Kinetics of endotoxin concentration and tumor necrosis factor-α, interleukin-1β, and interleukin-6 activities in the systemic and portal circulation during small intestinal ischemia and reperfusion in dogs

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Objective—To determine whether small intestinal ischemia and reperfusion induces bacterial translocation and proinflammatory cytokine response in either the systemic or portal circulation in dogs.

Animals—17 healthy adult Beagles.

Procedure—The superior mesenteric artery (SMA) was occluded for 0 (group-3 dogs), 30 (group-1 dogs), or 60 (group-2 dogs) minutes, followed by reperfusion for 180 minutes; serum lactate and endotoxin concentrations and tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 activities in the systemic and portal circulation and intramucosal pH were measured at various time points.

Results—In group-2 dogs, TNF-α activity was found to be significantly increased in the portal circulation, peaking at 60 minutes of reperfusion; TNF-α activity, in the systemic circulation, gradually increased from 60 minutes of reperfusion to the end of the experiment; however, the increase was not significant. In group-1 and -2 dogs, IL-6 activities significantly and gradually increased in the systemic and portal circulation during the reperfusion phase, and the magnitude of these increases was dependent on the duration of the ischemic phase. There were no significant changes in IL-1β activity or endotoxin concentration in any dog group.

Conclusions and Clinical Relevance—Results of the our study indicate that intestinal ischemia and reperfusion leads to significant increases of the circulating TNF-α and IL-6 activities, depending on the duration of the ischemia phase, in the absence of detectable endotoxin in the circulation. This finding suggests that intestinal ischemia and reperfusion induces a systemic proinflammatory cytokine response in dogs. (*Am J Vet Res* 2002;63:1680–1686)

The intestine has been proposed as a central organ in the pathogenesis of **multiple organ dysfunction syndrome (MODS)** in critically ill human patients.[1-3] The intestine is affected by ischemia in the earliest stage of various types of critical illness including shock, trauma, and sepsis.[4-6] Subsequent resuscitation results in reperfusion of the ischemic intestine. Recently, it has been shown that intestinal ischemia and reperfusion not only leads to an acute local intestinal injury, but can also cause distant organ injury and MODS.[1-3] In veterinary clinical practice, however, the intestine has as yet only been recognized as a target organ in critical conditions such as shock, and the concept[3] that the intestine is the principal organ of MODS has not been accepted. However, we believe that the management of critically ill patients that targets care of the intestine is also important in veterinary clinical practice, because several experimental studies[7,8] on dogs have revealed the development of distant organ injury in accordance with intestinal ischemia and reperfusion.

Over the past decade, the translocation of bacteria or their products such as endotoxin from the intestine, now termed bacterial translocation, has received considerable attention in research on the pathogenesis of distant organ injury following intestinal ischemia and reperfusion. More recently, however, attention has been focused on the systemic inflammatory response mediated by chemical mediators, such as proinflammatory cytokines, arachidonic acid metabolites, and platelet-activating factor, as the common pathophysiologic process of the development of intestinal ischemia-reperfusion-induced distant organ injury. Whereas proinflammatory cytokines are believed to play a pivotal role in this inflammatory response and in distant organ injury,[9] relatively few studies have attempted to define whether the proinflammatory cytokine response that accompanies intestinal ischemia and reperfusion is a localized phenomenon or whether other organs such as the liver and lung contribute to the synthesis of proinflammatory cytokines. Also, the exact mechanisms by which intestinal ischemia and reperfusion contribute to the systemic inflammatory response are unknown. In addition, especially in dogs, little information is available on the kinetics of proinflammatory cytokines in the circulation.

We believe that evaluating the temporal and spatial features of the proinflammatory cytokine response can help to elucidate the series of pathophysiologic changes that accompany intestinal ischemia and reper-

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fusion, namely, the intestinal ischemia-reperfusion systemic inflammatory response, MODS. In response to intestinal ischemia and reperfusion, we hypothesized that endotoxin and proinflammatory cytokines in the intestine results in an amplified release of proinflammatory cytokines into the systemic circulation by other distant organs, such as the liver and lung. The purpose of the study presented here was to determine whether small intestinal ischemia and reperfusion induces bacterial translocation and proinflammatory cytokine response in either the systemic or portal circulation in dogs. Experiments were conducted to investigate the kinetics of endotoxin and proinflammatory cytokines in the systemic and portal circulation and the role of intestinal ischemia and reperfusion in endotoxin and proinflammatory cytokine production. The correlation between the duration of the ischemic phase and the magnitude of the endotoxin translocation and proinflammatory cytokine responses in intestinal ischemia and reperfusion in dogs was determined by occlusion of the **superior mesenteric artery (SMA)** for 30 and 60 minutes.

Materials and Methods

Anesthesia and surgical preparation—This study was approved by the Bioethics Guidelines of Nippon Veterinary and Animal Science University. Seventeen healthy adult Beagles of both sexes, weighing 9 to 12 kg, were used. Food was withheld from the dogs for 18 hours prior to the experiment, and water was supplied ad libitum. Anesthesia was induced via inhalation of isoflurane[a] in 100% oxygen. After endotracheal intubation, the endotracheal tube was connected to a pressure-limited ventilator,[b] and the dog was mechanically ventilated with a fractional inspired oxygen value of 1.0. Spontaneous respiration was completely stopped by IV administration of a bolus of pancuronium bromide[c] (0.1 mg/kg), and throughout the experiment additional pancuronium bromide was administered as necessary. Minute ventilation was adjusted to maintain normocarbia ($Paco_2$, 40.0 ± 5.0 mm Hg). Anesthesia was maintained by inhalation of isoflurane in 100% oxygen. During the experiment, dogs received a continuous IV infusion of lactated Ringer's solution[d] (10 mL/kg/h) as fluid replacement. Body temperature was maintained between 36.5 and 37.5°C by use of a heating mat.

The left femoral artery was cannulated with a sterile catheter positioned in the abdominal aorta for arterial blood sample collection. The left cephalic vein was cannulated for fluid and drug infusions. A midline laparotomy was performed. The SMA was identified and isolated from surrounding tissue close to its origin from the aorta. A silastic vessel loop was positioned around the SMA for complete occlusion of the vessel. A splenic vein was cannulated, and a sterile catheter was inserted and positioned with its tip in the portal vein for blood sample collection. Subsequently, a small antimesenteric incision was made in the terminal of the descending part of the duodenum, and a tonometric catheter[e] was inserted and placed in the lumen of the proximal portion of the jejunum. The incision in the duodenum was closed with a purse-string suture, and the tonometric catheter was positioned. After all measurement systems were in place, the abdominal incision was sutured and closed, except for the portion necessary for the experimental procedures. The open area was covered with a sterile, moist gauze pad to prevent evaporation of body fluids. After surgical preparation was completed, 100% oxygen was switched to air gas for ventilation, and the fractional inspired oxygen value was reduced to 0.21. After preparation, a 60-minute period was allowed for all variables to stabilize before obtaining baseline measurements.

Experimental protocol—The 17 dogs were placed into 3 experimental groups. Group-1 dogs (n = 5) were subjected to 30 minutes of SMA occlusion and 3 hours of reperfusion after the occlusion was released. Group-2 dogs (n = 6) were subjected to 60 minutes of SMA occlusion and 3 hours of reperfusion after the occlusion was released. Group-3 dogs (n = 6) served as a sham-operated control group. In group-1 and -2 dogs, to prepare the ischemic phase, the SMA was completely occluded by adding a constant tensile strength to a silastic vessel loop positioned around the SMA. The reperfusion phase was achieved by opening the occlusion.

Arterial and portal blood sample collections were performed before SMA occlusion (baseline), immediately before the occlusion was relieved, and 15, 60, 120, and 180 minutes after the occlusion was relieved. Blood samples were centrifuged at 3,000 × g, 4°C, for 20 minutes. Serum samples were stored at -80°C until the cytokine, endotoxin, and lactate assays were performed. Tonometry was performed before SMA occlusion (baseline), immediately before the occlusion was relieved, and 30, 60, 90, 120, 150, and 180 minutes after the occlusion was relieved.

Tonometry—In the tonometer's silicone balloon, 2.5 mL of saline (0.9% NaCl) solution was allowed to equilibrate for 30 minutes. Thereafter, 1 mL of saline solution was aspirated and discharged; this volume represented the dead space of the tonometer. The remaining 1.5 mL was immediately aspirated, and P_{CO_2} of the aliquot was determined in a blood gas analyzer.[f] The correction factor (1.29) for the 30-minute equilibration period listed by the manufacturer was used to adjust the measured P_{CO_2} in the aliquot of the saline solution. This value, intramucosal P_{CO_2}, together with the simultaneously obtained arterial HCO_3^- concentration, was used in the Henderson-Hasselbalch equation ($pH = 6.1 + \log([HCO_3^-] / [P_{CO_2} \times 0.03])$) for the calculation of intramucosal pH.

Lactate assay—Serum lactate concentration was measured by the electrode method by use of a portable blood lactate test meter.[g]

Endotoxin assay—Serum endotoxin concentration was measured by use of an limulus ameobocyte lysate assay.[h] Briefly, for the sample pretreatment to remove nonspecific inhibitors, serum samples were diluted in pyrogen-free water in a ratio of 1:10 and heated at 75°C for 5 minutes, cooled on ice and used for the assay. A 200-mL portion of the pretreated sample and assay solution were mixed in a test tube[i] and incubated at 37°C in a tube reader.[j] Gelation time is defined as the time needed for the transmittance of the reaction mixture to reach a 5% decrease value. The endotoxin concentration was obtained by use of the gelation time of the sample in relation to the calibration curve of *Escherichia coli* UKT-B endotoxin.[k] All determinations were performed in duplicate.

Bioassay for tumor necrosis factor-α activity—Serum **tumor necrosis factor-α (TNF-α)** activity was determined by cytotoxicity bioassay, using WEHI 164 clone 13 murine fibrosarcoma cells.[l] The cytotoxicity bioassay was conducted using a minor modification of the method of Eskandari et al.[10] In brief, 100-µL volumes of serial 2-fold dilutions of serum samples or recombinant human TNF-α[m] in RPMI 1640 medium[n] were added to 96-well flat-bottomed tissue culture plates.[o] Then, 5×10^4 WEHI 164 cells were added on top of the samples in 100-mL volumes of RPMI 1640 medium containing 1 µg of actinomycin D/mL/well and incubated at 37°C and 5% CO_2 for 20 hours. After incubation, cytotoxicity was measured colorimetrically by use of tetrazolium salt. In brief, 50 µL of 2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide[p] solution (1 mg of solution/mL of RPMI 1640 containing 20 µL of phenazine methosulfate

[0.383 mg/mL][p]) 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.[q] Tumor necrosis factor-α activity was determined from the standard curve with recombinant human TNF-α. All determinations were performed in triplicate.

Bioassay for interleukin-1β activity—Serum **interleukin-1β (IL-1β)** activity was determined by cytotoxicity bioassay, using the human melanoma subclone A375S2.[l] The cytotoxicity bioassay was conducted by use of a minor modification of the method of Nakai et al.[11] In brief, 100-µL volumes of serial 2-fold dilutions of serum samples or recombinant human IL-1β[m] in MEM medium[n] were added to 96-well flat-bottomed tissue culture plates.[o] Then, 2×10^3 A375S2 cells were added on top of the samples in 100-mL 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 interleukin-6 activity—Serum **interleukin-6 (IL-6)** activity was determined by proliferative bioassay, using the IL-6-dependent murine hybridoma cell line 7TD1.[r] The proliferative bioassay was conducted using a minor modification of the method of Van Snick et al.[12] In brief, 100-µL volumes of serial 2-fold dilutions of serum samples or recombinant human IL-6[m] in RPMI 1640 medium[n] were added to 96-well flat-bottomed tissue culture plates.[o] Then, 5×10^3 7TD1 cells were added on top of the samples in 100-µL volumes of RPMI 1640/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.

Statistical analyses—All data were expressed as means (± SEM). The serum lactate, TNF-α, IL-1β, and IL-6 data were expressed in terms of change from the baseline values at each measurement point. To detect significant differences among the 3 experimental groups and from baseline measurements at each time point within a group of dogs, the Kruskal-Wallis test and the Friedman test were used, respectively. When a null hypothesis was rejected, a multiple comparison test was performed by use of the Fisher exact test. For all comparisons, $P < 0.05$ was regarded as significant.

Results

Tonometry—In group-1 and -2 dogs, the intramucosal pH as an indicator of intestinal oxygen metabolism was significantly ($P < 0.001$) reduced from 7.355 ± 0.025 to 6.916 ± 0.075 and 7.383 ± 0.043 to 6.798 ± 0.037, respectively, compared with the baseline value at the end of the ischemia phase (**Fig 1**). These intramucosal pH values increased gradually after the release of the occlusion, but not to baseline values. In addition, intramucosal pH was lower in group-2 dogs, compared with group-1 dogs at the end of the ischemia phase and during the reperfusion phase. In group-3 dogs, there were no significant changes in intramucosal pH throughout the experiment.

Lactate concentration—In group-2 dogs, serum lactate concentrations in the portal circulation were increased after reperfusion, reaching a peak value at 15 minutes of reperfusion (0.90 ± 0.24 mmol/L, $P = 0.001$ vs baseline, $P = 0.006$ vs group-3 dogs) followed by a gradual decrease, but not to baseline values. In the systemic circulation, serum lactate concentrations in group-2 dogs reached a peak increase at 60 minutes of reperfusion (0.43 ± 0.13 mmol/L, $P = 0.018$ vs group-3 dogs) followed by a rapid decrease to baseline values (**Fig 2**). In group-1 dogs, serum lactate concentrations in the portal circulation reached a peak increase at 15 minutes of reperfusion (0.44 ± 0.14 mmol/L, $P = 0.026$ vs baseline) followed by a rapid decrease to baseline values, whereas serum lactate concentrations in the systemic circulation were only slightly increased at 15 minutes of reperfusion (0.26 ± 0.13 mmol/L). In group-3 dogs, there were no significant changes in serum lactate concentrations in either the systemic or portal circulation throughout the experiment.

Endotoxin concentration—In all dog groups, serum endotoxin concentrations in the systemic and portal circulation remained stable below the cutoff value (10 pg/mL) throughout the experiment, indicating that translocation of endotoxin from the intestinal lumen into the circulation did not occur.

TNF-α activity—In group-2 dogs, TNF-α activities were increased significantly in the portal circulation, reaching a peak increase at 60 minutes of reperfusion (3.9 ± 1.5 U/mL, $P = 0.010$ vs baseline, $P = 0.003$ vs group-3 dogs), and remained increased until 120 minutes of reperfusion (2.9 ± 0.7 U/mL, $P = 0.006$ vs group-3 dogs; **Fig 3**). In contrast, in the systemic circulation, TNF-α activities gradually increased from 60 minutes of reperfusion to the end of the experiment, although the changes were not significant. In group-1 and -3 dogs, there were no significant changes in TNF-α activities in either the systemic or portal circulation throughout the experiment.

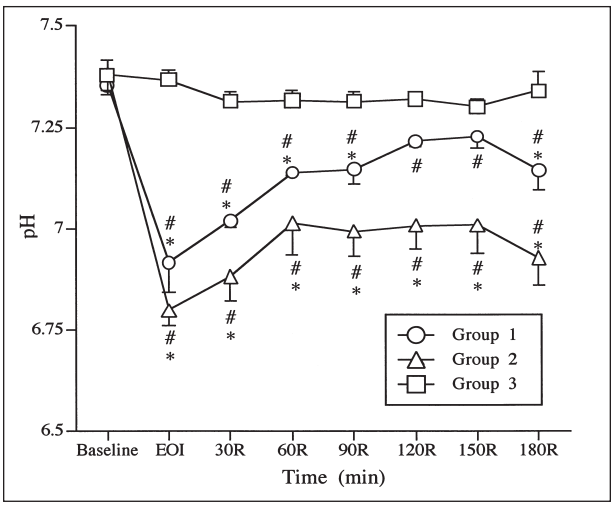

Figure 1—Mean (± SEM) intramucosal pH values in dogs in which the superior mesenteric artery was occluded for 30 minutes (group 1) or 60 minutes (group 2) and in sham-operated control dogs (group 3) versus the duration (minutes) of reperfusion (R) after occlusion of the artery was reversed. EOI = End of ischemic period. *Significant ($P < 0.05$) difference from group-3 dogs. #Significant ($P < 0.05$) difference from baseline value within dog group.

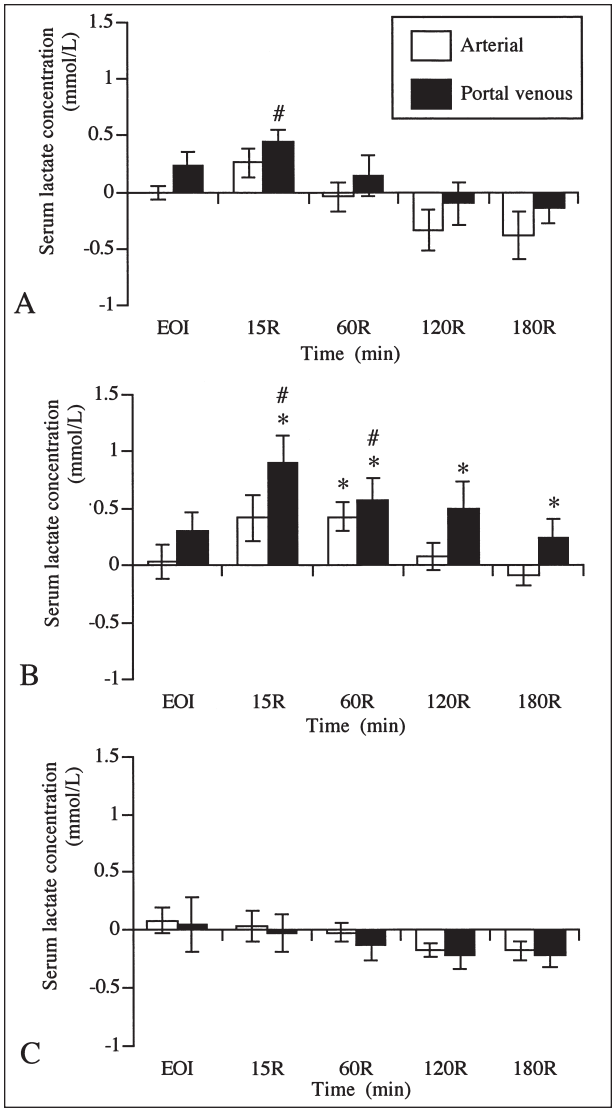

Figure 2—Mean (± SEM) serum lactate concentration expressed as a change from baseline value in group-1 dogs (A), group-2 dogs (B), and group-3 dogs (C). *See* Figure 1 for remainder of key.

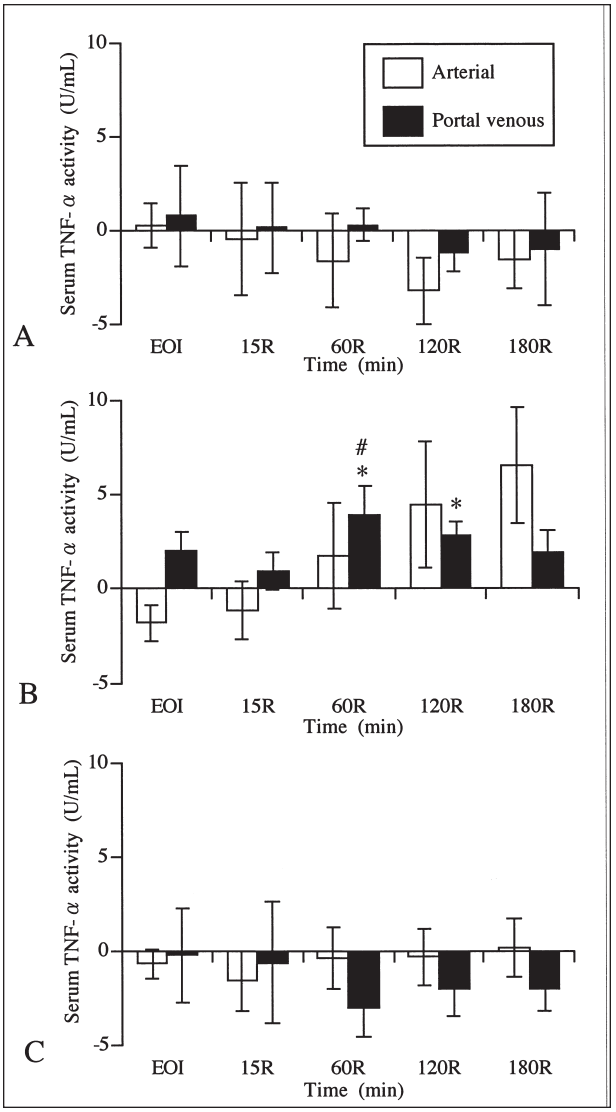

Figure 3—Mean (± SEM) serum tumor necrosis factor-α (TNF-α) activity expressed as a change from baseline value in group-1 dogs (A), group-2 dogs (B), and group-3 dogs (C). *See* Figure 1 for remainder of key.

IL-1β activity—There were no significant changes in any of the dog groups in IL-1β activities in either the systemic or portal circulation throughout the experiment.

IL-6 activity—In group-1 dogs, IL-6 activities in the systemic and portal circulation started to increase at 60 minutes of reperfusion (45.2 ± 23.4 and 36.4 ± 13.0 U/mL, respectively) and became significantly increased at 120 minutes of reperfusion (systemic circulation, 77.1 ± 23.3 U/mL, $P = 0.008$ vs baseline; portal circulation, 72.2 ± 18.6 U/mL, $P = 0.003$ vs baseline). In group-1 dogs, IL-6 activities in the systemic and portal circulation reached a peak at the end of the experimental period (systemic circulation, 98.8 ± 29.3 U/mL, $P = 0.001$ vs baseline; portal circulation, 90.4 ± 21.5 U/mL, $P < 0.001$ vs baseline).

In group-2 dogs, IL-6 activities in the systemic and portal circulations started to increase at 60 minutes of reperfusion (47.1 ± 26.9 and 63.3 ± 33.6 U/mL, respectively; portal circulation, $P = 0.045$ vs group-3 dogs), and both were significantly increased at 120 minutes of reperfusion (systemic circulation, 99.9 ± 25.7 U/mL, $P = 0.008$ vs baseline; portal circulation, 126.8 ± 47.9 U/mL, $P = 0.009$ vs baseline, $P = 0.017$ vs group-3 dogs). In group-2 dogs, IL-6 activities in the systemic and portal circulation reached a peak at the end of the experimental period (systemic circulation, 141.3 ± 38.7 U/mL, $P < 0.001$ vs baseline, $P = 0.007$ vs group-3 dogs; portal circulation, 138.1 ± 46.4 U/mL, $P = 0.005$ vs baseline, $P = 0.007$ vs group-3 dogs).

The magnitude of these increases was higher in group-2 dogs, compared with group-1 dogs, depending on the duration of the ischemic period (**Fig 4**). Additionally, in contrast to the TNF-α activities, the systemic and portal IL-6 activities were similar at all time points. In group-3 dogs, there were no significant

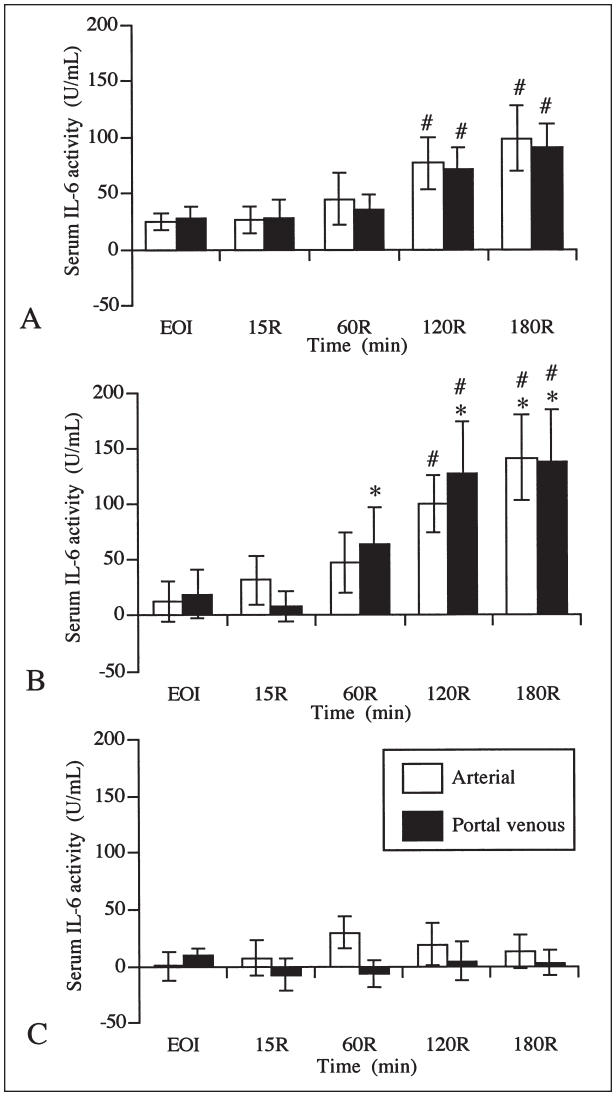

Figure 4—Mean (± SEM) serum interleukin-6 (IL-6) activity expressed as a change from baseline value in group-1 dogs (A), group-2 dogs (B), and group-3 dogs (C). *See* Figure 1 for remainder of key.

changes in IL-6 activities in either the systemic or portal circulation throughout the experiment.

Discussion

In our study, we attempted to determine whether intestinal ischemia and reperfusion induces bacterial translocation and a proinflammatory cytokine response in dogs. The main result of our study is that intestinal ischemia and reperfusion leads to significant increases in circulating TNF-α and IL-6 activities, depending on the duration of the ischemia phase. Moreover, these cytokine responses occurred in the absence of detectable endotoxin in the circulation. The validity and severity of the graded insult of the intestinal ischemia and reperfusion in the dogs of our study were confirmed by the reduction in intramucosal pH and the increase in serum lactate concentration.

Tumor necrosis factor-α is thought to be 1 of the proinflammatory cytokines that plays an important role in the pathogenesis of MODS. It is reported that the injection of TNF-α into rodents causes hemodynamic changes similar to septic shock and widespread tissue damage not unlike that seen in MODS.[13] In addition, results of other studies[14-16] indicate that TNF-α also contributes to the development of distant organ injury following intestinal ischemia and reperfusion. In group-2 dogs of our study, TNF-α activity increased significantly in the portal circulation, reaching a peak activity at 60 minutes of reperfusion, followed by a decline at the end of the experiment. In addition, at 60 minutes of reperfusion, the degree of increase in TNF-α activity was approximately 2-fold higher in the portal circulation, compared with the systemic circulation. These findings suggest that TNF-α was released from the intestine following reperfusion and entered the portal circulation. Furthermore, in the portal circulation, the peak serum concentration of lactate, which was produced in the ischemic intestine during SMA occlusion and washed out from the ischemic area into the portal circulation by subsequent reperfusion, occurred earlier than the peak activity of TNF-α (lactate at 15 minutes and TNF-α at 60 minutes of reperfusion) in group-2 dogs, indicating that the generation of TNF-α from the intestine occurred after reperfusion, but not during ischemia. This finding is in accordance with those of other studies in rodents,[17-19] which indicate that the synthesis of TNF-α is promoted after reperfusion rather than during ischemia, and that circulating TNF-α activity reaches its peak increase at an early stage of the reperfusion phase.

In our study, TNF-α activities continued to increase after 60 minutes of reperfusion until the end of the experiment (180 minutes of reperfusion) in the systemic circulation, in contrast to the changes in the portal circulation. Furthermore, in the latter stages of the reperfusion phase (120 and 180 minutes of reperfusion), the TNF-α activity was higher in the systemic circulation, compared with the portal circulation, whereas the TNF-α activity was higher in the portal circulation at 60 minutes of reperfusion. These results suggest that the major source of circulating TNF-α might change over time from the intestine to remote organs such as the liver and lung. This finding is supported by Jiang et al,[17] who, in rats with hemorrhagic shock, demonstrated a pattern of TNF-α appearance similar to that shown in our results. They speculated that the main source of TNF-α shifted from the intestine-associated macrophage in the lamina propria of the intestine to the hepatic macrophage (Kupffer cell).

It is well established that IL-6 mediates the host acute response in hemorrhage, trauma, and sepsis, and that high circulating amounts of IL-6 positively correlate with septic shock and with the mortality rate in a number of inflammatory diseases.[20-22] Furthermore, in IL-6 knock-out mice with splanchnic artery occlusion, it was recently reported that a deficiency of IL-6 attenuated the expression of the adhesion molecule and neutrophil-mediated tissue injury, leading to a reduction in the mortality rate.[23] This finding indicates the importance of IL-6 in developing MODS that accompanies intestinal ischemia and reperfusion. In our study, following reperfusion, a significant gradual increase in IL-6 activity was detected and, unlike TNF-α, the mag-

nitude of the IL-6 response in the portal circulation was almost equivalent to that in the systemic circulation, suggesting that IL-6 was generated mainly in the remote organs, but not in the intestine. This observation may be supported by a study by Yao et al[24] that revealed in rats with SMA occlusion a pattern of circulating IL-6 response similar to that seen in our results. These researchers surmised that the intestinal ischemia and reperfusion induced not only local intestinal inflammation but also systemic inflammatory response. However, the results of several other studies[19,25-27] indicate that the response to intestinal ischemia and reperfusion in the intestine becomes the main source of IL-6 production. Combined with these results, the remote organs and the local intestine seem to be related to the IL-6 response after intestinal ischemia and reperfusion in dogs, although the degree of involvement in IL-6 synthesis is uncertain. In addition, IL-6 activities were higher in group-2 dogs, compared with group-1 dogs, suggesting that the magnitude of the increase of circulating IL-6 activity after the intestinal ischemia and reperfusion is dependent on the duration of the ischemic phase.

Serum IL-1β activity did not change significantly in our study, although it has been recognized, as well as TNF-α, as 1 of the proinflammatory cytokines that play an important role in the pathogenesis of MODS. This result is different from the results of several studies[28,29] that reveal a remarkable IL-1β response following intestinal ischemia and reperfusion. By comparison, however, Gurley et al[30] reported that they failed to detect IL-1β activity in dogs with SMA occlusion, which was measured with a bioassay method by use of the A375 cell line as in our study. They speculated that a possible explanation for this result was a lack of assay detection. Therefore, despite our inability to detect circulating IL-1β, we believe that the involvement of IL-1β cannot be ruled out in intestinal ischemia and reperfusion in dogs.

In our study, endotoxin was not detected in either the systemic or portal circulation. This result differs from several studies[14,15,17,28] that indicate that intestinal ischemia and reperfusion could disrupt the intestinal barrier function and promote the translocation of endogenous endotoxin into the portal circulation, leading to a systemic cytokine response and the development of the septic state. However, in several experimental studies on hemorrhagic shock[18,19] and SMA occlusion[18,26] and in a clinical study[31] in trauma victims in human medicine, investigators were not able to document the translocation of endotoxin or bacteria in the portal circulation. Therefore, the role of endotoxin translocation in intestinal ischemia and reperfusion is still a matter of debate. However, several possible explanations for our observation that significant TNF-α and IL-6 responses occurred in the absence of endotoxin in the circulation must be considered. First, translocating bacteria or their products trapped in the lamina propria by local phagocytic cells might have initiated a local inflammatory response and induced or potentiated cytokine production by the intestine-associated lymphatic tissue after intestinal ischemia and reperfusion.[18,25] Second, translocation of endotoxin via a lymphatic route may be more important than translocation via a portal venous route. Third, in our study, we estimated bacterial translocation by endotoxin assay only, but not by bacteria culture or another bacterial component assays such as peptidoglycan. Therefore, translocation of whole bacteria or other unmeasured, immunomodulatory, bacteria-related factors within the intestine aside from endotoxin was responsible for the increased cytokine response. In fact, results of recent studies indicate that bacterial components, such as peptidoglycan,[32] bacterial peptide,[33] flagellin,[34] and bacterial DNA,[35] stimulate cytokine production directly via toll-like receptors. Fourth, ischemia or ischemia and reperfusion per se could be a trigger for cytokine synthesis by a mechanism independent of endotoxin. In this mechanism, oxygen-derived free radicals are produced following ischemia and reperfusion, and these oxygen-derived free radicals activate nuclear factors such as nuclear factor-kappa β, which bind to the enhancer sequences of the cytokine genes.[36] In addition, hypoxia or hypoxia-reoxygenation directly stimulates the expression of cytokines in several cell types.[37-39] For example, hypoxia activates the nuclear binding activity for the nuclear factor-IL-6 motif in cultured human endothelial cells,[40] and hypoxia-reoxygenation induces an increase of the transcriptional activity of the heme oxygenase gene in endothelial cells,[41] which could also be involved in the synthesis of cytokines. Finally, a lack of endotoxin assay detection may be the possible explanation for failure to detect endotoxin translocation in our study.

[a]Rhodia, Nagase Medicals, Osaka, Japan.
[b]A.D.S. 1000, Shin-ei Industries Inc, Saitama, Japan.
[c]Mioblock injection, Sankyo, Tokyo, Japan.
[d]Solulact, Terumo, Tokyo, Japan.
[e]Tnonomitor, Datex Ohmeda, Tokyo, Japan.
[f]Gastat 3, Techno Medica, Tokyo, Japan.
[g]Lactate Pro, Arkray, Kyoto, Japan.
[h]Limulus ES-II Test Wako, Wako Pure Chemical Industries, Osaka, Japan.
[i]Limulus test tube S, Wako Pure Chemical Industries, Osaka, Japan.
[j]Toxinometer ET-208 S, Wako Pure Chemical Industries, Osaka, Japan.
[k]Control standard endotoxin, Wako Pure Chemical Industries, Osaka, Japan.
[l]American Type Culture Collection, Manassas, Va.
[m]Boehringer Mannheim GmbH, Mannheim, Germany.
[n]Gibco BRL, Life Technologies Inc, Rockville, Md.
[o]Microtest tissue culture plate, Becton Dickinson, Frankin Lakes, NJ.
[p]Sigma Chemical Co, St Louis, Mo.
[q]Spectra Rainbow Thermo A-5002, Wako, Osaka, Japan.
[r]Riken cell bank, Ibaraki, Japan.

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