Effects of continuous low-dose infusion of lipopolysaccharide on expression of E-selectin and intercellular adhesion molecule-1 messenger RNA and neutrophil accumulation in specific organs in dogs

Yoko Sakaue, DVM; Yoshinori Nezu, DVM, PhD; Satomi Yanagisawa; Shinobu Komori, DVM, PhD; Yasushi Hara, DVM, PhD; Kimimasa Takahashi, DVM, PhD; Masahiro Tagawa, DVM, PhD; Ryo Ogawa, MD, PhD

Objective—To determine the effects of continuous low-dose infusion of lipopolysaccharide (LPS) on the expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) mRNA and neutrophil accumulation in the lungs, liver, spleen, small intestine, and pancreas in dogs.

Animals—11 healthy adult Beagles.

Procedure—Dogs received a continuous infusion of a low dose (10 µg/kg/h, IV) of LPS (*Escherichia coli* 055:B5) or saline (0.9% NaCl) solution (20 mL/kg/h, IV) for 8 hours. Activity levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) and the number of WBCs in circulation were examined before and 1, 2, 4, and 8 hours after the onset of LPS infusion. Expression of E-selectin and ICAM-1 mRNA and the number of neutrophils in each tissue were examined.

Results—After the onset of LPS infusion, serum TNF-α and IL-1β activities transiently increased. Thereafter, IL-6 activity increased, and high IL-6 activity was maintained throughout the experiment. In dogs in the LPS group, expression of E-selectin mRNA increased only in the lungs, and expression of ICAM-1 mRNA increased in the lungs and liver; the number of neutrophils in the tissue increased in the lungs and liver.

Conclusions and Clinical Relevance—Results suggested that expression of E-selectin and ICAM-1 mRNA increased during sepsis, particularly in the lungs and liver, and that this increase was associated with neutrophil accumulation. Hence, inhibiting the activation of endothelial cells in the lung and liver may decrease organ damage caused by accumulated neutrophils and help regulate multiple-organ dysfunction. (*Am J Vet Res* 2005;66:1259–1266)

Sepsis is a major cause of death in critically ill patients.[1–3] Sepsis is characterized by a systemic inflammatory response and multiple-organ dysfunction induced by **lipopolysaccharide (LPS)** and other bacterial products and inflammatory mediators such as cytokines produced by monocytes, macrophages, and endothelial cells in response to LPS.[1,2] These mediators induce microvascular dysfunction by activating endothelial cells and neutrophils; clinical effects include systemic hemodynamic dysfunction and fever.[1,2,4,5]

Endothelial cells activated by LPS express several adhesion molecules including E- and P-selectin and **intercellular adhesion molecule-1 (ICAM-1)**.[6–8] E- and P-selectin bind tetrasaccharide sialyl Lewis X to neutrophils and mediate the slow rolling of neutrophils along inflamed endothelial cells.[6,9] Thereafter, ICAM-1 expressed in endothelial cells binds integrins to neutrophils and mediates the firm adhesion of neutrophils to the endothelial cells.[7,10,11] Finally, chemokines and junctional adhesion molecules induce transendothelial migration of neutrophils into extravascular tissue.[10,12] Lipopolysaccharide also disrupts the endothelial cell function of balancing between metabolic and other demands in microcirculatory blood flow.[2] Endothelial cells activated by LPS induce activation of the extrinsic coagulation pathway triggered by tissue factors and increase production of plasminogen activator inhibitor-1, which suppresses fibrinolytic activity.[5,13]

Thus, in sepsis, organ dysfunction is caused by failure in the regulation of microcirculation, disseminated intravascular coagulation, and the direct cytotoxic effects of neutrophils.[2,5,13] On the basis of these findings, it appears that endothelial cells play a key role in sepsis. Moreover, because endothelial cells are found in organs throughout the body, they should be the targets of treatment for sepsis.

Endothelial cell function in sepsis has been studied in various animal models[14–16]; however, organ-specific endothelial cell function in the early stage of sepsis has not been clarified in dogs. Many experiments have been performed in which bolus infusions of lethal doses of LPS have been administered IV, but use of that technique is problematic in that it does not simulate the conditions of clinical sepsis.[17] Walvatne et al,[18] Tarnoky et al,[19] and Mitaka et al[20] continuously infused LPS at rates of 5, 10, and 15 µg/kg/h, respectively, to dogs. Results of those studies indicate that induction of sepsis by use of continuous infusion of low doses of LPS closely approximates actual clinical conditions and is a suitable technique for the study of sepsis.[17,21]

We hypothesized that organ specificity exists for

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From the Divisions of Veterinary Surgery (Sakaue, Nezu, Komori, Hara, Tagawa) and Veterinary Pathology (Yanagisawa, Takahashi), Nippon Veterinary and Animal Science University, 1-7-1 Kyonan-cho, Musashino-shi, Tokyo, 180-8602, Japan; and the Division of Anesthesiology, Nippon Medical School, 1-5-1 Sendagi, Bunkyo-ku, Tokyo, 113-8603, Japan (Ogawa). Dr. Komori's present address is the Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka-shi, Iwate, 020-8550, Japan.

endothelial cell activation during sepsis. Thus, the purpose of the study reported here was to determine the effects of continuous low-dose infusion of LPS on serum cytokine activity and expression of E-selectin and ICAM-1 mRNA and neutrophil accumulation in the lungs, liver, spleen, small intestine, and pancreas in dogs.

Materials and Methods

Dogs—Ten healthy adult male and female Beagles were used in the study. Dogs were 1 to 2 years old and weighed 8.5 to 11.0 kg. Food was withheld from dogs for 18 hours prior to the study; however, water was available ad libitum. The study was approved by the Committee on Bioethics of Nippon Veterinary and Animal Science University.

Anesthesia and surgical preparation—For anesthesia, a catheter was inserted into the left cephalic vein of each dog for administration of pentobarbital sodium[a] first as a bolus (30 mg/kg, IV), then as a constant low-dose infusion (2 mg/kg/h, IV); small supplementary doses of pentobarbital sodium were administered as required. An endotracheal tube was inserted and connected to a pressure-limited ventilator.[b] Each dog was mechanically ventilated with a **fraction of inspired oxygen (F_{IO_2})** of 1.0. Spontaneous respiration was completely stopped by administration of a bolus of pancuronium bromide[c] (0.05 mg/kg, IV); throughout the experiment, additional doses of pancuronium bromide were administered as required. Minute ventilation was adjusted to maintain normocapnia (P_{aCO_2} of 35 to 40 mm Hg). During surgical preparation, a continuous infusion of physiologic saline (0.9% NaCl) solution[d] (10 mL/kg/h, IV) and 5% dextrose[e] (5 mL/kg/h, IV) was administered to dogs as fluid replacement. Body temperature was maintained between 36.5°C and 37.5°C by use of a heating pad.

A catheter was inserted into the left femoral artery of each dog; catheters were positioned in the abdominal aorta to enable continuous collection of blood samples. A catheter was inserted in each femoral vein to enable infusion of fluids and LPS. After surgical preparation was completed, the flow of 100% oxygen was changed to air for ventilation, and the F_{IO_2} value was decreased to 0.21. After preparation, heart rate, blood pressure, and body temperature were permitted to stabilize during a 60-minute period before baseline blood samples were obtained.

Experimental protocol—Dogs were allocated to 2 groups. Five dogs received a continuous IV infusion (10 µg/kg/h, IV) of a low dose of LPS[f] (*Escherichia coli* 055:B5; LPS group); LPS was dissolved in physiologic saline solution (total volume, 40 mL), whereas the remaining 5 dogs received a continuous IV infusion of only saline solution (40 mL; control group). After baseline blood samples were obtained, the infusion of LPS was initiated. Concurrent with the onset of the LPS infusion, the continuous IV infusion of saline solution that was being administered for fluid replacement was increased from a rate of 10 mL/kg/h to 20 mL/kg/h; this rate was maintained throughout the experiment. In a preliminary study, we determined that this rate did not decrease preload.

Blood samples for serum cytokine (**tumor necrosis factor-α [TNF-α]**, **interleukin-1β [IL-1β]**, and **interleukin-6 [IL-6]**) assays and WBC counts were collected before (baseline) and 1, 2, 4, and 8 hours after the start of LPS infusion. After the last samples were obtained, all dogs were euthanatized by administration of an overdose of pentobarbital sodium, and tissue samples from the lung, liver, small intestine, spleen, and pancreas were immediately collected.

Bioassay for TNF-α activity—Serum TNF-α activity was determined by cytotoxicity bioassay by use of WEHI 164 clone 13 murine fibrosarcoma cells.[g] The cytotoxicity bioassay was performed by use of a minor modification of the method of Eskandari et al.[22] Bioassay is the established method and is characterized by measurement of biological activity. Briefly, 100-µL volumes of serial 2-fold dilutions of serum samples or recombinant human TNF-α[h] in RPMI 1640 medium[i] were added to 96-well flat-bottomed tissue culture plates.[j] Then, 5×10^4 WEHI 164 cells were added to the samples in 100-µL volumes of RPMI 1640 medium containing 1 µg of actinimycin D/mL per well and incubated at 37°C and 5% CO_2 for 20 hours. After incubation, cytotoxicity was measured colorimetrically by use of tetrazolium salt. Briefly, 50 µL of 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide[k] solution (1 mg of solution/mL of RPMI 1640 medium containing 20 µL of phenazine methosulfate[l] [0.383 mg/mL]) was added to each well. After an additional 4 hours of incubation at 37°C and 5% CO_2, color development was measured at 450 nm by use of a microplate reader.[m] Tumor necrosis factor-α activity was determined from the standard curve with recombinant human TNF-α. All determinations were performed in triplicate.

Bioassay for IL-1β activity—Serum IL-1β activity was determined by cytotoxicity bioassay by use of the human melanoma subclone A375S2.[n] The cytotoxicity bioassay was performed by use of a minor modification of the method of Nakai et al.[23] Bioassay is the established method and is characterized by measurement of biological activity. Briefly, 100-µL volumes of serial 2-fold dilutions of serum samples or recombinant human IL-1β[o] in **minimum essential medium[p] (MEM)** were added to 96-well flat-bottomed tissue culture plates.[j] Then, 2×10^3 A375S2 cells were added to the samples in 100-µL volumes of MEM/well and incubated at 37°C and 5% CO_2 for 92 hours. After incubation, cytotoxicity was measured colorimetrically by use of tetrazolium salt. Interleukin-1β activity was determined from the standard curve with recombinant human IL-1β. All determinations were performed in triplicate.

Bioassay for IL-6 activity—Serum IL-6 activity was determined by proliferative bioassay by use of the IL-6–dependent murine hybridoma cell line 7TD1.[q] The proliferative bioassay was performed by use of a minor modification of the method of Van Snick et al.[24] Bioassay is the established method and is characterized by measurement of biological activity. Briefly, 100-µL volumes of serial 2-fold dilutions of serum samples or recombinant human IL-6[r] in RPMI 1640 medium[i] were added to 96-well flat-bottomed tissue culture plates.[j] Then, 5×10^3 7TD1 cells were added to the samples in 100-µL volumes of RPMI 1640 medium/well and incubated at 37°C and 5% CO_2 for 68 hours. After incubation, proliferation was measured colorimetrically by use of tetrazolium salt. Interleukin-6 activity was determined from the standard curve with recombinant human IL-6. All determinations were performed in triplicate.

Quantitative reverse transcriptase polymerase chain reaction assay—Tissue samples ($5 \times 5 \times 5$ mm) collected from the lung, liver, small intestine, spleen, and pancreas were placed in a tissue storage reagent.[s] The tissues were incubated at 4°C for approximately 12 hours, then stored at –80°C before RNA isolation. Total RNA was extracted by use of a kit[t] according to the manufacturer's instructions. The isolated RNA was suspended in ribonuclease-free water,[u] quantified with a spectrophotometer,[v] and adjusted to a concentration of approximately 0.02 µg/µL.

Complementary DNA was synthesized from RNA by use of a **reverse transcriptase (RT)** method.[w] The RT reaction was performed in 50 µL containing 10mM of Tris-HCL (pH,

8.3); 50mM of KCl; 2.5mM of $MgCl_2$; 10mM of 2′-deoxyadenosine 5′-triphosphate, 2′-deoxyguanosine 5′-triphosphate, 2′-deoxythymidine 5′-triphosphate, and 2′-deoxycytidine 5′-triphosphate; ribonuclease inhibitor (2.5 U/μL); murine leukemia virus RT (2.5U/μL); random hexamers (2.5 U/μL); and 7.5 μL of total RNA. The thermal cycling conditions were as follows: 15 minutes at 42°C, 5 minutes at 99°C, and 5 minutes at 5°C. The RT reactions were performed with thermal cycler.[x]

Quantitative real-time RT-polymerase chain reaction assay—To evaluate the level of expression of the target genes, a quantitative RT-**polymerase chain reaction (PCR)** assay was performed with real-time RT-PCR technique by use of a sequence detector.[y] The dog-specific detection probes and primers were produced according to a study by Aihara et al[25] (**Appendix**). Dog-specific detection probes[z] were labeled with a reporter fluorescent dye, FAM(6-carboxyfluorescein), on the 5′ nucleotide and a quenching fluorescent dye, TAMRA(6-carboxy-tetramethyl-rhodamine), on the 3′ nucleotide. The amplification reaction (total, 50 μL) contained 13.5 μL of diethyl pyrocarbonate water, 25 μL of a master mix,[aa] 200 nmol/L (2.25 μL) of sense and antisense primer,[bb] 100 nmol/L (2 μL) of each detection probe, and 5 μL of cDNA. The thermal cycling conditions were 40 cycles of 20 seconds at 94°C for denaturation and 1 minute at 60°C for annealing and extension. Data were analyzed according to the manufacturer's instructions by use of the standard curve method. Briefly, 4 serial dilutions of a standard sample in which expression of target genes was already determined were analyzed and used to construct a standard curve. On the basis of the standard curve, the mRNA concentration of each sample was calculated by its **threshold cycle (CT)** value. The CT values corresponded to the cycle number at which the fluorescent emission monitored in real time reached the threshold, which was set at 10 SD above the mean of baseline emissions calculated from cycles 5 to 15. The expression levels of target genes were evaluated by the ratio of the number of target mRNA to β-actin mRNA because total RNA concentrations from each sample were normalized more certainly by the quantity of β-actin mRNA. A liver tissue sample collected from a dog in which LPS (2 mg/kg, IV bolus) had been infused was used as the standard sample. The dog, which was not in either of the experimental groups, was euthanatized by the administration of an overdose of pentobarbital sodium after 2 hours of LPS infusion. Tissue samples from the liver were immediately collected. All determinations were performed in triplicate, and the mean value of the triplicate runs was used for each determination.

The specimens were fixed in 10% formaldehyde, embedded in paraffin and sectioned, stained with H&E, and mounted on slides. To evaluate the neutrophil accumulation in tissues, the number of neutrophils in 0.059 mm^2 of tissue samples was measured by use of light microscopy (400X magnification). Mean values were determined from 10 randomly chosen microscopic fields for the lung, pancreas, spleen, and small intestine and from 10 portal areas and 20 intralobular areas for the liver. The number of WBCs was measured by use of an electronic cell counter.[cc]

Statistical analyses—All data were expressed as mean ± SEM values. The Mann-Whitney *U* test was used to detect significant differences in the serum cytokine activity, expression of adhesion molecule mRNA, and neutrophil accumulation between groups, and the Friedman test was used to detect significant differences in the serum cytokine activity from baseline measurements at each time within each group of dogs. When the null hypothesis was rejected, a multiple-comparison test was performed by use of the Fisher exact test. For all comparisons, values of $P < 0.05$ were considered significant.

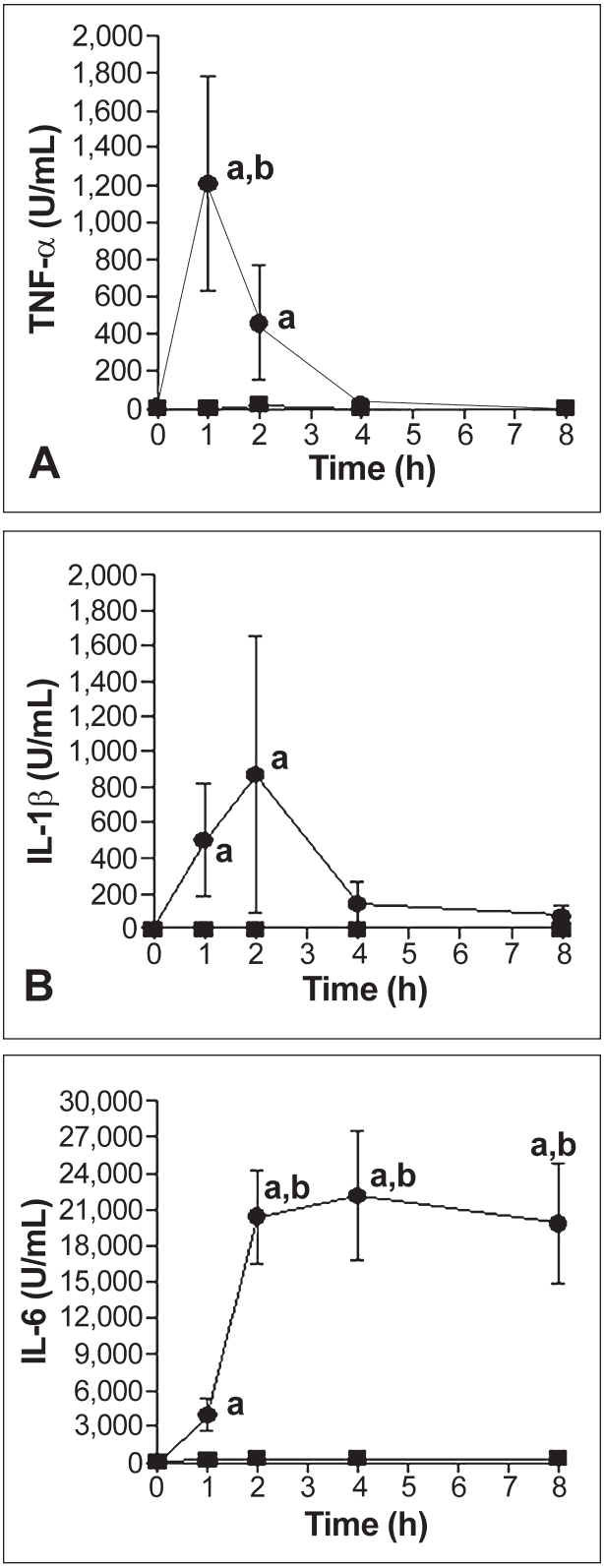

Figure 1—Mean ± SEM values for serum tumor necrosis factor-α activity (TNF-α ; panel A), interleukin-1β activity (IL-1β; panel B), and interleukin-6 activity (IL-6; panel C) in dogs receiving a continuous low-dose (10 μg/kg/h) infusion of lipopolysaccharide (LPS) solution (circles; n = 5) or saline (0.9% NaCl) solution (control group; squares; 5). [a]Significantly ($P < 0.05$) different from the value for the control group. [b]Significantly ($P < 0.05$) different from baseline (time 0) value.

Table 1—Mean ± SEM values for expression of E-selectin and intercellular adhesion molecule-1 (ICAM-1) mRNA in various organs of dogs receiving a continuous infusion of a low dose (10 µg/kg/h, IV) of lipopolysaccharide (LPS; n = 5) solution or saline (0.9% NaCl) solution (control; 5) 8 hours after the start of infusion.

		Organ		
Variable	**Group**	**Small intestine**	**Spleen**	**Pancreas**
E-selectin	LPS	16.13 ± 4.08	1.11 ± 0.37	1.00 ± 0.26
	Control	3.72 ± 2.19	0.88 ± 0.26	0.59 ± 0.15
ICAM-1	LPS	0.14 ± 0.06	0.76 ± 0.15	16.28 ± 0.99
	Control	0.30 ± 0.22	0.52 ± 0.28	7.14 ± 2.30

Results are expressed as the ratio of ICAM-1 or E-selectin mRNA to β-actin mRNA.

Table 2—Mean ± SEM values for the number of neutrophils (cells/0.059 mm^2) in tissues of various organs of dogs receiving a continuous infusion of a low dose (10 µg/kg/h, IV) of LPS (n = 5) solution or saline solution (control; 5) 8 hours after the start of infusion.

		Organ				
Variable	**Group**	**Lung**	**Liver**	**Small intestine**	**Spleen**	**Pancreas**
Neutrophils	LPS	372 ± 63[a]	381 ± 60[a]	73 ± 10	200 ± 58	8 ±1
	Control	163 ± 24	141 ± 24	69 ± 15	192 ± 20	5 ±1

[a]Significantly ($P < 0.05$) different from the value for the control group.

Results

In the LPS group, TNF-α activity increased 1 hour after the start of the LPS infusion (1,204.4 ± 578.8 U/mL), which was significantly different from that in the control group (0.8 ± 0.4 U/mL; $P = 0.009$) and the baseline value (8.8 ± 8.8 U/mL; $P = 0.009$; **Figure 1**). Tumor necrosis factor-α remained high 2 hours after the start of the LPS infusion (457.4 ± 304.6 U/mL; $P = 0.026$), compared with the control group (16.2 ± 15.5 U/mL). This was followed by a rapid decrease such that 8 hours after the onset of the LPS infusion, TNF-α activity was not detected.

In the LPS group, IL-1β activity increased 1 hour after the start of the LPS infusion (494.8 ± 319.9 U/mL), which was significantly ($P = 0.005$) different from the value for the control group (0.0 ± 0.0 U/mL). Interleukin-1β activity peaked 2 hours after the start of LPS infusion (866.4 ± 783.6 U/mL), which was significantly ($P = 0.005$) different from the control group (0.0 ± 0.0 U/mL). This was followed by a rapid decrease (Figure 1).

In the LPS group, IL-6 activity started to increase at 1 hour after the start of the LPS infusion (3,922.0 ± 1,370.4 U/mL vs 199.6 ± 31.8 U/mL for the control group; $P = 0.009$). This was followed by an additional increase; 2 hours after onset of the LPS infusion, IL-6 activity had increased by 20,398.4 ± 3,869.9 U/mL, which was significantly different from that of the control group (286.8 ± 60.5 U/mL; $P = 0.009$) and the baseline value (45.0 ± 13.5 U/mL; $P = 0.001$). In the LPS group, IL-6 activity continued to increase throughout the experiment; 4 hours after the onset of the LPS infusion, IL-6 activity was significantly higher in the LPS group (22,151.2 ± 5,323.4 U/mL) than in the control group (291.9 ± 126.4 U/mL; $P = 0.009$) or at baseline (45.0 ± 13.5 U/mL; $P < 0.001$). Eight hours after the onset of LPS infusion, IL-6 activity was significantly higher in the LPS group (19,841.4 ± 4,945.1 U/mL)

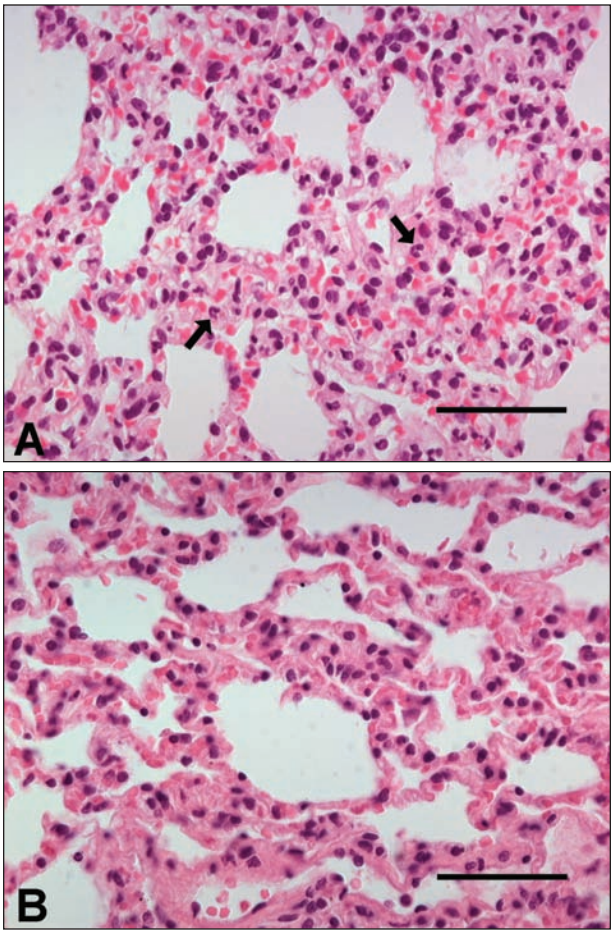

Figure 2—Photomicrographs of sections of lung tissue from a dog that received a continuous low-dose (10 µg/kg/h) infusion of LPS solution (A) or saline solution (control group; B) 8 hours after the start of infusion. Notice accumulation of neutrophils (arrows) in lung tissue from a dog in the LPS group. H&E stain; bar = 50 µm.

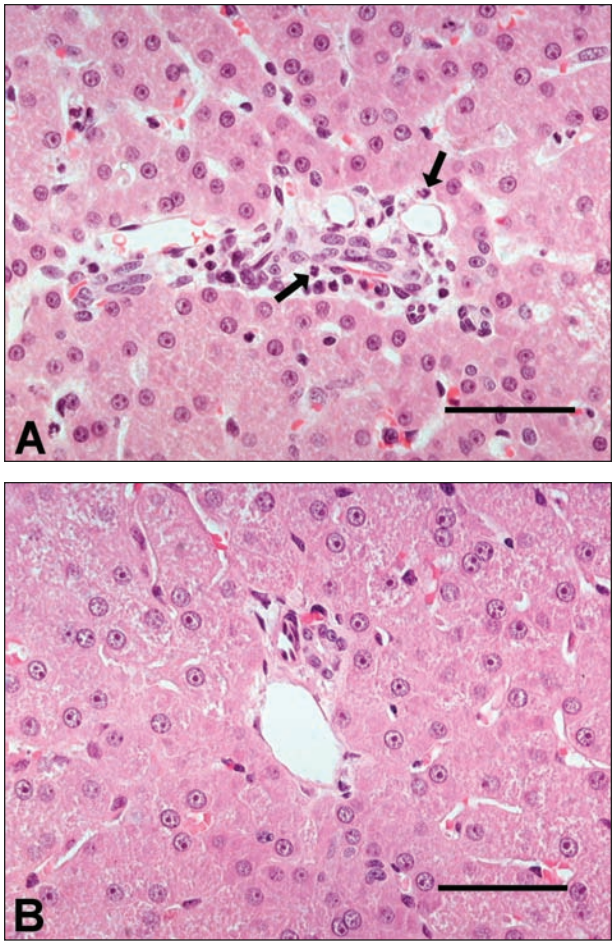

Figure 3—Photomicrographs of sections of liver of a dog that received a continuous low-dose (10 µg/kg/h) infusion of LPS solution (A) or saline solution (control group; B) 8 hours after the start of infusion. Notice the accumulation of neutrophils (arrows) in liver tissue from a dog in the LPS group. H&E stain; bar = 50 µm.

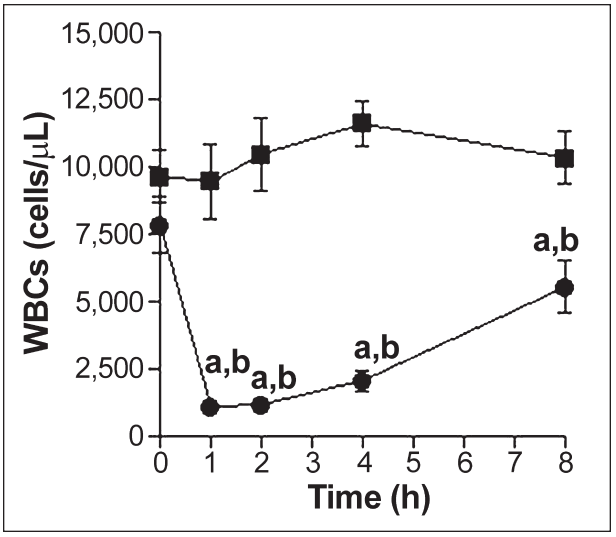

Figure 4—Mean ± SEM number of WBCs in dogs receiving a continuous low-dose (10 µg/kg/h) infusion of LPS solution (circles; n = 5) or saline solution (control group; squares; 5). *See* Figure 1 for remainder of key.

than in the control group (311.6 ± 86.1 U/mL; P = 0.009) and at baseline (45.0 ± 13.5 U/mL; P = 0.001; Figure 1). In the control group, there were no significant changes in serum cytokine activity throughout the experiment.

In the LPS group, the ratio of E-selectin mRNA to β-actin mRNA in the lungs (1.902 ± 0.457) had increased significantly (P = 0.034), compared with the control group (0.69 ± 0.085), after 8 hours of infusion. In the LPS group, the ratio of ICAM-1 mRNA to β-actin mRNA in the lungs (6.78 ± 5.19) had increased significantly (P = 0.009), compared with the control group (0.68 ± 0.16), after 8 hours of infusion.

In the LPS group, the ratio of ICAM-1 mRNA to β-actin mRNA in the liver (2.642 ± 0.52) had increased significantly (P = 0.047), compared with the control group (1.31 ± 0.19). There was no significant change in the ratio of E-selectin mRNA to β-actin mRNA between the LPS and the control groups. There were no significant differences in the expression levels of E-selectin and ICAM-1 mRNA in the small intestine, spleen, or pancreas between the LPS and control groups (**Table 1**).

The number of neutrophils in the lungs (372 ± 62 cells/0.059 mm^2) of dogs in the LPS group was significantly (P = 0.016) increased, compared with the control group (163 ± 24 cells/0.059 mm^2). The number of neutrophils in the liver (381 ± 59 cells/0.059 mm^2) of dogs in the LPS group was significantly (P = 0.009) increased, compared with the control group (141 ± 24 cells/0.059 mm^2; **Table 2**). Large numbers of neutrophils were detected in the alveolar walls of the lung and in the perivascular fibrous capsule and sinusoidal capillary of the liver (**Figures 2 and 3**). There was no significant difference in the number of neutrophils in the small intestine, spleen, and pancreas between the LPS and control groups.

The number of WBCs (1,100 ± 212 WBCs/µL) in dogs in the LPS group decreased significantly 1 hour after the start of LPS infusion, compared with baseline value (7,860 ± 1,038 WBCs/µL; $P < 0.001$) and the control group (9,480 ± 1,396 WBCs/µL; $P < 0.001$; **Figure 4**). The number of WBCs in dogs in the LPS group remained low throughout the experiment.

Discussion

We evaluated adhesion-molecule mRNA expression as an indication of endothelial cell activation and neutrophil accumulation in the lung, liver, small intestine, spleen, and pancreas in a model of sepsis in dogs by use of a continuous infusion of a low dose of LPS. The main finding of the study was that adhesion-molecule mRNA expression and the number of neutrophils were higher in the lung and liver in dogs that received LPS, compared with dogs that did not receive LPS. These results suggested that, during sepsis, endothelial cells in the lung and liver were selectively activated and that this was associated with neutrophil accumulation.

Serum cytokine activity via continuous infusion of a low dose of LPS was similar to that reported in other studies[26–28] in dogs and studies of pigs[21] and sheep.[29] Lipopolysaccharide stimulates macrophages, mono-

cytes, and endothelial cells via toll-like receptor 4, which leads to the production of inflammatory mediators and cytokines.[3,30] It is recognized experimentally and clinically that LPS or other bacterial products induce the rapid release of TNF-α and IL-1β, followed by the release of IL-6 from leukocytes or endothelial cells[4] resulting in a systemic inflammatory reaction.[4,13] In our study, the cytokine reaction was typical, and a systemic inflammatory reaction was induced. Hence, continuous infusion of a low dose of LPS appeared to be a suitable technique for the study of endothelial cell function and neutrophil accumulation during sepsis.

E-selectin is a cell adhesion molecule expressed on stimulated endothelial cells and is a ligand for tetrasaccharide sialyl Lewis X on leukocytes.[6] Expression of E-selectin is regulated by increased transcription after stimulation by LPS or inflammatory cytokines.[7,9,31] In our study, expression of E-selectin mRNA after 8 hours of LPS stimulation had increased only in the lung. Results of other studies[9,31] indicate that the peak time of increase in E-selectin mRNA expression and protein concentration is 3 to 6 hours after stimulation with LPS. Therefore, in our study, it is likely that expression of E-selectin mRNA in most tissues was induced predominantly between 0 and 8 hours after initial LPS stimulation. Results of our study suggested that expression of E-selectin mRNA in the lung was greater or more persistent than that in other tissues. Moreover, E-selectin is expressed only in endothelial cells stimulated by inflammatory mediators,[8] and reportedly E-selectin is expressed in response to LPS, TNF-α, and IL-1β stimulation.[32,33] Thus, in our study, expression of E-selectin mRNA may have been induced in response to LPS or to TNF-α or IL-1β induced by LPS.

Intercellular adhesion molecule-1 is expressed in endothelial cells and serves as a ligand for integrin that mediates binding to leukocytes.[6,7] Increased expression of ICAM-1 mRNA in endothelial cells reportedly begins several hours after various LPS stimuli and continues approximately 24 hours after onset of stimuli.[14,34–37] With regard to continuous expression, ICAM-1 mRNA was expressed in the lung and liver and was not expressed in the small intestine, spleen, or pancreas in this study.

Intercellular adhesion molecule-1 is expressed on endothelial cells,[14,34–37] macrophages,[38] monocytes,[36] and type I alveolar epithelial cells.[39] Therefore, in our study, ICAM-1 mRNA expression was not limited to endothelial cells. However, ICAM-1 has generally been recognized as an indicator of activation of endothelial cells[2,5–7,40]; therefore, it appears that at least part of the increased expression of ICAM-1 mRNA in our study may have been attributable to endothelial cells.

In our study, observations regarding adhesion-molecule mRNA expression suggested that endothelial cells, particularly in the lung and liver, are activated during sepsis. We did not directly evaluate protein expression, and results of 1 study[41] indicate that mRNA expression does not correlate with protein expression because mRNA translation does not occur or the gene product is not detectable. However, results of other studies[31,42] indicate that mRNA expression is associated with protein expression; therefore, the respective proteins may have been expressed in our study.

In the study reported here, the number of neutrophils in tissues was significantly increased in the lung and liver, which also had increased expression of adhesion-molecule mRNA. This finding indicated that adhesion-molecule proteins were likely expressed in our study. Additionally, the decrease in the number of circulating WBCs may indicate that leukocytes extravasate into lung and liver tissues via adhesion molecules. Localization of neutrophils was limited to the alveolar walls of the lung and the perivascular fibrous capsule and sinusoidal capillary of the liver, yet there were no injuries to the structure of the alveoli and hepatocytes. Interestingly, this result indicated organ failure in the early stage of sepsis, unlike the destructive cellular damage by neutrophil migration and extravasation in severe sepsis.

Reportedly,[12,43] endothelial cells in the lung and liver are highly influenced by LPS. Numerous macrophages exist in those organs because of contact with the environment and may be responsible for the induction of cytokine responses.[44,45] Moreover, the microvascular bed in the lung and liver is characterized by low blood pressure,[45] and those organs contain a large number of neutrophils[46,47]; therefore, neutrophils are easily trapped and accumulated. In addition, because the lung has a mechanism for hypoxic pulmonary vasoconstriction,[48] it is subject to ischemia by alveolar hypoventilation or hypoxia during sepsis, and ischemia and reperfusion thereafter may induce a cytokine response. Presently, the gastrointestinal tract is most affected by septic shock, and diarrhea, melena, and ileus are characteristic clinical signs.[49] Results of a previous study[50] using the same model of sepsis in dogs indicated that hepatosplanchnic circulation was disturbed earlier than systemic circulation. Thus, although the dysfunction of the gastrointestinal tract is important clinically, results of the study reported here suggested that activation of endothelial cells and accumulation of neutrophils in sepsis occur more severely in the lung and liver, compared with the gastrointestinal tract, similar to mice[11] and humans.[45]

a. Nembutal injection, Dainihon, Osaka, Japan.
b. ADS 1000, Shin-ei Industries Inc, Saitama, Japan.
c. Mioblock injection, Sankyo, Tokyo, Japan.
d. Isotonic sodium chloride solution, Terumo, Tokyo, Japan.
e. Glucose injection, Terumo, Tokyo, Japan.
f. Lipopolysaccharide, *Escherichia coli* 055:B5, Sigma Chemical Co, St Louis, Mo.
g. WEHI 164 clone 13 murine fibrosarcoma cells, American Type Culture Collection, Manassas, Va.
h. Recombinant human tumor necrosis factor-α, Boehringer Mannheim GmbH, Mannheim, Germany.
i. RPMI 1640 medium, Gibco BRL, Life Technologies Inc, Gaithersburg, Md.
j. Microtest tissue culture plate, Becton-Dickinson, Franklin Lakes, NJ.
k. 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide, Sigma Chemical Co, St Louis, Mo.
l. Phenazine methosulfate, Sigma Chemical Co, St Louis, Mo.
m. Spectra Rainbow Thermo A-5002, Wako, Osaka, Japan.
n. A375S2 cells, American Type Culture Collection, Manassas, Va.
o. Recombinant human interleukin-1β, Boehringer Mannheim GmbH, Mannheim, Germany.

p. Minimum essential medium (MEM), Gibco BRL, Life Technologies Inc, Gaithersburg, Md.
q. 7TD1 cells, Riken cell bank, Ibaraki, Japan.
r. Recombinant human interleukin-6, Boehringer Mannheim GmbH, Mannheim, Germany.
s. RNAlater, Takara-bio, Tokyo, Japan.
t. RNeasy kit, Qiagen KK, Tokyo, Japan.
u. RNase-free water, Gibco BRL, Life Technologies Inc, Gaithersburg, Md.
v. Ultrospec 3000pro, Amersham Biosciences Corp, Piscataway, NJ.
w. RNA PCR kit, Perkin-Elmer Applied Biosystems, Fremont, Calif.
x. GeneAmp PCR System 9600, Perkin-Elmer Applied Biosystems, Fremont, Calif.
y. 7700 Sequence Detection System, Perkin-Elmer Applied Biosystems, Fremont, Calif.
z. Detection probes, Perkin-Elmer Applied Biosystems, Fremont, Calif.
aa. TaqMan Universal Master Mix, Perkin-Elmer Applied Biosystems, Fremont, Calif.
bb. Sense and antisense primers, Sigma Aldrich Co, St Louis, Mo.
cc. Celltac α, Nihon Korden, Tokyo, Japan.

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Appendix—Nucleotide sequences of dog-specific primers and probes for real-time quantitative reverse transcriptase polymerase chain reaction assay for detection of E-selectin, intercellular adhesion molecule (ICAM)-1, and β-actin messenger RNA in dogs receiving a continuous infusion of a low dose (10 μg/kg/h, IV) of lipopolysaccharide solution or saline (0.9% NaCl) solution.

Gene	Sense primer	Probe	Antisense primer
E-selectin	AACGAAGGCTTCCTGATGC	AGCCCAAATTGAATGTACTGCACAAGG	ACAAACTGGGACTTGCTGACT
ICAM-1	AGAGAGGCTGCACTCCACAG	TGGCAGGAGAATGTGACCTTCTATAGCTTC	GCTCACTCAGGGTCAGGTTG
β-actin	CTCCATCATGAAGTGTGACGTT	CAAGGACCTCTATGCCAACACAGTGCT	ATCTCCTTCTGCATCCTGTCAG