Localization of Highly Purified Allergen and IgG-inducing Antigen in Adult *Dirofilaria immitis* Determined by Fluorescent Antibody Test

Koichiro FUJITA and Setsuko TSUKIDATE

Department of Medical Zoology, Nagasaki University School of Medicine, Nagasaki, Japan

Abstract: The localization of the allergen as well as the IgG-inducing antigen in the *Dirofilaria immitis* was determined by the fluorescent antibody test. The IgG-inducing antigen was located at the site of the musculature inside of cuticle and the lateral lines of the adult worm. On the other hand, the specific localized site of the allergen was not observed clearly in the worm body itself, but excretory and secretory (ES) products derived from the adult worm were determined to be important source of the allergen. A precipitine line was observed in Ouchterlony's gel diffusion method between ES antigen and the hyperimmunized anti-allergen rat serum. Microfilaria was determined to have no allergen at all, but some amounts of the IgG-inducing antigen. ES antigen either from a male or a female worm was mainly composed of the allergen, but contained relatively smaller amounts of IgG-inducing antigen.

INTRODUCTION

We have separated allergen in *Dirofilaria immitis* from IgG-inducing antigens (Fujita, 1975) and obtained allergen in the highly purified form recently (Fujita and Tsukidate, 1981). Physicochemical properties of the highly purified allergen as well as the IgG-inducing antigen were then studied in succession by us (Fujita, Ikeda and Tsukidate, 1979; Fujita, Tsukidate and Ikeda, 1981). In these studies, we demonstrated that the purified allergen had a molecular weight of 15,000 and a carbohydrate content 2%. The allergen was positively charged, and its determinant group was protein in nature. The IgG-inducing antigen was also purified in relatively pure form. It was shown that in the contrast to the allergen, the IgG-inducing antigen had a larger mo-

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lecular weight (ca, 600,000) and a higher content of carbohydrate (20%), but both the allergen and the IgG-inducing antigen were remarkably stable to various kinds of proteases, periodate digestion and other physicochemical treatments. However, in order to understand the relationship between host and parasite in filarial infection, it is necessary to know the localization of these antigens in Dirofilaria worm. In this study, we used the fluorescent antibody technique, and showed where the allergen as well as the IgG-inducing antigen exsisted in the adult Dirofilaria worm.

MATERIALS AND METHODS

Allergen and IgG-inducing antigen: The highly purified allergen was obtained by a combination of DEAE-Sephadex A-50 chromatography and Sephadex G-200 gel filtration as previously described (Fujita and Tsukidate, 1981). The IgG-inducing antigen was separated from the allergen fraction by chromatography on DEAE-Sephadex A-50 (Fujita and Tsukidate, 1977) and partially purified as already reported (Fujita, Tsukidate and Ikeda, 1981).

Hyperimmune rat sera against purified allergen (anti-Bd) and partially purified IgG-inducing antigen (anti-Da): Rats were injected intramuscularly with 1.5mg of the purified allergen, the IgG-inducing antigen and the crude extract (CE) from adult Dirofilaria worm, emulsified in Freund's complete adjuvant at 1 week interval, and bloods were taken 10 days after the 4th injections. Another group of rats were also immunized twice with 0.4mg of the allergen, the IgG-inducing antigen and CE previously mixed with aluminum hydroxide gel. Infected serum was obtained from the dog infected with *D. immitis*.

Fluorescent antibody test: Adult female Dirofilaria worms were obtained from the dogs in Kanazawa city and washed with physiological saline. Microfilariae (Mf) were collected from the blood of the infected dog. Blood containing Mf was added one to nine volumes of 3.8% sodium citrate solution. Mf was obtained by filter method as described in Santos, Santos and Azevedo (1976). Fresh ovalbumine from a chicken egg was mixed with 1g of the gum arabic powder. After the centrifugation of the mixture at 3,000rpm for 10 min., the supernatant was used for the envelopment of the adult worm and Mf. The adult female worm was mounted in egg-white and gum-arabic medium, and were frozen rapidly in dry ice and acetone. Frozen sections of 5 micron in thickness were prepared by a cold microtome, and were utilized as the antigen after treated with 95% ethanol for 8 minutes. Anti-rat IgG rabbit γ-globulin was conjugated with fluorescein isothiocyanate (FITC) and was used at 4 fold dilution. Indirect fluorescent antibody staining was conducted on cover slips with frozen sections. Hyperimmune rat sera against the purified allergen and the IgG-inducing antigen reacted to the sections of adult worm up to 64 fold dilution, but were used as 16 fold. These procedures were carried out according to the report by Ishii and Tanaka (1968). These

sections were also stained with hematoxylin and eosin as controls, and were examined by light microscopy. Staining Mf was carried out with the whole body.

Excretory and secretory products (ES antigen): Adult male or female worms were incubated into the physiological saline at 37°C for 2 hours, and ES antigen was obtained. Protein content in ES antigen was estimated, and the ES antigen was lyophylized and kept in a refrigerator until used.

Mf antigen: Mf were homogenized with glass and teflon homogenizers, disrupted by sonication (Insonator, 200M, Kubota, Tokyo) at 140 Watt for 5min and extracted in phosphate buffered saline at pH 7.2 for 2 days in a refrigerator. The emulsion was centrifuged at 13,000g for 20min. The supernatant was used as Mf antigen.

Ouchterlony's immunodiffusion test: Immunodiffusion plates were prepared with 1.2% Noble agar in veronal buffered saline, PH 8.2. Immunodiffusion was done at 4°C over a 48-hr period, after which the slide were washed and stained with amido black.

Indirect hemagglutination (IHA) test and passive cutaneous anaphylaxis (PCA) test: These tests were carried out according to the previous paper (Fujita, 1975).

RESULTS

Antibody titres in rats immunized with purified allergen, IgG-inducing antigen and crude extract, and in infected serum

As shown in Table 1, the rats immunized with allergen produced only PCA antibody, whereas the rats immunized with IgG-inducing antigen induced only IHA antibody. The rats accepted with CE as antigen produced both PCA and IHA antibody. However, when rats repeatedly immunized with each of antigens with Freund's complete adjuvant, all the rats induced only IHA antibody. The hyperimmune sera against CE, the purified allergen and the IgG-inducing antigen were designated as anti-CE, anti-Bd and anti-Da respectively. The IHA and PCA activity of infected serum was also assayed; the IHA titre was $1 : 3^{3.0}$ and the PCA was $1 : 2^{2.0}$.

Table 1. IHA and PCA antibody titres in rats immunized with the crude extract, the purified allergen and the IgG-inducing antigen

antigens	hyperimmunized with 1.5mg × 4 of antigen plus Freund's complete adjuvant		immunized with 0.4mg × 2 of antigen plus $Al(OH)_3$ adjuvant	
	IHA	PCA	IHA	PCA
crude extract	$3^{3.7} \pm 3^{2.1}$*	0	$2^{5.5} \pm 2^{0.6}$	$2^{5.0} \pm 2^{0.8}$
allergen	$3^{3.0} \pm 3^{0.5}$	0	0	$2^{4.5} \pm 0$
IgG-inducing antigen	$3^{9.5} \pm 3^{0.5}$	0	$2^{6.0} \pm 2^{0.8}$	0

* reciprocal IHA titre, mean ± S.E. among four rats immunized

Localization of purified allergen and IgG-inducing antigen determined by fluorescent antibody test

The hyperimmune sera, anti-Bd and anti-Da, were used to search for the located place of the allergen as well as the IgG-inducing antigen in the adult female Dirofilaria worm. First of all, the localization of the IgG-inducing antigen was examined. Intensity of the specific fluorescence was seen to be the strongest at the site of musculature inside of cuticle of the adult worm, and next to the musculature were lateral lines, as shown in Photo.3. Then the localized place of the allergen in the worm was also looked for by fluorescent antibody test. However, specific fluorescence was not observed clearly in the worm body itself, but sometimes seen in the internal cavity of the digestive canal of Dirofilaria, as shown in Photo.4. The immunofluorescent reactions obtained with the whole body of Mf made it impossible to identify the fluorescing antigenic sites, in any case that anti-Bd or anti-Da serum was used as the immune serum respectively (Photo. 5 and 6).

Localization of allergen determined by Ouchterlony's test

The preceding data indicated that the allergen might be located in excretory and secretory fluids of Dirofilaria worm. Ouchterlony's gel diffusion test was employed in

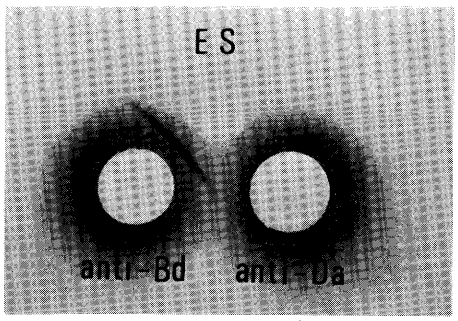

Fig. 1. Ouchterlony's gel diffusion test of ES antigen against anti-allergen (anti-Bd) and anti-IgG-inducing antigen (anti-Da) serum

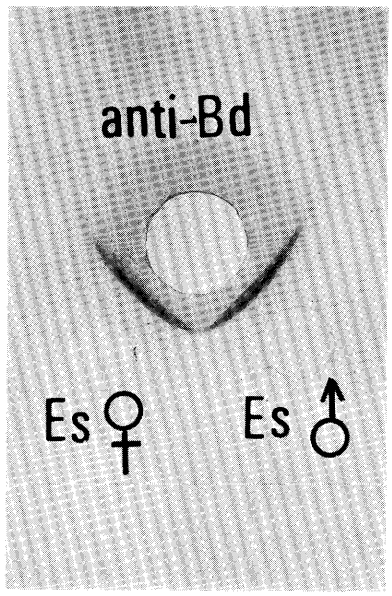

Fig. 2. Ouchterlony's gel diffusion test of ES antigen derived from the male (ES ♂) and the female (ES ♀) worms against anti-Bd serum

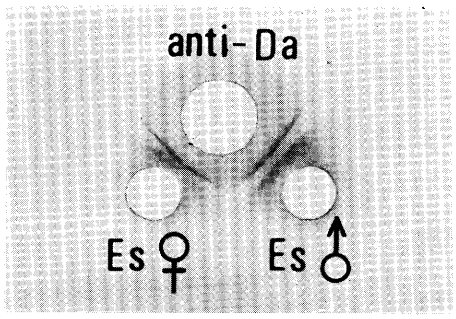

Fig. 3. Ouchterlony's gel diffusion test of ES antigen with three times higher concentration against anti-Da serum

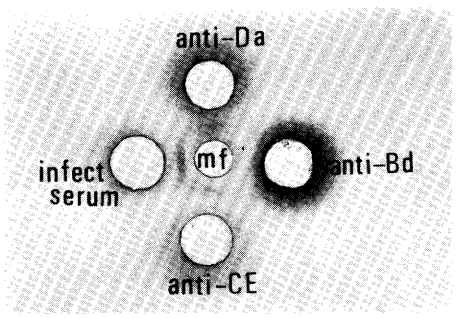

Fig. 4. Ouchterlony's gel diffusion test of Mf antigen against anti-Da, anti-Bd and anti-CE serum and infected dog serum

order to know the localization of the allergen. A definite precipitin line against ES antigen (0.3mg per hole used) was observed between anti-Bd serum, but no line between anti-Da serum, as shown in Fig.1. Common precipitin line was found in Ouchterlony's gel diffusion test between anti-Bd serum and the ES antigen derived from the male and the female worm, as indicated in Fig.2. These results lead to the conclusion that ES antigen was the important source of the allergen, and the allergen was localized in the ES antigen.

When the ES antigen with a higher concentration (0.9mg per hole) used in the test, the precipitin line was also observed between anti-Da serum (Fig.3); ES antigen was mainly composed of allergen, but contained relatively smaller amounts of IgG-inducing antigen. Then, the antigenicity of Mf was examined in Ouchterlony's test. Mf antigen of 0.3mg was put into a hole, and reaction was tested against anti-Da, anti-Bd and anti-CE serum, and infected serum. As shown in Fig.4, the precipitin line was observed in anti-Da, anti-CE serum and infected serum, but no line was seen in anti-Bd serum at all. Mf was determined to have no allergen but some amounts of IgG-inducing antigen.

DISCUSSION

The present study succeeded for the first time in determining the localization of the purified allergen as well as the IgG-inducing antigen in the Dirofilaria worm. The highly purified allergen obtained in our laboratory was located and concentrated in the excretory and the secretory products exhaused by adult male or female worm. On the other hand, the IgG-inducing antigen was located at the site of musculature inside of cuticle and lateral lines of the adult worm. Ishii, Matsuda, Kamiya and Kobayashi (1969) conducted fluorescent antibody test for the diagnosis of the cotton rat filariasis. In their study, it was demonstrated that the sera of infected cotton rats reacted also to

the site of musculature inside of cuticle, the lateral lines and the internal cavity of digestive canal. As the sera were supposed to contain much amounts of IgG antibody, these results would suggest the localized place of IgG-inducing antigen in the worm, which was consistent with our present result.

It was revealed by the present study that microfilaria had no allergen at all, but some amounts of IgG-inducing antigen. Therefore, microfilaria was supposed to be a good antigen for the diagnosis of human filariasis. Santos, Santos and Azevedo (1976) reported that the immunofluorescence test using microfilariae as antigen was excellent in specificity for the diagnosis of *Wuchereria bancrofti* filariasis. Lucasse (1962) described the technique of the immunofluorescence test for onchocerciasis. When microfilariae were used as antigen, the test became very specific, and he concluded that the test might be useful in serological diagnosis of Onchocerca patients where indication of the disease existed but the microfilariae could not be easily found.

Localization of the highly purified allergen in the worm was clearly demonstrated in this study, which would lead us the better understanding the important play of the IgE antibody production in the host-parasite relationship. The allergen as the important factor in the filarial infection was now under investigation in our laboratory.

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精製アレルゲンおよびIgG誘導抗原の犬糸状虫体内局在部位．螢光抗体法による証明

藤田紘一郎・月舘説子（長崎大学医学部医動物学教室）

犬糸状虫より単一の糖蛋白として精製されたアレルゲンおよびIgG誘導抗原の虫体内局在部位をアレルゲンおよびIgG誘導抗原で過免疫したラット血清を用い，間接螢光抗体法で調べた．IgG誘導抗原は虫体の筋層，とくに角皮内層突起部や側索に存在していた．しかし，アレルゲンの局在部位は虫体組織内ではそれほど明瞭ではなく，特異抗体が時々消化管内壁に見られるにすぎなかった．しかし，オクタロニー法によってアレルゲンは虫体の分泌・排泄液（ＥＳ抗原）中に多量に存在することが明らかにされ，アレルゲンが虫の分泌・排泄液中に局在していることが判明した．なお，ミクロフィラリアはIgG誘導抗原を有していたが，アレルゲンは全く含んでいなかった．ＥＳ抗原は，雄成虫あるいは雌成虫いずれから由来したものでも多量のアレルゲンを含んでおり，その他少量のIgG誘導抗原を有していた．

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Photo. 1: Cross section of adult female *Dirofilaria immitis* worm. Hematoxyline and eosin staining.

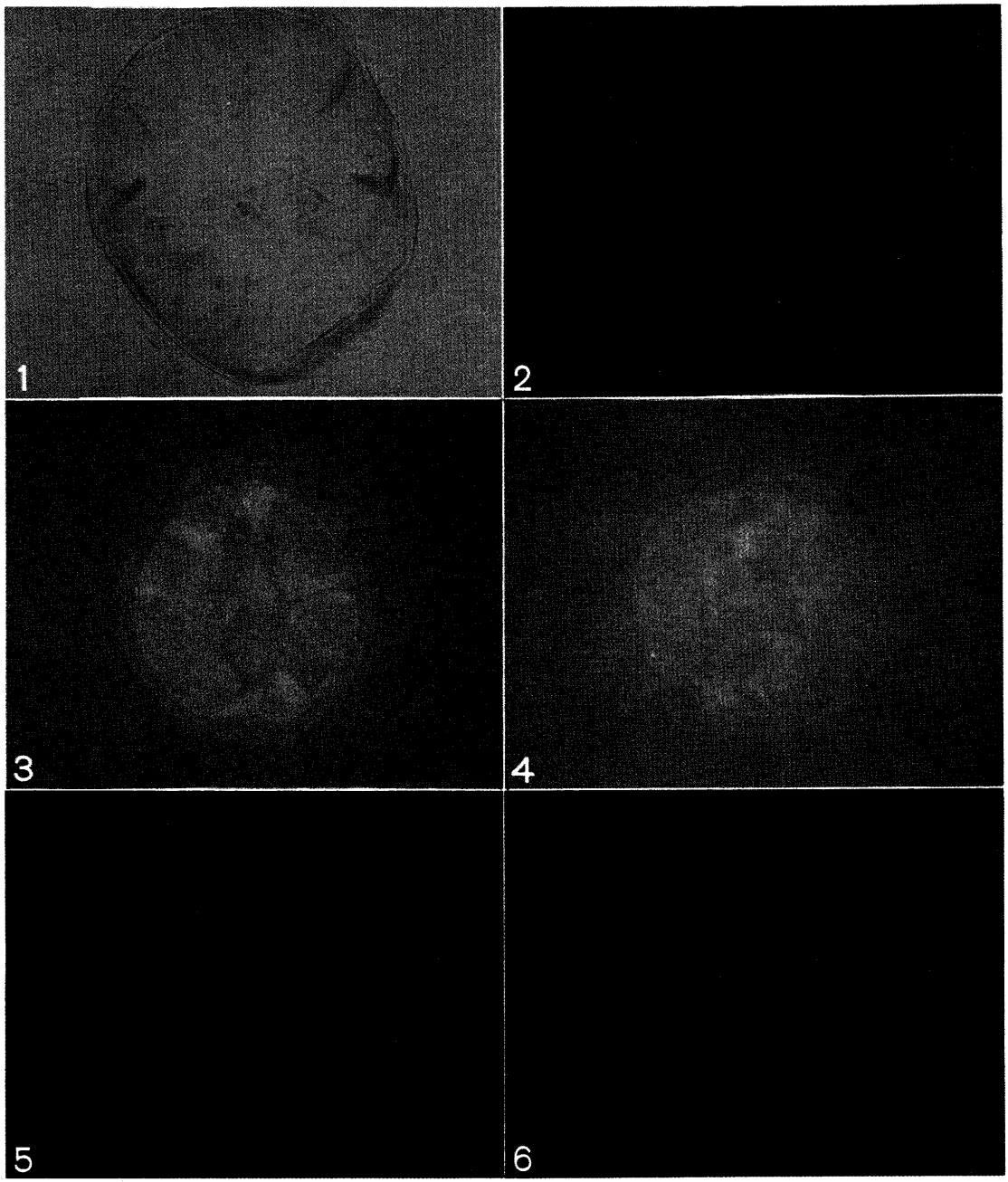
Photo. 2: Control cross section of adult female Dirofilaria worm by fluorescent antibody test.

Photo. 3: Localization of the IgG-inducing antigen in the worm. Intensity of the specific fluorescence was seen at the musculature and lateral lines.

Photo. 4: Localization of the allergen in the worm. Specific fluorescence was not observed clearly, but sometimes seen in the internal cavity of digestive canal.

Photo. 5: No fluorescent reaction was obtained with whole body of microfilaria. In the case of the anti-IgG-inducing antigen rat serum used.

Photo. 6: No fluorescence with whole body of microfilaria. In the case of the anti-allergen serum used.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