

# Role of histamine in heartworm extract-induced shock in dogs

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**Objective**—To determine whether heartworm (HW) extract-induced shock in dogs is consistent with anaphylactic shock by examining the role of histamine.

**Animals**—6 mixed-breed dogs (3 without and 3 with HW infections) and 4 specific pathogen-free (SPF) Beagles.

**Procedure**—Four experiments were performed as follows: 1) 6 mixed-breed dogs were treated IV with 2 ml of HW extract, and plasma histamine concentrations were determined; 2) 4 SPF dogs were treated IV with 2 ml of HW extract and examined for shock; 3) sera from 6 dogs of experiment 1 and from 4 SPF dogs of experiment 2 that were obtained before HW extract treatment were tested for heterologous passive cutaneous anaphylaxis (PCA), using rabbits during a sensitization period of 48 to 72 hours; and 4) mast cell degranulation by HW extract was tested, using rat mesentery and canine cultured mast cells.

**Results**—Experiment 1: 6 dogs developed shock, and plasma histamine concentrations increased significantly from  $0.3 \pm 0.2$  (mean  $\pm$  SD) ng/ml before HW extract treatment to  $44.6 \pm 68.9$  ng/ml at the onset of shock; experiment 2: all SPF dogs developed shock and had an increase in plasma histamine concentrations; experiment 3: sera from mixed-breed dogs without HW infection and from SPF dogs had negative PCA reactions; experiment 4: HW extract degranulated rat mesentery mast cells and released histamine directly from canine mast cells.

**Conclusions and Clinical Relevance**—Results of our study indicate that an unknown mast cell-degranulating substances contained in HW extract may degranulate mast cells directly, consequently releasing histamine that may participate in the onset of shock in HW extract-induced shock in dogs. (*Am J Vet Res* 2001;62:770–774)

It has been confirmed that *Dirofilaria immitis* extract induces shock immediately after IV administration to dogs.<sup>1-3</sup> Characteristics of the clinicopathologic findings are transient leukopenia and thrombocytopenia during circulatory collapse, along with increases in serum activities of alanine transaminase, alkaline phosphatase, lactate dehydrogenase, and creatine kinase.<sup>1,2</sup>

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Pathologic findings indicate that heartworm (HW) extract-induced shock may be a distributive shock, because the hepatosplanchnic vascular congestion leads to a reduction in venous return.<sup>3</sup> These findings are similar to those of endotoxin-induced shock,<sup>4</sup> anaphylactic shock,<sup>5</sup> and shock induced by administration of drugs such as diethylcarbamazine<sup>6-9</sup> and milbemycin D<sup>10</sup> in dogs with microfilaremic HW infection (microfilaremic-induced shock).

In our previous report,<sup>11</sup> we found that the cause and mechanisms of HW extract-induced shock may be different from those of endotoxin-induced shock, because serum tumor necrosis factor concentration, which was a pivotal mediator in endotoxin-induced shock, was minimally increased. Alternatively, mechanisms of microfilaremic-induced shock caused by administration of diethylcarbamazine has been proposed as an immunoglobulin E (IgE) mediated anaphylactic shock (an interaction of microfilariae antigen and IgE, which degranulates previously sensitized mast cells)<sup>9,12</sup> or a non-IgE mediated anaphylactic shock (direct mast cell degranulation).<sup>7</sup> In IgE and non-IgE mediated anaphylactic shock, chemical mediators such as histamine are liberated from mast cells.<sup>5,13</sup> Therefore, it is suspected that the pathophysiologic reactions of HW extract-induced shock may involve histamine release by mast cell degranulation. The purpose of the study reported here was to determine whether HW extract-induced shock in dogs is consistent with anaphylactic shock by examining the role of histamine.

## Materials and Methods

**Experiment 1: changes in plasma histamine concentrations in dogs with shock induced by IV administration of HW extract**—Six mixed-breed dogs ranging from 1 to 3 years old and weighing between 6.5 and 14.8 kg were used. Dogs were obtained from the local shelter. Three of the dogs did not have a HW infection, and 3 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  $408 \times g$  for 10 minutes, and then the sediment (0.1 ml) was examined as a wet mount under scanning magnification. To confirm the presence of adult worms, all 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) at the end of the study, and necropsy was performed. The study was conducted in a manner consistent with the Gifu University Guidelines for Animal Experimentation. Clinical signs of shock were transient and over quickly, so we made no attempt at alleviating them.

We have previously reported that female and male HW extract induces shock similarly in dogs.<sup>1</sup> To verify whether the onset of shock induced by these 2 types of HW extract

would involve histamine, we used female and male HW extract in this study. Briefly, 10 adult female or 20 adult male worms collected from HW infected dogs were washed several times with physiologic saline (0.9% NaCl) solution, cut into small fragments with scissors, comminuted by use of a mechanical mixer, and were sonicated for 10 seconds a total of 10 times in 20 ml of 5% glucose solution. The worm suspension was centrifuged at  $8,000 \times g$  for 20 minutes, and the supernatant solution was collected. All steps were conducted at 4 C. Two milliliters of female or male HW extract was equivalent to the volume extracted from a female adult HW or 2 male adult HW, respectively. Of 6 dogs, 3 were treated IV with 2 ml of female HW extract and the other 3 with 2 ml of male HW extract. In female and male HW extract, histamine concentrations were determined by the method described in the following section.

Systemic blood pressure was measured by use of the oscillometric method<sup>8</sup> 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.<sup>14</sup> All dogs 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 11 minutes after treatment and 30, 60, and 120 minutes after treatment. In the clinical examination, heart rate, respiratory rate, rectal temperature, mucous membrane color, cardiac sound, peristaltic sound, skin temperature by palpation, and general state were evaluated. Four-milliliter whole blood samples obtained at each time were immediately allocated to test tubes that contained potassium EDTA for WBC and platelet counts and sodium EDTA for plasma histamine assay. A whole blood sample was also obtained before treatment and allocated to an empty tube to separate serum for a **passive cutaneous anaphylaxis (PCA)** reaction in experiment 3. Plasma for histamine assay was separated by centrifugation at  $1,630 \times g$  for 10 minutes, and the plasma within approximately 1 cm above the buffy coat was not used to avoid possible contamination with leukocytes. Serum for the PCA reaction was separated by centrifugation at  $1,630 \times g$  for 10 minutes. Plasma and sera were stored at  $-80$  C. White blood cell and platelet counts were measured as reported previously.<sup>1,2</sup> Plasma histamine concentration was determined by high-performance liquid chromatography combined with fluorometric assay.<sup>15</sup> This assay is sensitive to 0.10 ng of histamine/ml.

**Experiment 2: induction of shock and changes in plasma histamine concentrations by IV administration of heartworm extract in specific pathogen-free (SPF) dogs**—The SPF dogs were 4 female Beagles 9 months to 1 year of age, weighing between 9.6 and 12.0 kg. Lack of HW infection was confirmed by no detection of circulating microfilariae and adult worms by use of the same methods as in experiment 1, respectively. Two milliliters of female HW extract was administered IV to each dog, and procedures were performed the same way as in experiment 1. To observe lesions, 2 of 4 dogs were euthanized by administration of a lethal dose of sodium pentobarbital at the onset of shock.

**Experiment 3: determination of heterologous passive cutaneous anaphylaxis reaction**—Heterologous PCA determinations were performed by the method of Hsu et al.<sup>16</sup> Test sera were obtained from blood samples of 6 dogs in experiment 1 and 4 SPF dogs in experiment 2 before treatment with HW extract. Serial 2-fold dilutions of test sera in 0.1-ml quantities were administered intracutaneously on the shaved abdomens and lateral sides of 4 Japanese White rabbits that were 16 to 20 weeks of age, 2.7 to 3.2 kg in body weight, and of both sexes. After a sensitization period of 48 to 72 hours, the rabbits were treated IV with 0.5 ml of HW

extract in 0.5 ml of 0.5% Evans blue solution. Positive control sera were obtained from 2 dogs infected with HW, which were not dogs used in our study, and which had been found to produce strongly positive PCA reactions in rabbits in a preliminary examination. Sterilized physiologic saline was a negative control. Thirty minutes later, the average diameter of blue spots was determined on the external side of the skin, and blue spots with a diameter 5 mm greater than those of the controls were regarded as positive reactions. Passive cutaneous anaphylaxis titers were recorded as the reciprocal of the maximum serum dilution that gave a positive reaction.

**Experiment 4: in vitro degranulation of mast cells in rat mesentery and canine mast cells**—A test involving the degranulation of mast cells in rat mesentery was performed by the method of Boreham.<sup>17</sup> Briefly, 10 Wistar strain rats that were healthy and 6 to 15 weeks of age were anesthetized by diethyl ether and euthanized by exsanguination. Small loops of intestine, with the mesentery intact, were spread in a petri dish and washed with PBS solution (pH 7.2). The mesentery was flooded with 2 ml of female or male HW extract, 2 ml of 5% glucose solution, from which HW were extracted, and 50  $\mu\text{g}/\text{ml}$  of concanavalin A<sup>b</sup> in 2 ml of distilled water as positive control or 2 ml of PBS solution as negative control and incubated at room temperature (approx 25 C) for 15 minutes. After removing the fluid and washing the mesentery with PBS solution, the tissue was fixed with formol alcohol (4 parts absolute alcohol, 1 part 10% formalin, pH 7.0) for 60 minutes at room temperature (approx 25 C) and stained with 0.05% aqueous toluidine blue for 10 minutes. The mesentery was carefully cut from the intestine, spread on a glass slide, cleared in xylene, and mounted by the conventional procedure. Five replicates of each test were performed, and 500 cells on each preparation were counted to determine the percentage of mast cells that degranulated.

For the in vitro testing of degranulation of canine mast cells, LuMC cells<sup>18</sup> derived from a spontaneous canine intestinal mast cell tumor were used. The LuMC cells were examined by bacteriologic cultural technique in RPMI 1640 medium<sup>c</sup> (2 mM l-glutamine, 50  $\mu\text{g}/\text{ml}$  gentamicin, 1.5  $\mu\text{g}/\text{ml}$  fungisone, 4.4  $\mu\text{l}/\text{l}$  lipoprotein) with 10% heat-inactivated fetal bovine serum<sup>d</sup> at 37 C in humidified air with 5% CO<sub>2</sub>. The LuMC cells were suspended at a concentration of  $1.0 \times 10^6$  cell/ml in Pipes-buffered saline (25 mM Pipes,<sup>b</sup> 117 mM NaCl, 5 mM KCl, 0.1% bovine serum albumin, 3 mM Ca<sup>2+</sup>, 0.4 mM Mg<sup>2+</sup>, pH 7.3). Test samples were extracted from 10 female or 20 male adult HW in 20 ml of Pipes-buffered saline by the procedure described in experiment 1 and diluted 3 to 6,561 times with Pipes-buffered saline. Calcium ionophore A23187<sup>b</sup> was used as positive control and Pipes-buffered saline as negative control. After 1 ml of cell suspension was centrifuged at  $408 \times g$  for 10 minutes, the supernatant fluid was removed, and the cell pellets were used for the mast cell degranulation test. One milliliter of each test sample or control at increasing concentrations was added to the cell pellets and incubated for 45 minutes at 37 C. After incubation, the cell suspension was centrifuged at  $408 \times g$  for 10 minutes at 4 C, and the supernatant was collected and added to 12% perchloric acid for an incubation period of 24 hours at 4 C. The supernatant was collected by centrifugation at  $1,630 \times g$  for 30 minutes, and the histamine released into the supernatant was measured by an automated fluorometric assay.<sup>19</sup> All treatments were performed in duplicate, and the **percentage of histamine release (HR%)** was calculated by the following equation:

$$\text{HR\%} = \left[ \frac{\text{HcTest sample} - \text{HcNegative control}}{\text{HcComplement} - \text{HcNegative control}} \times 100 \right]$$

where HcTest sample = histamine content released by the test sample, HcNegative control = the histamine content released by negative control, and HcComplement = the entire amount of histamine included in cell pellets.

**Statistical analysis**—Statistical analysis was performed, using 1-way ANOVA followed by Fisher protected least significant difference test for paired data. A *P* value of < 0.05 was considered significant.

## Results

**Experiment 1**—Reactions to female HW extract did not differ from those to male HW extract, so these data were combined. Characteristic signs of the reaction were lethargy, tachypnea, palpitation, and vomiting immediately after HW extract treatment, followed by circulatory collapse, pale mucous membranes, and intestinal hyperperistalsis at the onset of shock. Systolic blood pressures decreased significantly 3 to 11 minutes after HW extract treatment and began to return normal 30 minutes after treatment (Table 1). White blood cell and platelet counts decreased significantly at the onset of shock but increased to before-treatment values by 60 minutes after HW extract administration. Plasma histamine concentrations increased significantly from  $0.3 \pm 0.2$  (mean  $\pm$  SD) ng/ml before HW extract treatment to  $44.6 \pm 68.9$  ng/ml at the onset of shock. However, they decreased rapidly 30 minutes after HW extract treatment and reached almost before-treatment concentrations 120 minutes after treatment. Although plasma histamine concentration of each dog at the onset of shock varied between 0.85 and 181.75 ng/ml (1.44 to 648.8 times greater than before-treatment values), no differences were found between dogs with and without HW infection. Histamine was not detected in female and male HW extract.

**Experiment 2**—Systolic blood pressures decreased significantly 2 to 11 minutes after HW extract treat-

ment, and all SPF dogs developed shock (Table 2). Clinical signs were similar to those of dogs in experiment 1, and WBC and platelet counts decreased significantly at the onset of shock. Plasma histamine concentrations increased significantly from  $0.3 \pm 0.1$  (range, 0.2 to 0.4) ng/ml before HW extract treatment to  $6.2 \pm 1.6$  (range 4.04 to 7.47) ng/ml (10.1 to 35.6 times greater than before treatment values) at the onset of shock. On necropsy examination, main pathologic features were centralobular congestion in the liver, hemorrhage and edema in the gallbladder wall, and congestion and hemorrhage in mucous membranes of the gastrointestinal tract.

**Experiment 3**—Sera from mixed-breed dogs with HW infection had positive PCA reactions with a range of PCA titers between 16 and 32, but all sera from mixed-breed dogs without HW infection and SPF dogs had negative reactions.

**Experiment 4**—Upon the addition of female HW extract,  $79.6 \pm 11.8\%$  (mean  $\pm$  SD) of the rat mesentery mast cells degranulated, compared with  $79.2 \pm 16.5\%$  of the mast cells when male HW extract was added (Fig 1). A 5% solution of glucose degranulated the mast cells at  $40.6 \pm 21.0\%$ , PBS solution as negative control at  $15.0 \pm 10.8\%$ , and Concanavalin A as positive control at  $88.4 \pm 12.7\%$ . Degranulations by female and male HW extract were significantly greater than those by 5% glucose solution and PBS solution.

Female and male HW extract released histamine from canine LuMC cells dose-dependently (Fig 2). The highest HR% was 9.58% by female HW extract diluted 3 times and 13.57% by male HW extract diluted 3 times.

## Discussion

Anaphylactic shock is an immediate severe systemic reaction that results from the release and gener-

Table 1—Mean ( $\pm$  SD) clinical and hematologic findings and histamine concentration in mixed-breed dogs with shock induced by treatment with heartworm extract

Variables	No. of dogs	Before treatment	Time after treatment (min)			
			3 to 11*	30	60	120
Systolic blood pressure (mm Hg)	6	128 $\pm$ 15	36 $\pm$ 25†	91 $\pm$ 40†	111 $\pm$ 20	121 $\pm$ 14
WBC (X 10 <sup>3</sup> /μl)	6	13.7 $\pm$ 3.2	5.3 $\pm$ 2.8†	11.7 $\pm$ 3.2	13.9 $\pm$ 3.9	18.4 $\pm$ 3.6†
Platelet (X 10 <sup>3</sup> /μl)	6	354 $\pm$ 62	61 $\pm$ 21†	284 $\pm$ 77	368 $\pm$ 80	375 $\pm$ 90
Histamine (ng/ml)	6	0.3 $\pm$ 0.2	44.6 $\pm$ 68.9†	3.2 $\pm$ 4.0	1.2 $\pm$ 1.1	0.6 $\pm$ 0.5

\*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.

Table 2—Mean ( $\pm$  SD) clinical and hematologic findings and histamine concentration in specific pathogen-free dogs with shock induced by treatment with heartworm extract

Variables	Before treatment	Time after treatment (min)			
		2 to 11*	30	60	120
Systolic blood pressure (mm Hg)	154 $\pm$ 18 (4)	27 $\pm$ 14‡ (4)	140 $\pm$ 35 (2)	147 $\pm$ 7 (2)	139 $\pm$ 1 (2)
WBC (X 10 <sup>3</sup> /μl)	13.4 $\pm$ 1.3 (4)	2.8 $\pm$ 1.0† (4)	8.4 $\pm$ 4.0‡ (2)	12.9 $\pm$ 1.8 (2)	14.1 $\pm$ 6.0 (2)
Platelet (X 10 <sup>3</sup> /μl)	374 $\pm$ 51 (3)	110 $\pm$ 40† (3)	180 (1)	229 (1)	393 (1)
Histamine (ng/ml)	0.3 $\pm$ 0.1 (4)	6.2 $\pm$ 1.6† (4)	2.1 $\pm$ 1.8 (2)	0.8 $\pm$ 0.7 (2)	0.3 $\pm$ 0.1 (2)

Values in parentheses indicate the No. of dogs. No. of dogs examined decreased at 30 minutes after heartworm extract treatment, because 2 of 4 dogs were euthanatized in order to observe lesions.  
See Table 1 for key.

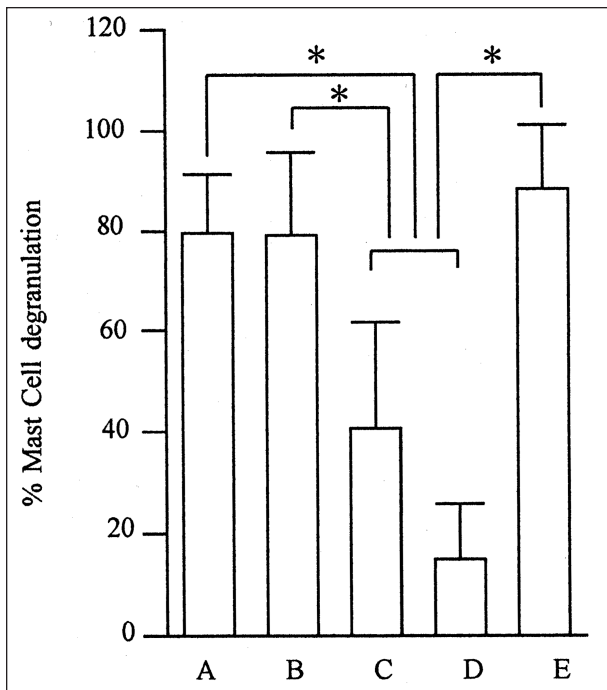


Figure 1—In vitro degranulation of mast cells in rat mesentery in response to the following: A—female heartworm (HWV) extract; B—male HWV extract; C—5% glucose solution; D—PBS solution; E—Concanavalin A. Column and vertical bar represent mean and 1 SD, respectively. Numbers of experiments were 12 in A, 8 in B, and 20 each in C, D, and E. Five replicates of each experiment were performed with 500 cells being counted on each preparation. \*Significantly ( $P < 0.01$ ) different from degranulation of mast cells by 5% glucose solution and PBS solution.

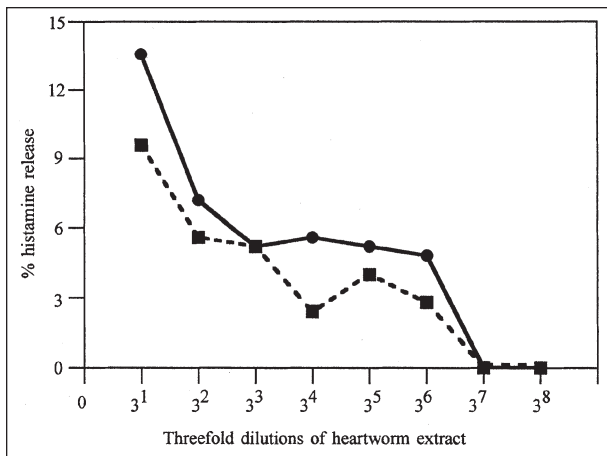


Figure 2—In vitro degranulation of canine mast cells. Closed squares = Female HWV extract. Closed circles = Male HWV extract. Female and male HWV extract released histamine from LuMC cells dose-dependently. The % histamine concentrations were mean values of 2 measurements, respectively. The highest % Histamine release was 9.58% by female HWV extract diluted 3 times and 13.57% by male HWV extract diluted 3 times.

ation of endogenous chemical mediators such as histamine, leukotrienes, and prostaglandins by exposure to foreign substances in mast cells.<sup>5,13</sup> This reaction is usually the result of an interaction between the antigen and IgE but can also result from a mast cell degranulation attributable to mechanisms that are not mediated by IgE.<sup>5,13</sup> The former reaction is the result of reexpo-

sure to the antigen, resulting in bridging or crosslinking of IgE molecules bound to Fc receptors on the surface of mast cells, followed by degranulating primary mediators and generating secondary mediators in mast cells.<sup>5,13</sup> The latter reaction is the result of 2 main mechanisms. One mechanism involves anaphylatoxins C3a and C5a, which are generated via activation of the complement cascade by either the classic or alternate pathway, causing a degranulation of mast cells. The other mechanism involves the degranulation of mast cells caused directly by drugs or other chemicals.<sup>5,13</sup> In HWV extract-induced shock, plasma histamine concentrations at the onset of shock increased 1.44 to 648.6 times that of before-treatment values. These increases in plasma histamine concentrations were observed in dogs with and without HWV infection and were not different between them. Plasma histamine concentrations decreased rapidly 30 minutes after HWV extract administration, and blood pressure, WBC count, and platelet count returned to within reference range values. In addition, histamine was not detected in HWV extract. Therefore, it was considered that histamine released from mast cells in vivo by administration of HWV extract may participate in the onset of shock in dogs with and without HWV infection.

Secondly, all SPF dogs not infected with HWV or other parasites that had a common antigenicity with HWV developed shock upon treatment with HWV extract. Clinical signs and main pathologic findings at the onset of shock in SPF dogs were similar to previously reported findings.<sup>1,3</sup> In addition, plasma histamine concentrations of SPF dogs at the onset of shock increased 10.1 to 35.6 times that of before-treatment values. In a heterologous PCA reaction for the detection of IgE,<sup>16</sup> all sera from mixed-breed dogs without HWV infection and SPF dogs that developed shock by the administration of HWV extract had negative reactions to HWV antigen. These findings indicate that anti-HWV IgE and IgG did not participate in the onset of shock and that mast cell degranulations caused by HWV extract were not mediated by anti-HWV antibody. Moreover, female and male HWV extract degranulated rat mesentery mast cells directly and induced histamine release from LuMC cells in a dose-dependent manner. This suggests that HWV extract may contain unknown mast cell-degranulating substances.

Our results indicate that an unknown mast cell-degranulating substance contained in HWV extract may degranulate mast cells directly; consequently, the released histamine may participate in the onset of HWV extract-induced shock. And because all of 3 HWV-infected dogs had positive PCA reactions, it seems that the reaction of these dogs to HWV extract may result from direct mast cell degranulation or a combination of mechanisms including direct mast cell degranulation and IgE-mediated anaphylactic reaction.

Peak plasma histamine concentrations at the onset of shock varied in each dog. We propose the following explanations for this finding. First, Silverman et al.<sup>20</sup> reported that histamine release was not observed in dogs that had physiologic changes in canine antigen-induced IgE-mediated anaphylactic shock. They thought this may be attributed to the involvement of

other chemical mediators such as prostaglandin D<sub>2</sub>, which are generated in mast cells, in the onset of a hypotensive reaction. Thus, varied histamine concentrations observed in HW extract-induced shock may be the result of the same cause. Alternatively, Boreham et al<sup>6</sup> reported that microfilaremic-induced shock attributable to the administration of diethylcarbamazine did not involve an immunologic reaction and that the cause of shock was the liberation by diethylcarbamazine of substances from microfilariae that were able to constrict the hepatic veins either directly or via the release of host factors. Therefore, it was suspected that the onset of HW extract-induced shock may involve unknown substances liberated from adult worms that were able to constrict the hepatic veins in the described manner along with the mechanisms of mast cell degranulation.

Boreham<sup>17</sup> also reported that adult HW and microfilariae extracts degranulated rat mesentery mast cells in vitro in a nonimmunologic manner. The mast cell degranulation substance of other nematodes was already isolated from *Ascaris suum* by Uvnäs et al,<sup>21</sup> Thompson,<sup>22</sup> and Greenspon et al.<sup>23</sup> This substance was a peptide (mast cell-degranulating polypeptide) with a molecular weight of 3,000 to 6,000, which was able to degranulate rat peritoneal mast cells on a nonimmunologic basis. However, the physiologic importance of mast cell-degranulating polypeptide has not yet been clarified. Results of our study indicate that HW extract may contain mast cell-degranulating substances and that these substances may be the cause of HW extract-induced shock. Isolation and identification of the substances from HW extract would be useful in elucidating the mechanisms of HW extract-induced shock, and it is suspected that an investigation of whether other nematodes, including *Ascaris suum*, contain the same substances may play an important role in elucidating the pathophysiologic mechanisms of helminthiasis.

Results of our study indicate that unknown mast cell-degranulating substances contained in HW extract may degranulate mast cells directly; consequently, the released histamine may participate in the onset of HW extract-induced shock. Varied histamine concentrations at the onset of shock suggested another mechanism; that is, that the onset of shock may involve unknown substances able to constrict the hepatic veins either directly or via the release of host factors.

<sup>a</sup>BP-8800, Nippon Colin Co Ltd, Komaki, Japan.

<sup>b</sup>Sigma Chemical Co, St Louis, Mo.

<sup>c</sup>GIBCO BRL, Life Technologies Inc, Rockville, Md.

<sup>d</sup>JRH Biosciences, Lenexa, Kan.

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