Pathophysiologic and Immunologic Changes in a Canine Endotoxemia over a Period of 24 Hours

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ABSTRACT. In this study, the pathophysiologic and immunologic parameters from a 24-hr of canine endotoxemia model by lipopolysaccharide (LPS) infusion were evaluated. For that, twelve healthy beagles received a continuous 24-hr IV infusion of low dose LPS (10 µg/kg/h, from *Escherichia coli* serotype O111:B4) dissolved in saline. Complete blood counts and serum biochemical analysis as well as histopathologic examination were performed to assess pathophysiologic changes such as neutrophil migration and organ injury. To evaluate immunologic parameters, the concentrations of plasma tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-10 were determined, and neutrophil activation was also evaluated based on cell surface expression of CD11b using flow cytometry analysis. As results, systemic signs of endotoxemia including fever, vomiting, and hemorrhagic diarrhea were observed within short time after LPS infusion. Severe leukopenia and increased fluorescent intensity of CD11b on neutrophils were observed at 3 hr while percent positive of CD11b was the maximum at 12 hr during the experiment. Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and creatinine phosphokinase (CPK) concentrations increased markedly, and organ damage was confirmed on histopathologic examination. Plasma TNF-α peaked at 3 hr and decreased rapidly, while the concentrations of IL-6 and IL-10 increased gradually until 6 hr and decreased thereafter. Using this canine endotoxemia model, we were able to determine the kinetics of pathophysiologic and immunologic parameters over a period of 24 hr. This study will enhance our understanding of their mechanisms underlying canine sepsis.

KEY WORDS: canine sepsis, CD11b, cytokine, endotoxemia, lipopolysaccharide.

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Canine sepsis is associated with substantial morbidity and mortality in veterinary medicine [1, 3, 13, 14, 17, 32]. Since Gram-negative bacteria are the most commonly isolated in sepsis [4, 27], experimental intravenous administration of the endotoxin, lipopolysaccharide (LPS): a cell membrane component of Gram-negative bacteria, *in vivo* animal studies rodents as well as canine have been done to emulate sepsis [7, 8]. However, current canine studies regarding immunopathophysiologic response have exclusively focused on early response (less than 8 hr) of immunologic and physiologic response to endotoxin [25, 26]. Very little is known about dynamic changes in cytokine response or neutrophil activation in a late phase of canine endotoxemia. Since the inflammatory response during naturally developing sepsis in dogs is much longer in duration [34], documenting dynamics of innate immune response in a chronic endotoxemia model will provide clues to the immunopathologic changes in naturally developing sepsis.

The goal of this study was to determine kinetics of the innate immune response to endotoxin over a 24 hr period

so that this information can be applied to clinical patients for selection of appropriate biomarkers in future clinical investigations.

We evaluated the pathophysiologic consequences of systemic endotoxemia by evaluating whether continuous infusion of LPS caused organ injury, based on physical parameters, blood cell counts, plasma biochemistry analysis and histopathologic examination. To evaluate immune response, we evaluated the production of cytokines and expression of CD11b, a member of α-chain integrin and a component of β2 integrins expressed on the surface of neutrophils upon activation.

MATERIALS AND METHODS

Experimental design: Animals. All aspects of this study were approved by the Committee on Bioethics at Chonbuk National University (CBU-2008-021). Twelve healthy adult beagles (eight females and four males, 8–12 kg, 1–2 years old) were used. Dogs were fed a standardized diet twice daily, and water was available *ad libitum*. Prior to the study, dogs were fasted for at least 12 hr.

Treatments. To induce endotoxemia, all dogs received a continuous intravenous infusion of a low dose of LPS (10 µg/kg/h, from *Escherichia coli* serotype O111:B4; Sigma,

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St. Louis, MO) dissolved in normal saline solution, as described previously [26]. Normal saline solution was infused at 10 ml/kg/h for the first 12 hr and then at 5 ml/kg/h during 12–24 hr of the experiment.

Sample collection. Clinical examinations including rectal temperature (T), heart rate (HR) were determined before starting endotoxin infusion (0 hr, baseline). Clinical signs including vomiting and diarrhea were recorded based on visual inspection. Two milliliters of EDTA-treated blood samples were used to obtain complete blood count (CBC), and neutrophil CD11b expression at 0, 3 (CBC only), 6, 12, and 24 hr. Plasma was acquired from heparinized blood samples at 0, 6, 12, and 24 hr for biochemistry or cytokine analyses. After the 24-hr experiment, four dogs were randomly selected and euthanized by tiletamine/zolazepam (Zoletil 50, Virbac) for tissue collection.

Pathophysiologic parameters: Hematologic analyses including white blood cell (WBC) count, hematocrit (Hct) and platelet (PLT) count were performed by an impedance cell counter (Vet ABC blood counter, ABX Diagnostics, Montpellier, France). Liver injury was assessed by measuring plasma concentrations of alanine aminotransferase (ALT); alkaline phosphatase (ALP), and total bilirubin (T-BIL) [12, 20]. Renal function was assessed by measuring plasma concentrations of blood urea nitrogen (BUN) and creatinine. Metabolic dysfunction was evaluated glucose measurement. The plasma biochemical analyses described above were performed using the VetScan VS2® (Abaxis, Union city, CA). Plasma lactate dehydrogenase (LDH), creatinine phosphokinase (CPK), aspartate aminotransferase (AST) were also measured using a dry chemistry analyzer (SPOTCHEM™ EZ SP-4410, ARKRAY Inc. Japan).

Tissue samples from the lungs, spleen, kidney, small intestine, and liver were collected on necropsy, fixed in 10% neutral-buffered formalin, and processed using standard histological paraffin methods. Tissue sectioned at 5 μ m was stained with hematoxylin and eosin (H&E) and examined by light microscopy. Histologic findings were described.

Immunologic parameters: Immunologic parameters were composed of two main measurements: cytokine mediated immune response; cellular (neutrophil) mediated immune response.

Cytokines. Plasma TNF- α , IL-6 and IL-10 concentrations were measured at baseline, 3 hr (TNF- α), 6 hr, 12 hr, and 24 hr using sandwich ELISA based on commercially available matched canine antibody pairs (Duoset ELISA development kit, R&D Systems, Minneapolis, MN, U.S.A.) [9]. Assays were performed in duplicate according to the manufacturer's instructions. The lower limit of detection of the assays for each cytokine as follows: 15.6 pg/ml for TNF- α , 62.5 pg/ml for IL-6, and 31.3 pg/ml for IL-10.

Measurement of CD11b expression on neutrophils. The expression of the cell surface molecule CD11b on neutrophils was measured from samples collected at baseline (0 hr), 3, 6, 12, 24 hr. A total of 100 μ l of heparinized whole blood was incubated with anti-canine CD11b (CA16.3E10 clone, AbD Serotec, Oxford, UK) for 20 min at room temperature [6]. Thereafter, cells were washed and incubated

in 100 μ l of FACS buffer (1% PBSA: PBS solution containing 1% bovine serum albumin) with RPE-conjugated secondary antibody (AbD Serotec, Oxford, UK). To exclude monocytes, FITC-conjugated anti-human CD14 (a marker of monocytes, LPS, and the LBP receptor) (M5E2 clone, BD Biosciences, San Jose, CA, U.S.A.) was also added to the same tube. Red blood cells were lysed and fixed with a commercial fix and lysis solution (FACS Lysing solution, BD Bioscience).

Strict neutrophil gating was performed based on forward and sideward scatter parameters and subsequent subtraction of FITC-CD14 positive cells, i.e., CD14⁺/CD11b⁺ cells [11]. Then, the geometric mean fluorescence intensity (GMFI) of RPE-CD11b and percent CD11b-RPE positive cells were scored from the strictly gated neutrophils (Fig. 1). The flow cytometric analysis was performed using a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) and data analysis was performed using FCS Express (FCS Express Version 3, De Novo Software). Data was acquired from a minimum of 10,000 events defined by light scatter gates to include the neutrophil population.

Statistical analyses: The statistical software packages SPSS (SPSS 17.0, SPSS Inc., Chicago, IL, U.S.A.) was used to perform the statistical evaluations. Values below the detection limit of the assay were assigned the default values of 0.016 (TNF- α), 0.063 (IL-6) and 0.031 (IL-10) (ng/ml) for statistical analyses. Data normality was analyzed using the Shapiro-Wilk test. Student's *t*-test (parametric: if the data was normally distributed) or the Mann-Whitney U test (non-parametric: if the data distribution was skewed) was used to detect significant differences from the baseline measurements at each time point. The correlation between neutrophil counts and CD11b expression (GMFI) was analyzed using Spearman's rho correlation test. The significance level for all statistical tests was predetermined at $P < 0.05$.

RESULTS

Overall observations: All dogs completed the study without mortality. After a 15–30 min LPS infusion, typical clinical signs of endotoxemia including vomiting and bloody diarrhea were observed (data not shown). Rectal temperature increased after 3 hr of LPS infusion and then decreased over the rest of the experiment ($P > 0.05$). HR gradually increased and peaked at 12 hr of the experiment ($P < 0.05$) (Fig. 2).

Pathophysiologic parameters: The mean baseline peripheral blood leukocyte counts was 10.38 ± 1.71 (mean $\pm$ SD, $\times 10^3/\mu$ l). Severe leukopenia ($P < 0.001$ vs. baseline) was observed after 3 hr of LPS infusion, followed by a gradual increase until the experiment ended at 24 hr. The major leukocyte component that decreased in the blood was granulocytes; while the proportion of monocytes stayed relatively constant. Rebound leukocytosis was observed at 24 hr ($P < 0.05$) (Fig. 3). Platelet count decreased gradually during the experiment, and severe thrombocytopenia was observed at 24 hr ($P < 0.001$ vs. baseline) (Table 1). Hct concentrations decreased slightly until 12 hr but all of the

Fig. 1. A dot plot of flow cytometric measurements of CD11b expression on neutrophils. Neutrophil analyses were selected based on forward-/side-scatter parameters (a) and FITC conjugated CD14 negative stained cells (b). Surface receptor expression was measured as the geometric mean fluorescence intensity (GMFI) or percent CD11b-PE positive cells (c). M1 region included less than 1% of the isotype controls in the histogram.

Fig. 2. Changes in rectal temperature (T, a) and heart rate (HR, b) in a canine endotoxemia model over a period of 24 hr. T increased suddenly at 3 hr ($P=0.051$ vs. baseline) and then decreased gradually over the next 21 hr. HR increased gradually and was maximal at 12 hr ($P<0.05$). Error bar indicates mean $\pm$ SD. (^a $P<0.05$ vs. baseline at each time point)

values were greater than 34.1% (data not shown).

The concentrations of most enzymes (LDH, CPK, AST, ALT and ALP) increased gradually (Fig. 4), but blood glucose concentration decreased in a time-dependent manner (data not shown). No significant increases in blood urea nitrogen, creatinine, or total bilirubin concentrations were observed during the experiment (Table 1).

On histopathologic examination, apoptosis of splenic lymphocytes and neutrophil infiltration in the liver were the most consistent observations in all four dogs. Neutrophil recruitment within the sinusoids and around/within the portal and central vein was observed in LPS treated dogs (4/4). Many apoptotic lymphocytes were detected in the splenic white pulp of LPS infused dogs (3/4). Severe

Fig. 3. Changes in white blood cell (WBC) counts (a) and relative composition (b) over a period of 24 hr in response to induction of endotoxemia. Severe leukopenia was observed at 3 and 6 hr after LPS infusion (a). The major decreased WBC component was neutrophils; the numbers of monocytes remained relatively constant (b). Error bar indicates mean $\pm$ SD. (^a P <0.05 vs. baseline; ^b P <0.01 vs. baseline; ^c P <0.001 vs. baseline at each time point)

Fig. 4. Changes in plasma biochemistry parameters over a period of 24 hr in our canine endotoxemia model. LDH and CPK concentrations (a) increased in a time dependent manner. AST, ALT and AST concentrations (b) also increased after low dose, continuous, LPS infusion. Error bar indicates mean $\pm$ SD. (^a P <0.05 vs. baseline; ^b P <0.01 vs. baseline; ^c P <0.001 vs. baseline at each time point)

alveolar congestion and perivascular edema were present in the lung of LPS treated dogs (2/4). Additionally, mild infiltration of neutrophils into glomeruli in 2/4 of the dogs was observed (Fig. 5). No pathological change was observed in the small intestine and pancreas.

Immunologic parameters: All of cytokine concentrations at baseline were always below the detection limit of the assay. The plasma concentration of TNF- α increased rapidly after 3 hr of LPS infusion (P <0.001 vs. baseline) and was undetectable after 6 hr. Plasma IL-6 and IL-10 concentrations increased gradually and peaked 6 hr after LPS infusion (P <0.001 vs. baseline for IL-6 and IL-10), fol-

lowed by a gradual decrease through the remainder of the experiment (Fig. 6).

The GMFI of CD11b in the neutrophil population was highest at 3 hr (P >0.05) and decreased gradually thereafter while percent positive of CD11b on neutrophils was the maximum at 12 hr (P <0.001 vs. baseline). The GMFI of CD11b expression showed a negative correlation with peripheral neutrophil counts ($r=-0.403$, $P=0.004$) (Fig. 7).

DISCUSSION

In this study, we observed not only pro-inflammatory but

Table 1. Changes in platelets counts (PLT), glucose, blood urea nitrogen (BUN), creatinine and bilirubin concentrations over a period of 24 hr after LPS infusion (data represent mean $\pm$ SD)

	0 hr	3 hr	6 hr	12 hr	24 hr
PLT ($\times 10^3/\mu\text{L}$)	265.08 $\pm$ 66.74	185.42 $\pm$ 63.52*	185.08 $\pm$ 45.75*	151.67 $\pm$ 45.14**	84.75 $\pm$ 32.92**
Glucose (mg/dl)	105.08 $\pm$ 12.70	n. a.	71.92 $\pm$ 19.53**	74.83 $\pm$ 14.58**	69.08 $\pm$ 19.73**
BUN (mg/dl)	15.08 $\pm$ 5.99	n. a.	10.42 $\pm$ 5.09	9.67 $\pm$ 4.39*	11.00 $\pm$ 3.38
Creatinine (mg/dl)	0.78 $\pm$ 0.20	n. a.	0.75 $\pm$ 0.17	0.68 $\pm$ 0.15	0.73 $\pm$ 0.19
Bilirubin (mg/dl)	0.33 $\pm$ 0.05	n. a.	0.38 $\pm$ 0.09	0.36 $\pm$ 0.09	0.36 $\pm$ 0.09

* $P < 0.05$; ** $P < 0.01$; n. a., not available.

Fig. 5. Histopathologic examination in a canine endotoxemia model over a period of 24 hr. Apoptosis in the spleen and infiltration of neutrophils in the liver were the most consistently observed after LPS infusion. (a) Neutrophil recruitment within the sinusoids and around/within the portal (arrow) ($\times 400$, scale bar=25 μm). (b) Apoptotic lymphocytes (arrow) in the splenic white pulp ($\times 400$, scale bar=25 μm). (c) Severe alveolar congestion and perivascular edema (arrow) in the lung ($\times 200$, scale bar=50 μm). (d) Mild infiltration of neutrophils into glomeruli (arrow). ($\times 400$, scale bar=25 μm)

also anti-inflammatory pathophysiologic and immunologic kinetic responses in a long term canine endotoxemia model (24 hr). Sudden increase in body temperature, increasing frequency of vomiting and hemorrhagic diarrhea was the initial clinical manifestation in this model. Endotoxin triggered increasing the GMFI of neutrophilic CD11b expression, resulting in severe leukopenia in the early stage. Biochemical and histopathologic analysis revealed neutrophil infiltration to tissues and organ injury in endotoxemia.

Endotoxin also stimulated systemic immunologic changes such as early TNF- α increment orchestrating IL-6/IL-10 release until 6 hr of LPS infusion. This cytokine activation profile obtained with CRI-LL is in accordance with our previous canine peripheral blood mononuclear cell (PBMC) over a period of 24 hr *in vitro* study [35].

The simultaneous increase in IL-10 with TNF- α and IL-6 increment can be explained a compensatory anti-inflammatory response. The initial hypercytokinemia is

Fig. 6. Changes in cytokine (TNF- α , IL-6 and IL-10) concentration in a canine endotoxemia model over a period of 24 hr. Maximum TNF- α production was observed at 3 hr, while peak IL-6 and IL-10 concentrations were observed at 6 hr. Cytokine concentrations at baseline were always below the detection limit. Error bar indicates mean $\pm$ SD. (^b $P < 0.01$ vs. baseline; ^c $P < 0.001$ vs. baseline at each time point)

tions, CD11b expression (GMFI) on neutrophils did not increase in the late stage (after 6 hr), despite of continuous LPS infusion. Plasma AST and ALT concentrations tended to decrease after 12 hr. Because sepsis is interpreted not only as a consequence of excessive pro-inflammatory cytokine production, but also as a consequence of immunoparalysis, this balance of the two types of cytokines may potentially be used to predict septic outcomes. We found predominant apoptosis in splenic lymphocytes from all of 4 dogs on histopathologic examination as well as peripheral lymphocytic apoptosis in a flow cytometry analysis using Annexin-V/ PI (propidium iodide) stain after completion of 24 hr LPS infusion (data not shown). Since it is known that substantially impaired immune response due to extensive death of immune effector cells is associated with poor outcome in sepsis [15, 18], this pathophysiologic change in endotoxemia explains the mechanism of immunoparalysis due to apoptosis of immune cells in sepsis.

However, in order to estimate the clinical patients' pro-/anti-inflammatory immune balance, it is not reasonable to measure early changes such as canine TNF- α and CD11b expression (GMFI) at once from clinical cases. TNF- α was detectable only within 3 hr of the start of the experiment and decreased to normal concentration within a few hours whereas IL-6 and IL-10 concentrations increased after 6 hr of onset and remained elevated for approximately 12 hr ($P < 0.01$ vs. baseline). These results consistent with our previous findings that cytokine concentrations increased during the later phase of inflammation and sustained for a

Fig. 7. Dot scatter plot showing the correlation between CD11b expression (geometric mean fluorescence intensity, GMFI) on neutrophil counts (a), box plots showing GMFI (a) and percent positive cells (b) of CD11b expressions on neutrophils over a 24-hr period of the experiment. (^a $P < 0.01$ vs. baseline; ^b $P < 0.001$ vs. baseline at each time point) GMFI CD11b expressions on neutrophils increased after 3 hr of LPS infusion ($P > 0.05$) and showed significant negative correlations to white blood cell counts ($r = -0.4$). Percent positive cells expressing CD11b on neutrophils elevated after 12 hr of LPS infusion ($P < 0.001$). Error bar indicates mean $\pm$ SD. The boundaries of the box are Tukey's hinges. The median is identified by a line inside the box. The length of the box is the interquartile range (IQR) computed from Tukey's hinges. Values more than three IQR's from the end of a box are labeled as extreme, denoted with an asterisk (*). Values more than 1.5 IQR's but less than 3 IQR's from the end of the box are labeled as outliers (●).

known to be accompanied by a compensatory response of raised concentrations of circulating anti-inflammatory cytokines (e.g., interleukin-10), which are associated with a down-regulated immune response (immunoparalysis) [5]. Thus aggressive clinical signs were rarely observed, and pro-inflammatory cytokine (TNF- α , IL-6) produc-

longer period of time could be better predictors of outcome in 28 canine systemic inflammatory response syndrome cases [34].

Circulating effector cells of the innate immune system such as neutrophils participate in the first line of host defense. It is known that many leukocytic mediators includ-

ing toll-like receptors, protease-activated receptors 1 and 4, TNF- α and interleukins initiate their activation including CD11b expression. It was also proposed that over-activated blood neutrophils led to organ injury via releasing proteolytic enzymes and oxygen metabolites after infiltration into tissue [23], or reduced in neutrophil number [10] and function [29] also led to serious infections and poor prognosis [22]. However, neutrophilic CD11b expression (GMFI) and leukopenia were found only in the early stage of the experiment in our study. One possible explanation for this phenomenon is neutrophils with high CD11b expression (GMFI) were migrated to tissues from peripheral circulation, and could not be detected in the whole blood samples. Thus the percent positive cells of CD11b expression regardless of its intensity might be low at 3 hr but elevated after 12 hr, anti-inflammatory phase. This is in accordance with previous reports that lower CD11b intensity in septic shock or multiple organ failure [24, 31] despite of its overall increase in septic insult. This study provides kinetics of CD11b expression on canine neutrophil and its relationship between neutrophil counts in endotoxemia.

Besides neutrophil changes, typical hematologic, biochemical scenarios of sepsis including thrombocytopenia, hypoglycemia and increased in liver enzymes were found in this experiment. The abnormal hemostatic function due to endotoxin stimulation might result in DIC (disseminated intravascular coagulation), which usually contribute to the hemorrhagic tendencies of sepsis. This might also contribute organ damage. But hemostatic functions were not investigated in this study, thus hemostatic indicators such as platelet activation, D-dimer concentration, aPTT (activated partial thromboplastin time), PT (prothrombin time) and AT (antithrombin) III should be evaluated in the future.

None of the dogs, however, showed increase in renal enzymes in this experiment, and which is consistent with the histopathologic result. In a previous report of LPS bolus injection to canine model caused increment of BUN and serum creatinine [33], but the BUN was decreased, assuming hemodilution, and creatinine were relatively constant in our study. Continuous infusion of saline with LPS could improve renal perfusion and prevent renal ischemic injury in this model. Indeed, mean arterial blood pressure (MAP) was also maintained levels greater than 90 mmHg via a continuous saline infusion [25] while the MAP after bolus injection has been reported to be less than 60 mmHg [2]. We infused saline for 24 hr in order to examine organ injuries with cytokine storming and neutrophil activation due to minimized vascular circulation and hypoperfusion. Factors that have the greatest effect on kidney injury during endotoxemia should be investigated in future studies.

We applied CRI-LL to induce canine endotoxemia in this study. Generally, bolus injection of LPS has been used to induce endotoxemia in dogs [2, 16], but it has been problematic in that it does not simulate the conditions of clinical sepsis [8]. Thus, Sakaue *et al.* established a canine endotoxemia model using a CRI-LL for an 8-hr of experimental period [25, 26]. It resulted in rapid peaking of AST, ALT and ALP (<8 hr), after which concentrations of these enzymes

decreased [33]. This indicates that organ damage was temporary and easily reversible shortly. Thus, we infused LPS continuously in this study as previously described [21, 25, 26, 28, 30], and our results confirm previous claims that induction of endotoxemia by CRI-LL closely approximates actual clinical conditions and thus is a suitable technique to investigate sepsis [8, 21]. Moreover, prior experimental studies in dogs have mostly been short-term (up to 8 hr) [19, 25, 26]; however, in our study, we maintained endotoxemia for 24 hr and were thus able to monitor both the initial and late stages of the pathophysiologic, and immunologic changes that occur during canine endotoxemia. Liver and lung damage in plasma biochemistry profile and histopathologic examination supports pathophysiologic consequence from a long term endotoxemia model. Keeping endotoxemia more than 24 hr might give us more information. However, our preliminary study showed cytokine changes and plasma biochemistry changes after 24 hr were not as dynamic as before 24 hr. Considering animal ethics and effectiveness of the experiment, we decided to keep endotoxemia for 24 hr.

In veterinary medicine, successful development of new diagnostic/prognostic biomarkers of sepsis depends on a good understanding of the pathophysiologic, immunologic changes associated with sepsis. This study may provide the essential information to allow translation of basic science advances into the successful treatment of human sepsis, while benefiting veterinary patients.

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