contribution of different intestinal cell types to the inflammatory process. Several different epithelial cell types exist, and all appear to play some role in the inflammatory process. For example, it has been reported that Paneth cells function as specialized defense cells in the small intestine (38). They also are important in vasculogenesis in the intestinal villi and modulating effect of gut microbiota (48). Paneth cells are also specialized intestinal epithelia that store antimicrobial peptides that may be released on exposure to LPS (40). It is possible that LPS mediates the release of TNF-α or other injurious or growth inhibitory substance from Paneth cells.

A recently completed multicenter trial of enteral Gln supplementation in 649 VLBW infants showed that there was not a difference in hospital-acquired bacteria culture positive sepsis, the primary outcome (50). However, evaluation of secondary outcomes demonstrated improved gastrointestinal function and, surprisingly, a significant decrease ($P = 0.01$) in grades 3 and 4 intraventricular hemorrhages or periventricular leukomalcia in survivors. Such outcomes are related to significant delays in neurodevelopment (51). The mechanism of these results remain unclear but may relate to the hypothesized susceptibility of the immature intestine to stimulation of the inflammatory response, with propagation of this response to distal organs such as the brain and its modulation via Gln administered to the lumen of the gastrointestinal tract.

Recent studies (27) have demonstrated that the route of nutritional supply can influence local and systemic inflammatory responses to intraperitoneal bacterial challenge. The survival rate of rats was significantly higher (60 vs. 22%) when rats received enteral nutrition instead of parenteral nutrition. Rats fed with enteral nutrition had lowered TNF-α levels in their bronchoalveolar lavage fluid, indicating a major effect of enteral nutrition on a distal organ. The role of Gln in the inflammatory response in the intestine has been suggested by several studies. One particularly intriguing study by Houdijk et al. (23), reported marked decreases in several types of infection including sepsis and pneumonia in adult trauma patients who were supplemented with Gln. Concurrent with decreased sepsis was a significant decrease in the plasma concentrations of soluble TNF receptors p55 and p75. These are now recognized as compounds that are detected very early in sepsis in both neonates and adults and correlate with subsequent outcome (33). Another recent study in colorectal cancer patients (2) demonstrated that oral Gln supplementation was effective in preventing mesenteric blood mononuclear cell activation and excessive production of cytokines. Gln has also been demonstrated to reduce IL-6 and -8 production in the intestines of healthy volunteers (8, 9). Furthermore, in an adult rat model of endotoxemia, Gln supplementation not only reduced cytokine release from the intestine, it also diminished distal organ damage and mortality (53). Similar to our proposed mechanisms for Gln action in VLBW infants (34), these authors speculated that the decrease in these proinflammatory cytokines was secondary to a blunted proinflammatory response from the gastrointestinal tract.

IL-8 is a cytokine/chemokine that recruits polymorphonuclear neutrophils, which, in turn, mediate tissue destruction and have been implicated in the pathogenesis of various forms of intestinal injury. Furthermore, lung injury and other distal pathology have been associated with increased IL-8 secretion from the intestine (14, 31). The interaction of these pathways with immunonutrients such as Gln is poorly defined in developing animals. This is important because several pathological entities such as chronic lung disease, necrotizing enterocolitis, cerebral palsy, and sepsis have been associated with elevated plasma cytokines (33). There are likely to be other cytokines and chemokines involved in the glutamine modulation of inflammation. Anti-inflammatory cytokines such as IL-4 and -10 have been implicated in control of intestinal inflammation. The effects of glutamine on IL-4 and -10 as well as other cytokine and chemokine secretion will need to be evaluated in subsequent studies.

There are very few in vivo studies that have compared the relative roles of Gln vs. Glu in the small intestine, and the need for such studies has previously been stated (1). However, the capability for metabolic Gln-Glu interconversion and synthesis to closely related functionally relevant metabolites may be a moot point when one considers other functions in which GLN is involved. In addition to its role as a nitrogen donor for the synthesis of purines and pyrimidines, roles recently ascribed to Gln include signaling processes (41–43) and a role in intercellular junction integrity (37). Thus it was important to determine whether Glu may play a role similar to that of Gln in modulation of intestinal inflammation. The results of this study suggest that overall Glu does downregulate the intestinal inflammation but not to the same degree as Gln when provided as a supplement in the same animal.

In summary, this study is the first to show Gln-mediated modulation of LPS-induced inflammation in the developing infant rat small intestine that is being nourished by gastrostomy. The intestine is the largest immune organ of the body and is a major nidus for inflammation that can spread to the entire body, causing systemic inflammation. Whether Gln provided under similar conditions can prevent inflammatory damage to organs distal to the intestine needs to be the subject of future investigations.

ACKNOWLEDGMENTS

This study was supported by National Institute of Child Health and Human Development Grant RO1-HD-38954

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