Immune-Enhancing Diet and Cytokine Expression During Chronic Sepsis: An Immune-Enhancing Diet Containing L-Arginine, Fish Oil, and RNA Fragments Promotes Intestinal Cytokine Expression During Chronic Sepsis in Rats

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Chronic feeding with enteral immune-enhancing diets (IEDs) provides benefits based on composition of the diet, route of feeding, and timing of feeding in relation to timing of trauma or surgery. Our prior studies of acute feeding in naïve rats demonstrated that IED promotes blood flow and proinflammatory cytokines in the ileum. We hypothesized that chronic feeding with IED would shift gut immune status to an anti-inflammatory state during chronic sepsis, resulting in an altered state of cytokine expression in the gut. Five days prior to feeding, gauze was implanted subcutaneously in the backs of male Sprague-Dawley rats, which were fed for 3 days with either control diet (CD, Boost; Mead-Johnson, Evansville, IL) or IED (Impact; Novartis) and randomly assigned to one of four groups: saline control (NS) + control diet (CD), sepsis (EC) + CD, NS + IED, or EC + IED. EC rats were inoculated with 10^9 CFU *Escherichia coli* and 10^9 CFU *Bacteroides fragilis* in 2 ml normal saline into the back sponge while NS rats received 2 mL normal saline alone. After 3 days, animals were anesthetized and gut tissue samples were harvested and frozen at –80°C. Tissue protein was extracted and ELISA was performed for interleukin (IL-1β, IL-5, IL-6, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. In saline controls, IED feeding decreased IL-1β, IL-5, IL-6, TNF-α, and IFN-γ and increased IL-10 compared with CD-fed animals. In septic animals, IED feeding increased IL-5 and IL-6, while decreasing IFN-γ and IL-10 in the distal third of the small intestine compared with CD-fed septic rats, whereas IL-1β and TNF-α levels were unchanged. Chronic IED feeding produced a anti-inflammatory state via decreased IFN-γ and increased IL-5 and IL-6, which both promote gut IgA class switching, suggesting that the gut is shifted toward humoral immunity during chronic IED feeding in septic rats. (J GASTROINTEST SURG 2006;10:46–53)

KEY WORDS: IL-1β, IL-5, IL-6, IL-10, interferon-γ, TNF-α, immunonutrition, GALT, MALT, gut blood flow

Despite advances in treatment and therapies, severe sepsis presents a major health problem in the United States with 215,000 deaths per year, or 9.3% of all deaths, and approximately $17 billion in estimated medical expenses.[1] Affected patients develop a postinjury hypermetabolic state that leads to negative nitrogen balance and the loss of lean body mass, which primes the development of multiple organ failure.[1] In the past 20 years, the role that nutrition plays in reducing septic complications in ICU patients has been examined, including route of nutrient delivery (parenteral versus enteral),[2,3] timing of initiation of therapy,[4] and composition of individual diets.[5–13] Early enteral feeding with immune-enhancing diets (IED) decreased the rate of septic complications in some patients. Immune-enhancing nutrients include glutamine, L-arginine, ω-3 fatty acids, and RNA fragments, and many IEDs

Presented at the Forty-Sixth Annual Meeting of The Society for Surgery of the Alimentary Tract, Chicago, Illinois, May 14–18, 2005 (poster presentation).

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Supported in part by Veterans Administration Merit Review funding.

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1091-255X/06/$—see front matter
doi:10.1016/j.gassur.2005.10.002

are available, such as Immun-Aid (McGaw, Irvine, CA), Nutren (Clintec, Chicago, IL), and Impact (Novartis, Minneapolis, MN).

The mechanisms whereby IEDs enhance immunity are diverse. Meta-analysis[14–16] of IEDs in the prevention of septic complications, length of hospital stay (LOS), and mortality concluded that IED reduced septic complications compared with standard diets in surgery patients.[14] In one study, critically ill and elective surgery patients who received IED had shorter LOS and decreased ventilator use compared with patients who received standard diets with comparable caloric and nitrogenous content. Interestingly, meta-analyses published to date have not demonstrated a benefit in mortality in IED-fed patients. How immunonutrients provide the benefits of decreased septic complications, decreased hospital stay, and decreased ventilator use remains a source of debate and the focus of intense basic science and clinical research.

Potential mechanisms of IED benefits include enhanced gut mucosal immunity, altered inflammatory processes, maintenance of intestinal tissue oxygenation and/or barrier function, and improved systemic nitrogen balance. We proposed that some of IED benefits might involve improved blood flow during nutrient absorption in the small intestine.[17–19] Postprandial hyperemia is dependent on nutrient composition and site of nutrient absorption.[20] The gut is the largest immune organ and contains approximately 80% of the immunoglobulin cells located in the gut-associated lymph tissue (GALT) of the ileum.[21] Adequate blood flow and nutrient delivery to the GALT tissue might help promote a well-directed immunological response to infection. In the present study, we hypothesized that chronic IED feeding in rats with chronic sepsis would stimulate an anti-inflammatory state by decreasing the Th1 proinflammatory cytokines interleukin (IL)-1β, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α and increasing the Th2 anti-inflammatory IL-10. We further proposed that chronic IED would increase IL-5 and IL-6 promoting antibody class switching to IgA and thus enhancing mucosal immunity. To produce chronic sepsis, we injected bacteria or saline into subcutaneous back sponges in Sprague-Dawley rats and randomly assigned these animals to be fed either IED or CD for 3 days prior to gut tissue harvest to measure cytokine expression in the small intestine (IL-1β, IL-5, IL-6, IL-10, TNF-α, and IFN-γ).

MATERIAL AND METHODS

Animal Care

Animals were maintained in a facility approved by the American Association for the Accreditation of Laboratory Animal Care. The research protocol was approved by the institutional Animal Care and Use Committee and Biohazard Safety Committee at the Louisville VA hospital. Thirty-two male Sprague-Dawley rats (200–220 g) were acclimated for 2 weeks prior to experimental use, during which time the animals received standard rat chow (20 g/day) and water ad libitum. Animal weights were recorded daily to ensure positive weight gain. Animals were housed in individual metabolic cages to prevent coprophagia, and a constant room temperature of 25 °C was maintained.

Chronic Sepsis Model

The current study used a model of chronic sepsis first described by Mela-Riker et al.[22] Over the years, our laboratory has adapted the model for use in microcirculation studies,[23,24] and it is briefly described here. Rats were anesthetized using intramuscular xylazine (60 mg/kg) and ketamine (150 mg/kg). Food, but not water, was withheld the evening prior to surgery. Using standard aseptic techniques, animals were prepped and draped in a designated survival surgical suite. An approximately 3-cm skin incision was made between the scapulas. Through a subcutaneous tunnel a sterile 5 × 5-cm^2 gauze sponge was placed near the base of the tail on the lower back. Incision was closed with interrupted 4-0 silk sutures. Animals were allowed free access to food and water during a 5-day recovery period. After the recovery period, a pooled bacterial inoculum consisting of 10^9 colony forming units (CFU) in 1 ml of *Escherichia coli* and 10^9 CFU in 1 ml of *Bacteroides fragilis* was injected percutaneously in the implanted sponge. Forty-eight hours following the first injection, animals received a second injection of pooled bacterial inoculum. Control animals were treated the same as septic animals but received 2 ml of saline percutaneous injections versus the bacterial inoculum. Experiments involving tissue harvest for cytokine assay were carried out 24 hours after the second sponge injection of either bacteria inoculum or saline (total of 72 hours sepsis or 72 hours saline control).

Feeding Regimen

Animals were fed 20 g/day of standard rat chow and water ad libitum for 5 days following sponge implantation. On the day of the first saline/bacteria injection, rats were fed 10 g of rat chow at 08:00. After bacteria or saline injections at 12:00, rats were fed either 30 ml of control diet (CD) or 30 ml of IED at 15:00. For the next 2 days, animals received a total of 60 ml of IED (1.00 kcal/ml) or 60 ml of CD

(1.01 kcal/ml) with half the dose (30 ml) at 08:00 and the other at 15:00. The dose of IED or CD given (60 ml/day) matched the caloric content of 20 g of standard rat chow (3.00 kcal/g) that our rats are normally fed on a daily basis. On the day of tissue harvest (72 hours after first bacteria/saline injection), rats were fed the 08:00 dose of IED or CD and then prepared for surgery/tissue harvest at 12:00. Table 1 shows the nutritional composition of the two diets. Boost CD was chosen because it is the best available match in nitrogen and caloric content of the IED used (Impact). Figure 1 outlines the experimental protocol timeline for sepsis, feeding regimen, surgery, tissue harvest, and cytokine assays.

Surgery and Tissue Harvest

At 12:00, 72 hours following the first injection of bacteria/saline, animals were anesthetized by intraperitoneal urethane (800 mg/kg) and α-chloralose (60 mg/kg) with supplemental urethane (25% of original dose) given as needed to maintain a surgical plane of anesthesia throughout the experimental protocol. Prior to surgery, animals received 1 ml of subcutaneous normal saline to maintain body fluid homeostasis. Body temperature was monitored and maintained at 37.0° ± 0.5°C using a heating pad and rectal thermistor. Using PE240 tubing, a tracheostomy was performed and animals spontaneously breathed room air. The right carotid artery was cannulated with PE50 tubing for measurements of mean arterial pressure (MAP) and heart rate (HR). Animals were allowed to equilibrate for 45–60 minutes following completion of the surgery. Following equilibration, animals' MAP and HR were recorded and averaged over a 20-minute period. Under deep surgical anesthesia, the small intestine was harvested and divided into thirds, and the gut contents removed and weighed. Tissue was placed in a 10% Triton X-100 reagent (1 ml/10 mg tissue), homogenized, and stored at −80°C until cytokine assay was performed. When assays were ready, samples were thawed and cytokine ELISAs for IL-1β, IL-5, IL-6, IL-10, IFN-γ, and TNF-α were performed. Protocols for the ELISAs followed the directions included in the individual kits (R&D Systems, Minneapolis, MN). Samples from the distal third segment of the small intestine (ileum) were assayed in triplicate with appropriate blanks and controls.

Table 1. Nutritional comparison of enteral diets used

Component (units)	CD (Boost)	IED (Impact)
Calories (kcal/ml)	1.01	1.00
Protein (mg/ml)	61	56
Carbohydrates (mg/ml)	139	132
Fat (mg/ml)	25	28
Sodium (mg/ml)	0.928	1.068
Potassium (mg/ml)	2.068	1.400
Osmolarity (mOsm/mg H_2O)	650	375
Immune-enhancing components	—	L-Arginine, ω-chain fatty acids, RNA fragments

Comparison of IED versus CD. Summary of composition differences between the control diet (CD, Boost; Novartis) and the immune-enhancing diet (IED, Impact; Novartis). Major differences include the presence of immune-enhancing components in the IED and the hyperosmolarity and increased potassium in the CD.

Statistical Analysis

Data are expressed as mean ± SEM, and differences between groups were determined by two-way analysis of variance (ANOVA). The null hypothesis was rejected a priori at $P < 0.05$. When differences were found using ANOVA, the post hoc Tukey-Kramer honestly significant different multiple range test was performed.

RESULTS

There were no significant differences in animal body weights found among the four groups of animals (Fig. 2). Some of the IED animals developed diarrhea during days 2 and 3 of feeding compared with no diarrhea in CD animals. We previously found that chronic diarrhea developed in all animals fed an IED diet during day 3 of feeding.[19] All animals had free access to water ad libitum and exhibited positive weight gain during the protocol. No differences in mean arterial blood pressure were observed between the four groups. As has been well described in the past, our chronic sepsis model resulted in a sustained tachycardia in both the IED and CD groups compared with saline controls. Similarly, septic animals had significantly increased spleen weights compared with saline controls (Fig. 2). Both tachycardia and increased spleen weights suggest a state of chronic inflammation produced by the sepsis model used.

Chronic feeding of CD and IED in animals given inoculums of bacteria or control injections of saline produced different patterns of cytokine expression in the small intestine. Figure 3 shows the levels of IL-1β, IL-5, IL-6, and TNF-α measured in the distal third of the small intestine, while Figure 4 shows the levels of IL-10 and IFN-γ in the distal, middle,

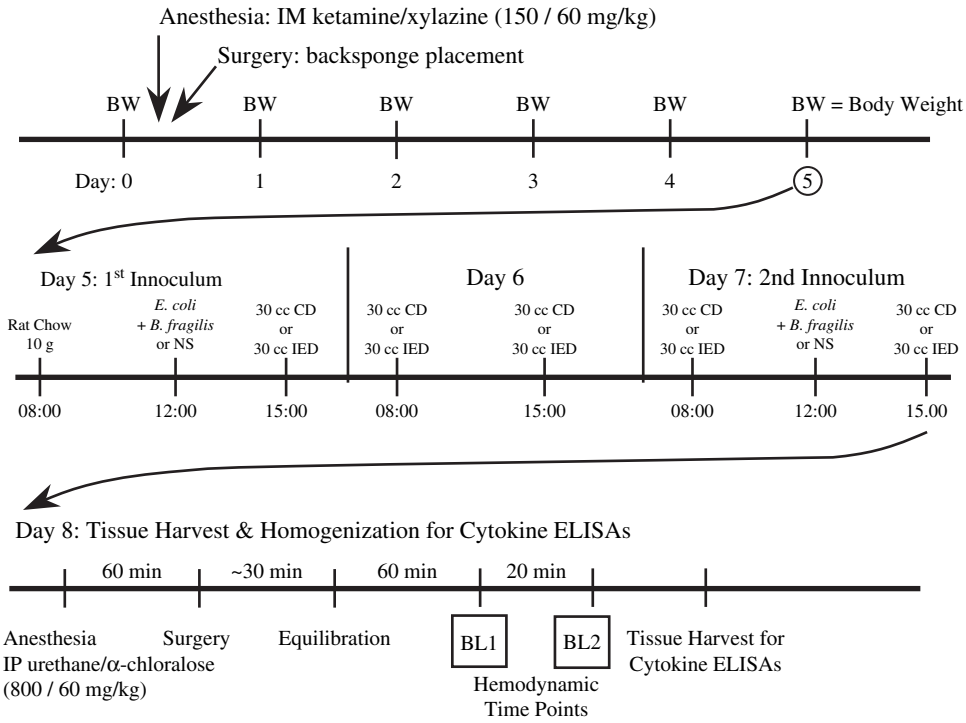

Fig. 1. Schematic of the protocol followed. *E. coli* = *Escherichia coli*, 10^9 CFU in 1 ml normal saline (NS); *B. fragilis* = *Bacteroides fragilis*, 10^9 CFU in 1 ml NS; BW = body weight measurement; CD = control diet, Boost, Novartis; IED = immune-enhancing diet, Impact, Novartis; IM = intramuscular; IP = intraperitoneal; BL = baseline.

and proximal thirds of the small intestine. IED saline decreased levels of IL-1β, IL-5, IL-6, and TNF-α in ileal segments when compared with CD-fed saline animals. IED saline significantly increased tissue levels of IL-10 in all three small intestine segments (Fig. 4, *A*) compared with CD saline. Conversely, IED saline IFN-γ tissue levels are decreased in all three segments versus CD saline (Fig. 4, *B*). IED sepsis increased IL-5 and IL-6 but remained unchanged in IL-1β and TNF-α compared with CD sepsis. IFN-γ levels remained unchanged in proximal and middle thirds; however, IED sepsis decreased IFN-γ in the distal third compared with CD sepsis. IL-10 levels were unchanged in proximal, but decreased in middle and distal, IED sepsis.

Sepsis and saline produced another set of distinct cytokine patterns in each individual diet. IED and CD saline IL-1β tissue levels remained unchanged compared with IED and CD sepsis (Fig. 3). Sepsis increased IL-5 and IL-6 in IED but significantly decreased tissue levels in CD-fed animals (Fig. 3, *C* and *D*). TNF-α levels remained unchanged by sepsis in CD animals and increased in IED-ed animals (Fig. 3, *B*). IL-10 levels in all three intestinal segments in CD animals remained unchanged by sepsis, with all three tissue levels being significantly lower in IED animals (Fig. 4, *A*). Conversely (Fig. 4, *B*) IFN-γ levels were significantly increased in IED saline versus IED sepsis in all tissue. IFN-γ levels remained unchanged by sepsis in CD-fed animals.

DISCUSSION

In a recent editorial opinion, Singer and Cohen[25] stated that despite advances in inflammation research, the application of these findings is complex, including with regard to the appropriate use of immunonutrition. The metabolic response to stress requires a physiologic insult to initiate cytokine release (IL-1β, IL-2, IFN-γ, and TNF-α), to activate cellular immunity, and promote the proinflammatory systemic inflammatory response syndrome (SIRS).[26] Simultaneous with upregulation is feedback inhibition by the inflammatory process to release anti-inflammatory cytokines (IL-4, IL-10, and IL-13). The pattern of cytokine secretion that predominates will determine immune competence to respond to trauma, illness, or surgery. This process is been called the mixed antagonist response syndrome (MARS).[27]

Recent studies focused on the ability of specific immune-enhancing nutrients to stimulate immunity

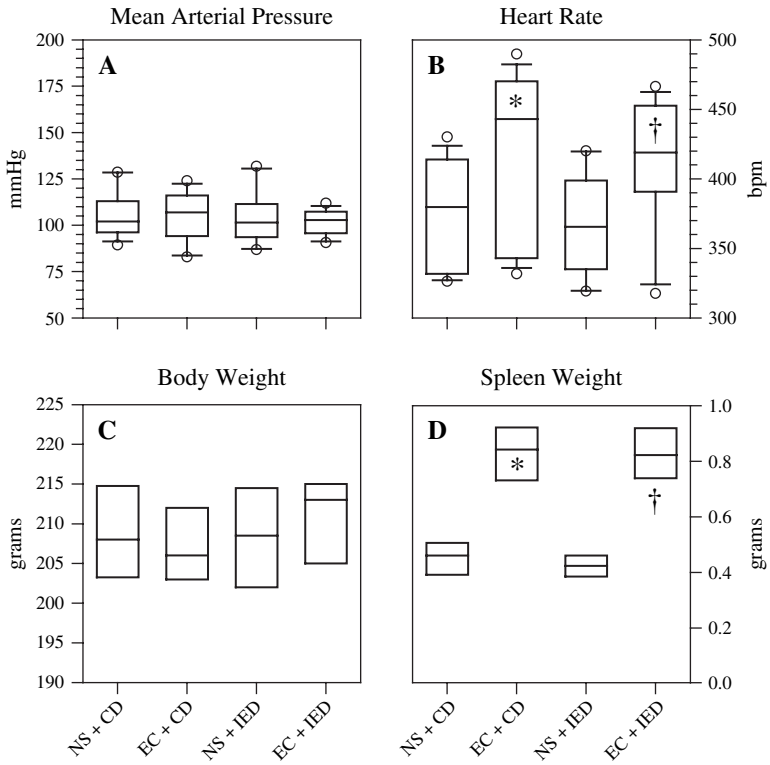

Fig. 2. (**A**) Mean arterial blood pressure, (**B**) Heart rate, (**C**) Body weight, and (**D**) Spleen weight in septic (EC) or control (NS) rats after 72 hours of feeding with either control diet (CD) or immune-enhancing diet (IED). *$P < 0.05$ versus corresponding saline control group.

to promote healing.[28,29] Nutrients that possess immune-enhancing properties include L-arginine, glutamine, taurine, ω-3 fatty acids from fish oil, messenger RNA fragments, antioxidant vitamins (vitamin A, vitamin E, and vitamin C), and trace minerals (zinc, selenium, and copper).[30] The use of immune-enhancing nutrients alone or in combination in immune-enhancing enteral diets (IEDs) has stimulated debate and research to elucidate the proper role of these IEDs in the treatment of critically ill patients. Suggested roles of IEDs range from use in acute illness or trauma to use in chronic debilitating illnesses such as inflammatory bowel disease and rheumatoid arthritis. Meta-analyses[14–16,29] assess the current literature with conclusions ranging from endorsements of IEDs to suggestions that IEDs might promote morbidity and mortality in some disease conditions and/or patient populations. These findings are not surprising. We[19] and others[25] have pointed out that the constant flux of immune cells and mediators results in a dynamic state that responds differently to stimuli depending on the stage of the disease process, the combination of specific immune-enhancing nutrients administered to various patient populations, and the dosage, timing, and route of the IED administration.

The response of the gut to physiologic insult is complex.[31] Mucosal immunity, inflammation, and tolerance to bacterial pathogens require $CD4^+$ T-lymphocytes (including both Th1 and Th2 subsets), $CD8^+$ cytotoxic T cells, and other T-cell subsets (T regulatory cells).[32,33] B-lymphocytes are also required and undergo commitment through $\mu \rightarrow \alpha$ class switching.[34] Interactions between B cells and $CD4^+$ T cells induce plasma cells to stimulate polymeric IgA production.[35] Mucosal immunity also depends on antigen-presenting cells ($CD4^+$ T cells, $CD8^+$ T cells, and epithelial cells) to produce cytokines to regulate the overall immune process as outlined above. The mix of cytokines produced in large measure determines the response of the system to shift toward humoral or cellular immunity. Naïve $CD4^+$ T regulatory cells, which have never encountered antigen, can differentiate into activated (effector) cells or memory cells.[32,33] The mucosal migration patterns of these three T regulatory cell subsets (naïve, effector, and memory) along with B cells form the basis of gut mucosal immunity. In the presence of foreign peptide antigens, naïve $CD4^+$ T regulatory cells and antigen-presenting cells become differentiated to effector $CD4^+$ T regulatory cells and, depending on the overriding cytokine milieu,

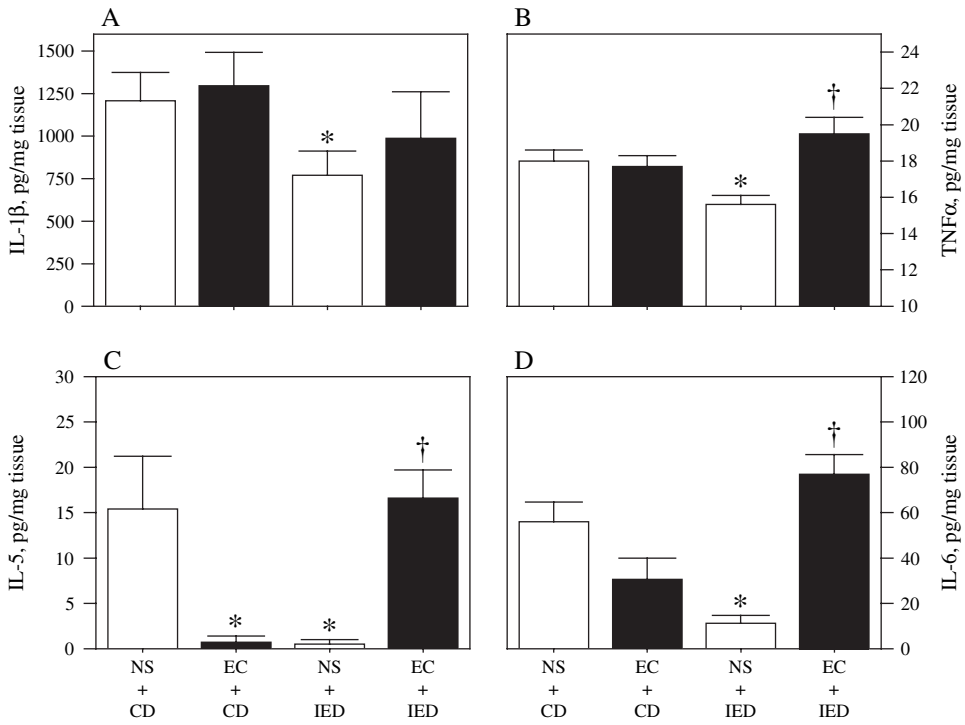

Fig. 3. (**A**) Interleukin-1β (IL-1β) protein levels expressed as picograms per milligram of tissue in the last third of the small intestine in the septic (EC) versus control (NS) groups fed either CD or IED. (**B**) IL-5 protein levels in the last third of the small intestine demonstrating decreased IL-5 in septic rats compared with saline control and decreased IL-5 in IED-fed rats compared with CD-fed saline rats. IED feeding in septic rats restored IL-5 to control levels. (**C**) IL-6 protein levels in the last third of the small intestine demonstrating a similar pattern to that observed for IL-5. Again, IED reversed the effects of sepsis on IL-6 level. (**D**) TNF-α protein levels in the distal third of the small intestine. IED decreased TNF expression in the saline groups while increasing TNF expression in the septic animals. *$P < 0.05$ versus NS + CD group. †$P < 0.05$ versus NS + IED.

promote either the cell-mediated Th1 immunity or the humoral-mediated mucosal antibody Th2 response.[32,33] Thus, the cytokine environment of the mucosa-associated lymphoid tissue (MALT) reservoirs of Th cells determines the induction of the cell subsets to stimulate humoral or cellular processes. Iwasaka and Noguchi[36] have shown that in human patients with severe sepsis, Th2 cell–mediated processes predominate (humoral immunity), which they suggest might lead to immunosuppression in these patients.

Furthermore, mucosal IgA is regulated by Th1 versus Th2 cell subsets.[37] As already noted, $CD4^+$ T helper cells are required for the mucosal IgA response to protein-based antigens. Th2 cells are more efficient at regulating this process, and significant cross-regulation exists between the two pathways. Th1 cells produce IFN-γ, which facilitates isotype switching from IgM to IgG_{2a} and inhibits IL-4-induced isotype switching, to inhibit Th2 cell proliferation.[34] Conversely, Th2 cells produce IL-4 despite enhanced, ongoing Th1 responses to inhibit Th1 cytokine secretion.[38] The presence of IL-12 promotes the continuation of the Th2 response.[39] In the present study, we focused on the pattern of cytokine protein levels in the tissue to assess the functional response to septic shock of the terminal ileum, the site of most GALT in the gastrointestinal system. In order to characterize the GALT response to sepsis and IED, studies are needed to analyze the subclass response of IgG, IgE, and IgA in this model.

In the present study, we describe the gut cytokine response after chronic feeding with IED or CD in septic versus saline control rats. These data demonstrate a decrease in proinflammatory cytokines (IL-1β, IL-6, IFN-γ, and TNF-α) in animals fed IED compared with CD and an increase in the anti-inflammatory cytokine IL-10. The cytokines measured in this study have multiple effects.[40] In the gut, IL-1β from macrophages activates the vascular endothelium and lymphocytes and increases the proliferation of effector cells, with the end result of producing fever and increasing IL-6. IL-6 from macrophages stimulates lymphocyte activation and increases

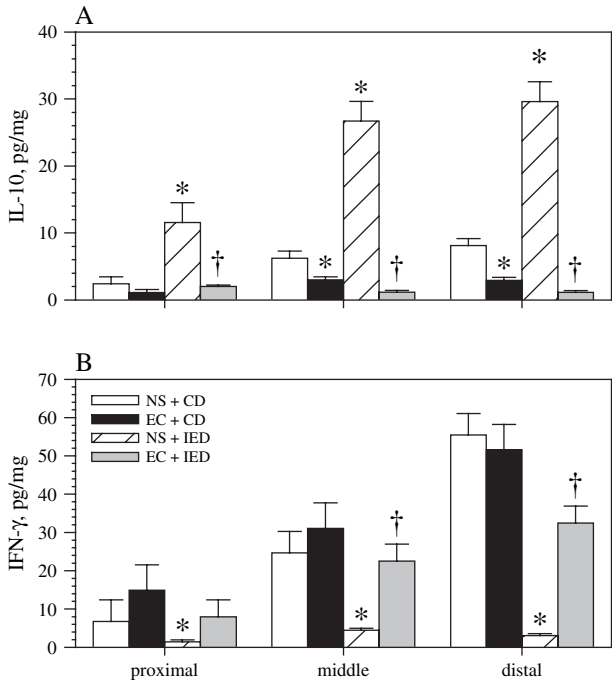

Fig. 4. (**A**) IL-10 protein levels in the small intestine of rats. IED stimulates IL-10 production in all three segments of the small intestine compared with CD-fed rats, but sepsis reverses this finding. (**B**) IFN-γ protein levels suggesting that IED protects IFN-γ production in septic rats in the terminal ileum (distal third). *$P < 0.05$ versus NS + CD group. †$P < 0.05$ versus NS + IED.

antibody production, which also promotes fever and induces acute-phase protein reactants. TNF-α from macrophages and Th1, some Th2, and cytotoxic T cells activate and induce nitric oxide production (inducible nitric oxide synthase) and activate the vascular endothelium. Overall, these three cytokines activate complement opsonization, promote phagocytosis, decrease viral and bacterial replication (through increased body temperature), increase antigen processing, and facilitate the adaptive immune response to antigen. IL-5 from Th2 cells promotes differentiation of B cells and production of IgA as well as eosinophil growth and differentiation from hematopoietic bone marrow stem cells. Th2 cells also produce IL-10, which promotes switching to MHC class II in B cells, inhibits Th1 cells, inhibits cytokine release from macrophages, and stimulates mast cell growth from hematopoietic cells. Th1 and cytotoxic T cells produce IFN-γ to activate macrophages and promote MHC class I and class II in macrophages and somatic cells as well as promoting B-cell differentiation and IgG_{2a} synthesis, inhibiting T cell growth, activating natural killer cells, and promoting other antiviral processes. In our study, sepsis decreased IL-10 and IFN-γ in the IED-fed rats compared with the saline control IED-fed rats and also reversed IL-5 and IL-6 expression in the terminal ileum, suggesting that IED promotes IgA class switching in the terminal ileum, presumably at the site of MALT.

CONCLUSIONS

IEDs have received significant interest in recent years, including the 1995 study of Bower et al,[41] which reported that in septic patients who received adequate doses of Impact, an IED, the IED imparted equal safety, decreased rate of infectious complications, and reduced length of hospital stay compared with standard liquid diets. In our study, we examined the small intestinal production of proinflammatory (IL-1β, IL-6, IFN-γ, and TNF-α) versus anti-inflammatory (IL-10) cytokines in septic rats chronically fed either IED containing L-arginine, mRNA fragments, and ω-3 fatty acids or an isonitrogenous, isocaloric control diet lacking immune-enhancing nutrients. We found that septic rats fed IED for 72 hours after the onset of sepsis had decreased GI production of proinflammatory cytokines, increased anti-inflammatory cytokines, and enhanced IgA class switching in B cells via increased IL-5. Thus, the cytokine milieu of the IED-fed septic rats promoted gut mucosal humoral immunity and immunoglobulin production via a predominantly Th2 pathway.

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