Canine Acute Phase Response: Relationship between Serum Cytokine Activity and Acute Phase Protein in Dogs

Kazuto YAMASHITA, Toru FUJINAGA¹⁾, Toru MIYAMOTO¹⁾, Mitsuyoshi HAGIO¹⁾, Yasuharu IZUMISAWA, and Tadao KOTANI

Department of Veterinary Surgery 1, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069 and ¹⁾Department of Veterinary Surgery, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

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ABSTRACT. The changes in serum activity of interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) were studied in dogs with acute inflammation. Dogs with local inflammation induced by an intramuscular injection of turpentine oil showed clinically a typical progression in the inflammatory response, recovering on day 14 after the treatment. Serum concentrations of C-reactive protein (CRP) and α_1 acid glycoprotein (α_1 AG) increased, and the albumin concentration decreased in all dogs during the acute phase response. These values each returned to the normal range from day 14 to 21. Serum IL-6-like activity was detected from 2 hr to day 6 after treatment. Serum TNF-like activity in the treatment group was detected at a low level from 3 to 24 hr after treatment, but there was no statistically significant difference compared with the control group. The temporal changes in serum IL-6 and TNF-like activities preceded those in serum concentrations of CRP, α_1 AG, and albumin. No dogs showed a detectable rise in serum IL-1-like activity after treatment.—**KEY WORDS:** acute phase protein, canine, IL-1, IL-6, TNF.

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The systemic reaction of the acute phase response is characterized by fever, leukocytosis, increases in the erythrocyte sedimentation rate and secretion of ACTH and glucocorticoids, decreases in serum levels of iron and zinc, a negative nitrogen balance, and dramatic changes in the concentration of acute phase proteins [13, 25]. The local reaction at a site of injury is characterized by a number of responses, such as aggregation of platelets and clot formation, dilatation of blood vessels, and the accumulation and activation of granulocytes and mononuclear cells, which in turn release cytokines [13]. In the dog, C-reactive protein (CRP), α_1 -acid glycoprotein (α_1 AG), haptoglobin, ceruloplasmin, and fibrinogen were proposed to be acute phase proteins [3, 4, 6, 7, 29].

Recently, a macrophage-derived cytokine, interleukin (IL)-6 was shown to modulate production of the acute phase proteins by human hepatocytes [2]. Pullicino *et al.* [26] reported a rapid increase in concentration of serum IL-6 during the acute phase response accompanying elective surgery in man. In dogs, however, there is little information regarding the role of IL-6 during the acute phase response induced by inflammatory stimuli. We have shown that bioassays with human melanoma A375S1, IL-6 dependent murine hybridoma MH60.BSF2 and WEHI 164 murine sarcoma subclone 28-4 were useful for measuring IL-1, IL-6 and TNF-like activities in canine sera, respectively [33].

The purpose of the present study was to show changes in serum IL-1, IL-6, and TNF-like activity during the acute phase response in relation to the serum concentration of the acute phase proteins in dogs.

MATERIALS AND METHODS

Experimental animals: Ten clinically normal adult beagles (4 males and 6 females) weighing 6.0–10.0 kg were used. Local inflammation was induced by an injection of 1 ml turpentine oil (Nacalai Tesque, Inc., Kyoto) into the right femoral muscle of 5 dogs (2 males and 3 females in the treatment group). The other 5 dogs received no treatment (control group).

Physical examination: General clinical symptoms and rectal temperature were examined every day before and after the treatment.

Blood collection: Five ml of blood was collected from the jugular vein. Immediately, 1.0 ml of the blood was mixed with the anticoagulant EDTA-2K for blood analysis. The rest of the blood was put into a sterile tube, centrifuged at $1,580 \times g$ for 15 min at room temperature (20–22°C) and the serum was collected aseptically. Sera were stored at -80°C until required.

Blood examination: The following factors were determined in a System 9000 Automated Hematology Cell Counter (Serono-Baker Diagnostics, Inc., Allentown, U.S.A.): red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), hematocrit (Ht), hemoglobin (Hb), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular volume (MCV). The differential white blood cell count was done on Giemsa-stained blood smears.

Serum biochemical examination: Determination of serum total protein concentration (TP) by biuret test and serum iron concentration by the method of Eisenweiner *et al.* [8] were performed with a COBAS MIRA S (F. Hoffmann-La Roche & Co., Ltd., Basel, Switzerland).

The serum albumin concentration was calculated from TP and the albumin percentage in serum determined by cellulose acetate membrane electrophoresis. The serum CRP concentration was determined by the single radial immunodiffusion (SRID) method [18] with anti-canine CRP goat serum and 444 $\mu\text{g/ml}$ canine CRP positive serum (kindly donated by Dr. M. Naiki, National Institute of Health, Tokyo). The serum $\alpha_1\text{AG}$ concentration was determined by the SRID method with a kit for quantitative analysis of canine $\alpha_1\text{AG}$ (kindly donated by Dr. K. Tamura, Saikinkagaku Institute, Sendai).

Bioassay for IL-1: IL-1-like activity in samples was assessed by cytolytic assay with the human melanoma subclone A375S1 (kindly provided by Dr. Y. Hirai, Otsuka Pharma. Co., Ltd., Tokushima) [33]. The cytolytic assay was conducted by the method of Nakai *et al.* [22]. The cells (2×10^3) were cultured together with recombinant human (rh) IL-1 α (final concentration 0.01–10 U/ml; provided by Dr. Y. Hirai) diluted serially for a standard curve, or with 0.01 ml of samples in a final volume of 0.2 ml/well with Eagle's Minimal Essential Medium (Nissui Pharma. Co., Ltd., Tokyo) supplemented with 10% fetal bovine serum in a 96-well flat-bottomed tissue culture plate (Becton Dickinson & Company, New Jersey, U.S.A.). After 96 hr incubation at 37°C in 5% CO₂, 0.05 ml of 0.05% neutral-red dye (Wako Chem. Ind., Ltd., Osaka) was added to each well, and the plate was incubated for 2 hr at 37°C. After washing the plate with phosphate buffered saline (PBS), 0.1 ml of 50% ethanol in 0.05 M NaH₂PO₄ was added to each well for extraction of the dye incorporated into viable cells. The optical density (O.D.) was read at an excitation wavelength of 540 nm. All determinations were performed in triplicate.

Bioassay for IL-6: IL-6-like activity was assessed by proliferative assay with an IL-6 dependent murine hybridoma clone, MH60.BSF2 (kindly provided by Dr. T. Kishimoto, Institute for Molecular and Cellular Biology, Osaka University, Suita) [33]. The proliferation assay was conducted by the method of Matsuda *et al.* [20]. The cells (1×10^4) were cultured together with rhIL-6 (final concentration 0.00076–0.2 U/ml; provided by Dr. T. Kishimoto), or with 0.01 ml of serially diluted samples in a final volume of 0.2 ml/well with RPMI1640 (Gibco, New York, U.S.A.) supplemented with 10% fetal bovine serum and 5×10^{-5} M 2-mercaptoethanol (Sigma Chem. Co., St. Louis, U.S.A.) in a 96-well flat-bottomed tissue culture plate for 48 hr. Each well was pulsed with 18.5 kBq/well [³H]-thymidine ([³H]-TdR; Amersham, Buckinghamshire, U.K.) during the last 6 hr of incubation. The cells were then harvested onto glass filter paper and radioactivity was determined in a scintillation counter. The results were expressed as counts per minute (cpm) of [³H]-TdR incorporation. All determinations were performed in triplicate.

Bioassay for TNF: TNF-like activity was assessed by cytolytic assay with the WEHI 164 murine sarcoma subclone 28–4 (kindly provided by Dr. Y. Yokomizo,

National Institute of Animal Health, Tsukuba) [33]. The cytolytic assay was conducted by a minor modification of the method of Eskandari *et al.* [9]. The cells (5×10^4) were cultured together with rhTNF α (final concentration 0.0012–10 U/ml; provided by Dainippon Pharma. Co., Ltd., Osaka) or with 0.01 ml of samples in a final volume of 0.2 ml/well with RPMI1640 supplemented with 10% fetal bovine serum and 0.5 $\mu\text{g/ml}$ actinomycin D (Sigma Chemical Co., St. Louis, U.S.A.) in a 96-well flat-bottomed tissue culture plate for 20 hr. MTT, 3-(4,5-dimethylthiazolyl) 2, 5 diphenyl tetrazolium bromide (Sigma) was prepared at 5 mg/ml in PBS and filter sterilized. After incubation, 0.1 ml of the supernatant was removed from each well and the well pulsed with 0.025 ml of MTT. Following a 2 hr incubation, 0.1 ml of 50% N,N-dimethylformamide (Wako) containing 0.7 M sodium dodecyl sulfate (Wako) was added to each well and pipetted. The O.D. was read at an excitation wavelength of 550 nm. All determinations were performed in triplicate.

Statistical analysis: The value for each treatment group was compared with that for the control group. The data obtained were statistically analyzed by Student's *t* test according to variance equivalence. For non-normally distributed data, Welch's method was used. A difference was considered statistically significant when the *p*-value was smaller than 0.005.

Fig. 1. Changes in rectal temperature, blood cell counts, hematocrit, and hemoglobin concentration after an injection of turpentine oil. Error bars show standard deviation. ●: Treatment group, *n*=5; ○: Control group, *n*=5.

RESULTS

Clinical findings: In the control group, none of the dogs showed clinical signs during the course of this study. In the treatment group, all dogs exhibited clinical signs of local inflammation. The signs were most severe on day 3. Rectal temperature rose significantly from 2 hr to day 5, reaching the maximum on day 3 (Fig. 1). The exudate at the site of local injury induced by the injection of turpentine oil was drained by a 2 cm skin incision on day 6. The wound was washed with warmed sterilized saline and covered with dry dressings. No antibiotics were administered to the dogs. Inflammatory signs improved rapidly after drainage. No clinical symptoms were observed in either the control group or the treatment group after day 14 post treatment.

Hematological findings: The results of blood examinations are shown in Fig. 1. In the treatment group, WBC increased significantly from 6 to 24 hr and from day 4 to day 7 after treatment. The faster increase showed hyperhypercytosis of polymorphonuclear neutrophil. Normoskeocytosis was found during the later increase. RBC and Ht values decreased significantly from day 4 to day 10. Hb values decreased significantly from day 5 to day 10. MCHC increased significantly compared with the control group from 24 hr to day 5 after treatment, but the value was within normal limits. PLT decreased significantly from day 4 to day 7.

Serum biochemical findings: As shown in Fig. 2, a typical change in the concentration of serum acute phase proteins and iron was found in the treatment group during the acute phase response. The serum CRP concentration increased significantly and reached its maximum value on day 2 ($431.5 \pm 31.3 \mu\text{g/ml}$). The serum $\alpha_1\text{AG}$ concentration increased significantly and reached its maximum value on day 3 ($2.63 \pm 0.2 \text{ mg/ml}$). The serum albumin concentration decreased significantly and reached its

Fig. 2. Changes in serum concentrations of acute phase proteins and iron after an injection of turpentine oil. Error bars show standard deviation. Key see to Fig. 1.

Fig. 3. Changes in serum interleukin-6 (IL-6) and tumor necrosis factor (TNF)-like activities after an injection of turpentine oil. Error bars show standard deviation. Key see to Fig. 1.

minimum value on day 5 ($2.38 \pm 0.33 \text{ g/dl}$). The serum iron concentration decreased significantly and reached its minimum value on day 2 ($19.2 \pm 6.0 \mu\text{g/ml}$).

Serum IL-1, IL-6, and TNF-like activities: The titer (U/ml) of cytokine activity in the serum was calculated from the standard curve [33]. As shown in Fig. 3, increase in serum IL-6 was induced following a local injury. In the treatment group, serum IL-6-like activity increased dramatically by 4 hr ($16.1 \pm 16.1 \text{ U/ml}$), reaching its maximum value at 12 hr ($148.9 \pm 55.5 \text{ U/ml}$). The activity was significantly higher than that of the control group until day 6. Serum TNF-like activity of the treatment group tended to be higher than that of the control group following injury and reached its maximum value 24 hr after treatment ($1.2 \pm 0.8 \text{ U/ml}$). However, there was no statistical difference between the treatment and control groups. Changes in the serum IL-6 and TNF-like activities after treatment were followed by those of CRP, $\alpha_1\text{AG}$, and albumin concentrations in serum.

No dogs showed a detectable increase in serum IL-1-like activity.

DISCUSSION

The acute phase response is a biophylactic reaction during the early stage against infection, tissue injury, neoplastic growth, or immunological disorders [13]. Cytokines are protein mediators of cell-to-cell communication and play an important part in the acute phase response [13, 15, 16]. IL-1 and TNF have many biological functions such as pyrogenic action, activation of neutrophils, osteoclast activation, induction of IL-6, and induction of synthesis of some acute phase proteins [15, 23]. IL-6 also fulfills many biological roles such as induction of B cell differentiation, activation of T cells and thymocytes, induction of acute phase proteins, stimulation of hemopoietic precursor cell growth and differentiation, pyrogen action, inhibition of TNF production, and induction of adrenocorticotrophic hormone [16]. In dogs, however, there is only a little information on the cytokine network of the acute phase response. A better knowledge of the pathophysiological mechanisms of the acute phase response will lead to more effective treatment of inflammatory diseases in dogs.

Expression of IL-6 can be induced in various cells including fibroblasts, monocytes/macrophages, T cells, B cells, endothelial cells, epidermal cells, synovial cells, keratinocytes and diverse tumor cells. The production of IL-6 is further amplified by other cytokines, especially IL-1 and TNF, that also generated in response to infectious agents [16]. In the present study, the serum IL-6-like activity increased dramatically after inflammatory stimulation. In dogs, as in man, it is suggested that IL-6 can exert broad systemic effects in the acute phase response.

Increases in serum TNF and IL-1 activity are induced by endotoxic shock and are observed before an increase in serum IL-6 activity [14, 17, 18, 21]. Serum TNF-like activity was also detected at an early stage of the acute phase response in the treatment group. However, the value was lower than that seen in acute phase sera from dogs [17, 33] and other animals [1, 12, 14, 18, 21] with endotoxic shock. IL-1-like activity could not be detected in sera of either group, although the A375S1 assay used in the present study could detect IL-1-like activity in acute phase sera from dogs with endotoxic shock induced by an intravenous injection of lipopolysaccharide [33]. In human subjects with elective surgery or accidental injury, no detectable rise in the serum concentration of TNF was shown in acute phase sera [26]. It was suggested that the amount of TNF and IL-1 production were very low in local inflammation. During the acute phase response of local inflammation, expression of IL-1 and TNF seems to be different from that in endotoxic shock.

It seems that IL-6 shows less acute toxicity than some other cytokines, such as IL-1 and TNF, produced during the acute phase response [15, 16]. The ability of IL-6 to inhibit TNF production suggests that the generation of IL-6, which is increased by TNF, may eventually lead to a decrease in the toxic effects due to TNF [16]. In the

present results, no serum TNF-like activity was detected after day 2, while high serum IL-6-like activity was observed from 6 hr to day 2. Also in dogs, although there is no information about the biological half-life of canine TNF, increased IL-6 may have the ability to inhibit TNF production.

In the present study, all dogs with local injury showed a typical inflammatory systemic reaction involving fever, leukocytosis, and mild anemia during the acute phase response. IL-1 is known as endogenous pyrogen and TNF also induces fever [15]. IL-1 causes a mobilization of mature neutrophils from the bone marrow into the peripheral blood, resulting in neutrophilia [15]. The proliferation and development of hemopoietic progenitor cells are regulated by a number of cytokines. IL-6 is capable of supporting the proliferation of some mouse granulocyte/macrophage progenitors and stimulates myelopoiesis and erythropoiesis *in vivo* [16, 27]. The faster leukocytosis, which showed an increase in polymorphonuclear neutrophils, might be induced by IL-1 released from activating inflammatory cells, although no IL-1 activity could be detected in serum. The later increase, which showed normoskeocytosis, might be induced by IL-6 because the increase followed the dramatic increase in serum IL-6-like activity. Throughout the course of the acute phase response, the anemia remained normocytic and normochromic and was identified as iron deficiency anemia. Feldman *et al.* [11] reported that anemia of inflammatory disease was caused by a disordered iron metabolism evidenced by a depressed serum iron concentration, total iron binding capacity, percentage saturation of transferrin, and a decrease in the number of bone marrow sideroblasts. They also reported that bone marrow reticuloendothelial iron and hepatic nonheme iron were increased. In the present study, recovery from anemia and thrombocytopenia followed an increase in serum IL-6-like activity. The recovery, therefore, seems to be caused by the biological activity of IL-6 in stimulating erythropoiesis and myelopoiesis [16].

Changes in the serum concentration of CRP and α_1 AG, so-called acute phase proteins, were similar to those previously reported in dogs [4, 6, 7], cattle [5], man [25, 28], and horses [31, 32]. These syntheses *in vitro* have been shown to be increased by inflammatory stimuli, while albumin synthesis is decreased [27]. In the present results, the serum albumin concentration decreased as a negative acute phase protein.

The beginning of these changes lagged behind the inflammatory stimulation. The cause of this delay is regarded to be a cascade of reactions including an activation of inflammatory cells and the formation, secretion, and transport of acute phase mediators from the site of local injury to the liver, which is a main source of the acute phase proteins [10, 13]. In the present study, the dramatic increase in serum IL-6-like activity preceded the changes in the serum concentration of acute phase proteins. Nishimoto *et al.* [24] and Pullicino *et al.* [26] reported similar results in human patients undergoing

elective surgery. Some important *in vitro* studies have shown that IL-6 can initiate the synthesis of many acute phase proteins [2, 16, 27].

In conclusion, serum cytokine activity changes faster than acute phase proteins and other reactants after local injury in dogs. Particularly, serum IL-6-like activity increases dramatically, therefore, it may have major roles in acute phase response. Furthermore, the change in serum cytokine activity induced by local injury seems to be different from that induced by endotoxic shock.

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