Background. The objective of this study was to evaluate the effects of lipopolysaccharide (LPS) endotoxemia and enteral arginine (ARG) supplementation on intestinal structural changes, enterocyte proliferation, and apoptosis in rat.

Methods. Male Sprague–Dawley rats, weighing 250–280 g, were divided into three experimental groups: control rats, LPS rats treated with lipopolysaccharide given ip at a dose of 10 mg/kg every 24 h (two injections), and LPS-ARG rats treated with enteral arginine given in drinking water (2%) 72 h before and following injection of LPS. Intestinal structural changes, enterocyte proliferation, and enterocyte apoptosis were determined on day 3 following the first LPS injection.

Results. LPS rats demonstrated a significant decrease in bowel weight in duodenum, mucosal weight in duodenum, jejenum, and ileum, mucosal DNA and protein in jejenum and ileum, and villus height in jejenum and ileum compared to control animals. LPS rats also had a significantly lower cell proliferation index in jejenum and ileum and a higher apoptotic index in jejenum and ileum compared to control rats. LPS-ARG animals demonstrated greater duodenal bowel weight, duodenal and ileal mucosal weight, ileal mucosal DNA and protein, ileal villus height, and jejunal and ileal cell proliferation index compared to LPS animals.

Conclusions. LPS endotoxemia impairs the integrity of the gastrointestinal mucosa in rat. Decreased cell proliferation and increased apoptosis may be considered the main mechanisms responsible for the decreased cell mass. Enteral arginine administration decreases the mucosal injury caused by lipopolysaccharide. © 2004 Elsevier Inc. All rights reserved.

Key Words: sepsis; lipopolysaccharide; immunonutrition; arginine; bacterial translocation; intestine.

INTRODUCTION

Immunonutrition includes specific nutrients (arginine, glutamine, omega-3 fatty acids, and nucleotides) that improve immune function in critically ill patients suffering from trauma, sepsis, or major surgery [1, 2]. Clinical studies show that immune-enhancing enteral diets improve the immunometabolic response, decrease the infection rate, and shorten the length of hospital stay after the start of enteral feeding [1–3]. The exact mechanisms of action of immune-modulating ingredients are poorly understood. Several in vitro and in vivo studies suggest that their effects include the promotion of T-cell blastogenesis, enhancement of cellular immunity, and increased concentration of the trienoic eicosanoids [4–6]. Other mechanisms involve selective stimulation of intestinal perfusion [7] and improvement in gut barrier function.

Bacterial translocation, which is defined as a migration of bacteria and endotoxin across the intestinal mucosa, has been postulated as an important factor in development of multiple organ failure [8, 9]. Mechanisms responsible for bacterial translocation depend on factors related to the micro-organisms and the host defenses. A considerable number of animal experiments have been performed to elucidate the pathogenesis of impaired gut barrier function to find a rationale
for new therapeutic options. A complex of several immunological and nonimmunological factors maintains the barrier function of the gastrointestinal tract, which includes salivary secretions, gastric acid, bile, pancreatic secretions, mucus production, intact intestinal mucosa, and a normally functioning local immune system [9]. The integrity of the gastrointestinal mucosa is a key element in maintaining gut barrier function. Mucosal injury has been considered key in the translocation process. Therefore, identification of those factors that maintain mucosal integrity of the gastrointestinal tract will suggest new therapeutic strategies for improvement in gut barrier function.

Arginine is a nonessential amino acid that plays a central role in the modulation of immune system activation. The mechanism by which arginine activates the immune system is poorly understood. Its importance for macrophage and T-lymphocytes has been reported recently by Bansal et al. [10]. In the last 2 decades, arginine has attracted major interest since it has been identified as the natural substrate of nitric oxide (NO) and is now recognized to have a major role in many regulation processes. Arginine and NO are critical to the normal physiology of the gastrointestinal tract. Several studies have suggested that endogenous formation of nitric oxide maintains the mucosal integrity of the intestine and protects the gut from injuries from blood-borne toxins and tissue-destructive mediators.

The purpose of the present study was to evaluate the effect of oral arginine supplementation on structural mucosal changes in the small bowel induced by lipopolysaccharide endotoxemia in rat.

MATERIALS AND METHODS

Surgical procedures and animal care were conducted in compliance with the guidelines established by the Guide for the Care and Use of Laboratory Animals, Rappaport Faculty of Medicine, Technion (Haifa, Israel).

Male Sprague–Dawley rats weighing 250–280 g were used for the study. Animals were housed in individual wire-bottom Plexiglas cages, kept at 21°C on 12-h day and night cycles, acclimated to their environment for at least 7 days with free access to water, and pair fed with standard chow. During 72 h of the experiment fluid and food intake was measured.

Experimental Design

Animals were randomly assigned to one of three experimental groups: (1) control rats (Group A, CONTR, n = 14), (2) rats treated with lipopolysaccharide (LPS) given ip once a day at a dose of 10 mg/kg for 48 h (two doses) (Group B, LPS, n = 14), and (3) rats with LPS endotoxemia treated with oral arginine given in drinking water (2%) 72 h before and following injection of LPS (Group C, LPS-ARG, n = 13). On the morning of the third day following the first LPS injection, animals were anesthetized with ip sodium pentobarbital (45 mg/kg) and sacrificed by inducing open pneumothorax.

Intestinal Morphology Analysis

The small intestine was removed and divided into three segments: duodenum, jejenum (10 cm from the Treitz ligament) and terminal ileum (10 cm from the ileocecal junction). Each segment was washed with ice-cold saline, dried, and weighed. For each segment, overall bowel weight was calculated per centimeter of bowel length. The segments were opened lengthwise, and the diameter was measured at three equidistant places as described by Dowling [13]. The surface area of the intestinal segment was calculated as the circumference multiplied by the segment length and expressed per centimeter of bowel length. Mucosa was scraped off, weighed, and snap frozen in liquid nitrogen. Mucosal samples (100 mg) were homogenized using a Kontes Tfenbroek Tissue Grinder. DNA and protein were extracted using TRIZOL reagent as described by Chomczynski [14]. The DNA concentrations were recorded spectrophotometrically and calculated per centimeter of bowel length. Final protein concentration was measured spectrophotometrically using a commercially available kit (Bio-Rad, Protein Assay) and was calculated per centimeter of bowel length.

Histological sections were prepared from the jejunal and ileal remnants. Pieces of proximal jejenum and terminal ileum near the ileocecal junction were placed in 5% phosphate-buffered Formalin, washed with absolute alcohol, embedded in paraffin, cut to 5 μ thickness, and stained with hematoxylin–eosin. The five longest villi and crypts were selected for the microscopic analysis, using a 10 × 4 magnifying lens. Histological images were loaded on a 760 × 570 pixel resolution buffer using a computerized image analysis system composed of a trichip RGB video camera (Sony, Japan) installed on a light microscope (Zeiss, Germany) and attached to an IBM-compatible personal computer (Pentium III, MMX, 450 MHz, 125 MB RAM), equipped with a frame grabber. Images were captured, digitized, and displayed on a high-resolution monitor. The villus height and crypt depth were measured using the Image Pro Plus 4 image analysis software (Media Cybernetics, Baltimore, MD, USA).

Histological features of Peyer’s patches located in the ileal segment of the small bowel were assessed.

Measurement of Crypt Cell Proliferation and Villus Cell Apoptosis

Crypt cell proliferation was assessed using 5-bromodeoxyuridine (5-BrdU). Standard BrdU labeling reagent (Zymed Laboratories, Inc., San Francisco, CA, USA) was injected intraperitoneally at a dose of 1 ml/100 g of body weight 2 h before sacrifice. Tissue slices (5 μ) were deparaffinized with xylene, rehydrated with graded alcohol, and stained with a biotinylated monoclonal anti-BrdU antibody system provided in a kit (Zymed Laboratories). An index of proliferation was determined as the ratio of crypt cells staining positively for BrdU per five crypts.

The terminal deoxyuridine nick-end labeling (TUNEL) immunohistochemical assay was used to identify apoptotic cells using the LS Cell Death Detection kit (Boehringer Mannheim GmbH, Mannheim, Germany). Five-micrometer-thick paraffin-embedded sections were deparaffinized, rehydrated in graded alcohol, and microwave pretreated in 10 mM citrate buffer (pH 6.0). After washing in PBS, the specimens were incubated in buffer containing a nucleotide mixture with fluorescein-labeled deoxy-UTP and TdT at 37°C for 1 h. After washing, the slides were incubated with blocking solution (3% H₂O₂ in methanol) for 10 min and stained with antifluorescein antibody, Fab fragment from sheep, conjugated with horseradish peroxidase (converter-POD) at 37°C for 30 min. AES substrate (Zymed Laboratories) was applied for color development. For each group, the number of stained cells was counted in at least five villi in areas without necrosis. The apoptotic index was defined as the number of apoptotic TUNEL-positive cells per five villi.

All measurements were performed by a qualified pathologist blinded as to the source of intestinal tissue.
Statistical Analysis

The data are expressed as the mean ± SEM. Nonparametric Kruskal–Wallis ANOVA tests were used for statistical analysis, with \( P \) less than 0.05 considered statistically significant.

RESULTS

Body Weight and Mortality Rate

There were no differences in fluid intake among the three experimental groups. LPS injections were accompanied by decreased appetite. Food intake also decreased in LPS groups compared to control animals but did not change following oral arginine administration. Figure 1 demonstrates a final body weight expressed as a percentage of initial weight. As expected, LPS endotoxemia (Group B) resulted in an approximately 18\% \((P < 0.05)\) decrease in final body weight compared to control animals (Group A). Exposure to oral arginine (Group C) did not significantly change final body weight compared to the LPS animals (Group B).

All animals in Group A survived. Injection of LPS (Group B) resulted in a 25\% mortality rate (3 of 12 rats died). Pretreatment with arginine resulted in a 17\% mortality (2 of 12 rats died) (NS versus LPS group).

Mucosal Surface Area

Three days after LPS injection, intestine appeared decreased in both thickness and diameter. Compared to control animals (Group A), LPS rats (Group B) showed a significantly lower surface area per unit of intestinal length in duodenum (7.6 ± 0.2 versus 9.2 ± 0.1 cm\(^2\)/centimeter of length, \( P < 0.05 \)), jejunum (7.3 ± 0.2 versus 8.6 ± 0.2 cm\(^2\)/centimeter of length, \( P < 0.05 \)), and ileum (7.4 ± 0.1 versus 8.1 ± 0.3 cm\(^2\)/centimeter of length, \( P < 0.05 \)) (Figure 2). Exposure to enteral arginine (Group C) did not significantly change mucosal surface area in any of the three intestinal segments compared to LPS animals (Group B).

Total Intestinal and Mucosal Weights

As shown in Figure 3, overall total intestinal weight expressed as grams/centimeter of length/100 g of body weight decreased significantly in duodenum (23.5 ± 0.1 versus 28.8 ± 2.1 mg/centimeter of length/100 g of body weight, \( P < 0.05 \)) in LPS rats (Group B) compared to control animals (Group A). LPS-ARG rats (Group C)
LPS rats. Versus control animals (Group A) (Figure 4). Additionally, LPS weight, \( P < 0.05 \) versus 23.5 \( \pm \) 1 mg/centimeter of length/100 g of body weight, \( P < 0.05 \) compared to control animals. Enteral arginine (Group C) resulted in a significant increase in ileal mucosal DNA (8.0 \( \pm \) 1.1 versus 5.6 \( \pm \) 0.2 \( \mu \)g/centimeter of length/100 g of body weight, \( P < 0.05 \)) and protein (39.0 \( \pm \) 7.3 versus 24.3 \( \pm \) 2.4 \( \mu \)g/centimeter of length/100 g of body weight, \( P < 0.05 \)) content compared to LPS-non-treated animals (Group B).

**Histological Findings**

LPS rats (Group B) showed a significant decrease (compared to control animals) in villus height in jejunum (413 \( \pm \) 22 versus 531 \( \pm \) 31 \( \mu \)m, \( P < 0.05 \)) and ileum (274 \( \pm \) 25 versus 357 \( \pm \) 12 \( \mu \)m, \( P < 0.05 \)) and a trend toward a decrease in jejunal and ileal crypt depth; however, this trend did not achieve statistical significance (Figure 5). Following exposure to enteral arginine, LPS rats (Group C) demonstrated significantly taller villi in ileum (339 \( \pm \) 15 versus 274 \( \pm \) 25 \( \mu \)m, \( P < 0.05 \)) than LPS animals (Group B), suggesting increased enterocyte mass. Although the jejunum and ileum of LPS-ARG rats manifested a tendency toward longer, cytoarchitecturally preserved crypts, when compared to LPS rats, this trend did not achieve statistical significance.

**Cell Proliferation and Apoptosis**

A significant decrease in cell proliferation was seen following LPS injection (Group B) compared to control animals (Group A) in jejunum (186 \( \pm \) 33 versus 250 \( \pm \) 7 BrdU-positive cells/5 crypts, \( P < 0.05 \)) and ileum (176 \( \pm \) 10 versus 253 \( \pm \) 13 BrdU-positive cells/5 crypts, \( P < 0.05 \)) (Figure 6). Following exposure to enteral arginine, LPS

**Mucosal DNA and Protein**

A significant decrease in mucosal DNA was observed in jejunum (6.9 \( \pm \) 0.8 versus 10.8 \( \pm \) 1.6 \( \mu \)g/centimeter of length/100 g of body weight, \( P < 0.05 \)) and ileum (5.6 \( \pm \) 0.2 versus 8.9 \( \pm \) 1.1 \( \mu \)g/centimeter of length/100 g of body weight, \( P < 0.05 \)) in LPS rats (Group B) compared to control animals (Group A) (Figure 4). Additionally, LPS rats demonstrated a significant decrease in mucosal protein content in jejunum (31.2 \( \pm \) 3.1 versus 49.5 \( \pm \) 3.0 \( \mu \)g/centimeter of length/100 g of body weight, \( P < 0.05 \)) and ileum (24.3 \( \pm \) 2.4 versus 43.4 \( \pm \) 2.8 \( \mu \)g/centimeter of length/100 g of body weight, \( P < 0.05 \)) compared to control animals. Enteral arginine (Group C) resulted in a significant increase in ileal mucosal DNA (8.0 \( \pm \) 1.1 versus 5.6 \( \pm \) 0.2 \( \mu \)g/centimeter of length/100 g of body weight, \( P < 0.05 \)) and protein (39.0 \( \pm \) 7.3 versus 24.3 \( \pm \) 2.4 \( \mu \)g/centimeter of length/100 g of body weight, \( P < 0.05 \)) content compared to LPS-non-treated animals (Group B).

**Microscopic Bowel Appearance**

A significant decrease in cell proliferation was seen following LPS injection (Group B) compared to control animals (Group A) in jejunum (186 \( \pm \) 33 versus 250 \( \pm \) 7 BrdU-positive cells/5 crypts, \( P < 0.05 \)) and ileum (176 \( \pm \) 10 versus 253 \( \pm \) 13 BrdU-positive cells/5 crypts, \( P < 0.05 \)) (Figure 6). Following exposure to enteral arginine, LPS
rats (Group C) demonstrated a significant increase in the jejunal (234 ± 21 versus 186 ± 3 BrdU-positive cells/5 crypts, P < 0.05) and ileal (218 ± 11 versus 176 ± 10 BrdU-positive cells/5 crypts, P < 0.05) proliferation rates compared to LPS animals (Group B).

After lipopolysaccharide injection, a significant increase in enterocyte apoptosis was observed in jejunal (21.32 ± 3.4 versus 9.2 ± 3.3 apoptotic cells/5 villi, P < 0.05) and ileal (28.2 ± 3.4 versus 16 ± 5 apoptotic cells/5 villi, P < 0.05) compared to control animals (Figure 6). Exposure to ARG did not significantly change the apoptosis rates in jejunal and ileal compared to those in LPS-untreated animals.

**DISCUSSION**

Sepsis and endotoxemia are frequent complications in severely sick and injured patients and are closely associated with the development of adult respiratory distress syndrome and multiple organ failure. The gut is thought to be the source of this septic state [15] and the first step in a “gut–liver–lung axis” [16]. Bacterial translocation has been shown to occur in different animal models of shock, trauma, and sepsis. Bowel mucosal injury has been implicated in the pathophysiology of bacterial translocation in many of these models [17]. A complex of several immunological and nonimmunological processes maintains the barrier function of the gastrointestinal tract. The combination of an intact intestinal mucosa and a normally functioning immune system provides adequate barrier function. The immune system of the gastrointestinal tract, which includes Peyer’s patches, lymphoid cells located within the intestinal lamina propria, intraepithelial lymphocytes, and aggregated lymphoid tissue within mesenteric lymph nodes, plays a major role in protecting the host from the external environment. However, growing evidence suggests that several nonimmunological mechanisms also protect the gastrointestinal tract from enteric microflora and bacterial products. These include epithelial cell turnover, tight intracellular junctions between epithelial cells, salivary secretions, gastric acid, mucus production, intestinal motility, and bile salts.

The dynamic process of epithelial cell turnover is a function of the rates of crypt cell proliferation, migration along the small bowel crypt–villus axis, differentiation, and cell death via apoptosis. This process may be affected by nutritional status, the route of feeding, and the adequacy of specific nutrients in the diet. Programmed cell death, or apoptosis, is the most common form of eukaryotic cell death [18]. Apoptosis is a cascade of catabolic reactions leading to cell death by “suicide” that preserves cell homeostasis. This process can be initiated by different signaling pathways and is altered by many factors, including radiation, inflammation, hypothermia, and bowel resection [19]. Recent evidence demonstrates that apoptosis increases in sepsis [20, 21]. The identification of factors that promote growth and regeneration of the intestinal epithelium and reduce apoptosis will suggest new therapeutic strategies for maintaining gut integrity.

Arginine is one of the most versatile amino acids in animal cells. It was first isolated from lupine seedlings in 1886 and subsequently found to be a major amino acid in the basic proteins of many mammalian cells and tissues. Arginine is a nonessential amino acid processed metabolically by the urea cycle. The metabolism of arginine is determined by the expression of the arginine metabolizing enzymes, inducible nitric oxide synthase, and two arginase isoforms (arginase I and II). The physiological significance of arginine metabolism extends far beyond its incorporation as an amino acid into proteins. Arginine plays an important role in many physiological and biological processes, including release of several hormones, collagen synthesis during wound healing, immune response, tumor biology, and the regulation of inflammation [4, 22]. L-Arginine improves the oxygen partial pressure of the internal organs and significantly increases the survival rate of rats with traumatic shock [23]. Arginine may stimulate wound healing [24]. Arginine has beneficial effects on radiation-induced tissue injury in rat small bowel [25].

L-Arginine is converted to nitric oxide and citrulline by the enzyme nitric oxide synthase (NOS). NO is a diffusible, multifunctional, transcellular messenger that has been implicated in numerous physiological and pathological conditions, including immune regulation, host defense, neurotransmission, and vascular homeostasis [26].
There is growing interest in the potential roles of arginine and NO as regulators of cell proliferation and apoptosis in general and in the gastrointestinal tract in particular [27]. Several studies have suggested that endogenous formation of nitric oxide from L-arginine maintains the mucosal integrity of the intestine. However, not all investigators support this concept. It is apparent that in chronic injury, NO becomes detrimental by combining with reactive oxygen species to form potent free radicals. In contrast, inhibition of NO synthesis after acute injury may exacerbate damage and inflammation.

In a recent study, Gookin et al. reported that iNOS-derived NO is a key mediator of early villous reepithelialization following acute injury caused by deoxycholate in porcine ileal mucosa [28]. The mechanisms of this positive effect are still unclear; however, a suppressive effect of appropriate amounts of NO on apoptotic cell death in the gastrointestinal tract may be considered one of them.

In the present study, we evaluated the effect of lipopolysaccharide endotoxemia and oral arginine supplementation on small intestine morphology and cell turnover. Alterations in bowel and mucosal weights, mucosal DNA and protein contents, and histological appearance were measured in this study. In addition, cell proliferation and apoptosis were measured to characterize enterocyte turnover. Mucosal DNA and protein were calculated per centimeter of bowel weight. Although bowel length may change due to spasm or bowel distension, the calculation per unit of bowel length is considered the gold standard in describing structural changes in intestine. We observed that LPS endotoxemia caused mucosal injury in small bowel. This conclusion is supported by the observed decreased mucosal surface area in all three bowel segments of intestine, decreased bowel and mucosal weight in duodenum, jejenum, and ileum, and decreased mucosal DNA and protein in jejenum and ileum. LPS rats also demonstrated lower villus height in jejenum and ileum compared to their control counterparts, suggesting a decrease in absorptive surface area. This study does not address whether impaired structure is associated with altered function. However, in our conventional thinking of intestinal function, it is assumed that a decrease in villus height is accompanied by impaired nutrient absorption. Accordingly, reduced weight gain was observed in LPS animals. Although decreased food intake and malnutrition may affect gut mass, the calculation of mucosal parameters per 100 g of body weight minimizes the effect of decreased body weight and malnutrition on calculated mucosal parameters. The morphological changes in small intestine were consistent with the decrease in the cell proliferating index and increased cell apoptosis, which suggests that lipopolysaccharide decreases in enterocyte proliferation, affects the inherent cell turnover, and decreases the total number of enterocytes. Although lipopolysaccharide itself may cause a poorly controlled systemic inflammatory response, impaired mucosal integrity and the concomitant bacterial translocation may be responsible for the increased mortality following LPS injections.

The present study has shown that dietary arginine protects the intestinal mucosa from damage caused by LPS endotoxemia. LPS rats treated with arginine demonstrated a significant increase in mucosal weight without changes in mucosal surface area and overall bowel weight, suggesting than mucosal hyperplasia rather than bowel enlargement or intestinal muscle hypertrophy is responsible for the increased intestinal mass. Increases in mucosal DNA and protein in ileum along with hypertrophy of the individual cells, which we have demonstrated morphometrically, are characteristic of tissues undergoing increased cell proliferation or repair. Because the changes in DNA and protein contents are directly proportional to bowel and mucosal weight changes and to changes in microscopic bowel appearance, this measurement rules out such factors as edema or vascular engorgement as responsible for differences in mucosal weight. Histologically, marked increases in villus height in ileum, a close correlate of cell number, change in direct proportion to mucosal DNA and protein. The present data also suggest that oral arginine increases mucosal proliferation in functioning intestine, as demonstrated by the increased cell proliferation index. Our study also demonstrates that the cell apoptosis rate did not change significantly following oral arginine administration, suggesting that increased enteroctye proliferation rather than enterocyte death via apoptosis is responsible for the increased cell mass. Although the mortality rate decreases with exposure to oral arginine, this change was not statistically significant.

CONCLUSIONS

Lipopolysaccharide endotoxemia caused a marked intestinal mucosal injury in rats. Decreased enterocyte proliferation and increased cell apoptosis may be responsible for this negative effect. Exposure to oral arginine has a protective effect on the intestinal mucosa from damage caused by LPS endotoxemia. Increased enterocyte proliferation rather than apoptosis is responsible for this beneficial effect. The results of the present study add to the body of evidence that suggests that maintaining the mucosal integrity of the gastrointestinal tract with concomitant improvement in gut barrier function may be the reason for the positive effect of oral arginine in septic patients.

REFERENCES


