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Reaginic and Hemagglutinating Antibody Production in Dogs Infected with *Dirofilaria immitis*

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Abstract. The kinetics of reaginic and hemagglutinating antibody productions in dogs experimentally infected with *Dirofilaria immitis* was studied by passive cutaneous anaphylaxis reaction and indirect hemagglutination test. Reaginic antibody was first detected on the 65th day of infection, which coincided with the fourth molt of worm development. Although the antibody transiently disappeared on the 97th day, it reappeared, thereafter, and boosted when microfilaremia became evident. The reaginic activity of sera was completely deleted by heating at 56°C for 60 min and by reduction and alkylation procedures. Sephadex G-200 gel filtration analysis indicates that reaginic activity was recovered in the ascending portion of the second (IgG) peak, suggesting molecular weight slightly greater than that of IgG. Hemagglutinating antibody was first observed on the 19th day of infection and then, steadily increased until the end of the study. Hemagglutinating antibody responses showed the pattern of two distinct peaks which coincided both with the fourth larval molt and the occurrence of microfilaremia.

Immunological responses of dogs to *Dirofilaria immitis* have been studied not only for the immunodiagnostic purpose [3–6, 13, 14, 17, 23, 25, 29] but also for the elucidation of the kinetics of humoral antibody production [1, 7, 17, 20, 27]. In these reports, hemagglutinating antibody production has been extensively studied by indirect hemagglutination (IHA) test. In regard to reaginic antibody, however, only a few information has been available on antibody production in dogs following *D. immitis* [7–9, 11, 26] as well as some other parasitic helminth [2, 8, 28] infections and also ragweed polenosis [21, 22].

Previously Hayasaki et al. [7] pointed out the sufficient production of reaginic antibody as well as hemagglutinating antibody in dogs experimentally infected with *D. immitis*, and they also suggested that these antibody productions would possibly be correlated with the fourth molting of the migrating larvae. The purpose of the present study is to elucidate the production of reaginic and hemagglutinating antibodies in relation to the worm development in the host and also to determine the physicochemical properties of canine reaginic antibody.

Materials and Methods

Dogs: Seven mongrel dogs aged about three months, housed in a mosquito-proof room, were used for *D. immitis* infection. Two adult dogs, white and light yellow haired, were employed as recipients for PCA reaction. They were maintained in good health for at least 6 months until use and kept mosquito-free and helminth-free.

Experimental infection: The mosquitoes, *Aedes togoi*, were infected by feeding on a dog with about 200 circulating microfilarial (Mf) counts per 20 μl of blood. Infective larvae (L_3) were collected from the proboscises of mosquitoes 10 to 14 days after infection and suspended in saline. Seven dogs were

divided into two groups. Three dogs were subcutaneously injected into the inguinal region with 457, 100 and 10 of L_3, respectively. Four dogs were used as uninfected controls.

Serum samples: All dogs were bled at intervals of approximately 2 weeks and sera were separated and stored at −40°C until use.

Peripheral Mf counts: Dogs were screened for Mf by concentration method [16] and microfilarial density in 20 μl of blood was measured by counting Mf on methylene blue stained smears.

Antigen: Phosphate buffered saline (PBS; pH 6.4) extracts of *D. immitis* adult worms and intrauterine microfilariae were prepared as previously described [6]. The protein concentration of the antigen was determined by the method of Lowry et al. [12].

IHA test: IHA test was performed as previously described [6].

Passive cutaneous anaphylaxis (PCA) reaction: Before the skin sensitization, the recipients were anesthetized with sodium pentobarbital (Somnopentyl, Pitman-moore, Inc. N.J.) and then shaved over the back. Intradermal injections of 0.1 m*l* of homologous antisera were made by using a 1 m*l* syringe fitted with 22-gauge needle at 2.5 to 3 cm intervals. Each pool of test sera was injected undiluted and at dilutions of 1:4, 1:16, 1:64, 1:128, 1:256 and 1:512 in saline into two recipients. PCA reaction was induced 72 hr after sensitization. Antigen challenge was performed by the intravenous injection of the adult worm antigen (1.0 mg proteins/kg body weight) together with an adequate amount of 0.5% Evans' blue (1.0 m*l*/kg body weight). The reactions were read 60 min after the antigen injection. They were defined as positive when the area of cutaneous bluing was greater than 5 mm in diameter. The reciprocal of the highest dilution showing a positive reaction was taken as the PCA antibody titer.

Treatment of heat, reduction and alkylation: Samples (0.5 m*l*) of PCA positive serum were treated with heat or reduction by 2-mercaptoethanol (2-ME) and alkylation by iodoacetamide (IA) as described by Rockey and Schwarzman [22]. Briefly, the samples were dialyzed against 500 m*l* of 0.15M 2-ME in PBS (pH 7.2) for 2 hr at a room temperature, and subsequently against 0.02M IA in PBS (pH 7.2) for 2 hr at 4°C and then against several changes of PBS (pH 7.2) for 24 hr at 4°C. Controls were treated in an identical manner except that 2-ME was omitted.

Similar samples were heated at 56°C in a water bath for 30 to 60 min. Untreated samples served as controls.

Gel filtration: Chromatography was performed essentially as described by Zvaifler and Becker [30]. A column of 2.5×90 cm was packed with Sephadex G-200 which had been equilibrated with PBS (pH 7.2). One m*l* of whole dog serum was applied. Fractions of 5 m*l* were collected at a flow rate of 10 m*l* per hr. Protein profiles were determined by measuring OD at 280 mμ.

Gel-diffusion technique: Double diffusion studies were performed by the micromethod of Ouchterlony [19] using anti-dog IgG rabbit serum (Miles Lab., Inc. Elkhart, Ind.), to determine IgG positive fractions among various eluates from the Sephadex

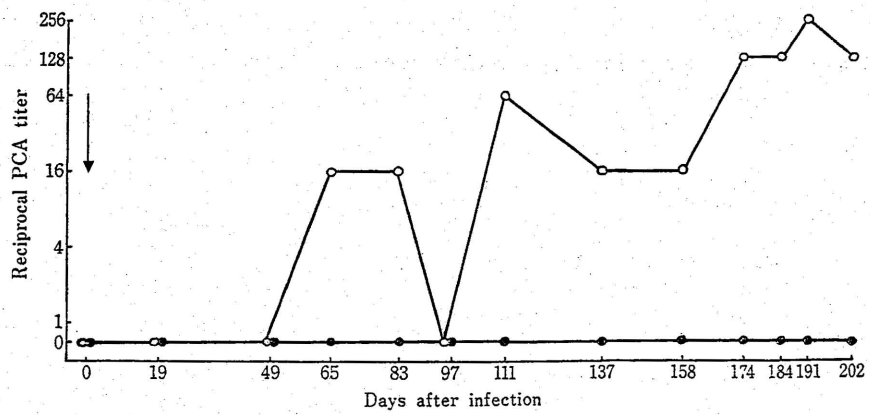

Fig. 1. PCA titers in sera from dogs experimentally infected with *D. immitis*.
○——○ : Mean PCA titer in infected dogs.
●——● : Mean PCA titer in uninfected dogs.
Arrow indicates infection.

G-200 gel filtration.

Results

Progressive changes of the reaginic antibody production in *D. immitis* infected dogs were monitored by PCA reaction (Fig. 1). Reaginic antibody was first detected in the sera of infected dogs on the 65th day of infection and then persisted until the 83rd day. The reaginic antibody disappeared on the 97th day, but thereafter, the antibody reappeared on the 111th day and persisted in relatively high titers until the end of the observations. When microfilaremia became evident, the reaginic antibody titers were boosted. No reaginic antibody was observed in uninfected controls throughout the course of the study.

PCA positive-pooled sera from infected dogs were examined for the reaginic activity after heating at 56°C for 30 or 60 min, or after the reduction by 2-ME and then alkylation by IA (Table 1). Reaginic activity of the sera was completely deleted by heat treatment for 60 min. Treatment of IA alone also resulted in a partial inactivation of reaginic activity, which might be due to a technical artifact produced during serum treatment. A single sample obtained from infected dogs on the 191st day of infection

Table 1. Effect of heat, and reduction and alkylation on PCA activity of anti-*D. immitis* sera from dogs

Treatment	Reciprocal PCA titer of sera obtained on	
	Day 174	Day 184
Unheated	128	129
Heated at 56°C for 30 min	16	16
60 min	0	0
2-mercaptoethanol and iodoacetamide	0	0
Iodoacetamide only	16	64
Dialysis only	ND	128

PCA: Passive cutaneous anaphylaxis.
ND: Not done.

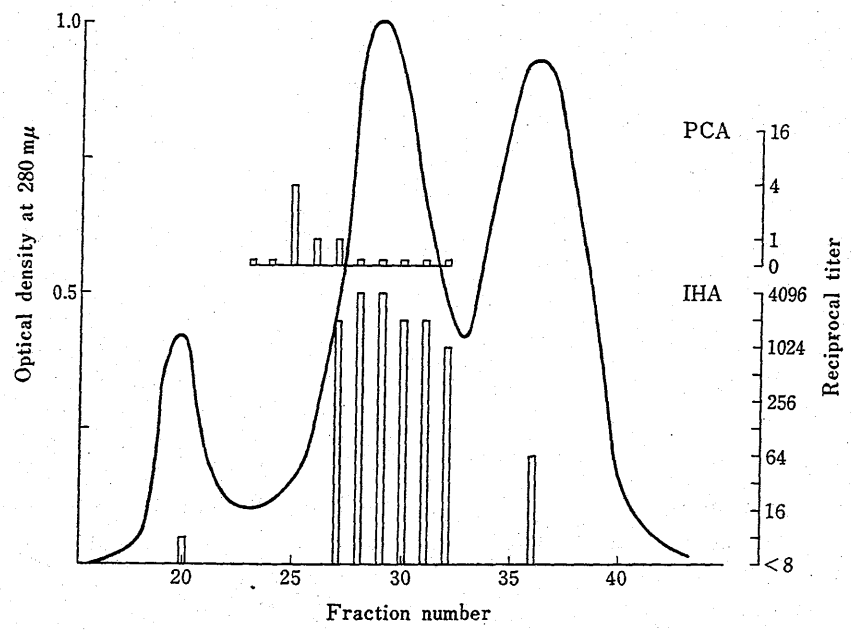

Fig. 2. PCA and IHA activities of the eluates from Sephadex G-200 gel filtration profile of pooled serum obtained on the 191st day of infection.

PCA: Passive cutaneous anaphylaxis. IHA: Indirect hemagglutination.

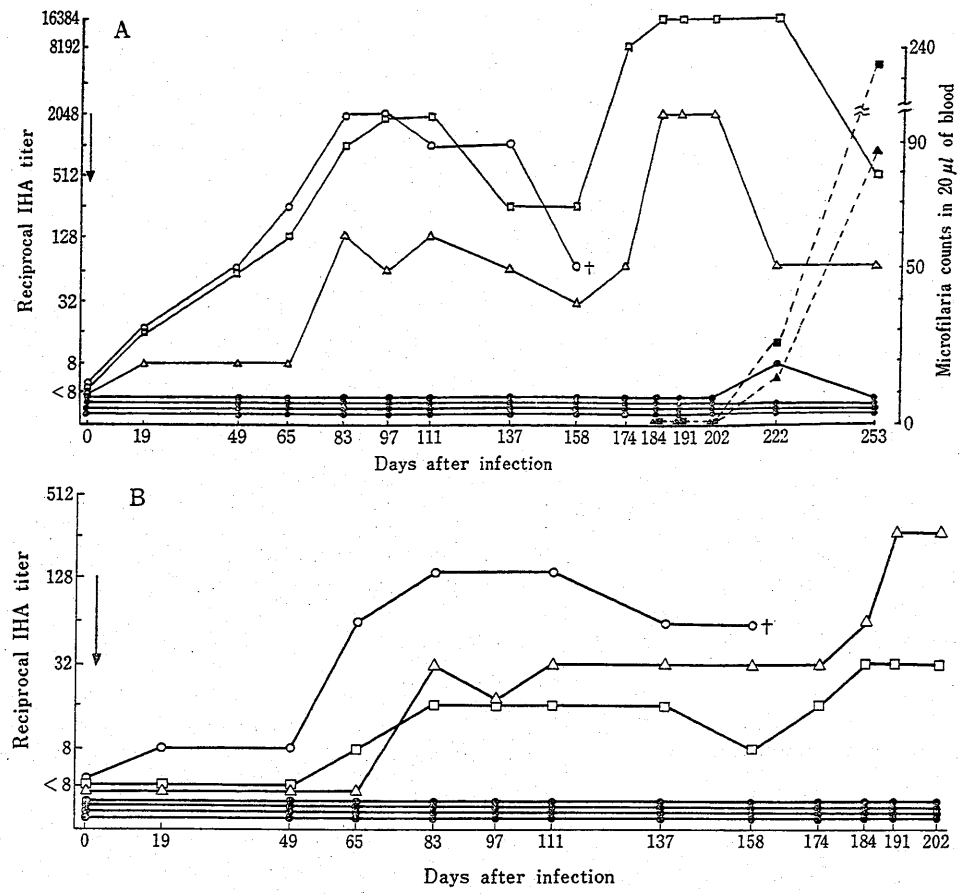

Fig. 3. IHA titers and microfilaria counts of dogs experimentally infected with *D. immitis*.

A: IHA antibody titers of individual infected dogs, determining by IHA test with intrauterine microfilarial antigen.

B: IHA antibody titers of individual infected dogs, determining by IHA test with adult *D. immitis* antigen.

○——○: Dog No. 1 (457L_3), □——□: Dog No. 2(100L_3), △——△: Dog No. 3 (10L_3), ●——●: Four control dogs.

Peripheral microfilarial count of individual infected dogs. ■——■: Dog No. 2, ▲——▲: Dog No. 3.

Arrow indicates infection. †: Necropsy.

was fractionated by the gel filtration with Sephadex G-200 (Fig. 2). The results indicate that reaginic activity was recovered in the ascending portion of the second peak (IgG) of gel filtration chromatography, whereas maximum hemagglutinating activity was recovered in the central portion of the peak. Gel diffusion assay clearly indicated that IgG antibody was involved in the second peak.

The kinetics of hemagglutinating antibody production in infected dogs, as assessed by IHA tests both with intrauterine Mf and adult *D. immitis* antigens, is shown in Fig. 3. Hemagglutinating antibody was first observed in sera from all three dogs on the 19th day of infection, when assessed by the IHA test with intrauterine Mf antigen, and

Table 2. Worm burdens of dogs experimentally infected with *D. immitis*

Group	Dog No.	Number of L_3	Period (Days) at necropsy after infection	Number of adult worm (M/F)	Recovery rate (%)
Infected dog	1	457	161	99 (65/34)	22
	2	100	253	64 (28/36)	64
	3	10	253	5 (2/ 3)	50
				Mean	45

L_3 : Third stage larva.
M/F : Male/female.
No worm was detected in four controls necropsied at the 253rd day of infection.

its titer then steadily increased and reached the first peak about 83 to 111 days post-infection. Following a gradual decrease of the antibody levels up to the 158th day, the titers reincreased and reached the second peak. Thereafter the titers again decreased until the end of the study. The shape of two distinct peaks was clearer in the IHA test with intrauterine Mf antigen than that in the test with adult worm antigen. In contrast, uninfected controls had consistently very low antibody titers of less than 1:8, indicating a negative IHA reaction, although a single dog yielded an antibody titer of 1:8 on the 222nd day after infection.

Experimental dogs were sacrificed for worm counts on the 253rd day of infection, except for a single dog (No. 1) necropsied on the 161st day. Worm recovery data are shown in Table 2. The mean recovery rate in infected dogs was 45%. To the contrary, no worm was recovered from four control dogs.

Peripheral Mf was first detected on the 184th day and its levels increased rapidly from the 222nd to 253rd day (Fig. 3).

Discussion

Reaginic antibody production in *D. immitis* infected dogs has been studied by some investigators using PCA reaction [7–9, 11, 26]. Some [9, 11, 26] of them have dealt with the reaginic antibody responses during the patent period of spontaneously or experimentally infected dogs. With regard to the prepatent period of the disease, little information is available for the kinetics of reaginic antibody responses except for our previous paper [7], although Hayashi et al. [8] demonstrated the short-term (4–5 hr) skin sensitizing antibody in experimentally infected dogs.

In the present study, the appearance of reaginic antibody coincided with the timing of the fourth molt (between the 60th and the 70th day) of migrating larvae [18], and the maximum PCA titer was noted at the time of the appearance of the peripheral Mf. Therefore, it was considered that the reaginic antibody production might be strongly stimulated by the molting worms and the Mf released from female worms. Unexpectedly, reaginic antibody disappeared on the 97th day of infection, while hemagglutinating antibody steadily increased and reached the first peak of the titer. Although the reason for this is still unknown, the feedback regulation [24] by the augmented hemagglutinating antibody production might be involved in this transient disappearance of reaginic antibody. However, such transient disappearance has not been observed in our previous paper [7].

The present result also revealed that the reaginic antibodies from experimentally infected dogs lost completely their activity

for PCA reaction by reduction and alkylation and were destroyed by heating at 56°C for 60 min. Kobayashi et al. [11] described that the reaginic antibody from the dogs spontaneously infected with *D. immitis* was destroyed by heating at 56°C for 60 min, and Williams et al. [28] also stressed that the canine reaginic antibody from the dogs experimentally infected with *Echinococcus granulosus* was destroyed by heating at 56°C for 30 min. These data resemble the present results. Hsu et al. [9] reported, however, that the reaginic activity of the sera from dogs naturally infected with *D. immitis* could not be destroyed by heating at 56°C for 1 hr, but completely destroyed by 6 hr-heat treatment. Unfortunately, they have not studied the physico-chemical properties of the PCA positive sera other than heat lability and thus, further comments cannot be made. The present results produced by Sephadex G-200 gel filtration revealed that the reaginic activity existed on the fractions which were eluted earlier than IgG, suggesting that the molecular weight is slightly greater than that of IgG. From the above, the physico-chemical properties of canine reaginic antibody are closely similar to human IgE [10].

Hemagglutinating antibody production persisted in *D. immitis* infected dogs and showed the pattern of two peaks of antibody titers. Such a two-peak development of hemagglutinating antibody production has been mentioned by Pacheco [20] and by Allain et al. [1], but, according to their reports, the dog showing two-peak pattern was found out only in one of three experimental animals, and also one of six experimental animals. It appears likely that hemagglutinating antibody responses, which were initially observed at the 19th day and boosted at the 65th to the 83rd day of infection, might possibly be correlated with the third (about the 10th day) and fourth larval molt during the tissue migration [18]. Therefore it is conceivable that the hemagglutinating antibody increases as well as the initial reaginic antibody production were probably associated with some metabolic substances released from molting larvae. It has been known about a nematode that the metabolic components released from molting larvae showed a marked antigenic activity [15].

It is of interest to notice that the IHA titers decreased after the 111th day of infection, which was the time when young adult worms penetrated into the pulmonary arteries. Although the reason for this is still unknown, it may be conceivable that the depression in titers is probably due to a low antigenicity of young adult worms, or due to the absorption of antibody by young adult worms in the blood vessels. The second peak of IHA titers, as noted from the 184th to the 202nd day (dog No. 3) or from the 184th to the 222nd day (dog No. 2) of infection, coincided with the appearance of Mf in peripheral blood. This result indicates that the released Mf newly stimulated not only the reaginic but also the hemagglutinating antibody productions.

It is noteworthy that the IHA antibody titers rapidly decreased when the peripheral Mf steadily increased. The explanation of such a phenomenon has been attempted by Pacheco [20], who presumed that these antibodies were absorbed by the Mf augmented in the blood circulation.

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要　約

犬糸状虫感染犬におけるレアギン様抗体ならびに赤血球凝集抗体の産生について：早崎峯夫（東京農工大学家畜内科学教室）——犬糸状虫人工感染犬のレアギン様抗体および赤血球凝集抗体の動態を受身皮膚アナフィラキシー反応と間接赤血球凝集反応により検討した．レアギン様抗体は，虫体の第4脱皮期に一致して，感染後65日に初めて認められ，その後，一過性に消失したが，再び出現して ミクロフィラリア血症の発現時期には増高した．レアギン活性は，56℃，60分の熱処理および還元・アルキル化処理により完全に失活した．また，レアギン活性は Sephadex G-200 によるゲル汎過の第2ピーク (IgG) の上昇部分に検出され 本抗体の分子量が，IgG のそれよりもわずかに大きいことを示した．赤血球凝集抗体は，感染後19日に初めて検出され，以後，実験終了時まで漸増した．赤血球凝集抗体価は，2峰性に推移し，各ピークは，それぞれ第4脱皮期およびミクロフィラリア血症の発現時期と一致した．