Influence of Immune Treatments against *Dirofilaria immitis* Infection in Dogs

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ABSTRACT. Mongrel dogs were inoculated with two kinds of antigenic substances. The first was a phosphate buffered saline extract of whole *Dirofilaria immitis* mixed with aluminum hydroxide gel (group 1), and the second was an orally administered live *Metastrongylus apri* infective larvae (L_3) (group 2). Both groups were then infected with *D. immitis* L_3. Indirect hemagglutination (IHA) tests showed that the antibody was produced by these inoculations before the infection was introduced, even in dogs inoculated with *M. apri*. This suggests a cross-reactivity between *D. immitis* and *M. apri*. The initial passive cutaneous anaphylaxis (PCA) antibody production was markedly delayed by about 70 days in group 2 compared with the production in the infected control dogs (group 3). The appearance of microfilaremia was also delayed by about one month in group 2 compared with that in the above control group. All dogs were sacrificed after the termination of the observation and worms recovered from the right ventricle and pulmonary arteries were counted and measured. The results indicated that immunization resulting from the homologous worm-somatic antigen might accelerate the growth of the infected larvae, whereas immunization resulting from the heterologous worm antigen, but cross-reactive to *D. immitis*, might disadvantageously affect the growth.—KEY WORDS: *Dirofilaria immitis*, immune treatment, *Metastrongylus apri*.

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Many previous studies have been made in attempts to develop a vaccination against parasitic infection. These studies were reviewed by Clegg and Smith [1]. In the case of filariasis, Wong *et al.* [12] reported the excellent protection of dogs against *Dirofilaria immitis* infection by using irradiated infective larvae as a live vaccine substance. With the exception of this report, little information about the development of the vaccine against *D. immitis* infection is available. The present report evaluates the protective effect of immune treatments to filarial infection.

MATERIALS AND METHODS

Dogs: Mongrel dogs, aged about three to five months and free from *D. immitis* infection, were used. They have never passed the infectious season (June to November) in Japan after birth, until they were used in this study. There was no evidence of *D. immitis*-specific antibody in their sera when they were assessed by the indirect hemagglutination test (IHA), according to the method of Hayasaki [2]. The dogs were divided into four groups: groups 1 and 2 were the immunized groups, group 3 was an infected control and group 4 was a noninfected control group. All four groups were housed in a mosquito-proof room throughout the experiment.

Immunization and experimental infection: The dogs from groups 1 and 2 were immunized with two kinds of antigenic substances before the experimental infection with *D. immitis*.

The four dogs of group 1 were twice immunized with an antigenic mixture every a week. The mixture consisted of 20 mg of crude antigen protein extracted with a phos-

phate buffered saline (PBS) from an equal number of adult males and females of *D. immitis,* incorporated with 40 mg of aluminum hydroxide ($Al(OH)_3$) adjuvant gel in 4 m*l* of saline, based on the method of Hektoen and Welker [5]. This mixture was divided into three equal parts and injected subcutaneously, intramuscularly and intraperitoneally in order to avoid stimulating the injection sites by the antigenic solution. Of these four dogs, two dogs (dog Nos. 1 and 2) were further immunized on day 71 after infection which coincided with the 4th molting stage (day 60–70 of infection) of the larvae [6].

In group 2, three dogs were orally inoculated thrice with live infective larvae (L_3) of the swine lung worm, *Metastrongylus apri,* every a week. The oral doses of L_3 were 3876, 3456 and 14514, respectively. Thus, the number of L_3 in each dose varied because of the variation in the number of the recovered *M. apri* L_3. These infective larvae were obtained as follows: Female adult worms of *M. apri* were cut into small fragments and suspended in saline. This suspension was added to the soil where earthworms, *Eisenia foetida,* were cultured under laboratory conditions at 26–28°C with an adequate amount of distilled water added *ad lib* to moisturize the soil. About one month later *M. apri* larvae were recovered from the upper 1/2–1/3 of the body of earthworms, where the larvae are known to be concentrated, by digesting these earthworm fragments with 2% pepsin solution (5,000 U. pepsin 2 g, HCl 0.2 m*l*, saline 100 m*l*) at 30°C for 2.5 hr. The larvae recovered were then suspended in saline. The 7 dogs of groups 1 and 2 were infected with *D. immitis* L_3 after immunized. The 5 dogs of group 3 were also infected with *D. immitis* L_3 as infected controls. In these groups, infective larvae, collected from infected mosquitoes, *Aedes togoi,* were subcutaneously injected according to the method of Hayasaki [4]. Group 4 with 6 dogs was used as an uninfected control group.

Serology: All dogs were bled postinfection at various intervals in order to monitor *D. immitis*-specific antibody production. The time-course changes in antibody titers were assessed by means of the IHA test and passive cutaneous anaphylaxis (PCA) reaction with PBS-extracted antigen of the whole adult worms as described previously [3, 4].

Peripheral microfilaria count and worm recovery: The microfilariae in 20 μl of blood were counted by staining blood smears with methylene blue. All dogs were sacrificed at the termination of the experiments and the adult worms parasitic in the right ventricle and pulmonary arteries were counted.

Statistic analysis: The recovery rate of the worms and their body length were statistically analyzed based on Student's *t* test.

RESULTS

IHA antibody production in the infected controls (group 3) was strongly stimulated both when the fourth larval molt terminated and when microfilaremia appeared, and showed a two-peak pattern throughout the experiment (Fig. 1). The PCA antibody, however, was first detected at the fourth molting period and thereafter persisted in high titer until the end of the experiment.

In groups 1 and 2, substantial IHA antibody production was observed before the infection with *D. immitis* L_3. This suggests that the immune treatment stimulated a certain antibody production. Particularly, the IHA titer of group 2 immunized with *M. apri* L_3 was similar to that of group 1 immunized with PBS extract of adult *D. immitis.*

After the infection, the time course changes of the antibody titer of group 1 showed a two-peak pattern similar to that of the infected control (group 3). On the

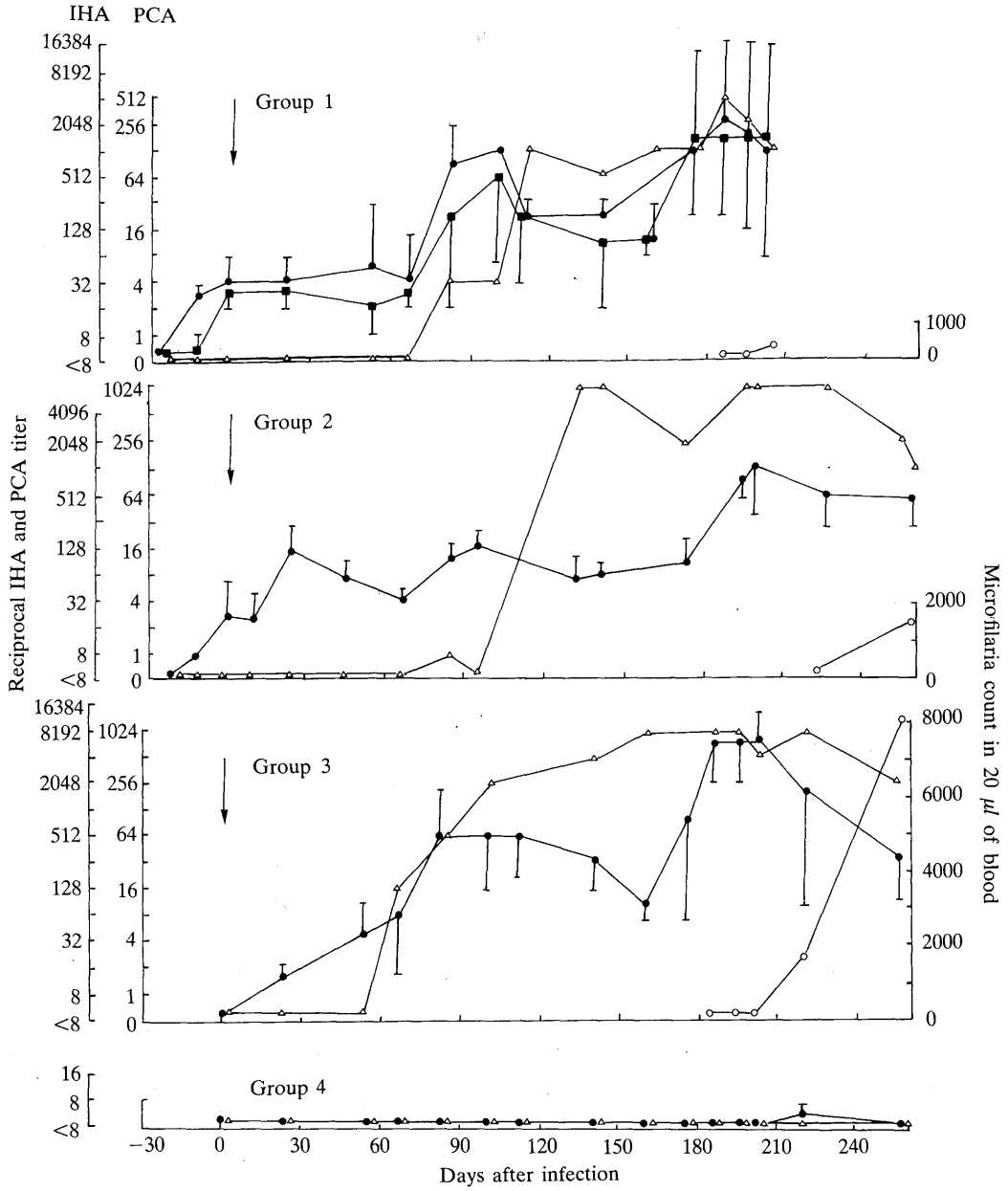

Fig. 1. Time-course changes in IHA (●) and PCA (△) antibody production and circulating microfilarial count (○) in groups 1–4, especially IHA titer of group 1 was expressed by subgroup A (●) and subgroup B (■). Arrows show the inoculation with *D. immitis* L_3.

contrary, group 2 did not indicate an obvious two-peak pattern. The initial PCA antibody production was observed in group 1 at the fourth molting period. However, in group 2, the appearance of the PCA antibody was markedly delayed by about 70 days and the appearance of microfilaremia was also delayed by about one month; in

Table 1. Effect of immunizations to worm recovery in *D. immitis* infected dogs

Group	Dog No.	No. of antigen inoculation	No. of L_3	Period (Days) at necropsy after infection	No. of worms (F/M) recovered	Total No. of worms and sex ratio (F/M)	Worm recovery rate (%)		
							individual	Mean (SD)	Difference
1A	1	3	101	207	79 (56/23)	243 (1.85)	78.2	81.1 (2.9)*a*	*a* vs *b* $p<0.01$
	2	3	195	207	164 (102/62)		84.1		
1B	3	2	106	207	69 (41/28)	122 (1.65)	65.1	58.2 (6.8)	
	4	2	103	253	53 (35/18)		51.4		
2	5	3	109	277	53 (21/32)	83 (0.84)	48.6	26.6 (16.7)	
	6	3	100	277	8 (5/3)		8.0		
	7	3	94	276	22 (12/10)		23.4		
3	8	—	10	253	5 (3/2)	201 (1.09)	50.0	47.4 (9.2)*b*	
	9	—	98	253	37 (20/17)		37.7		
	10	—	100	277	41 (22/19)		41.0		
	11	—	100	253	64 (36/28)		64.0		
	12	—	121	276	54 (24/30)		44.6		

Four dogs of group 1 were divided into two subgroups of two dogs each.
1 A: subgroup A. 1 B: subgroup B.

addition, the number of microfilariae was small, as compared to that in group 3.

The count and recovery rate of the worms in groups 1–3 are shown in Table 1. For a comparative evaluation, group 1 was divided into 2 subgroups: subgroup A included dog Nos. 1 and 2 while subgroup B included dog Nos. 3 and 4. Subgroup A was immunized thrice and subgroup B was immunized twice. The mean worm-recovery rate in subgroup A (81.1%) was higher than that of subgroup B (58.2%), although there was no significant difference between the 2 subgroups. Subgroup A showed the significantly higher recovery ($p<0.01$) than that of group 3; in particular, dog No. 2 showed the highest recovery rate (84.1%).

On the contrary, the mean worm-recovery rate in group 2 was 26.6% and was distinctly reduced compared to that of the infected control, and the lowest worm recovery rate was 8% in dog No. 6. The statistical difference ($p<0.1$), however, was not significant between the 2 groups.

No worm was detected in uninfected control dogs necropsied on day 253 and 276.

The body length of the recovered worms was measured (Table 2). The mean length of the worms in subgroups A and B was significantly greater than that of group 3. Furthermore, between the 2 subgroups, the worms from subgroup A were longer ($p<0.001$) than those from subgroup B by about 1 cm in males and 2 cm in females. In contrast, the mean worm length in group 2 was statistically smaller ($p<0.001$ in male, $p<0.05$ in female) than that in group 3.

DISCUSSION

In previous studies, Hayasaki [3, 4] indicated that, in dogs, the antibody response (mainly IgG) to *D. immitis* infection showed a characteristic two-peak pattern of antibody production throughout the prepatent and early patent periods. The antibody production was stimulated by the metabolites released both by the larvae at the fourth molting period on days 60 to 70 after infection and by the newborn microfilariae around day 180 after infection. Thereafter, the antibody titer decreased inversely when

Table 2. Comparison of recovered worms in body length

Group	Worm length (cm)					
	Male			Female		
	No. of worms determined	Mean (SD)	Difference	No. of worms determined	Mean (SD)	Difference
1 A	40	14.7(0.4)*a*	*a* vs *d* p<0.001 *a* vs *b* p<0.001	40	24.5(1.3)*e*	*e* vs *h* p<0.05 *e* vs *f* p<0.001
1 B	37	13.4(1.2)*b*		40	22.5(1.5)*f*	*f* vs *h* p<0.05
2	39	12.2(0.9)*c*	*c* vs *d* p<0.001	34	22.4(1.7)*g*	*g* vs *h* p<0.05
3	81	13.5(1.5)*d*		75	23.6(2.6)*h*	

1A and 1B: see Table 1.

the number of circulating microfilariae steadily increased. The reaginic antibody (IgE) assessed by the PCA reaction was first detected at the fourth molting period and the antibody titers were gradually increased throughout the experiment.

The antibody response of group 1 showed a pattern similar in IgG and IgE to that of the infected dogs in group 3, and the appearance of microfilaremia was coincident in both groups. Though the experiment of group 1 ended just before day 210, the subsequent changes in the number of circulating microfilariae could be expected to indicate a curve resembling that for group 3. The recovery rate, sex ratio and body length of the worms in subgroup A were greater than those in the infected control and subgroup B. These data may indicate that the immune treatment advantageously affected the survival rate and growth of the worms, particularly the female worms. Therefore, it is likely that the third inoculation in subgroup A may play an important role in eliciting the present results.

It is conceivable that the antibody produced by the immune treatment was not functional because it could not induce larvicidal activity. It seems likely that the host immune system failed to recognize the worm as an immunological foreign body. This in turn means that the larvae would be protected from the host immune response. Such a phenomenon is known to occur in schistosome and cestode infections [11, 13], and it was indicated that the antibody molecules bound to the body surface of the worms and covered over it and, therefore, the host immune system could not recognize the worms. We could not confirm this phenomenon in the present study, however, further experiment is necessary to verify the existence of antibody molecules on the body surface of *D. immitis*.

In group 2, there was a common antigenicity between *M. apri* and *D. immitis* since the antibody was detected by the IHA test with a *D. immitis*-derived antigen. However, this cross reactivity was confirmed only by the IHA test, therefore, further studies using other serological tests, such as gel diffusion or immunofluorescence, are needed.

Following the infection, a moderate IHA titer persisted. This may indicate the loss in quantity of the antigenic substances released from the migrating larvae because of the suppression of the larval growth by the immunization. The suppression of worm growth was evidently indicated by a low recovery rate of the worms and their reduction in body length. In particular, the sex

ratio of worms recovered suggested that the female worms were more suppressed in growth than the males.

Worm recovery rates in dog No.2 (84.1%) in group 1 and dog No.6 (8.0%) in group 2 deviated obviously from the normal recovery rates indicated not only in group 3, but also in our previous data on experimental infection of dogs with *D. immitis* [3, 4]. Therefore, these extreme values are possibly indicated by the immune treatment, although the recovery rates varied among individual dogs of each group.

In this study, the experimental dogs were sacrificed between days 207 and 277 of infection. This elicites a question whether the recovery rates would be altered if all the dogs were sacrificed on day 277, because it is theoretically possible that some worms reach the final location later than others, or that some died after they reached the location. On this regard, several studies have reported that no immature worm was recovered from the intermediate parasitic location in the dog after day 90 [10], day 120 [7] or day 142 [6] of infection. Kotani [6] also suggested that the larval migration to the heart probably does not persist beyond day 110 after infection. Furthermore, previous reports [8, 9] on the life span of *D. immitis* revealed that 13 of 18 experimentally infected dogs sacrificed before or at 4 years and 2 months of infection harbored no dead worms in the heart. Three of 18 experimentally infected dogs sacrificed at 4 years and 11 months of infection harbored many dead worms together with a few live worms. Two of 18 experimentally infected dogs sacrificed at 5 years and 5 months and at 6 years and 5 months, respectively, of infection harbored dead worms only. A similar study also revealed that only dead worms were detected in dogs sacrificed after 8 years of infection [8]. These reports suggest that the life span of the adult *D. immitis* is about 5 to 6 years. Considering these findings, it is possible that the final recovery rate would not be altered by the time lag of 70-days at autopsy.

It is reasonably concluded that, in *D. immitis* infection of dogs, immunization with homologous worm somatic antigen does not play a significant role in inducing a lethal immune response against the parasites. The results obtained in this study showed that the immunization with a homologous antigen might actually protect the larvae from the host immune response by forming an antibody-covering over the worm body. Thus, the immunization with a homologous antigen was considered rather advantageous for the wormd to grow than to protect the infection, whereas that with a heterologous antigen induced a protective immunity against *D. immitis* infection of dogs by the cross-reactive antigenicity between *D. immitis* and *M. apri.*

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要　　約

犬糸状虫感染に対する免疫処置の影響：早崎峯夫・大石　勇（東京農工大学農学部家畜内科学教室）——寄生虫由来抗原物質による免疫処置が犬糸状虫の感染に与える影響について検討した．実験群は免疫群2群と感染対照群1群の3群である．免疫処置は，糸犬状虫成虫のPBS抽出抗原と水酸化アラムゲルの混合液の注射（第一群），生きた *Metastrongylous apri* 感染幼虫の経口投与（第二群）によった．これら2群はともに免疫処置により犬糸状虫特異間接赤血球凝集抗体の産生がみられ，犬糸状虫と *M. apri* の間に交差反応の存在することが示唆された．第二群では，受身皮膚アナフィラキシー抗体の産生が，対照群より約70日遅延してみられた．実験犬を安楽死処置後，右心室・肺動脈より虫体を回収し，虫体数，体長について計測した．その結果，対照群と比較して，第一群では虫数多く，体長長く，同種抗原による免疫処置が幼虫の発育に有利に作用したことが示唆された．また第二群では虫数少なく，体長短く，交差反応性を有する異種抗原が感染幼虫の発育に不利に作用したことが示唆された．