Circulating Antigens and Antibodies of *Dirofilaria immitis* in the Dog Detected by Counterimmunoelectrophoresis

Masahiro TAGAWA, Kazuyoshi UEMATSU, Kazuo KUROKAWA,
and Hiroshi TANAKA¹⁾

First Department of Surgery, Nippon Veterinary and Zootechnical College, Musashino, Tokyo 180, and

¹⁾Department of Parasitology, Institute of Medical Science, University of Tokyo, Minato, Tokyo 108

(Received 27 February 1982/Accepted 28 February 1983)

ABSTRACT. An experiment was carried out to clarify the rise and fall of soluble circulating antigen (SCA) in relation to the fluctuation of antibody levels in dogs experimentally infected with *Dirofilaria immitis*. For detection of SCA, counterimmunoelectrophoresis (CIEP) was performed continually in these dogs at given intervals. Besides CIEP, the double diffusion technique (DD) was used for examining the antibody level. When CIEP was conducted on the serum of dogs experimentally infected with *D. immitis*, SCA was first detected as early as 1 month after infection, and either SCA or antibody in the blood showed a rise and fall with the lapse of time, but antigen and/or antibody were always detectable in the course of infection. On the other hand, antibody appeared inconstantly in each infected dog in the course of infection when examined by DD. The results indicated that a high level of SCA was present in the circulating blood of dogs infected with *D. immitis*. As a result, CIEP was regarded as a highly reliable and useful diagnostic tool for examining infection with *D. immitis*, since it could detect both SCA and antibody simultaneously and the procedure was completed in a shorter time than DD or immunoelectrophoresis.

The immunological diagnosis of canine filariasis is made chiefly by detecting antibody in the blood of the host. Many papers have been published on the methods of antibody detection. None of these methods, however, is satisfactory, since the response to antigen is not constant in the infected dogs.

Tagawa *et al.* [13] have been studying the antibody against *Dirofilaria immitis* in dogs experimentally infected with this parasite by performing the gel precipitation reaction during the course of the infection. In their observations of infected dogs over a period of a year, it was noted that some of these dogs exhibited no constant antibody response or possessed no detectable antibody, but all harbored a number of worms at autopsy. Such inconsistent appearance of antibody was considered to have been induced

by the neutralization or absorption of antibody during the state of antigen excess.

Desowitz and Una [4] demonstrated the presence of soluble circulating antigen (SCA) in dogs infected with *D. immitis* by counterimmunoelectrophoresis (CIEP). The present investigation was carried out to clarify the rise and fall of SCA in the course of infection with *D. immitis*. In this study, CIEP was evaluated as a method of choice for diagnosis of infection with *D. immitis*.

MATERIALS AND METHODS

1. Experimental infection of dogs

Fifteen puppies, 3–4 months old and free from infection with *D. immitis*, were used. They were infected experimentally with *D. immitis* by subcutaneous inoculation of 100–242 third-stage infec-

tive larvae collected from experimentally infected mosquitoes, *Aedes togoi*. Dogs were raised in a kennel covered with amosquito net during the experimental period to prevent them from accidental bites. It was confirmed that every experimental dog was free from intestinal parasites over a period from birth to the time of autopsy.

2. Gel precipitation reaction

a. Antigen preparation

D. immitis antigen was prepared from adult worms recovered from naturally infected dogs. Adult worms collected were freeze-dried and homogenized. The homogenate was extracted with a 100-fold volume of phosphate buffered saline (pH 7.2) to the dry weight of worm (PBS extract). The PBS extract was placed in collodion bags (Sartorius membrane filter) and concentrated to 1/10 volume to make a 1:10 dilution of worm antigen. The concentrated antigen, 0.5 ml, was introduced in small test tubes and stored in a freezer at -70°C until use.

b. Serum samples

Serum samples were collected continually from the experimentally infected dogs. Positive control serum was obtained from a rabbit immunized with the PBS extract mixed with an equal amount of Freund's complete adjuvant. To each serum sample was added sodium azide to 0.01% immediately after collection. All serum samples were stored in a freezer at -70°C until use.

c. Immunological assay

Counterimmunoelectrophoresis (CIEP): CIEP was performed following the method of Desowitz and Una [4]. Difco special agar Noble was dissolved at 1.0% in veronal buffer (pH 8.6, $\mu=0.05$). To detect antigen in each serum sample, canine serum and rabbit antiserum were placed on the cathodic and anodic sides of the

plate, respectively. Similarly, the presence of antibody in serum samples was assessed by CIEP using PBS extract. Electrophoresis was conducted by allowing 2 mA of direct current per centimeter of the width of agar to flow for an hour. The plate was then allowed to stand at room temperature for an hour to complete the reaction. To remove excess protein, the plate was placed in physiological saline for 48 h, washed with distilled water for 24 h and finally dried. The plate was stained with Coomassie brilliant blue and examined for precipitin lines.

Double diffusion technique (DD): Ouchterlony's test was performed on a 1% agar gel base. Six wells, 3 mm in diameter, were punched equidistant from each other around a 15-mm diameter circle with a central well of the same size. After serum reactions for 48 h, the agar plate was washed, dried and stained in the same manner as in CIEP. When one or more precipitin lines were detected, the reaction was regarded as positive.

RESULT

1. Recovery of filarial worms

The infected dogs were sacrificed in a period between 90 days and 2 years after inoculation. They were examined for infection with *D. immitis* and the number of infesting worms. As shown in Table 1, in 13 of 15 autopsy cases, 4–90 immature and adult worms (average, 29) were recovered from the right atrium and ventricle and the pulmonary artery. The remaining two dogs were positive for microfilariae (mf) and for adult worms as revealed angiocardigraphically in the pulmonary artery.

2. CIEP reactions of canine serum

Fig. 1 shows the results of reactions by CIEP between the serum from the ex-

Table 1. Experimental infection of dogs with *D. immitis*

Dog No.	Number of larvae inoculated	Number of worms recovered	Sacrificed days post-infection
12	100	4*	90
15	100	4	150
9	200	8	180
1	100	9*	105
3	100	11	2 years
14	100	12	135
4	100	13*	105
6	100	32	210
13	200	32	120
18	242	35	120
5	100	48	180
16	100	76	2 years
8	200	90	180
20	100	ne**	2 years
21	100	ne**	2 years

* Immature worms.

** ne: Dog not sacrificed.

Fig. 1. CIEP patterns of canine serum and immune rabbit serum.

IRS : Immune rabbit serum.

DIA: *Dirofilaria immitis* antigen.

PBS: Phosphate buffered saline.

CS : Canine serum.

perimentally infected dogs and *D. immitis* antigen. In CIEP reactions, one to seven (antibody) distinct precipitin lines appeared between *D. immitis* antigen and infected canine serum, one or two (SCA) lines between immune rabbit serum and infected canine serum, and eight (antibody) lines between *D. immitis* antigen and immune rabbit serum.

3. CIEP and DD reactions with serum of experimentally infected dogs

To examine the serum of the infected dogs for antigen and antibody, CIEP and DD were performed on infected canine serum at given intervals. They were bled three to 21 times over a period from preinfection to 90 days or 2 years after infection.

As shown in Table 2, CIEP revealed that antibody alone was detected in five of the 15 dogs (33.3%) and antigen alone in one dog (6.7%).

When CIEP was applied to a total of 146 serum samples, antigen alone was detected in 76 samples (52.1%), antibody alone in 107 samples (73.3%), and both

Table 2. The frequencies of positive precipitin reactions among samples by CIEP and DD tests on sera from dogs experimentally infected with *D. immitis*

Dog No.	Number of samples**	CIEP				DD
		Ag+*	Ab+*	Ag+	Ab+	Ab+
1	6	0	5	0	0	0
4	4	0	3	0	0	0
6	5	4	0	0	0	0
9	7	2	3	1	1	0
13	5	0	4	0	0	0
14	4	0	3	0	0	0
15	6	2	5	2	2	0
18	3	0	2	0	0	0
5	6	5	2	2	2	3
8	10	3	8	2	2	2
12	4	2	3	2	2	3
16	22	11	20	10	10	11
3	21	18	19	16	16	2
20	22	18	16	13	13	10
21	21	11	15	6	6	13
Total	146	76	104	54	54	44

Ag: Antigen, Ab: Antibody.

* Showing the number of Ag+, or Ab+ serum samples.

** The number of serum samples taken in the course of infection.

antigen and antibody in 54 samples (37.0%). The result also indicated that CIEP detected antigen and/or antibody in all the 146 samples, while DD demonstrated the appearance of precipitin lines in 44 of the 146 samples (30.1%).

To study daily changes in SCA and antibody, blood samples were collected daily from three infected dogs for 8 consecutive days after the appearance of mf. In dog No. 3, SCA was detected constantly on all 8 days and antibody on 6 days by CIEP, but no antibody was detected by DD (Table 3) on these days. In dog No. 16, by CIEP, antibody was detected on all 8 days and SCA on 4 days only, and by DD antibody was detected on 4 days (Table 4). In dog No. 20, by CIEP, SCA was detected on 6 days and antibody 7 days, and by DD, antibody on only 1 day (Table 5).

DISCUSSION

The initial purpose of the present investigation was to clarify the rise and fall of SCA of *D. immitis* in relation to the fluctuation of antibody level in dogs infected with this parasite. As a result, it was found that a high level of SCA was often detected in the circulating blood in these dogs. Furthermore, in detection of antibody by CIEP, it was revealed that both antigen and antibody were coexisting in the serum. In other parasitic infections, Berggren and Weller [3], Gold, *et al.* [7], and Nash *et al.* [9] reported the presence of SCA in dogs infected with *Schistosoma mansoni*. Desowitz and Una [4] demonstrated the presence of SCA in dogs infected with *D. immitis* by CIEP.

Several workers have reported the constancy of antibody in dogs in the course of infection with *D. immitis* [2, 6,

Table 3. Progressive changes in circulating antigens and antibodies in dog No. 3 experimentally infected with *D. immitis*

Time course	CIEP		DD
	Antigen	Antibody	
Initial	—	—	—
1 (month)	—	+	—
2	—	+	—
8	+	+	+
10	+	+	+
11	+	+	—
12	+	+	—
13	+	+	—
14	+	+	—
15	+	+	—
22	+	+w	—
23-1*	+	+w	—
2	+	+w	—
3	+	+w	—
4	+	+	—
5	+	+w	—
6	+	+	—
7	+	—	—
8	+	—	—
25	+w	+	—
26	+w	+	—
Total positives	18	18	2

w: weak.

* Examinations on 8 consecutive days.

Table 4. Progressive changes in circulating antigens and antibodies in dog No. 16 experimentally infected with *D. immitis*

Time course	CIEP		DD
	Antigen	Antibody	
Initial	—	—	—
1 (month)	+	+	+
6	+	+	+
8	+	—	—
9	+	+w	—
10	+	+	+
11	+	+w	+
20-1*	+s	+s	—
2	+s	+s	+
3	—	+	—
4	—	+	+
5	—	+	+s
6	+w	+	+s
7	+	+	—
8	—	+	—
21	—	+	—
22	—	+w	+
23	—	+	+w
25	+w	+	+w
Total positives	11	17	11

w: weak, s: strong.

* Examinations on 8 consecutive days.

10-12]. Tagawa *et al.* [13] ascribed the cause of such inconstancy to the neutralization or absorption of antibody by SCA. Wong [14] attributed this cause to masking of antibody by a massive amount of circulating antigen. And Ah *et al.* [1] and Pacheco [10] asserted that the cause of such inconstancy might be the absorption of antibody by mf. The present investigation indicated that anti-*D. immitis* antibody and the corresponding antigen were not always detectable in the circulating blood of the infected dogs. ES antigens, secreted and excreted by adult *D. immitis*, would be the principal antigenic substances responsible for stimulating antibody formation. The quanti-

tative balance of antigen and corresponding antibody may be thus a critical factor regulating the presence of either antigen or antibody alone or simultaneous presence of both of them in the circulating blood. For instance, enormous release of ES antigens would first result in the immune-complex formation with circulating antibody and then might lead to antigen excess when the antibody was exhausted. This excessive ES antigen would be bound to newly formed antibody and in turn the antibody excess would be brought about shortly. This type of antigen or antibody excesses might cause the inconstant presence of anti-*D. immitis* antibody and corre-

Table 5. Progressive changes in circulating antigens and antibodies in dog No. 20 experimentally infected with *D. immitis*

Time course	CIEP		DD
	Antigen	Antibody	
Initial	—	—	—
5 (month)	—	+w	+
7	+w	+	+
8	+w	+s	+
9	+w	+	+
10	+w	+	+s
18–1*	—	+s	+w
2	—	+	—
3	+w	+	—
4	+w	+	—
5	+w	+	—
6	+w	+	—
7	+	+w	—
8	+	—	—
19	+	—	—
20	+	—	—
21	+	+	+
23	+	+	+
Total positives	14	14	8

w: weak, s: strong.

* Examinations on 8 consecutive days.

sponding circulating antigen, as observed in the current study.

At present, the immunological diagnosis of *D. immitis* is made by the detection of antibody in the circulating blood, although this method has not always been reliable, as mentioned above. The present study has made it clear that various degrees of SCA, sometimes up to a high level, were demonstrated in the circulating blood of dogs infected with *D. immitis*, and that SCA and/or antibody could be detected in the course of infection. Therefore, it is considered that diagnosis of infection with *D. immitis* can be made more exactly by detecting antibody and antigen by CIEP than by other methods.

The second purpose of the present investigation was to determine the sensi-

tivity of CIEP for diagnosis of infection with *D. immitis*. It has been reported that CIEP was effectively applicable to the detection of antibody in some paratitic infections. For example, Krupp [8] mentioned that CIEP was the most effective method in gel diffusion techniques for diagnosis of amebiasis. Despomier *et al.* [5] pointed out that CIEP was a sensitive and specific method for diagnosis of trichinosis. In the present investigation, it was confirmed that CIEP was a reliable method for diagnosis of infection with *D. immitis* by detecting antigen and/or antibody in the circulating blood simultaneously on the same agar plate. Advantages with CIEP were the high sensitivity and faster reactions than double diffusion or immunoelectrophoresis. In conclusion, CIEP is considered to be an effective technique for diagnosis of infection with *D. immitis*.

ACKNOWLEDGMENTS. The authors wish to show their gratitude to Professor Kaoru Noda of the University of Hawaii for correcting the manuscript and to Dr. Akiko Shirasaka of the Department of Parasitology, Institute of Medical Science, University of Tokyo, for supplying the mosquitoes.

This work was supported in part by Grant-in-Aid No. 376174 from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Ah, H. S., Peckham, J. C., Mitchell, F. E., and Thompson, P. E. (1972). Studies of *Dirofilaria immitis* infection in dogs relative to immunizations and antigen-antibody interaction. In, *Canine Heartworm Disease—The Current Knowledge*, Bradley, R. E., Editor, University of Florida, Gainesville, 55–67.
2. Allain, D. S., Kagan, I. G., and Schlotthauer, J. C. (1972). Serologic studies on dogs experimentally infected with *Dirofilaria immitis*. In, *Canine Heartworm Disease—The Current Knowledge*, Bradley, R. E., Editor, University of Florida, Gainesville, 69–76.
3. Berggren, W. L., and Weller, T. H. (1967). Immuno-electrophoretic demonstration of specific circulating antigen in animals infected with *Schistosoma mansoni*. *Am J. Trop. Med.*

- Hyg. **16**, 606–612.
4. Desowitz, R. S., and Una, S. R. (1976). The detection of antibodies in human and animal filariasis by counterimmunoelectrophoresis with *Dirofilaria immitis* antigens. J. Helminthol. **50**, 53–57.
5. Despommier, S., Müller, M., Jenks, B., and Fruitstone, M. (1974). Immunodiagnosis of human trichinosis using counterimmunoelectrophoresis and agar gel diffusion techniques. Am. J. Trop. Med. Hyg. **23**, 41–44.
6. Ellsworth, J. H., and Johnson, A. H. (1973). Precipitin test and soluble antigen fluorescent antibody technique for detecting *Dirofilaria immitis* in dogs. Am. J. Vet. Res. **34**, 689–692.
7. Gold, R., Rosen, F. S., and Weller, T. M. (1969). A specific circulating antigen in hamsters infected with *Schistosoma mansoni*. Am. J. Trop. Med. Hyg. **18**, 545–552.
8. Krupp, I. M. (1974). Comparison of counterimmunoelectrophoresis with other serologic tests in the diagnosis of amebiasis. Am. J. Trop. Med. Hyg. **23**, 27–30.
9. Nash, T. E., Prescott, B., and Neva, F. A. (1974). The characteristics of a circulating antigen in schistosomiasis. J. Immunol. **112**, 1500–1507.
10. Pacheco, G. (1966). Progressive changes in certain serological response to *Dirofilaria immitis* infection in the dog. J. Parasitol. **52**, 311–317.
11. Shichinohe, K., Tagawa, M., Kurokawa, K., Fujita, K., and Tanaka, H. (1973). Studies on the diagnosis of canine filariasis with special reference to the comparison of hemagglutination test and skin test. Japan J. Vet. Sci. **35**, 1–10.
12. Tagawa, M., Shichinohe, K., and Kurokawa, K. (1972). Experimental studies on the diagnosis of canine filariasis. I. Immunological and hematological findings on dogs infected with *Dirofilaria immitis* or subjected to transplantation with adult worm. Bull. Nippon Vet. Zootech. Coll. No. **21**, 193–201.
13. Tagawa, M., Kurokawa, K., and Tanaka, H. (1980). Analysis of immunoelectrophoretic patterns on dogs artificially infected with *Dirofilaria immitis*. Bull. Nippon Vet. Zootech. Coll. No. **29**, 14–22.
14. Wong, M. M. (1974). Experimental occult dirofilariasis in dogs with reference to immunological responses and its relationship to tropical eosinophilia in man. Southeast Asian J. Trop. Med. Publ. Health **5**, 480–486.

要 約

Counterimmunoelectrophoresis による *Dirofilaria immitis* の流血抗原および抗体の検出について：多川政弘・植松一良・黒川和雄・田中 寛¹⁾（日本獣医畜産大学第一外科学教室，¹⁾東京大点医科学研究 所 寄生虫研究部）——*Dirofilaria immitis* 人工感染における流血抗原 soluble circulating antigen (SCA) の推移を counterimmunoelectrophoresis (CIEP) を用いて経時的に検査し，また，同一血清について，CIEP と二重免疫拡散法 (DD) による抗体の推移もしらべた。*D. immitis* 人工感染犬血清においては，CIEP による SCA または抗体は，早いもので感染後 1 カ月に認められた。抗原あるいは抗体の示す沈降線は経過とともに消長したが，常にいずれか一方または両方が検出された。しかし，DD による抗体の検出成績は，すべての感染犬において不安定であった。*D. immitis* 感染犬の流血中心は高い頻度で SCA が存在し，また，*D. immitis* 感染犬で SCA と抗体の両方を同時に検出できるので，CIEP の信頼度は高く，DD や immunoelectrophoresis にくらべて早く判定できる，など有用性も高いことがわかった。