GLUTAMINE DECREASES INTERLEUKIN-8 AND INTERLEUKIN-6 BUT NOT NITRIC OXIDE AND PROSTAGLANDINS E₂ PRODUCTION BY HUMAN GUT IN-VITRO

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Background: Glutamine modulates cytokine production in various tissues but its effects on the production of other inflammatory mediators such as eicosanoids and nitric oxide have not been investigated in human gut. Aim: To evaluate the influence of glutamine on interleukin (IL)-8, IL-6, nitric oxide and prostaglandin E₂ production by human gut. Methods: Ten fasted volunteers received either enteral glutamine or isonitrogenous amino acids over 6 h in a cross-over design. Series of duodenal biopsies were frozen or cultured for 24 h with 0.5 or 5 mM of glutamine or amino acids. IL-6, IL-8 and PGE₂ were measured in culture media by ELISA and nitrites by Griess assay. mRNA levels for IL-6, IL-8, Cyclooxygenase-2 and NO synthase-2 were assessed in biopsies by RT-PCR. Results in percent, (median [range]) were compared by Wilcoxon test. Results: Glutamine decreased IL-8 and IL-6 in-vitro production: 63 [2–173] vs 100 [19–177] and 37 [5–489] vs 100 [33–431], both P<0.05. IL-8 mRNA level also decreased in biopsies cultured with 5 mM glutamine: 26 [13–142] vs 92 [34–215], P<0.05. Nitrites and PGE₂ concentrations were not significantly affected by glutamine. Conclusion: Glutamine has a specific inhibitory effect on pro-inflammatory cytokine production in the gut and may contribution to the modulation of intestinal inflammation.

Inflammatory bowel diseases (IBD), encompassing ulcerative colitis and Crohn’s disease, are characterized by chronic intestinal inflammation, which is possibly related to an imbalance of immune response. Indeed, the gut mucosa of patients with IBD has been reported to produce high amounts of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8 or TNFα, in contrast to a less marked increase in production of anti-inflammatory cytokines such as IL-10. Other mediators such as prostaglandins (PGs) and nitric oxide (NO) are also involved in the pathophysiology of IBD. These mediators are produced by cyclooxygenase (COX) and nitric oxide synthase (NOS), respectively. The inducible isoforms of these enzymes (COX-2 and iNOS) can be up-regulated by cytokines and their expression were found to increase in IBD, with a concomitant increase of their products, PGE₂ and NO.

Glutamine, the preferred substrate for both enterocytes and other rapidly dividing cells, such as immune cells, maintains gut barrier and reduces bacterial translocation in animals and stimulates gut mucosal proliferation in-vitro and gut protein synthesis in vivo. In addition, glutamine-supplemented parenteral nutrition supports gut morphology and barrier function in patients with IBD. Finally, glutamine reduces IL-8 and TNFα intestinal production in rats with experimental colitis whereas it maintains IL-4 intestinal production, an anti-inflammatory cytokine during total parenteral nutrition. In rats, glutamine can also modulate whole body NO production. In a previous study, we reported that glutamine decreased in-vitro IL-8 and IL-6 production by human duodenum from healthy subjects as compared to saline. However, more data on the specificity of glutamine effects on gut inflammatory response are needed to support its evaluation on cytokine response in human IBD.
The aims of this new study were to evaluate (1) whether the effects of glutamine on cytokine production are specific to this substrate in comparison to an isonitrogenous mixture of amino acids; (2) the influence of the dose of glutamine; and (3) whether the production of other mediators implicated in the inflammatory response (NO and PGE₂) is also affected by glutamine.

RESULTS

Histological examination of duodenal biopsies revealed no signs of inflammation in any subjects. Examination for H. pylori infection was also negative in all subjects. No Peyer’s patches were observed in these biopsies.

Uncultured biopsies

The relative abundance of mRNA in biopsies is displayed in Table 1. In uncultured biopsies, IL-8 and iNOS mRNA levels were not affected by glutamine, and the mRNA of IL-6 and COX-2 remained undetectable in both conditions.

Cultured biopsies

Cytokine, PGE₂ and nitrites concentrations in culture media are displayed in Table 2, as a percentage of control, i.e. A/A0.5. IL-8 and IL-6 concentrations decreased significantly in culture media of G/G0.5 compared to A/A0.5 biopsies, and G/G5 compared to A/A5 biopsies (both, P<0.05). There was no significant difference for IL-8 and IL-6 concentration between G/G5 and G/G0.5 biopsies (Table 2). Nitrites release in culture media decreased markedly in some subjects after glutamine in vivo infusion (Fig. 1A), but the difference was not statistically significant (Table 2; P=0.066). In contrast, IL-8 concentration decreased in all but one subjects (Fig. 1B). PGE₂ concentration was not influenced by in vivo and in-vitro conditions (Table 2).

The mRNA levels for IL-6, IL-8, COX-2 and iNOS (Fig. 2) increased compared to mRNA levels in uncultured biopsies (Table 1; P<0.05). IL-8 mRNA

| TABLE 1. Expression of IL-8, IL-6, COX-2, and iNOS mRNA in human duodenal biopsies |
|-----------------------------------|-----------------------------------|------------------|------------------|
|                                  | Relative mRNA (%)                 |                  |                  |
|                                  | IL-8                             | IL-6             | COX-2            |
| Uncultured biopsies              |                                  |                  |                  |
| Aa                               | 50 (19–98)                       | nd               | nd               |
| Gln                              | 53 (35–206)                      | nd               | nd               |
| Cultured biopsies                |                                  |                  |                  |
| A/A0.5                           | 100 (44–190)†                    | 100 (36–210)†    | 100 (16–221)†    |
| G/G0.5                           | 82 (11–147)†                     | 78 (59–303)†     | 84 (42–132)†     |
| A/A5                             | 92 (34–215)†                     | 78 (73–153)†     | 89 (18–250)†     |
| G/G5                             | 26 (13–142)*                     | 93 (29–155)†     | 79 (34–142)†     |

Nine duodenal biopsies were performed in each volunteers perfused with glutamine (Gln) or an isonitrogenous mixture of amino acids (Aa). Three biopsies were immediately frozen after sampling without culture. After glutamine infusion, three biopsies were cultured with 0.5 mM glutamine (G/G0.5) and three others with 5 mM glutamine (G/G5). After amino acids infusion, three biopsies were cultured with 0.5 mM amino acids (A/A0.5) and three others with 5 mM amino acids (A/A5). The mRNA levels were normalized to constant glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Results, expressed as percentage of the median of the control A/A0.5 series, are median (range) of 10 series of experiments.*P<0.05 vs A/A5 and †P<0.05 vs uncultured biopsies (Wilcoxon paired test); nd: not detectable.

| TABLE 2. Concentrations of IL-8, IL-6, PGE₂ and nitrites in culture media of human duodenal biopsies |
|-----------------------------------|-----------------------------------|------------------|------------------|
|                                  | Concentration in culture media (%) |                  |                  |
|                                  | IL-8                             | IL-6             | PGE₂             |
| Culture conditions               |                                  |                  |                  |
| A/A0.5                           | 100 (19–177)                     | 100 (33–431)     | 100 (36–158)     |
| G/G0.5                           | 63 (2–173)†                      | 37 (5–489)†      | 80 (13–161)      |
| A/A5                             | 97 (18–130)                      | 78 (21–411)      | 85 (33–155)      |
| G/G5                             | 46 (13–138)*                     | 42 (15–321)*     | 69 (42–161)      |

Six duodenal biopsies were performed in each volunteers perfused with glutamine or an isonitrogenous mixture of amino acids. After glutamine infusion, three biopsies were cultured with 0.5 mM glutamine (G/G0.5) and three others with 5 mM glutamine (G/G5). After amino acids infusion, three biopsies were cultured with 0.5 mM amino acids (A/A0.5) and three others with 5 mM amino acids (A/A5). Concentrations were normalized to biopsies weight. Results, expressed as percentage of the median of the control A/A0.5 series, are median (range) of 10 series of experiments. †P<0.05 vs A/A0.5 and*P<0.05 vs A/A5 (Wilcoxon paired test).
level decreased in G/G5 biopsies compared to A/A5 biopsies (Table 1; \( P < 0.05 \)), whereas it was not different between G/G0.5 and A/A0.5 biopsies. The mRNA levels for IL-6, COX-2, and iNOS were not influenced by glutamine (Table 1).

**DISCUSSION**

Several studies have shown that glutamine may modulate inflammatory response in various cell types.\(^{14,15}\) In the present study, glutamine decreased *in-vitro* production of pro-inflammatory cytokines, but had no effect on PGE\(_2\) and NO production and on the mRNA of the corresponding enzymes.

This study was performed in an organ culture model. Although cultured biopsies were not obtained from patients with IBD, it seems that some degree of inflammation was already induced in this model since we observed an increase of mRNA levels for inflammatory mediators in cultured as compared to uncultured biopsies (Table 1).

COX-2 and iNOS are up-regulated by pro-inflammatory cytokines and have been reported to be enhanced in inflamed intestinal mucosa,\(^3,4\) together with an increase of PGs and NO produced by these respective enzymes. In the present study, COX-2 mRNA level and PGE\(_2\) production are not influenced by glutamine, which is in accordance with indirect data gained from a study during PGE\(_2\)-induced hyperthermia.\(^{16}\) Whether glutamine could affect COX-2 expression in a more severe state of inflammation deserves further investigation. NO is a product of the enzymatic conversion of arginine to citrulline by NOS and is a major messenger involved in the control of inflammation. As glutamine may be a precursor for arginine and consequently NO production, assessing the effect of glutamine on NO synthase and NO production seems relevant. Indeed, a basal NO production seems critical to limit gut injury, while excess NO production can be deleterious.\(^{17,18}\) In fact, NO can react with oxygen free radical to form peroxynitrites and the consequences of overproduction of NO by iNOS may not only result in
pathological vasodilatation but also in tissue damage. In the present study, iNOS mRNA level and nitrites production, used as an index of NOS activity, were not different after glutamine or amino acids treatment. In contrast, in later analyses performed on mRNA remaining from our previous study, enteral glutamine reduced iNOS mRNA level as compared to saline (data not shown). Thus, glutamine and amino acids may have a similar limiting effect on iNOS expression, as compared to the absence of enteral nitrogen infusion.

An increased production of IL-6 and IL-8 is a major feature of IBD. IL-6 plays a key role in the regulation of the inflammatory response, especially in the transcription of several acute phase proteins genes in the intestine. In addition, in-vitro and in vivo studies have shown that glutamine could modulate IL-6 production. In the present study, glutamine reduced IL-6 production by human duodenal mucosa, probably by a post-transcriptional pathway, according to our previously reported results.

An abundant infiltration of neutrophils in intestinal mucosa is frequently observed during IBD and considered to be driven mainly by the chemokine IL-8, which is a potent chemoattractant of neutrophils. IL-8 also decreases neutrophils apoptosis by an inhibition of the pro-caspase 3 expression, and may thus contribute to the perpetuation of inflammation and to the constitution of tissue lesions. Thus, modulating IL-8 production may constitute a key target in future therapies for inflammatory digestive diseases.

In the present study, treatment with glutamine specifically decreased in-vitro production of IL-8 by human duodenal mucosa as compared to amino acids treatment. This finding is in accordance with our previous observation when glutamine was compared to saline, and with the decreased IL-8 plasma concentration and intestinal production observed in rats with experimental colitis pre-treated with glutamine. Furthermore, in the present study, IL-8 mRNA level was decreased after culture with 5 mM but not 0.5 mM glutamine. In contrast, in our previous study, culture with 2 mM glutamine failed to influence IL-8 mRNA level. Thus, the inhibitory effect of glutamine on IL-8 mRNA expression may be dose-dependent and need a high glutamine concentration. In uncultured biopsies, glutamine was not different from control as far as cytokine mRNA expression is concerned, indicating that the modulating effect of glutamine on cytokine production is mainly detectable in states of increased inflammation.

In summary, our results suggest that glutamine may modulate the inflammatory response in intestine to some extent through a specific decrease of the pro-inflammatory cytokines IL-8 and IL-6, while it has no detectable effect on PGE_2 and NO intestinal production under these conditions. The inhibitory effect of glutamine on IL-8 may also contribute to down-regulate the inflammatory response and to protect intestinal mucosa, by limiting neutrophil infiltration in mucosa. These results provide additional rationale for the evaluation of glutamine enteral supplementation to modulate intestinal inflammation in various clinical conditions.

**SUBJECTS AND METHODS**

**Study protocol**

Ten healthy volunteers (aged 20–24 years) gave informed consent to participate in the study. This study was approved by the Ethical Committee of Rouen University Hospital. Subjects were studied on two occasions, with a 2-week interval between studies. After an overnight fast, subjects received at 8 a.m. an intragastric infusion of either glutamine solution (25.95 g/l of L-glutamine with 3.8 g/l NaCl, infusion rate 4.5 ml/kg \( \pm 1 \)h \(^{-1} \) providing 0.8 mmol/kg \( \pm 1 \)h \(^{-1} \) or an isonitrogenous isosmolar mixture of amino acids (gly/ala/ser/pro/asp/lys: 2/2/4/4/7/10/10 g) over 6 h, in a random order. Solutions were freshly prepared immediately before use. This dosage of glutamine has well documented effects on whole body protein synthesis in humans, and reduces IL-8 intestinal production compared to saline infusion. At the end of the infusion, an endoscopy was performed and multiple biopsy specimens (mean weight 6.7 ± 1.5 mg) were taken from the distal duodenum. Two biopsies were fixed in formalin for histological assessment; three biopsies were transferred to vials containing guanidium solution and stored at −80°C until mRNA analysis; six biopsies were immediately transferred in preweighed tubes containing cooled Hank’s salt solution (Eurobio, France) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml; Sigma, Germany) and processed within one hour for organ culture as previously described. Biopsies were weighted immediately after sampling. Whole biopsy specimens were used, without cell separation procedures. Thus, cultured biopsies contained both enterocytes and gut immune cells, as verified by histological examination of preliminary experiments. However, routine cell counts, as well as surface area measurements, could not be performed in this study because of the culture and RNA extraction procedure. Biopsies were placed in plastic culture dishes and bathed in 1 ml of RPMI 1640 (Eurobio, France) supplemented with 10% foetal calf serum. RPMI 1640 contained glutamic acid (20 mg/l) and arginine (200 mg/l) but without glutamine. In preliminary experiments, we checked that the release of LDH in culture medium did not increase by more than 10%, indicating that intestinal tissue remained viable. Biopsies from subjects perfused in-vivo with amino acids were cultured either with 0.5 mM amino acids (cultures referred to as A/A0.5, \( n = 10 \)) or with 5 mM amino acids (A/A5, \( n = 10 \)) in the culture medium. Biopsies from subjects perfused in-vivo with glutamine were cultured either with 0.5 mM glutamine (G/G0.5, \( n = 10 \)) or with 5 mM glutamine (G/G5, \( n = 10 \)). The culture dishes were placed in chamber equilibrated with 5%
CO₂ and incubated at 37°C. After 24 h, biopsies were transferred to vials containing guanidium solution and stored at −80°C until mRNA analysis.

**Analytical methods**

**Cytokine and PGE₂ immunoassays**

Culture media were assessed for concentrations of IL-6, IL-8 and PGE₂ using a specific sandwich enzyme linked immunosorbent assay (ELISA, R&D Systems, United Kingdom). These assays have a sensitivity of 0.7, 7 and 8.25 pg/ml for IL-6, IL-8 and PGE₂, respectively. Assays in samples and standards were run simultaneously according to the recommendations of the manufacturer. The optical density was read at 450 nm for cytokines and at 405 nm for PGE₂ in a Titertek Multiskan Plus photometer. Immunoassays were performed on duplicate samples, with a coefficient of variation less than 10%. Results of cytokine and PGE₂ concentration in culture media were expressed as pg/mg tissue wet weight.

**Nitrites assays**

Culture media were assessed to determine nitrites production as an index of NOS activity. Culture medium (50 μl) was transferred in 96-well plates. Griess reagent (50 μl) was added in each well. The resultant colour change was quantified by spectrophotometry (550 nm). Nitrites levels were determined in duplicate using a sodium nitrites standard curve (0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 μmol/l) and expressed as μmol/l.

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

Mucosal RNA were extracted from biopsies by a modified-extraction method as previously described. The quality and quantity of total RNA were determined by spectrophotometry using the absorbance at A₂₆₀/A₂₈₀ nm. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed respectively by 24 (IL-8, COX-2, iNOS and GAPDH) or 30 cycles (IL-6) consisting of denaturation for 90 s at 95°C, primer annealing for 40 s at 60°C and primer extension for 40 s at 72°C using a thermal cycler MJ Research (PTC 200).

The PCR products were electrophoresed on a 6% polyacrylamide gel. After 2 days of exposure the autoradiogram was processed using non-parametric analysis with Wilcoxon paired T test. For cytokine, PGE₂ and nitrites assays in culture media and for each RNA, statistical analysis was performed using the mean of duplicates or triplicates (coefficient of variation 5–10%), respectively. The level of statistical significance was fixed at P ≤ 0.05.

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