

Comparison of heartworm extract-induced shock and endotoxin-induced shock in dogs by determination of serum tumor necrosis factor concentrations

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Objective—To compare the mechanisms of heartworm (HW) extract-induced shock and endotoxin-induced shock in dogs by determination of serum tumor necrosis factor (TNF) concentrations.

Animals—11 mixed-breed dogs (7 without and 4 with HW infections).

Procedure—Eight dogs were treated with 2 ml of HW extract IV, and 3 dogs were given endotoxin (*Escherichia coli* lipopolysaccharide [LPS]) at 40 or 400 µg/kg of body weight, IV. Changes in clinical and hematologic findings and serum TNF concentrations were examined from before treatment to 120 minutes after treatment in dogs given HW extract or from before treatment to 180 minutes after treatment in dogs given LPS. Tumor necrosis factor concentration was determined by cytotoxic assay, using WEHI-164 murine sarcoma cells, and plasma endotoxin concentration was determined in 2 dogs treated with HW extract, using the endotoxin-specific chromogenic test.

Results—Eight dogs developed shock 3 to 16 minutes after HW extract treatment. Rectal temperature did not change during examination. Serum TNF concentration was detected at a low concentration only 60 and 120 minutes after HW extract treatment, and plasma endotoxin was not detected during examination. In dogs treated with LPS, rectal temperature increased to > 40 C in 2 of 3 dogs, and serum TNF concentration began to increase 30 minutes after LPS treatment, reaching a maximum concentration by 60 minutes.

Conclusions—The cause and mechanism of HW extract-induced shock may be different from those of endotoxin-induced shock, because TNF, which was a pivotal mediator in endotoxin-induced shock, increased minimally in serum of dogs treated with HW extract. (*Am J Vet Res* 2001;62:765–769)

Intravenous administration of *Dirofilaria immitis* extract induces shock in dogs.^{1,3} In addition, shock resulting from heartworm (HW) infection may occur when adult worms are injured during surgical removal from the host.⁴ It is also well known that shock is induced by administration of prophylactics for HW

infection such as diethylcarbamazine,^{5,6} milbemycin D,⁷ and ivermectin⁸ in dogs with microfilaremic HW infection (ie, microfilaremic-induced shock). Moreover, in humans, microfilaremic-induced shock after treatment of onchocerciasis and lymphatic filariasis with diethylcarbamazine and ivermectin is common.^{9,10} The mechanisms of shock attributable to disrupted adult HW and microfilaremic-induced shock have not yet been clarified. Clinicopathologic and pathologic findings of HW extract-induced shock are fundamentally similar to those associated with microfilaremic-induced shock.^{1,3} Therefore, investigation of the mechanisms of HW extract-induced shock would be useful in elucidating the mechanisms of shock caused by injured adult worms and microfilaremic-induced shock. In addition, the shock-inducing substances in HW extract may have an important role in the pathophysiologic mechanisms of HW disease.

The main clinicopathologic findings of HW extract-induced shock are temporal leukopenia and thrombocytopenia and increases in serum activities of enzymes such as alanine transaminase and alkaline phosphatase during circulatory collapse with decrease in blood pressure.^{1,2} A characteristic pathologic finding is hepatosplanchnic vascular congestion.³ These clinicopathologic and pathologic findings resemble those of endotoxin-induced shock. We hypothesized that HW extract may induce shock by the same mechanisms as endotoxin-induced shock.

Endotoxin-induced shock is the result of severe pathophysiologic reactions caused by lipopolysaccharide (LPS), a structural component of the outermost layer in the outer membrane of gram-negative bacteria.¹¹⁻¹⁴ Lipopolysaccharide directly affects mononuclear phagocytes, neutrophils, platelets, and vascular endothelial cells and stimulates complement and kallikrein-kinin systems.¹¹⁻¹⁴ Consequently, a large variety of chemical mediators are produced and released, including cytokines such as tumor necrosis factor (TNF) and interleukin-1, arachidonic acid pathway metabolites (eicosanoids) such as prostaglandins and leukotrienes, platelet activating factor, nitric oxide, anaphylatoxins, and bradykinin.^{11-13,15} In addition, complicated interactions of these mediators are presumed to result in the pathophysiologic changes of endotoxin-induced shock.^{11-13,15} Among these mediators, TNF is thought to have a pivotal role in the pathogenesis of endotoxin-induced shock.¹¹⁻¹³ Tumor necrosis factor, which is produced primarily by mononuclear phagocytes, elicits other cytokines such as interleukin-1,

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interleukin-6, and interleukin-8 and activates the arachidonic acid pathway and complement system.^{13,16,17} Intravenous administration of small quantities of TNF induces circulatory collapse in the dog, with the same hallmark signs observed in endotoxin-induced shock.^{18,19} In experimental endotoxin-induced shock, serum concentrations of TNF increase greatly,^{12,17,20,21} along with plasma endotoxin concentrations.^{20,22,23} The purpose of the study reported here was to compare the mechanisms of HW extract-induced shock and endotoxin-induced shock in dogs by determination of serum TNF concentrations.

Materials and Methods

Dogs—Eleven mixed-breed dogs ranging from 1 to 3 years old and weighing between 5.7 and 14.8 kg were used in our study. Dogs were obtained from the local shelter. Seven of the dogs did not have a HW infection, and 4 had a HW infection. Heartworm infections were confirmed by detection of circulating microfilariae and adult worms. To detect circulating microfilariae, an acetone concentration technique was used in which 1 ml of blood hemolyzed in 9 ml of solution (5% acetone, 0.025% methylene blue, 0.2% sodium citrate) was centrifuged at 408 × g for 10 minutes, and then the sediment (0.1 ml) was examined as a wet mount under scanning magnification. The presence of adult worms was confirmed during necropsy at the end of the study.

Shock was induced in 8 of the 11 dogs by the administration of HW extract. Five were treated with 2 ml of female HW extract IV, which is equivalent to the volume extracted from a female adult HW, and the other 3 dogs were treated with 2 ml of male HW extract IV, which is equivalent to the volume extracted from 2 male adult HW. In the remaining 3 of 11 dogs, shock was induced by endotoxin (*Escherichia coli* LPS).² One dog was treated with LPS at 40 µg/kg (total 240 µg, low dose) IV, and the other 2 dogs were treated with LPS at 400 µg/kg (total 2,760 and 3,000 µg, respectively, high dose) IV. The study was conducted in a manner consistent with the Gifu University Guidelines for Animal Experimentation. Clinical signs were transient and over quickly, so we made no attempt to alleviate them. At the end of the study, dogs were euthanatized with a lethal dose of sodium pentobarbital IV (120 mg/kg for the first 4.5 kg of body weight and 60 mg/kg thereafter).

Preparation of heartworm extract and endotoxin—Heartworm extract was prepared from female or male adult worms by use of a previously reported procedure.¹ Lipopolysaccharide was dissolved in sterilized physiologic saline (0.9% NaCl) solution at 4 mg/ml and stored at -30 C. Stored solution was prepared in 2 ml of sterilized physiologic saline at each concentration.

Procedure—Systemic blood pressure was measured successively by use of the oscillometric method^b at the tail head or the axillary region. The onset of shock was defined as the time when the systolic blood pressure decreased below 90 mm Hg or > 40 mm Hg below baseline.²⁴ Eight dogs treated with HW extract underwent clinical examination and blood collection for laboratory tests before administration of HW extract and at the following times: at the onset of shock observed 3 to 16 minutes after administration of HW extract; and 30, 60, and 120 minutes after treatment. Three dogs treated with LPS underwent clinical examination and blood collection for laboratory tests before treatment with LPS and at the following times: 1 dog treated with a low dose of LPS at 15 minutes after treatment, at the onset of shock (37 minutes after treatment) and 60, 120, and 180 minutes after treatment; and 2 dogs treated with a high dose of LPS at the

onset of shock (1 and 13 minutes after treatment, respectively) and 30, 60, 120, and 180 minutes after treatment.

Blood samples were allocated to test tubes containing the following: potassium EDTA for hematologic examinations and platelet count, an empty tube for determination of serum TNF concentration, and an endotoxin-free tube containing sodium EDTA for determination of plasma endotoxin concentration in 2 dogs treated with HW extract. Samples for determination of serum TNF concentration and plasma endotoxin concentration were prepared by centrifugation at 1,630 × g for 10 minutes, and stored at -80 C. Plasma for determination of endotoxin concentration was transferred to an endotoxin-free tube. White blood cell and platelet counts were measured by the same method reported previously.¹² Rectal temperature was determined.

Tumor necrosis factor assay—Serum TNF concentration was determined by a cytotoxic assay, using WEHI-164 murine sarcoma subclone 28-4 (WEHI-164).^c In brief, WEHI-164 cells were examined by bacteriologic cultural technique in RPMI 1640 medium^d containing 10% fetal bovine serum, supplemented with 2 mM L-glutamine, 25 mM HEPES, 1 mM sodium pyruvate, and 3 µg of gentamicin/ml at 37 C and 5% CO₂. The cytotoxic assay was conducted by a slightly modified method of Espevik et al.²⁵ Briefly, 100-µl amounts of serial 2-fold dilutions of test serum or recombinant human TNF-α^e by RPMI 1640 medium were pipetted into the wells of 96-well flat-bottomed tissue culture plates.^f Next, 5 × 10⁴ WEHI-164 cells were added to each well in 100 µl of RPMI 1640 containing 1 µg of actinomycin D/ml (to produce a final concentration of 0.5 µg/ml) and incubated at 37 C and 5% CO₂ for 18 hours. After incubation, surviving cells were determined, using the tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)^g method according to a modified Mosmann assay.²⁶ In brief, tetrazolium salt was prepared at 5 mg/ml in PBS solution and filter sterilized; 25 µl of the solution was added to each well. After an additional 2 hours of incubation at 37 C, 100 µl of the supernatant was aspirated from the wells. One hundred microliters of extraction buffer (12.5% SDS, 50% dimethylformamide, pH 4.7) was added to each well, and after incubation overnight at 37 C, color development was measured at 570 nm, using a microplate reader.^h Tumor necrosis factor concentrations were determined from the standard curve with recombinant human TNF-α. The detectable limit was 0.015 U/ml. All determinations were performed in triplicate.

Endotoxin assay—Plasma endotoxin concentration was determined, using the endotoxin-specific chromogenic test.²³ⁱ The detectable limit was 3.0 pg/ml.

Statistical analysis—In dogs treated with HW extract and LPS, 1-way ANOVA and Fisher protected least significant difference were used to compare values before treatment with those at each time point after treatment. A 2-way ANOVA with repeated measurement and Student *t*-test were used for comparison of values between 8 dogs treated with HW extract and 3 dogs treated with LPS at each time point. A value of *P* < 0.05 was considered significant for all analyses.

Results

Clinical and hematologic findings—Reaction to HW extract and endotoxin did not differ between dogs without HW infection and dogs with HW infection, and reaction to female HW extract did not differ from reaction to male HW extract, so these results were combined. In 8 dogs treated with HW extract, blood pressure decreased remarkably 3 to 16 minutes after

Table 1—Mean (\pm SD) clinical and hematologic findings in dogs treated with heartworm extract

Variables	No. of dogs	Before treatment	Time after treatment (min)			
			3 to 16*	30	60	120
Systolic blood pressure (mm Hg)	8	134 \pm 18	35 \pm 22‡	103 \pm 43†	120 \pm 24	126 \pm 14
WBC (X 10 ⁶ / μ l)	8	13.7 \pm 2.7	4.6 \pm 2.8‡	10.9 \pm 3.1	13.7 \pm 3.4	17.3 \pm 3.6†
Platelet (X 10 ⁶ / μ l)	7	353 \pm 56	72 \pm 36‡	269 \pm 80†	358 \pm 77	378 \pm 83
Rectal temperature (C)	8	39.2 \pm 0.2	39.3 \pm 0.3	39.1 \pm 0.4	39.1 \pm 0.4	38.9 \pm 0.4

*At the onset of shock. †Significantly ($P < 0.05$) different from before-treatment values. ‡Significantly ($P < 0.01$) different from before-treatment values.
Undetectable systolic blood pressure (< 20 mm Hg) was designated as 20 mm Hg.

HW extract treatment but increased to > 100 mm Hg at 30 minutes after treatment and returned to nearly before-treatment values 120 minutes later (Table 1). White blood cell and platelet counts decreased significantly 3 to 16 minutes after HW extract treatment and returned to before-treatment values by 60 minutes. Rectal temperature did not change significantly during the study period. Endotoxin was not detected in the plasma of the 2 dogs that were tested.

By comparison, in the dog treated with a low dose of LPS, blood pressure decreased to 34 mm Hg at 37 minutes after LPS treatment, and WBC and platelet counts were decreased by 15 minutes after treatment and reached minimum values at 37 and 60 minutes after treatment, respectively. In the 2 dogs treated with a high dose of LPS, blood pressure and WBC and platelet counts decreased 1 and 13 minutes after LPS treatment, respectively. Rectal temperatures of 1 dog treated with a low dose of LPS and of 1 dog treated with a high dose of LPS began to increase 30 minutes after treatment and increased to > 40 C by 60 and 120 minutes after treatment, respectively. However, in the other dog treated with a high dose of LPS, rectal temperature mildly increased 180 minutes after treatment.

Serum tumor necrosis factor concentration—In 8 dogs treated with HW extract, serum TNF concentrations were not detectable until 30 minutes after HW extract treatment. At 60 minutes, the mean (\pm SD) TNF concentration (0.25 ± 0.32 U/ml) was low but significantly increased, compared with before treatment values. At 120 minutes after treatment, the mean TNF concentration (0.06 ± 0.13 U/ml) was detectable but was not significantly increased from before treatment values.

In the 3 dogs treated with LPS, the serum TNF concentration of each dog increased similarly regardless of LPS dose. The mean serum TNF concentration began to increase 30 minutes after LPS treatment and reached a significantly greater than before treatment concentration (40.20 ± 16.78 U/ml) at 60 minutes after treatment, remaining at a high concentration (18.19 ± 14.67 U/ml) until 180 minutes after treatment. Moreover, mean serum TNF concentrations in dogs treated with LPS were significantly higher than those in dogs treated with HW extract 30, 60, and 120 minutes after treatment.

Discussion

Results of our study indicate that TNF was not detected in serum at the time when all dogs treated with HW extract developed shock at 3 to 16 minutes

after treatment, and extremely low concentrations of TNF were detected in serum only at 60 and 120 minutes after treatment, when the dogs had recovered from shock. These results indicated that TNF may not participate in the onset of HW extract-induced shock and may not have a very important role in the pathophysiologic mechanisms of the shock.

In contrast, 3 dogs given LPS had remarkable increases in serum TNF concentrations at 30 to 180 minutes after LPS treatment, regardless of LPS dose, and these TNF concentrations were significantly higher than those in dogs treated with HW extract at 30, 60, and 120 minutes after treatment, respectively. Although only 3 dogs were treated with LPS in our study, other investigators have also reported that serum or plasma TNF concentrations increased remarkably after LPS treatment in dogs.^{20,27,28} LeMay et al²⁸ reported that all dogs given LPS at 0.1, 1.0, 10, and 40 μ g/kg, respectively, had high plasma TNF concentrations that appeared to increase in an all-or-none dose response and had clinical signs of vomiting, diarrhea, fever, and death associated with increases in plasma TNF concentrations. However, dogs treated with LPS at 0.01 μ g/kg had no detectable concentration of plasma TNF and no accompanying clinical signs. Their results indicate that TNF plays a key role in the pathophysiologic changes in endotoxin-induced clinical signs.

In addition, results of our study revealed that dogs treated with HW extract had no detectable concentrations of plasma endotoxin. Because a high concentration of endotoxin in plasma has been reported following IV injection of even a small amount (2 or 25 μ g/kg) of LPS to dogs,²²⁻²³ it seems that HW extract may contain minute amount of endotoxin or no endotoxin at all. Therefore, we concluded that the cause and mechanisms of HW extract-induced shock may be different from those of endotoxin-induced shock.

It is thought that an experimental IV bolus of LPS in dogs induces pathophysiologic changes characterized by cardiovascular collapse. The complement system or arachidonic acid cascade activation occurs immediately after an IV injection of LPS and, consequently, generates mediators such as anaphylatoxin C3a and C5a, platelet activating factor, and eicosanoids that are thought to cause rapid pathophysiologic changes.^{13,15,20,29-32} Tumor necrosis factor and other cytokines elicited by TNF such as interleukin-1, interleukin-6, and interleukin-8 are detectable in serum 30 minutes after an IV injection of LPS and cause the later changes.^{13,16,17,20-22} Moreover, inducible nitric oxide synthase, induced in macrophages, vascular smooth mus-

cle cells, and endothelial cells in response to TNF and other cytokines, may be responsible for nitric oxide production and much of the cardiovascular response, including delayed peripheral vascular failure.^{16,33} In our study, because HW extract-induced shock occurred immediately after HW extract treatment, it may be induced by mediators produced rapidly such as anaphylatoxin, platelet activating factor, and eicosanoids or the direct result of unknown substances contained in the HW extract.

Although rectal temperature did not increase in our dogs treated with HW extract, in dogs treated with LPS, especially 2 dogs with high serum concentrations of TNF, it increased to > 40 C. A proposed reason for hyperthermia in endotoxin-induced shock is that TNF and other mediators elicited by TNF such as interleukin-1 and prostaglandin E₂ may produce fever as an endogenous pyrogen.¹² Furthermore, endotoxin-induced shock is often accompanied by disseminated intravascular coagulation (DIC) as a result of activation of the coagulation cascade, and it is reported that the central role in this activation process may be caused by TNF.¹¹ The common pathway of coagulation is activated via the extrinsic pathway when TNF is administered to healthy humans.¹¹ Furthermore, endotoxin acting on monocytes and macrophages express tissue factor procoagulant activity, which is the initiator of the extrinsic coagulation pathway on the surface of endothelial cells and monocytes, and consequently induces DIC.³⁴ However, in our study, HW extract administration did not exert an LPS-like action. The HW extract did result in some TNF production but at low concentrations. Thus, blood coagulopathy complicated by HW extract-induced shock is not induced by DIC but by results from an inhibition of coagulation factor activities.^{2,3}

From the aforementioned results, the pathophysiologic reaction of HW extract-induced shock did not involve TNF, thus confirming that the cause and mechanisms of HW extract-induced shock were different from those of endotoxin-induced shock. Because HW extract-induced shock occurred immediately after HW extract treatment, it may be induced by mediators generated rapidly such as anaphylatoxin, platelet activating factor, and eicosanoids or the direct result of unknown substances contained in the HW extract.

^aLipopolysaccharide, *E coli* O127 serotype B8, Difco, Detroit, Mich.
^bBP-8800, Nippon Colin Co Ltd, Komaki, Japan.
^cProvided by Dr. Yasuyuki Mori, National Institute of Animal Health, Tsukuba, Japan.
^dGIBCO BRL, Life Technologies Inc, Rockville, Md.
^eGenzyme Corp, Minneapolis, Minn.
^fNunc No. 167008, Nunc A/S, Roskilde, Denmark.
^gSigma Chemical Co, St Louis, Mo.
^hImmuno MINI NJ-2300, Nippon InterMed Co Ltd, Tokyo, Japan.
ⁱEndospey method, Seikagaku Kogyo Co Ltd, Tokyo, Japan.

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