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原　　著

Enteric Lipopolysaccharide Raises Plasma IL-6 Levels in the Hepatoportal Vein During Non-Inflammatory Stress in the Rat

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Abstract It has long been known that plasma interleukin (IL)-6 levels elevate during non-inflammatory, physico/psychological stresses such as immobilization (IMB) and electric foot shock (FS). We previously demonstrated that an IMB-induced rise in plasma IL-6 in the rat was caused, at least partly, by an increased production of IL-6 in hepatic reticulo-endothelial cells which were induced by enteric flora-derived lipopolysccharide (LPS). This study investigated whether such enteric flora-derived LPS may produce IL-6 also in the mesentery and the mesenteric lymphoid nodes (MLN) before it reaches the liver. We found a rise in the IL-6 levels in the hepatoportal vein (PV) within 30 min during FS, while the IL-6 levels in the jugular vein showed a smaller and delayed rise with slower recovery. Plasma IL-6 levels near the exit of the hepatic vein in the inferior vena cava was highest at both control and stressed conditions, compared with those in the PV and any other extra-hepatic circulation. The stress-induced IL-6 elevation in the PV was abolished by an *in vivo* neutralization of LPS with continuous infusion of polymyxin B. Furthermore, the amount of LPS as assessed by its bioactivity increased rapidly in the mesentery, the MLN and the liver within 15min after the initiation of FS. Finally, fluorescent dye-labeled LPS infused into the lumen of the ileum was found in the extra-intestinal tissues and systemic vein, and FS increased the optical density in them. The findings suggest that lymphoid tissues in the gut associated lymphoid organs are continuously exposed to LPS at the basal condition, and that FS facilitates the LPS/bacterial translocation across the intestinal wall and thereby increases the production of IL-6 before LPS reaches the liver.

Key words : interleukin-6, lipopolysaccharide, enteric bacterial flora, non-inflammatory stress, bacterial translocation

Introduction

Interleukin-6 (IL-6) is a pro-inflammatory cytokine, which plays a critical role in host defense by its pleiotropic activities such as antibody production[6)], synthesis of acute phase proteins[17)], stimulation of the hypothalamic-pituitary-adrenocortical axis[9)15)23)], modulation of pain and fever[2)26)]. IL-6 is released not only during infection and inflammation but also during non-inflammatory stress such as immobilization (IMB) and exposure to an open field[14)24)36)]. We have demonstrated that the IMB-induced rise in plasma IL-6 in the rat is inhibited by partial hepatectomy, but not by splenectomy, hypophysectomy or adrenalectomy. This suggests that the liver is the primary source organs for the increase in blood IL-6 during IMB[24)]. In support of this, IL-6

mRNA, as measured by competitive RT-PCR, increased in the liver of mice in response to IMB for one hour[13)24)]. Both an *in situ* hybridization and an immunohistochemistry revealed an increase in IL-6 mRNA and IL-6 respectively in the hepatic Kupffer cells and sinusoidal endothelial cells of rats after one hour IMB stress[25)]. Furthermore, the IMB-induced elevation of plasma IL-6 levels was completely abolished by an *in vivo* neutralization of circulating lipopolysaccharide (LPS) with polymyxin B or endotoxin neutralizing protein[25)]. This indicates that endogenous LPS is responsible for the IMB-induced IL-6 production. In fact, FS and IMB stress increased the concentration of LPS in the PV in rats, which was higher than that observed in the jugular vein[32)33)34)]. The non-inflammatory stress-induced LPS is most likely to be derived from the enteric bacterial flora, because we found that fluorescein isothiocyanate (FITC)-labeled LPS injected into the intestinal lumen was detected in greater numbers of Kupffer cells and sinusoidal endothelial cells in the liver of IMB-stressed rats. Furthermore, about 80% of FITC-positive cells were stained with an anti-IL-6 antibody[26)]. These findings, taken together, suggest that the IMB stress enhances the translocation of LPS from the intestinal lumen and such LPS stimulates the reticuloendothelial system in the liver to produce IL-6, thereby increasing the plasma IL-6 levels in the general circulation.

However, a possibility remains that the enteric flora-derived LPS may also act on the gut associated lymphoid tissues (GALT) to synthesize IL-6 before it reaches the liver. In the present study, we investigated whether IL-6 was produced in the mesentery and the mesenteric lymphoid nodes (MLN) using electric foot shock (FS) stress model in rats and, if so, whether the response was mediated by LPS derived from the enteric flora.

Materials and Methods

Subjects

Adult male wistar rats weighing 300 ~400g were housed two per cage in a room maintained at 23 °C with artificial illumination from 07 : 00 to 19 : 00. Food and water were provided ad *libitum*.

Intravascular cannulation.

Under anesthesia with pentobarbital sodium (50~75mg/kg, i.p.) or Ketamine (100mg/kg, i.p.), rats received either one of the following combinations of intravascular cannulation, i.e. (1) the hepatoportal vein (PV) and the jugular vein (JV) at the root of the inferior vena cava, (2) the JV and the inferior vena cava near the branch of hepatic vein (IVC-HV), where the tip of tubing reached by being advanced by about 10cm from the right femoral vein, and (3) the JV and the inferior vena cava peripheral to the hepatic vein (Below IVC-HV), where the tip of tubing reached by being advanced by about 7cm from the right femoral vein.

For the PV cannulation, a polyethylene tube (i.d. 0.5mm×o.d. 0.8mm) filled with physiological saline was inserted into the main trunk of the PV via the gastroduodenal vein and fixed with ligatures and a tissue-adhesive glue. The tube was mounted on the back of animals, and connected to a disposable sheavel kit (DS-10, BRC, Nagoya, Japan) with a protective wire spring (HS-10, BRC, Nagoya, Japan). To avoid the coagulation in the tubing, sterilized physiological saline was continuously infused (12ml/day) using an infusion pump.

For the cannulation of the JV and the IVC, a silicone tube (i.d. 0.5mm and o.d.

1.0mm) filled with sterilized physiological saline containing heparin (50U/ml, Sigma) were inserted into the JV, IVC-HV and Below IVC-HV, as described above. The blood taken from the JV and the IVC-HV were considered to represent the mixed venous blood and the hepatic venous blood, respectively. These tubings were mounted on the back of animals, and reached the outside of the home cage so that the blood sample could be taken without restraint. After surgery, the rats received a prophylactic injection of penicillin G (5,000U, i.p.). They were then returned to the home cages and housed individually. During a post-surgical recovery period of at least 6 days, all the rats were placed in a foot shock cage (MTB-001, TOYO SANGYO CO., Tokyo, Japan, 25cm long×25cm wide×30cm deep) without giving shock for 3 hours daily to habituate them to the experimental environment.

The positions of all tubings were examined after the experiments. If the tips of them were incorrectly positioned, the data from such animals were discarded.

Electrical foot shock (FS)

As a non-inflammatory stress, electric foot shock (FS, 1mA in current, 1sec in duration, and 11 times/min) was delivered for 10min. On the experimental day, the animals were placed in the experimental cage at least one hour without FS. The blood samples (200μl) for the measurement of plasma levels of IL-6 were taken from the indwelling tubes at scheduled time interval. After each blood sampling, the same amount of physiological saline solution was injected to compensate the blood loss. The blood volume taken from one animal did not exceed 10% of the whole blood.

Measurement of plasma IL-6 concentration

Plasma IL-6 levels were measured by a bioassay using B9 cell, which is the IL-6 dependent murine hybridoma subclone cell. B9 cells were maintained in RPMI 1640 with 25mM HEPES containing 10% heat-inactivated (56 °C, 30min) fetal bovine serum and a 1% antibiotic-antimycotic solution in the presence of 20 hybridoma growth units/ml recombinant murine IL-6 (Boehringer Mannheim Biochemica, Germany). A2μl plasma sample was placed in a single well of a 96 well plate contained 198μl culture medium without IL-6, and was serially diluted to 8-fold the original concentration of the first well. In addition, each plate had a standard diluted line containing the recombinant murine IL-6, in which the concentration in the first well was 5pg/ml. 2-4×10^4/ml washed B9 cells in culture medium were added to each well (100μl), and the cells were incubated in 5% CO_2 at 37 °C for 72 hour. Bioactivity of IL-6 in samples was measured fluorometrically using Alamar Blue, a fluorometric growth indicator obtained from Alamar Bio-Sciences Inc (Sacrament, CA, USA). We added 20μl of Alamar Blue to all tested wells, and again incubated all plates for 8 hours in 5% CO_2 at 37 °C. Fluorescence was measured with Fluoroskan Ascent FL (LABOSYSTEM OY, Helsinki, Finland) with an excitation of IL-6 in original samples was 1pg/ml. Specificity of B9 cell bioassay for bioactivity of IL-6 was well-established *in vitro* and *in vivo* experiments[12)23)].

In vivo neutralization of circulating LPS

In order to block the action of endogenous LPS, polymyxin B (PMB, SIGMA, 2μg in 1ml of physiological saline) was continuous-

ly infused for 15min starting from 5min before the FS was given through the JV using an infusion pump. IL-6 levels of the PV and the JV in PMB-infused rats were compared with those of saline-infused rats at each scheduled time points.

Measurement of gut-derived fluorescent isothiocyanate (FITC)-labeled LPS in the general circulation and in the liver

In order to determine whether LPS in the intestine actually cross the intestinal wall during FS, we injected LPS originated from *Escherichia coli*, which was labeled with fluorescent isocyanate (FITC-LPS, SIGMA, St. Louis) into the lumen of the ileum and measured its concentration in the jugular vein after FS. The injection was performed through a thin vinyl tube (i.d. 0.25mm× o.d. 0.8mm), which was chronically implanted at 7cm oral side from the end of ileum. It is known that the rate of bacterial translocation from this portion to the extra-intestinal tissues is significantly higher than that in the cecum and ascending colon[5)7)]. The tube was wrapped with serous membrane, and fixed with 5-0 silk thread. The other end of the tubing was mounted on the back skin until experiment. Defecation and change of body weight in cannulated animals were observed for a week after the surgery whether the motility of the gut was maintained.

After a week, FITC-labeled LPS at 1mg/5ml was injected for 5min. Blood samples were taken serially (0, 15, 30min) from the jugular vein. The plasma samples prepared by centrifugation (500G, at 4 °C for 10min) were diluted by an equivalent volume of 20% glycerin solution containing 1 mM EDTA and 0.01% aprotinin (SIGMA) and stored in the refrigerator (−20 °C) overnight. The optical density (OD) in samples were measured by Fluoroskan Ascent FL (LABOSYSTEMS OY, Helsinki, Finland) with an excitation at 485nm and an emission at 538nm. The OD value means the FITC specific fluorescent counts.

In addition, we took the liver from the same animals 30min after the initiation of FS. The tissue contents of FITC-LPS in the liver with or without FS were also measured.

Measurement of bioactive LPS levels in tissues

In a separate series of study using an endospecy method, we measured tissue levels of bioactive LPS in the liver, spleen, the mesentery and the mesenteric lymph nodes (MLN) before (0min) and after (30min) the initiation of the FS. Under anesthesia with ketamine (10mg in 0.5ml physiological saline, i.v.), the thoracic cavity was opened and the inferior vena cava was exposed in rats. A physiological saline containing 5mM EDTA and 0.1% aprotinin (5ml) followed by LPS free physiological saline (50ml) (Otsuka, Osaka, Japan) was perfused through the left ventricle of the heart. This perfusion could block the motility of the gut by chelate of the Ca^{2+} ions (EDTA) and degeneration of the bioactive LPS by protease inhibitor (aprotinin), and finally exclude the contamination of the circulating LPS derived from open wound. After washing out the whole blood, we aseptically opened the abdominal cavity using sterilized operation materials. The tissue samples (50～100mg wet volume) of the liver, the spleen, the mesentery and the mesenteric lymph nodes (MLN) were put into the tubes with silica/ceramic matrix (fast RNA tubes with green caps, BIO 101, Inc., CA, USA) containing 400μl of LPS free physiological saline solution at 4 °C. Espe-

cially, the mesentery and the mesenteric lymph nodes were taken from the ileo-cecal region and a region along by the superior mesenteric vein, respectively. In addition, we removed section of the ileum in the rest conditions and measured LPS tissue contents to compare gut barrier systems. Tissue sample were aseptically homogenized using Cell Disruptor (FP120 FastPrep, BIO 101, CA, USA) to get the tissue suspension. After centrifugation at 15000rpm/min for 10min at 4 °C the supernatant were taken for LPS assay or FITC-LPS assay as described previously. The LPS activity was measured by a highly sensitive chromogenic LPS determination system (Endospecy, Seikagaku-kogyo Co., Tokyo, Japan)[28)29)]. The sensitivity of this system was less than 1pg/ml in standard LPS solution. The tissue samples (5μl) were duplicately put into wells in a 96 well LPS-free plate (Toxipet Plate 96F, Seikagaku-kogyo Co.). After adding 20μl of distilled water, plate was incubated at 37 °C for 10min. One hundred μl of main reagent of Endospecy, a mixture of *lymlus* lysate and chromogenic substrate, was added and further incubated at 37 °C for 45min in a incubator installed in a microplate reader (Microplate Reader MR5000, Dynatech Labs, VA, USA). Subsequently, 50μl of 0.04% $NaNO_2$, 50μl of 0.3% ammonium sulfate, and 50μl of 0.07% N-(1-Napphthylo)-ethylenediamine (DIA-MP set, Seikagaku-kogyo Co.) were added into all wells. The absorbance was measured at 550nm against a reference of 630nm. The absorbance curve was obtained serial dilution of the standard LPS solution (Et-2, *E. Coli* O111 : B4, 0.424 EU (176pg)/ml, Seikagaku-kogyo Co.).

Statistical analysis

The data were presented as the mean± SE. We first analyzed all groups by two-way (group and time factor) analysis of variance (ANNOVA). When the p value was <5% in both factors, we checked the significance of the difference between each value at each sampling using *Turky* test (one-way ANOVA) or Student t test.

Results

Foot shock (FS)-induced plasma IL-6 increase in the hepatic portal vein (PV) and in extra-hepatic sites of systemic vein

Plasma IL-6 levels of the PV and different sites in the systemic veins at the basal condition.

The effects of FS stress (10min) on the plasma levels in the following four sites, i.e., the PV, the JV, the inferior vena cava near the exit of hepatic vein (IVC-HV) and the IVC 3cm peripheral to IVC-HV (Below IVC-HV), were studied in "double" cannulated rats (rats cannulated with (1) the JV and the PV, (2) the JV and IVC-HV and (3) the JV and below IVC-HV). The changes in plasma IL-6 levels of the JV before and after application of FS were quite similar, in terms of the magnitude and time course, among the three groups of animals (two-way ANOVA, F=0.580) (Fig. 1 and 2). This ensures the validity of the data on plasma IL-6 levels from different preparations of rats. The mean values of plasma IL-6 in the JV at the basal condition (−30 and 0min) ranged from 36.7±6.1pg/ml to 40.9±6.7pg/kg among the three groups and they did not differ from each other significantly. The comparison of plasma IL-6 levels in the JV, the PV, the IVC-HV and the below IVC-HV at the basal condition revealed that the IL-6 concentration of the IVC-HV (−30min : 77.7±9.6pg/ml, 0min : 76.7±6.2) was always higher than those of

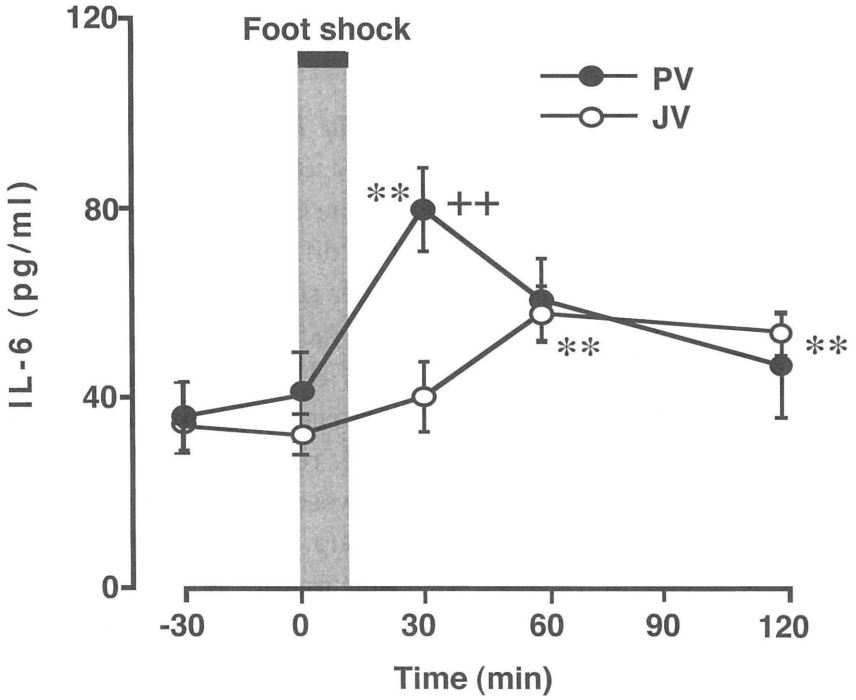

Fig. 1 FS-induced plasma IL-6 increase in the hepatoportal vein (PV) and in the jugular vein (JV).
Horizontal bar indicated the period of FS (0～10min). Each point represents the mean±SE (n=9). $**p<0.01$ compared to the value at −30min. $++p<0.01$ compared between PV and JV.

the remaining three sites ($p<0.01$) (Fig. 1 and 2).

The FS-induced changes in IL-6 levels in the PV and the JV

In the rats (n=9) having cannulation in both the PV and the JV, FS stress for 10min raised the plasma IL-6 levels in both sites (group factor: d.f.=1, F=11.82, $p<0.01$; time factor: d.f.=4, F=10.58, $p<0.01$; Fig. 1). Plasma IL-6 levels in the PV before application of FS (−30min: 38.2±5.8pg/ml and 0min: 42.6±10.1pg/ml) did not differ significantly from those in the JV (−30min: 37.8±3.4pg/ml and 0min: 36.7±6.1pg/ml). FS produced a rapid rise in IL-6 levels in the PV which reached a peak (80.0±8.6pg/ml, $p<0.01$ in comparison with both basal level and the value of JV at the same time) 30min after the beginning of FS and then quickly returned to the basal level. On the other hand, IL-6 levels in the JV after application of FS showed a slower and longer-lasting increase, which attained a statistical significance level at 60min (69.4±4.9pg/ml, $p<0.01$) and remained high even at 120min (60.6±5.7pg/ml, $p<0.01$).

The FS-induced changes in IL-6 levels in the IVC-HV and the JV

In the rats (n=5) having cannulation in the IVC-HV and the JV, FS induced an elevation of plasma IL-6 levels in both sites, but with different magnitudes and time courses (group factor: d.f.=1, F=129.91, $p<0.01$ and time factor: d.f,=4, F=13.35, $p<0.01$, Fig. 2 upper). The IL-6 concentration in the IVC-HV at the basal levels start-

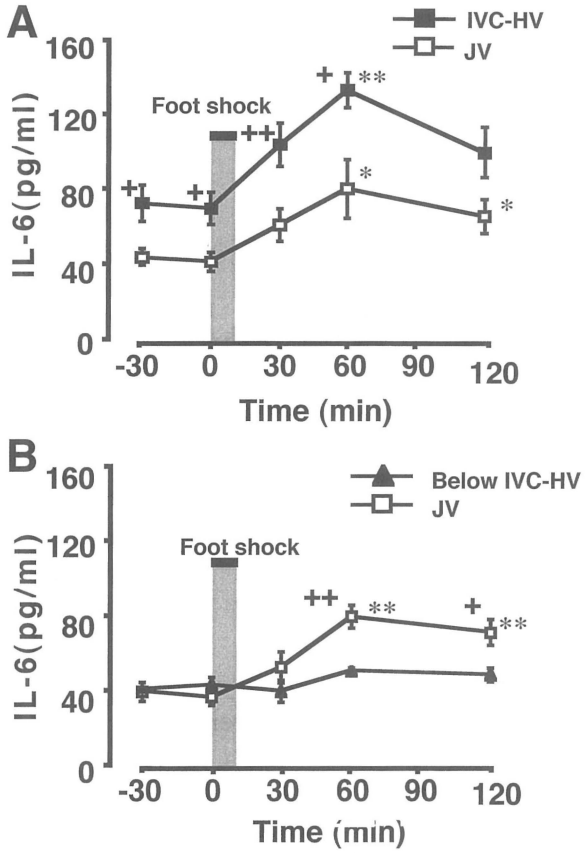

Fig. 2 FS-induced plasma IL-6 increase in the inferior vena cava near the branch of hepatic vein (IVC-HV) (A) or below IVC-HV (B) against juglar vein (JV).
Horizontal bar indicated the period of FS (0～10min). Each point represents the mean±SE (A, n=5; B, n=5). *$p<0.05$, **$p<0.01$, vs. the value at −30min. +$p<0.05$, ++$p<0.01$ compared between IVC-HV or Below IVC-HV and JV.

ed to increase within 30min (113.6±8.8pg/ml, $p<0.01$ vs. the basal levels), reached a peak (137.9±10.1pg/ml, $p<0.01$ vs. the basal levels) and returned rapidly to the basal level within 120min. In contrast, the IL-6 levels of the JV increased slowly, reached a peak (60.1±5.6pg/ml, $p<0.05$ vs. the basal levels) and remained high (58.7±6.2pg/ml, $p<0.05$ vs. the basal levels) even at 120min. The response pattern of IL-6 in the JV to FS is quite similar, in terms of magnitude and time course, to those of the JV in the other types of double cannulated rats.

The FS-induced changes in IL-6 levels in the Below IVC-HV and the JV

The basal levels of the below IVC-HV did not differ significantly from those of the other sites except for the IVC-HV. FS did not increase the IL-6 levels in the below IVC-HV, while those in the JV significantly increased (Fig. 2 lower). FS-induced plasma IL-6 response was significantly different between the below IVC-HV and the JV (group factor: d.f.=1, F=6.14, $p<0.05$ and time factor: d.f.=4, F=6.81, $p<0.01$). The plasma IL-6 levels at 60min (78.5±6.9pg/ml) and 120min (70.2±15.2pg/ml) in the JV were significantly increased than basal levels (−30min: 38.3±4.9pg/ml and 0min: 36.3±4.3pg/ml). Those values at 60min and 120min in the JV were significantly higher than the values in the below IVC-HV (60min: 50.2±1.7pg/ml, 120min: 48.2±3.8pg/ml), respectively.

Inhibition of FS-induced elevation of plasma IL-6 levels by polymyxin B (PMB)

In order to determine whether the FS-induced rise in plasma IL-6 in the PV and the JV are dependent on the circulating LPS, the effects of *in vivo* neutralization of LPS by continuous infusion of PMB before and during FS stress were examined in the rat. In accordance with Fig. 1, the plasma IL-6 levels in the PV and the JV of the saline-treated rats increased with characteristic patterns (Fig. 3). These IL-6 responses were abolished by continuous infusion of PMB at 2μg/ml for 15min starting from 5min before application of FS. The plasma IL-6 levels in the PV and JV treated with saline (PV-saline and JV-saline) were

compared with those in FS groups (group factor: d.f.=3, F=8.32, $p<0.01$ and time factor: d.f.=4, F=9.92, $p<0.01$, Fig. 3). The peak levels of IL-6 in the PV at 30min (94.4±8.6pg/ml) and in the JV at 60min (73.6±12.6pg/ml) were significantly attenuated when treated with PMB (PV-PMB group (30min), 48.9±8.1pg/ml; $p<0.05$ and JV-PMB (60min): 37.7±6.4pg/ml, $p<0.01$). The inhibition of FS-induced increase in plasma IL-6 in the JV were still observed at 120min (JV-saline: 63.1±9.7pg/ml vs. JV- PMB: 27.7±6.4pg/ml, $p<0.05$), but those levels of plasma IL-6 in the PV were not significantly diminished expect at 30min from the initiation of the FS.

FS-induced translocation of enteric FITC-labeled LPS into the systemic circulation and the liver

FS-induced translocation of enteric LPS into the general circulation was confirmed in rats. Animals were infused FITC-LPS (1mg in 5ml saline) into the lumen of the ileum 5min before the initiation of FS. The both values in 0min were not different from those in vehicle-injected animals, which indicated the level of auto-fluorescent for 538nm. Furthermore, the levels of auto-fluorescence in plasma were not significantly changed by FS stress (data not shown). FS increased OD value at 15min (OD: 0.136±0.005 vs. 0min: 0.120±0.004, $p<0.01$). Those levels of OD were also significantly elevated than same time course of non-FS group (15min: 0.118±0.004, $p<0.01$.) (Fig. 4A).

In addition, to confirm the LPS transloca-

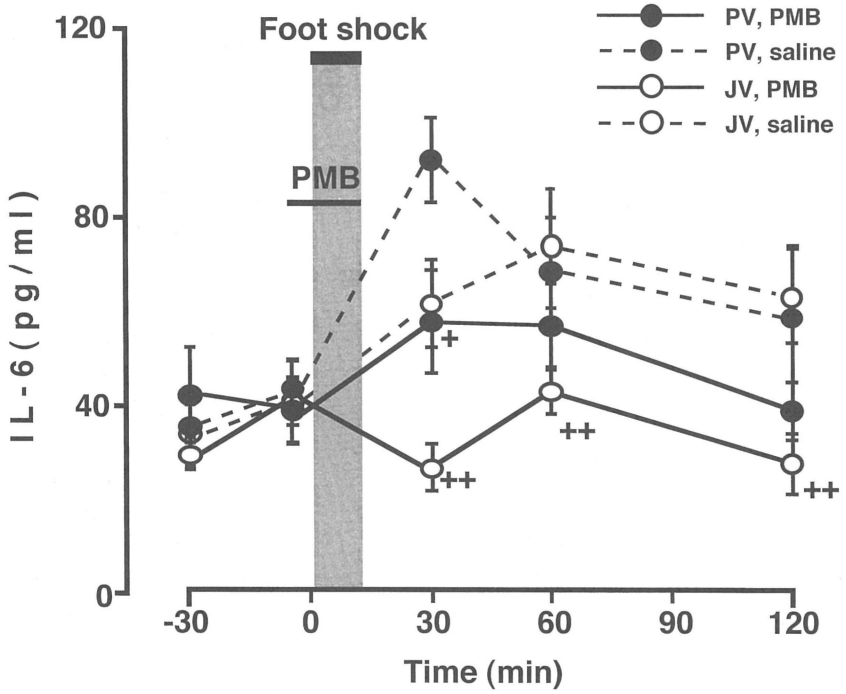

Fig. 3 Effect of continuous infusion of PMB on FS-induced plasma IL-6 increase in the hepatoportal vein (PV) and jugular vein (JV).
Horizontal bar indicated the period of FS (0~10min). Circulating bioactive LPS were neutralized by PMB (2μg in 1 ml saline) before 5min to 10min after initiation of FS. Each point represents the mean±SE (n=6). +$p<0.05$, ++ $p<0.01$ vs. each control group.

tion into the extra-intestinal organ, we detected the FITC-LPS in the liver taken from the same rats with or without FS. OD values in the liver taken from FS-exposed animals were higher than those in non-FS animals (FS (+): 0.432±0.054, FS (-): 0.294±0.043, $p<0.05$ between two groups).

Tissue contents of bioactive LPS in gut-associated organs during FS

To determine the translocation of enteric LPS to the gut-associated organs induced by FS stress, LPS tissue contents in the liver, the spleen, the mesentery and the MLN were measured before and after FS (group factor: d.f.=3, F=30.91, p<0.01 and time factor: d.f.=2, F=39.25, p<0.01, Fig. 5). LPS tissue contents both in the mesentery (344.3±60.1 pg/ml) and the MLN (281.1±16.3 pg/ml) were significantly higher than those in the liver (202.8±26.1 pg/ml vs. the mesentery, p<0.05 and MLN ; p<0.05) and spleen (212.8±26.1pg/ml, vs. the mesentery, p<0.05, MLN, p<0.05). LPS tissue contents in the liver, the mesentery, and the MLN were significantly elevated 15min after the beginning of FS (the liver: 502.1±54.4pg/ml, p<0.01, the mesentery; 722.6±55.9pg/ml, p<0.01 and the MLN, 636.6±49.2pg/ml, p<0.01), but not in the spleen. LPS tissue contents in the liver at 30min (199.21±54.4pg/ml) was lower than 15min levels (502.1±54.4pg/ml, p<0.01), but those levels of mesentery (518.8±69.8pg/ml) and MLN (520.8±454.4pg/ml) were still significantly higher than rest levels of LPS tissue contents, respectively. In additional study, we also measured LPS concentrations of the contents in the terminal ileum (1783±559ng/ml, n=7) in non-stressed condition.

Discussion

The present findings showed that peak

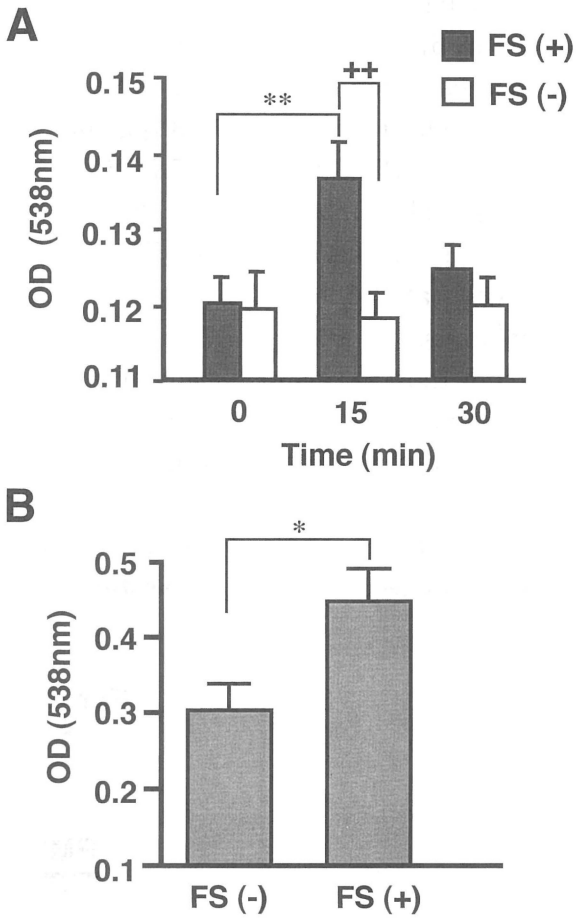

Fig. 4 Translocation of gut-derived FITC-LPS into the general circulation (A) and the liver (B) induced by FS.
A: FITC-LPS (1 mg in 5ml saline for 5min) continuosly infused into the lumen of the ileum 5min before the initiation of FS. FITC specific fluorescent counts (OD, optical density) in plasma taken from the general circulation were measured before and after initiation of FS. Each point represents the mean±SE (n=7). $**p<0.01$, vs. 0min. $++p<0.01$, compared between FS (+) and FS (−). B: The liver was taken from the same animals 30min after FS. OD values in supernatant of the homogenized tissue were measured by fluorescent plate reader (see Materials and Methods). $*p<0.05$, compared between FS (+) and FS (−).

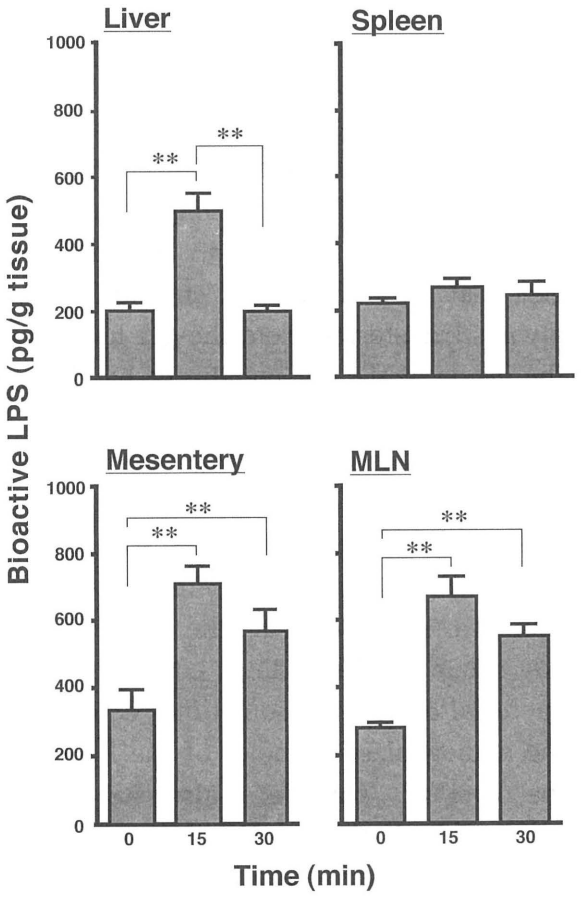

Fig. 5 The tissue content of bioactive LPS in gut-associated organs before and after FS.
The tissue content of bioactive LPS in the liver, the spleen, the mesentery, and the MLN were measured before and after FS. Each point represents the mean±SE (n=7). $^{**}p<0.01$ compared between groups.

levels of plasma IL-6 in the hepatoportal vein (PV) reached a maximum more rapidly, 30-min after the initiation of the FS, than that in the jugular vein (JV), which was observed 60min after the initiation of the FS. Furthermore, a peak level of plasma IL-6 in the PV was higher than that in the JV after the initiation of FS. These results indicated that source organs of plasma IL-6 elevations were different between in the PV and in the systemic circulation. In case of the systemic circulation, several reports were demonstrated that the liver might be a probable source of peripheral plasma IL-6 elevations after psycho-emotional stress[13)23)]. Highest levels of IL-6 tissue contents were existed in the liver than those in other site of the tissues such as brain, lung and heart etc, from the rest conditions in mice[25)], supported peripheral plasma IL-6 levels were released throughout the hepatic veins to the systemic circulation after FS. Indeed our present data showed that plasma IL-6 levels in the inferior vena cava near the hepatic vein (IVC-HV) were significantly higher than those levels in the JV before and after FS except for 120min. But plasma IL-6 levels Below IVC-HV did not show significant change of plasma IL-6 levels before, during and after FS. These results indicated that majority of systemic plasma IL-6 elevations was appeared throughout the liver during and after the FS stress. However, source organs of plasma IL-6 increased in the PV were not well elucidated, yet.

Our previous report showed IL-6 contents in the gut-associated organs as well as the liver were higher than those in the other possible source of plasma IL-6 such as the spleen and the brain in mice[24)]. Furthermore, bioactive LPS in the PV during immobilization (IMB) were increased about 3 times higher than that in the pre-IMB level within 30min in rat[26)]. IMB induce IL-6 mRNA expression in the mesentry and the MLN were increased as in the liver after IMB in mice[13)25)]. Although plasma IL-6 elevation in the JV was abolished by *in vivo* neutralization of circulating LPS using PMB and endotoxin neutralizing protein[25)], in the present study, plasma IL-6 elevation not only in the JV but also in the PV was abolished by continuous injection of PMB (2μg in 1ml for 15min) before and during FS

stress. All data together suggest that rapid translocation of enteric LPS were occurred by mild non-inflammatory stress, and thereby triggered the release and/or production of IL-6 in the gut-associated organs such as the mesentry and the MLN.

In the present study, We intended to confirm whether LPS increase in circulation induced by non-inflammatory stresses was derived from enteric flora, or not. We have traced FITC-labeled LPS, which was injected into the inside of the lumen of the terminal ileum just before FS stress. As expected, optical density specified for the FITC fluorescent color in plasma was increased 15min after the initiation of FS, while it was decreased at 30min. The tissue content in the liver with FS was significantly higher than that without FS at 30min after the initiation of FS. Since it is well known that the liver is the primary organ to exert the circulating LPS into the bile[16], circulating FITC-LPS translocated from the gut lumen might be trapped in the liver 30min after FS. Actually, more than 85% of the dose of ^{51}Cr-labeled LPS intravenously injected was found to be trapped in the liver within 5min after intravenous injection in mice[35)39)].

We also assessed the degree of the LPS translocation in the different way using highly sensitive chromogenic LPS determination system[25]. The sensitivity of this assay method is >1pg/ml, and the value means the potent to activate immune competent cells depending on the lipid A portion of LPS[28)29)]. Our data showed that a difference of the LPS levels between inside of the gut lumen and the tissues in the gut-associated organs was more than 5,000 times. Since the mean LPS levels in the JV and PV are about 2 and 3pg/ml[25)33)34)], a difference between gut-associated tissues and plasma in circulations was about one hundreds times. These differences indicated the function of barrier system against the LPS in the host body. As shown in Fig. 5, basal levels of the bioactive LPS both in the mesentery and MLN were higher than those in the liver and the spleen. The data suggest that some amount of enteric LPS was always transferred from the gut lumen into the inside of the gut membrane barrier system.

Tissue contents of a bioactive LPS were significantly elevated at 15min after the initiation of FS, except for the spleen. It is generally known that a bacterial translocation into the spleen from the gut lumen will not occur in the ordinal condition, while bacterial colony are sometimes detective in the homogenized-samples of the MLN and the liver[9)21)]. Our data also suggest the spleen exist in the outside of the major LPS scavenger system in the gut, mesentery, and the liver. Since stress-related substances such as complements[22], LPS binding proteins (LBP)[30], amyloid A[30], apoprotein A-1[20], etc, which can inactivate the LPS activity, are induced by stresses in the hepatocytes, tissue contents of the bioactive LPS in the liver might be decreased to the basal levels within 30min after FS.

Recently, LPS receptor and its signal transduction to initiate the cytokine cascade are going to be clarified. Toll-like receptor (TLR)-4 is believed to be the specific LPS receptor associated with CD14 on the surface of the cell membrane, which recognized the complex of LPS and LBP[1)3)]. However, Bellezzo et al. demonstrated that LPS-mediated NF-κB activation in Kupffer cells, but not in splenocytes, could be induced independently of CD14[4]. The CD14 independent pathway in the Kupffer cells was activated by very low concentra-

tion of LPS (1ng/ml) within 15min[39]. They speculated that this CD14 independent pathway might be essential for scavenging the gut-derived LPS in normal condition[19)27)31)]. Although IL-6 producing cells in this models is still unclear, we have to think about the contribution of immune-competent cells such as abdominal macrophages, monocytes in the GALT and/or MLN, which might be activated via both CD14-depended and CD14-indipended pathway[18)21)37)]. Further, since LPS-induced IL-6 production in kupffer cells was enhanced by co-incubation with noradrernalin[23)]. LPS-induced IL-6 production could be modified with other stress-related humoral factors or peritoneal macrophage[8)]. The physiological significance of the minimal change of plasma IL-6 levels in the PV will be clarified in the future investigation.

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References

1) Aderem A and Ulevitch RJ: Toll-like receptors in the induction of the innate immune response: nature 406: 782-787, 2000.
2) Akira S, Taga T and Kishimoto T: Interleukin-6 in biology and medicine. Adv Immunol, 54: 1-78, 1993.
3) Akira S, Toll-like receptors and innate immunity: Adv Immunol 78: 1-56, 2001.
4) Bellezzo JM, Britton RS, Bacon BR and Fox ES: LPS mediated NF-$\varkappa$B activation in rats Kupffer cells can be induced independently of CD14. Am J Physiol. 270 (Gastroinstest. Liver physiol. 33): G956-G961, 1996.
5) Berg RD: Bacterial translocation from the gastrointestinal tract. Trends in Microbiology 4: 149-154, 1995.
6) Carl DR and Gauldie J: Role of cytokine in acute phase response. Human cytokine; their role in disease and therapy. Ed. B. B Aggerweal & K. K. Duse, blachwell science, Cambridge 253-269, 1995.
7) Deitch EA, Bridges WM, Ma JW, Ma L, Berg RD and Speciman RD: Obstructed intestin as a reservoir for systemic infection. The Am. J. Surgery. 159: 394-401, 1990.
8) Goodrich ME and Mcgee DW: Regulation of mucosal Bcell immunoglobulin secretion by intestinal epithelial cell-derived cytokine. Cytokine 10 (12): 948-955, 1998.
9) Goujon E, Parnet P, Laye S, Combe C, Kelley KW and Dantzer R: Stress downregulates lipopolysaccharide-induce expression of proimflammatory cytokine in the spleen, pituitary, and brain of mice. Brain, behavior & immunity 9 (4): 292-303, 1995.
10) Jacob AI, Goldberg PK, Bloom BSN, Degenshein GA, and Kozinn PJ: Endotoxin and bacteria in portal blood. Gastroenterology 72: 1268-1270, 1977.
11) Jiang J, Bahrami S, Leichtfieried S, Red IH, Ohlinger, W and Schlag G: Kinetics of endotoxin and tumor necrosis factor appearance in portal and systemic circulation after hemorrhagic shock in rats. Ann of surg. 211 (1): 100-106, 1995.
12) Jifang L, Joan AK, Wendy E, Scalers SLK and Matthew. JK: Role of corticosterone in TNF and IL-6 production in isolated perfused rat liver. Am. J. physiology 268 (Regulatory integrative comp. physiol 37): R699-R706, 1995.
13) Kitamura H, Konno A, Morimatsu M, Jung BD, Kimura K and Saito M: Immobilization stress increases hepatic IL-6 expression in mice. Bio & Biophy Research Communications. 238 (3): 707-711, 1997.
14) Lemay LG, Vander AJ and Kluger MJ: The effects of physiological stress on plasma interleukin-6 activity in rats. Physiology & Behavior. 47 (5): 957-961, 1990.
15) Lencozowski MJP, van Dam AM, Poole S, Larrick JW and Tilders FJH: Role of circulating endotoxin and interleukin-6 in the ACTH and corticosterone response to intraperitoneal LPS: Am. J. physiol. 273

(Regulatory Integrative Comp. Physiol. 42): R1870-R1877, 1997.
16) Mimura Y, Sakisaka S, Harada M, Sata M and Tanikawa K: Role of hepatocyte in direct clearance of lipopolysaccharide in rats. Gastroenterology. 109 (6): 1969-1976, 1995.
17) Mosage H: Cytokine and the hepatic acute phase response. J. Pathology 181: 257-266, 1997.
18) Olofsson P, Nylander G and Olsson P: Endotoxin: route of transport in experimental peritonitis. Am J of Surg. 151: 443-446, 1986.
19) Osman NE, Westrom B and Karlsson B: Serosal but not mucosal endotoxin exposure increases intestinal permeability *in vitro* in rats. Sand J Gastroenterol 33: 1170-1174, 1998.
20) Park CT and Wright SD: Plasma lipopolysaccharide-binding protein is found associated with a particle containing apolipoprotein A-1, phospholipid, and factor H-related protein. J Biol Chem. 26: 271 (30): 18054-18060, 1996.
21) Pirosaka A, Ilona S, Zsuzsanna B and Zsuzanna B: Bacterial translocation after cold stress in young and old mice. Acta Microbiologica Hungarica. 37 (3): 289-294, 1990.
22) Pollack M, Ohl CA, Golenbock DT, Di PF, Wahl LM, Koles, NL, Guelde G and Monks BG: Dual effects of LPS antibodies on cellular uptake of LPS and LPS-induce Proinflammatory functions. J. Immuno. 159 (7): 3519-3530, 1997.
23) Takaki A, Huang QH, Somogyvari-vigh A and Arimura A: Immobilization stress may increase plasma interleukin-6 via central and peripheral catecholamines. Neuroimmunomodulation 1 (6): 335-342, 1994.
24) Takaki A, Hang QH and Arimura A: Is immobilization-induced plasma IL-6 elevation regulated by hepatic innervation? In shimazu (ed), Liver innervation, p213-219, T John libbey London, 1996.
25) Takaki A, Shioda S, Ito K, Maeda M, Kanemitsu Y, Yagi S, Arimura A and Hori T: Non-inflammatory stress and brain-gut-liver-immune axis, In Oomura Y and Hori T (ed): Brain and biodefence. p155-159, Scientific Societies Press Karger Tokyo Japan, 1998.
26) Takaki A, Hori T, Yagi S, Oka K, Kanemitsu Y, Sudo N, Sioda S and Arimura A: Plasma IL-6 and its pyrogenicity during non-inflammatory stress, In Yamashita H (ed): Mechanisms of stress and emotion, p173-177, Eisvier science B. V. 1999.
27) Tamion F, Vincent R, Said L, Maryvonne D, Bonmarchand G, Leroy J, Thuillez C and Lebreton JP: Gut ischemia and mesenteric synthesis of inflammatory cytokines after hemorrhagic or endotoxic shock. Am. J. Physiol. 273 (Gastrointest. Liver physiol. 36): G314-321. 1997.
28) Tamura H, Arimoto Y, Tanaka S, Yoshida M, Obayashi T and Kawai T: Automated kinetic assay for endotoxin and (1-3) β-D-glucan in human blood. Clin Chim Acta 226: 109-112. 1994.
29) Tanaka S, Nakamura T, Morita T and Iwanaga S: Limulus anti-LPS factor: ananticoagulant which inhibits the endotoxin-mediated activation of Limulus coagulation system. Biochem biophys Res Commun. 105: 717-723, 1993.
30) Anita CEV, Mieke AD, Patricia AM, Jan-Willem MG and Wim AB: Lipooplysaccaride binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acuta phase response. Journal of immune, 163: p2792-2798. 1999.
31) Tokyay R, Zeigler ST, Loick HM, Heggers JP, Garza PDI, Traber DL, and Herndon DN: Mesenteric lymphadectomy prevent postburn systemic spread of tranlocated bacteria. Arch Surg. 127: 384-388, 1992.
32) Triger DR, Boyer TD and Levin J: Portal and systemic bacteraemia and endotoxaemia in liver disease. Gut, 19: 935-939, 1978.
33) Yagi S, Takaki A, Ito K, Maeda M and Hori T: Change in IL-6 and LPS levels in the hepatoportal vein during non-inflammatory stress in rats. 27 th Annual Meeting, Society for Neuroscience Abstract 23 (part 1): 713, 1997.
34) Yagi S, Takaki A and Hori T: Interleukin-6 and endotoxin in the hepatoportal vein increase during non-inflammatory stress. Jpn J Physiol, 47 (Suppl 2): S226,

1997.
35) Yamaguchi Y, Yamaguchi K, Babb JL and Gans H: *In vivo* quantiation of the rats livers ability to eliminate endotoxin from portal vein blood. J of the Reticuloendotherial Society 32: 409-422, 1982.
36) Zhou D, Kusnecov AW, Shurin MR, Depaoli M and Rabin BS: Expose to Physical and psychological stressors elevates plasma interleukin-6: Relationship to the activation of hypothalamic-pituitary-adrenal axis. Endocrinology 133 (6): 2523-2530, 1993.
37) Zhu GF, Chancellor-Freeland C, Berm AS, Kage R, Leeman S, Beller DI and Black PH: Endogenous substance P mediates cold water stress-induced increase in interleukin-6 secretion from peritonial macrophages. The Journal of Neuroscience, 16 (11): 3745-3752, 1996.
38) Zhu XI, Zellweger R, Zhu XH, Ayala A and Chaudry IH: Cytokine gene expression in splenic macrophages and Kupffer cells following haemorrange. Cytokine 7 (1): 8-14, 1995.
39) Zlydaszyk JC and Moon RJ: Fate of 51Cr- labeled lipopolysaccharide in tissue culture cells and livers of normal mice. Infect Immun. 14: 100-105, 1976.

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(和文抄録)

ラット非炎症性ストレス時に腸管由来のLPSが肝門脈血中のIL-6濃度を増加させる

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インターロイキン6 (IL-6) 濃度は，炎症のみならず，身体拘束 (IMB)，フットショック (FS) のような非炎症，身体依存的なストレスの時にも上昇する事が知られている．我々はすでに，IMBストレス時に見られる血中IL-6の上昇の少なくとも一部は腸管由来のリポポリサッカライド (LPS) により刺激された肝臓の類洞内皮系細胞により産生される事を報告した．本研究では，このような腸管由来のLPSが肝臓に到達する以前に腸間膜やそのリンパ節ですでにIL-6を産生している可能性を証明する．FSを与えると，肝門脈血中 (PV) のIL-6濃度は30分にてピークを迎えるのに対して，頸静脈を介して右房近傍に留置したチューブより採血したIL-6濃度は遅れて上昇し，その後に基礎値にゆっくり戻る．下大静脈—肝静脈合流部 (VCH-HV) 付近では安静時でもストレス負荷時でも肝門脈血 (PV)，下大静脈—肝静脈合流部下部 (Below VCH-HV)，右房近傍より採血した混合静脈血 (JV) のIL-6濃度より高値を呈する．ストレス負荷にて上昇する肝門脈血中のIL-6濃度はポリミキシンB (PMB) の持続注入にてLPS活性を中和することにより抑制される．LPS濃度はFS負荷後15分で肝臓，腸間膜，そのリンパ節にて増加する．腸管内に持続注入したFITCでラベルしたLPSがFS負荷後に循環血中や腸管外の組織へ移行する．この現象は，腸管周囲のリンパ節周囲組織が平素よりLPSに暴露されており，FS負荷が引き金となりLPSの腸管壁の通過を促進し (LPS/bacterialtranslocation) 肝臓に到達する以前に腸管周囲組織でIL-6の流出に携わる事が示唆された．