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Generation of Pro-inflammatory and Anti-inflammatory Cytokines in the Gut in Zymosan-induced Peritonitis

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ABSTRACT

In major systemic inflammation such as severe peritonitis, various pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, play important roles in the development of multiple organ dysfunction syndrome (MODS). The purpose of this study was to investigate the outflow of pro-inflammatory and anti-inflammatory cytokines from the efferent mesenteric lymphatic vessels under peritonitis. Mesenteric lymph samples were collected from adult male rats at 2, 4, 6, 8 and 10 hr after an intraperitoneal injection of zymosan at a dosage of 0.1 mg/g (non-lethal dose) or 0.5 mg/g (lethal dose). Blood samples were obtained at 10 hr after zymosan administration. The amounts of drained TNF-α and IL-6 in the lymph peaked at 2–4 hr and 4–8 hr after zymosan administration, respectively. The amounts of drained IL-10 in the lymph gradually increased until 10 hr. The amounts of drained TNF-α and IL-10 in the mesenteric lymph were significantly correlated with the dosage of zymosan.

In conclusion, under intraperitoneal inflammation, pro-inflammatory cytokines (TNF-α and IL-6) increased in the mesenteric lymph and were drained into circulation. IL-10, one of the anti-inflammatory cytokines, also increased in the mesenteric lymph after several hours' delay and its increase was remarkable in severe inflammations. These findings suggested that the gut might be one of the pro-inflammatory and anti-inflammatory cytokine-generating organs under peritonitis. The lymph-drained amounts of each cytokine under peritonitis are considered to differ with the time or severity of inflammation, which may cause different conditions in patients due to the imbalance of pro-inflammatory and anti-inflammatory cytokines.

Key words: *TNF-α, IL-6, IL-10, Zymosan-induced peritonitis*

In patients with severe infections, multiple organ dysfunction syndrome (MODS) is a common cause of death. Although the pathophysiology of MODS is likely to be due to multiple factors, various pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6), play important roles in the pathogenesis of septic shock and sepsis-related MODS. In these conditions, several organs may produce cytokines and the gut is considered to be one of the major cytokine generating organs, since the gut-associated lymphoid cells or gut-associated lymphatic tissue (GALT) comprises approximately two-thirds of the lymphoid cells of the body and is therefore constantly exposed to potential stimuli[15,21].

Goris et al[12,13] originally described a rodent model of MODS induced by the intraperitoneal injection of sterile zymosan. Zymosan, a cell wall preparation derived from the yeast *Saccharomyces cerevisiae*, is a potent activator of macrophages and other mononuclear cells. *In vivo* injection of zymosan increases circulating concentrations of various metabolism mediators. When this nonbacterial and nonendotoxic agent is injected intraperitoneally, it produces an intense inflammatory response that results in functional and structural changes in remote organs, such as the liver, intestine, lung and kidneys[4,13,14,24,26]. Most rodents with 0.5 mg/g zymosan administration were reported to die of severe inflammation (lethal dose) and those with 0.1 mg/g (non-lethal dose) were not[13,17]. Thus, the different doses of injected zymosan result in the different magnitudes of intraperitoneal inflammation in the rodent model.

Several studies have already demonstrated cytokine generation by intraperitoneal injection of zymosan[12,13,17]. Using this model, Mainous et al[17]

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reported that the gut may be capable of producing pro-inflammatory cytokines such as IL-6 and TNF-α in response to an inflammatory stimulus, even in the absence of portal or systemic spread of bacteria. However, under such inflammation, the relationship of both pro-inflammatory and anti-inflammatory cytokine generations by the gut remains unknown. In the present study, we chose to focus on interleukin-10 (IL-10) as a representative cytokine among the several anti-inflammatory cytokines. IL-10 is a potent nonspecific anti-inflammatory cytokine. IL-10 is a powerful anti-inflammatory cytokine, down-regulating the production of TNF-α, IL-1β and interferon-γ, stimulating the production of IL-4, suppressing the production of Th1 helper cells, and inhibiting neutrophil function[2,3,6,9,11,18,22,27,28]. IL-10, in modulating the inflammatory response, is expressed in animal sepsis models[10] and in human sepsis[19]. Previous observations have demonstrated a protective role for IL-10 in animal models of the septic inflammatory response[20,23,25]. Ferrer et al[8] showed that late IL-10 administration reduced morbidity and mortality in an intraperitoneal zymosan shock model in mice. In the murine cecal ligation-and-puncture (CLP) model, Kato et al[16] also showed that IL-10 administration reduced mortality from severe peritonitis in mice. Thus, IL-10 is important for regulating the host response under these severe inflammations.

The exact purpose of this study is to evaluate the generation of pro-inflammatory and anti-inflammatory cytokines from the gut under intraperitoneal inflammation. To achieve this, we investigated these cytokines in the efferent mesenteric lymph using the zymosan-induced peritonitis model.

MATERIALS AND METHODS

Animals

Specific pathogen-free Wistar male rats (Japan SLC, Inc. Shizuoka, Japan) weighting 260–400 g were used. They were housed under barrier-sustained conditions and maintained in accordance with the recommendations of the Guide for Care and Use of Laboratory Animals. This study was carried out after permission from the Committee of Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine.

Experimental design

To determine the time course and magnitude of cytokine release, the efferent mesenteric lymphatic vessel was cannulated in a sterile fashion in 27 rats. To increase mesenteric lymph for identification of the mesenteric lymphatic vessels, ninety minutes before the operation, olive oil (6 ml/kg) was administered into the stomach using a steel cannula. These animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg) and a laparotomy was performed. The efferent mesenteric lymphatic vessel was cannulated using a sterile polyethylene tube, SP 10 (Natume Co., Tokyo. Japan), which was brought out through a stab wound in the right flank. A sterile gastrostomy tube (Central venous catheter 18G, Arrow Co. Tokyo, Japan) was then placed in the greater curvature of the stomach through the stab wound in the left lower quadrant of the abdomen and the top of this tube was passed through the pylorus into the duodenum. Then, an intraperitoneal injection of zymosan was performed at a dosage of 0.1 mg/g (non-lethal dose, n=9), or 0.5 mg/g (lethal dose, n=9), respectively. In the present study, we defined rats with 0.1 mg/g administrations as moderate peritonitis models and those with 0.5 mg/g administrations as severe peritonitis models. Rats that received an intraperitoneal injection of saline solution were used as a control group (n=9). The laparotomy incision was then closed with 3–0 braided silk. The animals were placed on a rubber mat under anesthesia with an intramuscular injection of pentobarbital sodium. A constant infusion of 5% glucose solution was begun via the gastrostomy tube at a rate of 10 ml/kg/hr with use of an infusion pump (TOP-5200, TOP Co.). Lymph was collected into a glass tube that contained EDTA-2Na in an ice bath. Lymph samples, collected at 2, 4, 6, 8 and 10 hr after zymosan challenge, were measured for IL-6, TNF-α and IL-10 concentrations. The amounts of each drained cytokine in the efferent mesenteric lymph were defined as the product, which was multiplied by each cytokine concentration by collected lymph volume at each time point. In our preliminary experiments on rats with the lethal dose of zymosan, sufficient amounts of drained lymph to assay cytokines could not be collected 10 hr after administration. Thus, in the present study, we investigated these rats until 10 hr after administration. At 10 hr after zymosan challenge, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (80 mg/kg), and then aortic blood samples were obtained for the assay of the same cytokines.

Cytokine assays

Lymph and serum samples were stored at –80°C prior to assay. Each cytokine concentration was measured with a double sandwich enzyme-linked immunosorbent assay technique (ELISA kits: Cytoscreen™, Rat IL-6, Rat TNF-α and Rat IL-10, BioSource International, Inc. Camarillo, California). An antibody specific for each rat cytokine has been coated onto the wells of the microtiter strips provided. Samples, including standards of known rat cytokine content, control specimens, and unknowns, are pipetted into these wells. During the first incubation, each cytokine antigen binds to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody at a second site. After removal of

excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of rat cytokines present in the original specimen.

Statistical methods

Data are presented as means ± standard error of the mean (SEM). Comparisons between groups were analyzed using the Bonferroni/Dunn test or analysis of variance. Values at $p < 0.05$ were considered to be statistically significant.

RESULTS

Effect of zymosan on the amounts of drained IL-6, TNF-α and IL-10 in the mesenteric lymph

As shown in Fig. 1, the amounts of drained IL-6 were significantly elevated in the mesenteric lymph between 4 and 10 hr and peaked at 4–6 hr (lethal dose of zymosan, 0.5 mg/g) or 6–8 hr (non-lethal dose of zymosan, 0.1 mg/g). There were no significant differences in cytokine release between the rats challenged with lethal and non-lethal doses of zymosan at any time point studied.

As shown in Fig. 2, in the mesenteric lymph, the amounts of drained TNF-α were significantly elevated at 2–4 hr in rats with the lethal dose of zymosan (0.5 mg/g) but not elevated in those with the non-lethal dose (0.1 mg/g). There was a significant difference between the rats challenged with lethal and non-lethal doses of zymosan at 2–4 hr and 4–6 hr. The amounts of drained TNF-α in the mesenteric lymph appear to correlate with the magnitude of the inflammatory insult. Interestingly, the amounts of drained TNF-α at 8–10 hr significantly decreased in rats with the lethal dose of zymosan (0.5 mg/g).

As shown in Fig. 3, in the rats challenged with the lethal dose of zymosan, the amounts of drained IL-10 in the lymph were already significantly elevated at 2–4 hr, and gradually increased

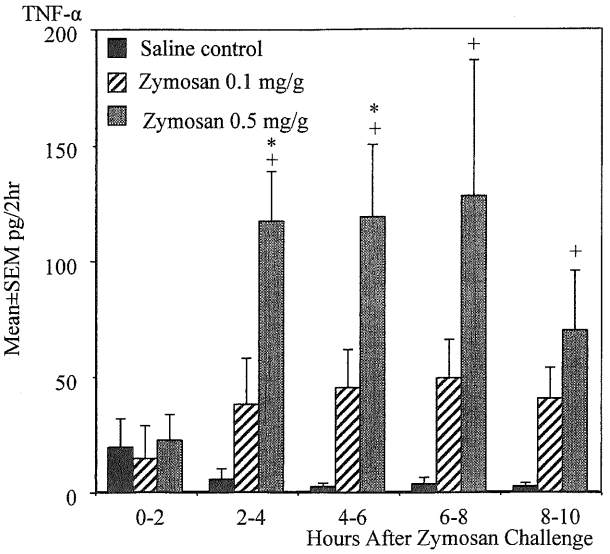

Fig. 2. The amounts of drained TNF-α in the efferent mesenteric lymph after challenge with saline or zymosan.
N = 9 in each group at each time point.
*$p < 0.05$: zymosan 0.5 mg/g vs. zymosan 0.1 mg/g.
+ $p < 0.05$: zymosan 0.5 mg/g vs. saline control.

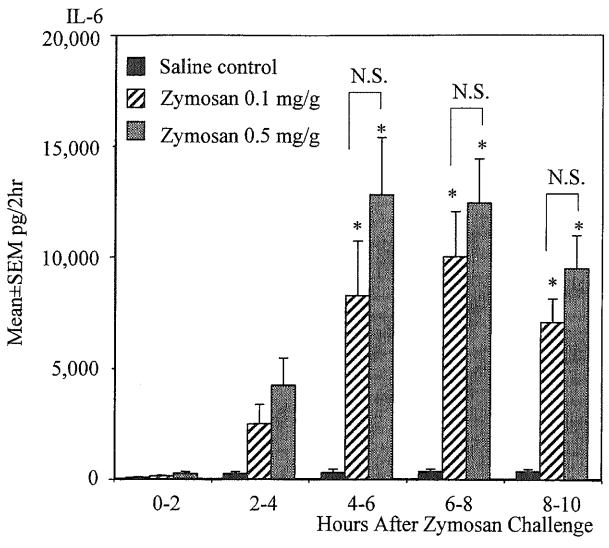

Fig. 1. The amounts of drained IL-6 in the efferent mesenteric lymph after challenge with saline or zymosan.
N = 9 in each group at each time point.
*$p<0.05$: zymosan 0.1 mg/g or zymosan 0.5 mg/g vs. saline control.

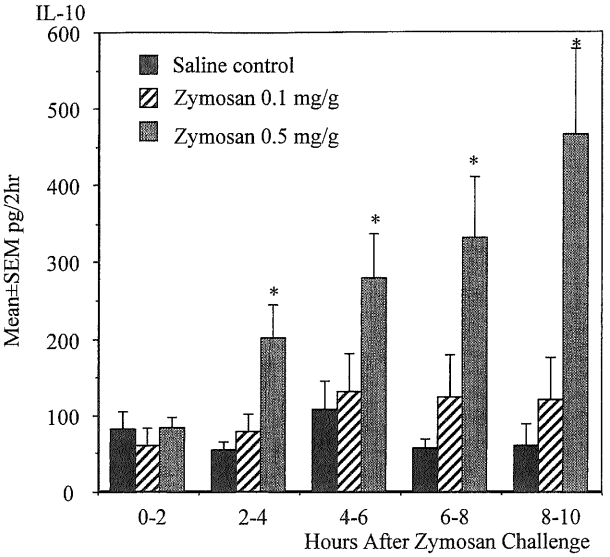

Fig. 3. The amounts of drained IL-10 in the efferent mesenteric lymph after challenge with saline or zymosan.
N = 9 in each group at each time point.
*$p < 0.05$: zymosan 0.5 mg/g vs. zymosan 0.1 mg/g or saline control.

until 8–10 hr. There was a significant difference between the rats challenged with lethal and non-lethal doses of zymosan at 2–4 hr, 4–6 hr, 6–8 hr and 8–10 hr. Thus, the amounts of drained IL-10 in the mesenteric lymph were also correlated with the magnitude of the inflammatory insult, and the peak of IL-10 output in the mesenteric lymph was considered to exist at 10 hr or later.

Comparison of each cytokine concentration between the mesenteric lymph and serum at 10 hr after zymosan challenge

As shown in Fig. 4, in rats at 10 hr after being challenged with non-lethal and lethal doses of zymosan, IL-6 concentration in the efferent mesenteric lymph was significantly greater than the serum concentration (17812.0 ± 1948.1 pg/ml, vs. 1187.3 ± 319.0 pg/ml, $p < 0.0001$, and 23947.2 ± 1773.8 pg/ml, vs. 6602.7 ± 2021.4 pg/ml, $p < 0.0001$, respectively). In rats challenged with the lethal dose of zymosan, IL-10 concentrations in the efferent mesenteric lymph were significantly greater than those in the serum (714.6 ± 124.0 pg/ml, vs. 38.6 ± 5.9 pg/ml, $p < 0.0001$). TNF-α concentrations were high in the mesenteric lymph and serum but there were no significant differences between them. These findings suggest that

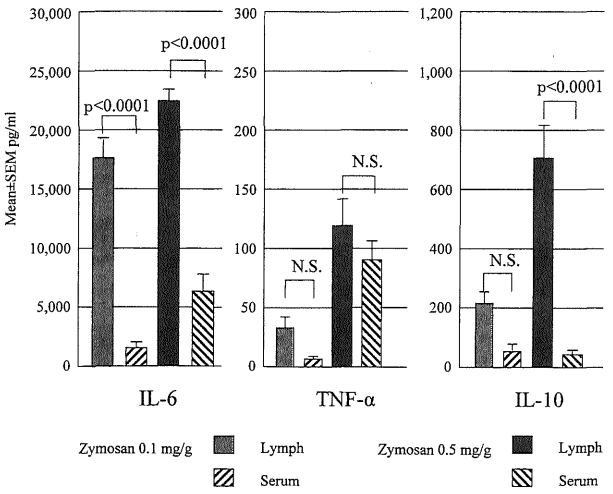

Fig. 4. Comparison of each cytokine concentration between the mesenteric lymph and serum after 10 hours challenge with zymosan at 0.1 mg/g and 0.5 mg/g.
N = 9 in each group. In rats 10 hours after challeng with zymosan at 0.1 mg/g, IL-6 concentration in the efferent mesenteric lymph was significantly greater than the serum concentration. In both TNF-α and IL-10 concentrations there were no significant differences between mesenteric lymph and serum. In rats with 0.5 mg/g zymosan challenge, both IL-6 and IL-10 concentrations in the efferent mesenteric lymph were significantly greater than those in the serum. On the other hand, TNF-α concentration showed no significant difference between mesenteric lymph and serum.

IL-6 and IL-10 were produced in the gut in zymosan-induced peritonitis and their outputs were different due to the severity of the peritonitis. In the gut, IL-6 is released easily during moderate peritonitis, while IL-10 is released only during severe peritonitis.

DISCUSSION

Several studies have revealed that pro-inflammatory cytokines, such as IL-6 and TNF-α, are released from the gut in shock or inflammation[5,17]. The present study revealed that, as well as these pro-inflammatory cytokines, anti-inflammatory cytokines such as IL-10 are also released from the gut under zymosan-induced peritonitis, and the time course and magnitude of their generation in the gut depended on the kind of cytokine and the magnitude of peritonitis.

The time course of IL-6, TNF-α and IL-10 secretions in the efferent mesenteric lymph suggested that TNF-α is generated first, IL-6 is generated next, and the increase of IL-10 occurs later. The magnitudes of these cytokine generations indicated that a significant increase of IL-6 secretion occurred in rats with the non-lethal dose of zymosan, while significant increases of IL-6, TNF-α and IL-10 secretions occurred in rats with the lethal doses. Thus, in moderate peritonitis modeled by a non-lethal dose of zymosan, the gut may mainly generate pro-inflammatory cytokines, which may contribute to the host defense. On the other hand, in severe peritonitis modeled by a lethal dose of zymosan induced peritonitis, pro-inflammatory cytokines including TNF-α and IL-6 increase to higher levels than in moderate peritonitis, which may induce the generation of IL-10 significantly to regulate the functions of these pro-inflammatory cytokines. In these severe inflammations, hypercytokinemia, named as SIRS (systemic inflammatory response syndrome), is considered to occur easily. Then, anti-inflammatory cytokines such as IL-10 are also induced in the gut to regulate the functions of the pro-inflammatory cytokines. Since the peaks of these pro-inflammatory cytokines are earlier than those of the anti-inflammatory cytokines, hypercytokinemia of anti-inflammatory cytokines may occur after the peaks of pro-inflammatory cytokine generation. This condition is considered as CARS (compensatory anti-inflammatory response syndrome) proposed by Bone[1], which induces suppression of the immune response and susceptibility of organ failure. The gut-release of anti-inflammatory cytokines followed the high-dose induction of pro-inflammatory cytokines only in severe peritonitis suggests that CARS may occur only in severe infections and follow the phase of hypercytokinemia of pro-inflammatory cytokines (severe SIRS). Thus, in the pathophysiology of severe infections, the imbalance of pro-inflammatory and

anti-inflammatory cytokines may contribute to the various conditions such as SIRS and CARS observed in patients.

A recent observation was that late IL-10 administration reduces morbidity and mortality in the intraperitoneal zymosan shock model in mice[8]. Early administration of IL-10, immediately following the initial insult, showed only a non-significant trend toward improved survival from the acute inflammatory response, but failed to influence the later development of organ dysfunction. In the murine CLP model, Kato et al[16] showed that exogenous IL-10 could protect animals in septic states from lethal processes, but the timing of IL-10 administration was very important because they could not show any effects when it was administered before or soon after induction of septic peritonitis. It is well known that the early pro-inflammatory cytokines, such as TNF-α and IL-1β, are needed for normal host defense against such septic states. Early administration of IL-10 might have blocked the induction of TNF-α and IL-1β and would not be beneficial in hosts with sepsis. This assumption is in accordance with the finding that anti-TNF antibody pretreatment cannot prevent lethality in the CLP model[7]. These observations also suggest that pro-inflammatory cytokines such as TNF-α and IL-1β play a major role during the early phase while anti-inflammatory cytokines such as IL-10 play a major role later.

The presence of cytokines in the efferent mesenteric lymph is not conclusive evidence for the gut being the only source of these cytokines. Mainous et al[17] reported that lymph originating from the gut first passes through the mesenteric lymph node complex, prior to entering the efferent mesenteric lymphatic vessel. The lymphoreticular cells of the mesenteric lymph node complex may generate cytokines and cytokines generated by the peritoneal macrophages might also enter the mesenteric lymphatic vessels. In this zymosan intraperitoneal injection model, it is difficult to determine the relative contributions of the GALT, the lymphoreticular cells of the mesenteric lymph node complex and the peritoneal macrophage to the each cytokine level in the efferent mesenteric lymph. Another potential source of cytokines appearing in the mesenteric lymph is via gut clearance of cytokines present in the systemic circulation. In the present study, however, the IL-6 and IL-10 concentrations in mesenteric lymph were significantly higher than in the serum of rats challenged with zymosan at 0.5 mg/g, supporting, at least, the impression that the lymphatic cells of the GALT, the mesenteric lymph node complex and peritoneal macrophage are major sources of IL-6 and IL-10. However, there was no significant difference in TNF-α concentrations. Although there is a possibility of another potential source of TNF-α, it seems natural that TNF-α generation in the gut occurred at an earlier phase and was already diminished at 10 hr after zymosan administration.

The present study showed that the gut is capable of generating not only pro-inflammatory cytokines but also anti-inflammatory cytokines under peritonitis. In severe peritonitis, each cytokine is at the peak of production at a different time. The imbalance between pro-inflammatory and anti-inflammatory cytokines may be very important in molding the various conditions in patients with severe peritonitis.

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