Dirofilaria immitis: Physicochemical Properties of IgG-inducing Antigen with Special Reference to the Comparison with Highly Purified Allergen

Koichiro FUJITA and Setsuko TSUKIDATE

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

Teruaki IKEDA

Department of Medical Zoology, Kanazawa Medical University, Ishikawa, Japan

Abstract: A soluble somatic preparation of adult *Dirofilaria immitis* was prepared and was found to contain potent antigens for the production of IgG antibody in rats as well as various strains of outbred or inbred mice without any detectable IgE antibody, in spite of the use of aluminum hydroxide as adjuvant. The IgG-inducing antigen was purified in relatively pure form from the soluble somatic preparation, and its physicochemical properties were studied and compared with those of the highly purified allergen. Both the IgG-inducing antigen and the allergen were remarkably stable to various kinds of proteases, periodate digestion and other physicochemical treatments, such as heat, vibration, acid and alkali, but very sensitive to pronase digestion. They had almost no significant enzymatic activities. In contrast to the allergen, IgG-inducing antigen had a larger molecular weight (ca, 600,000) and a higher content of carbohydrate (20%).

INTRODUCTION

Studies on the antigenic composition of *Dirofilaria immitis* have shown, as expected, a multiplicity of somatic and metabolic antigens (Fujita and Tsukidate, 1977). The nature and the immunological relevance of these antigens are mostly unknown since the majority of the experiments have been performed with rather crude extracts (Fujita, Tanaka, Sasa, Tagawa, Naito and Kurokawa, 1970). Ouchterlony's classical studies of diffusion in gel offer a powerful procedure for comparing identity and partial identity of a limited number of antigens (Ouchterlony, 1958). However, animal parasites offer

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complex systems of antigens which in crude form pose difficulties in analysis.

In order to know the nature and the role of antigens in host-parasite relationship, we have separated the IgG-inducing antigens from *D. immitis* and partially characterized them as already reported (Fujita and Tsukidate, 1977). Among various antigens, the antigen inducing IgG antibody in rats and in mice was a glycoprotein with carbohydrate content of 20 per cent. We have also obtained a highly purified allergen from *D. immitis* (Fujita, Ikeda and Tsukidate, 1979, Fujita and Tsukidate, 1981). The allergen was assessed as pure by disc electrophoresis and by immunodiffusion test, and it had a low carbohydrate content and small molecular weight.

This paper characterizes the IgG-inducing antigen and compares its physicochemical nature with that of allergen.

MATERIALS AND METHODS

Animals: The Wistar strain of male rats (120 to 200g) and the ddY strain of male mice (15 to 17g) were used throughout the experiments. Outbred ICR mice, inbred mice of C57BL/6($H\text{-}2^b$), BALB/c, DBA/2($H\text{-}2^d$) and C3H/He($H\text{-}2^k$) and two of their hybrids(C57BL/6×DBA/2)F_1 and (BALB/c×DBA/2)F_1 were also used.

Hyperimmune rat sera: Rats were injected intramuscularly with 1.5mg of crude extract of *D. immitis* (CE), fractions from DEAE-Sephadex A-50 or purified IgG-inducing antigen (Frac. D-a) and allergen (Frac. B-d), emulsified in Freund's complete adjuvant at 1 week intervals, and bloods were taken 10 days after the 5th or 4th injections.

Immunization with allergen or IgG-inducing antigen: Rats were injected twice intraperitoneally with 0.1 to 1.5mg protein of Frac. B-d or Frac. D-a previously mixed with aluminum hydroxide gel or Freund's complete adjuvant. Mice were also immunized with 1.25 to 100γ of Frac. D-a previously mixed with aluminum hydroxide. Two outbred, four inbred and two hybrids mice were immunized with Frac. D-a plus Freund's complete adjuvant, and their IgG antibody inducing activity was compared.

Indirect hemagglutination (IHA) test and passive cutaneous anaphylaxis (PCA) test: The methods of IHA test were carried out according to the previous paper (Fujita, 1975). A part of sera was tested by IHA test after 2-mercaptoethanol treatment. PCA test was carried out in normal rats, and the challenge antigen was given 48hrs later by intravenous injection of CE (1.5mg of protein) in one ml of 1% Evans Blue solution, but in cases in mice sera, the challenge was carried out 4hrs later. After 30min, the diameters of blue spots produced were recorded. Blue spots larger than 0.5cm in diameter were regarded as positive. Positive sera were tested again by PCA test after heat treatment at 56°C for 30min or 2-mercaptoethanol treatment: all of these positive sera became negative after treatments.

Determination of IgG antibody: IHA positive sera which was obtained from the rats immunized with Frac. D-a were tested again with IHA test after 2-mercaptoethnaol treatment. These sera were also fractionated by gel filtration on Sephadex G-200 (Pharmacia, Uppsala) column (1.6×90cm). On the other hand, a part of the sera was also fractionated by zone electrophoresis on a starch block (1.5×10×40cm) equilibrated with Veronal buffer at pH 8.6 (μ=0.05) with a constant current of 200mA for 20hrs at 5 °C. The pooled eluates from the gamma region were concentrated and dialyzed against 0.02M phosphate buffer at pH 8.0. The dialyzed gamma globulin fraction was then placed on a diethylaminoethyl (DEAE) cellulose column (2.2×26cm) equilibrated with the same buffer as used in dialysis.

Immunoelectrophoresis (IEP): The sample was electrophoresed on a 7×9cm glass slide covered with 10ml of 1.2% Noble agar in Veronal buffer pH 8.2, μ=0.1. The run was carried out for 3.5hrs at 4°C and at 1.2mA/cm. At the end of the run, the slides were washed in saline for 3 days, dried and stained with 0.5% amino black 10B.

Polyacrylamide gel electrophoresis: Disc electrophoresis using polyacrylamide gel was carried out according to Davis (1964) in 7cm long tubes for 90min by applying a current of 5mA per tube.

Physicochemical properties of allergen and IgG-inducing antigen: Molecular weights of IgG-inducing antigen and allergen were estimated by Sephadex G-200 gel filtration calibrated with reference substances. Estimation of carbohydrate content and protease digestions were carried out according to the previous paper (Fujita, 1975). Periodate oxidation and acid or alkaline treatment were also done according to previous paper (Fujita and Tsukidate, 1977). Digestion with pancreatic DNase and RNase was carried out basically the same as reported by Holley, Everett, Madison and Zamir (1965).

RESULTS

Separation of IgG-inducing antigens from allergen fractions

IgG-inducing antigens were partially separated from allergen fractions by chromatography on DEAE-Sephadex A-50 as previously reported (Fujita and Tsukidate, 1977). CE was fractionated by DEAE-Sephadex A-50 into five peaks which were designated as Frac. A, B, C, D and E. Four rats per group were immunized with each fraction and IHA as well as PCA antibodies induced were determined. Rats immunized with Frac. D or E mixed with aluminum hydroxide induced only IHA antibody but never induced PCA antibody. On the other hand, Frac. B induced PCA antibody but not IHA antibody in rats by this immunization procedure. However, when rats repeatedly immunized with each of these fractions with Freund's complete adjuvant, all the rats induced only IHA antibody but never PCA antibody as shown in Table 1.

Table 1 IHA and PCA antibody titers in rats immunized with fractions from chromatography on DEAE-Sephadex A-50.

fractions	immunized with 1.5 mg × 2 of antigen plus Al $(OH)_3$		hyperimmunized with 1.5 mg × 5 of antigen plus Freund's complete adjuvant	
	IHA*	PCA	IHA	PCA
CE	$2^{5.5} \pm 2^{0.62}$	$2^{5.0} \pm 2^{0.80}$	$3^{8.7} \pm 3^{2.1}$	0
Frac. A	$2^{3.8} \pm 2^{0.42}$	$2^{5.0} \pm 2^{0.58}$	$3^{5.0} \pm 0$	0
Frac. B	0	$2^{5.5} \pm 2^{0.87}$	$3^{5.0} \pm 0$	0
Frac. C	0	$2^{0.3} \pm 2^{02.5}$	$3^{7.5} \pm 3^{0.71}$	0
Frac. D	$2^{5.0} \pm 2^{0.37}$	0	$3^{9.2} \pm 3^{0.29}$	0
Frac. E	$2^{4.0} \pm 2^{0.41}$	0	$3^{9.7} \pm 3^{1.15}$	0

*reciprocals of IHA titer, mean ± S.E. among four rats immunized.

Antigen analysis

By using these hyperimmune sera, the analysis of antigens from *D. immitis* were carried out. The CE was studied in IEP against anti-Frac. A, B, C, D and E rat serum and patterns are shown in Fig. 1. Number of antigens from CE was difficult to calculate in crude form. However, when CE was studied against each of anti-fraction serum, the analysis of antigens seemed to be rather easy. Main antigens could be seen as 6 against anti-Frac. A, 5 against anti-Frac. B and against anti-Frac. C and 6 against anti-Frac. D and against anti-Frac. E serum. The comparing identity and partial identity of these limited number of antigens was, however, still difficult to analyse.

Partially purification of IgG-inducing antigen

Since the preceding data indicate that Frac. D as well as Frac. E induced only IHA antibody in rats, and they were composed of many kinds of proteins, Frac. D and

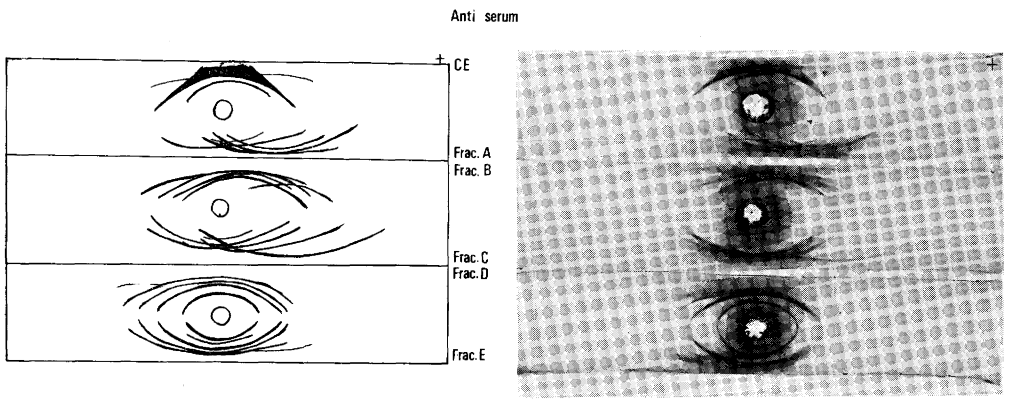
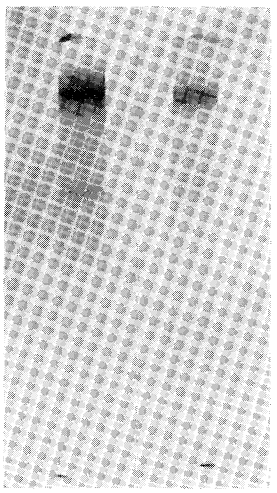


Fig. 1. Immunoelectrophoresis patterns of crude antigen (CE) against rat anti-CE, anti-Frac. A, anti-Frac. B, anti-Frac. C, anti-Frac. D and anti-Frac. E serum

E were further fractionated by double column of Sephadex G-200 as already reported (Fujita and Tsukidate, 1977). Among various fractions, Frac. D-a or Frac. E-a was most effective to induce IHA antibody in rats as well as in mice. Disc electrophoresis patterns of these fractions were shown in Fig. 2. Frac. D-a had two main protein bands very similar to those of Frac. E-a. Frac. D-a shared with the common bands with Frac. E-a and was considered to be composed of the same substances with Frac. E-a. The immunological responses of these IgG-inducing antigens were, then, examined and the results were summarized in Table 2. The rats immunized with these fractions produced only IHA antibody but never PCA antibody, even in the use of aluminum hydroxide gel as adjuvant. On the other hand, the rats immunized with highly purified allergen (Frac. B-d) or partially purified allergen (Frac. A-d) produced only PCA antibody. The rats hyperimmunized with these allergens, however, produced only IHA antibody, but the titers of IHA was rather lower than those of the rats hyperimmunized with Frac. D-a or Frac. E-a, as shown in Table 2.



D-a E-a

Fig. 2. Disc electrophoresis patterns of partially purified IgG-inducing antigens (Frac. D-a and Frac. E-a)

Table 2 IHA and PCA antibody titers in rats immunized with partially purified IgG-inducing antigen and allergen.

fractions	immunized with 0.4 mg × 2 of antigen plus Al $(OH)_3$		hyperimmunized with 1.5 mg × 4 of antigen plus Freund's complete adjuvant	
	IHA*	PCA	IHA	PCA
Frac. D—a	$2^{6.0} \pm 2^{0.82}$	0	$3^{9.5} \pm 3^{0.5}$	0
Frac. E—a	$2^{5.8} \pm 0$	0	$3^{9.0} \pm 0$	0
Frac. A—d	0	$2^{4.0} \pm 2^{0.20}$	$3^{3.5} \pm 3^{0.5}$	0
Frac. B—d	0	$2^{4.5} \pm 0$	$3^{3.0} \pm 0$	0

*reciprocals of IHA titer, mean ± S.E. among four rats immunized.

Determination of IgG antibody

The IHA positive sera from the rats immunized with Frac. D-a were tested again with IHA test after 2-mercaptoethanol treatment. All sera showed same positive titers of IHA after the treatment. These sera were also gel filtrated by Sephadex G-200 and

separated into three peaks. Each fraction was tested by IHA test. IHA activity was localized only in second peak, and no PCA activity was shown in this peak at all. A part of sera was fractionated by zone electrophoresis on a starch block, and the gamma region was obtained. The pooled eluates was then placed on a DEAE cellulose column, and only one peak was obtained. IHA activity was still observed in this fraction and no PCA activity was shown also in this case. This fraction was determined to IgG by the IEP technique using anti-rabbit IgG serum. It was determined by above mentioned results that the sera from the rats immunized with Frac. D-a had the IgG antibody against them.

Physicochemical properties of IgG-inducing antigen and highly purified allergen

The molecular weights of Frac. D-a and E-a were estimated on a calibrated Sephadex G-200 column. From the correlation between the elution volume and the molecular weights of two reference proteins (horse apo-ferritin and rat macroglobulin), the molecular weights of Frac. D-a and E-a were estimated to be same, being approximately 600,000. The molecular weight of the highly purified allergen (Frac. B-d) was also estimated on a calibrated Sephadex G-200. From the ellution volume and the molecular weights of two references (myoglobin, trypsin), the molecular weight of allergen was estimated about 20,000.

The carbohydrate contents in percentage of total protein in the IgG-inducing antigen (Frac. D-a) as well as the allergen were studied. The IgG-inducing antigen had carbohydrate in 20%, whereas the allergen only in 2%.

The comparison of these above mentioned properties of the IgG-inducing antigen with those of the allergen is summarized in Table 3.

Table 3 Comparison of the properties of IgG-inducing antigen with those of allergen.

	IgG-inducing antigen (Frac. D-a)	the highly purified allergen (Frac. B-d)
molecular weight	600,000	20,000
carbohydrate content	20%	2%
active site	protein	protein
kinds of protein	2	1
charge	+～−	+

Effect of cleavage of peptide bond by chymotrypsin, pepsin, trypsin or pronase digestion and effect of DNase or RNase and periodate oxidation.

In order to see and compare some physicochemical properties between the IgG-inducing antigen and the allergen, these antigens were digested with chymotrypsin, pepsin, trypsin or pronase. As shown in Table 4, both antigens were resistant to chymotrypsin, pepsin or tryptic digestion, but very sensitive to pronase digestion.

Table 4 Comparison of the physicochemical properties of the IgG-inducing antigen with those of allergen

treatment of antigen with	reciprocal of	
	IHA titer of the rats immunized with the treated IgG-inducing antigen (0.4mg×2)	PCA titer of the rats immunized with the treated allergen (0.4mg×2)
control	$2^{6.0}$*	$2^{4.5}$*
chymotrypsin	$2^{5.0}$	$2^{4.0}$
pepsin	$2^{5.5}$	$2^{3.5}$
trypsin	$2^{3.5}$	$2^{2.8}$
pronase	0	0
periodate oxidation	$2^{5.0}$	$2^{5.0}$
DNase	$2^{5.5}$	$2^{4.0}$
RNase	$2^{4.0}$	$2^{5.0}$
incubation in 37°C for 1hr	$2^{5.0}$	$2^{4.5}$
vibration in 37°C for 1hr	$2^{5.5}$	$2^{4.0}$
heat (80°C, 1hr)	$2^{5.0}$	$2^{6.0}$
heat (100°C, 1hr)	$2^{6.5}$	0
acid (pH 2.5, 37°C, 1hr)	$2^{5.5}$	$2^{4.5}$
alkali (pH 11.0, 37°C, 1hr)	$2^{5.5}$	$2^{4.5}$

*mean titer of four rats immunized.

In order to confirm the active site of the IgG-inducing antigen and the allergen, these antigens were then treated with pancreatic DNase or RNase. The activities of both antigens were recovered unaltered after either of the digestions. Periodate oxidation also resulted in almost no effect to these antigens. These results lead to the conclusion that the determinant group of the IgG-inducing antigen as well as the allergen is suggested to be protein but not carbohydrate nor nucleic acids in nature.

Effect of denaturing conditions on the antigenic activity

The effects of various denaturing conditions on the stability of antigens were studied and summarized in Table 4. The IgG-inducing antigen appeared remarkably stable to vibration at 37°C, to heating at 80°C or even at 100°C, or treatment with acid or alkali. The allergen was also resistant against these treatments, but the activity was completely destroyed after treatment with 100°C 1hr, which was different from the IgG-inducing antigen.

Immunological responses of IgG-inducing antigen

Partially purified IgG-inducing antigen (Frac. D-a) was then studied on the immunological responses of rats and mice. Wistar strain rats were immunized with various doses of Frac. D-a mixed with aluminum hydroxide, and IHA and PCA antibodies were assayed. With doses up to 1.5mg×2 from 0.1mg×2, IHA antibody of relatively

high titers was observed, whereas the titers of PCA antibody were very low, as shown in Fig. 3. Rats were also immunized with Frac. D-a plus Freund's complete adjuvant. The rats produced only IHA antibody also in this case, as shown in Fig. 4.

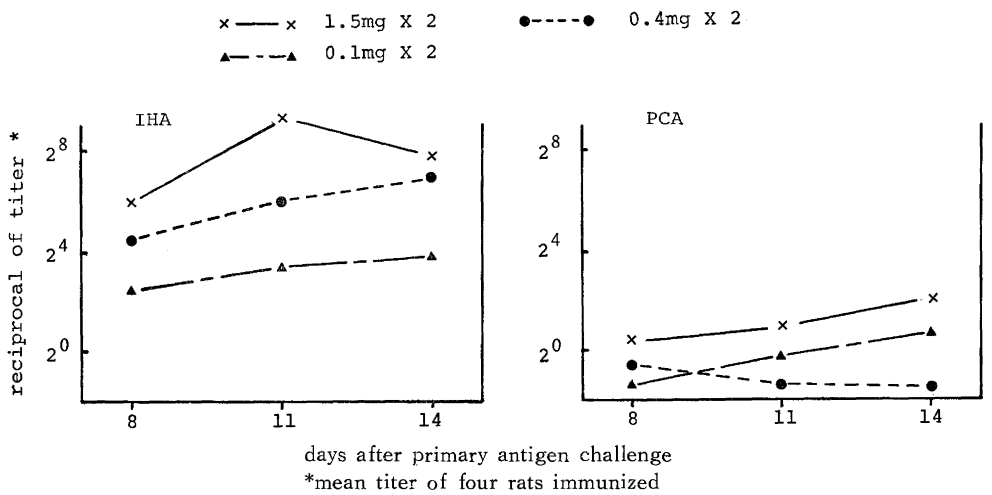


Fig. 3. Relationship between antigen dose and production of IHA and PCA antibodies in the rats immunized with partially purified IgG-inducing antigen (Frac. D-a) plus aluminum hydroxide adjuvant

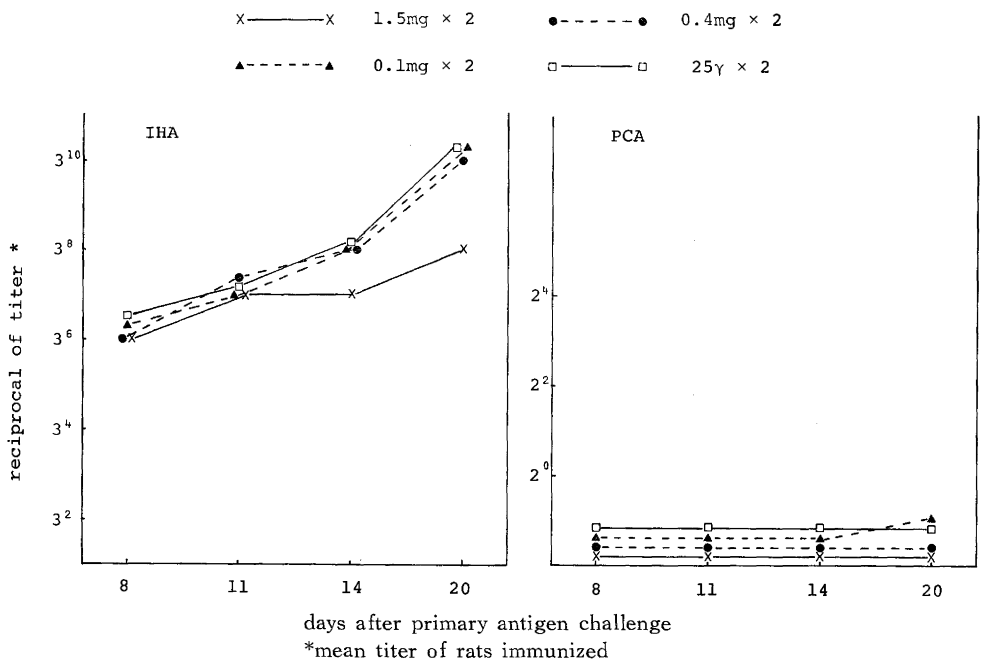


Fig. 4. IHA antibody production of the rats immunized with Frac. D-a mixed with Freund's complete adjuvant.

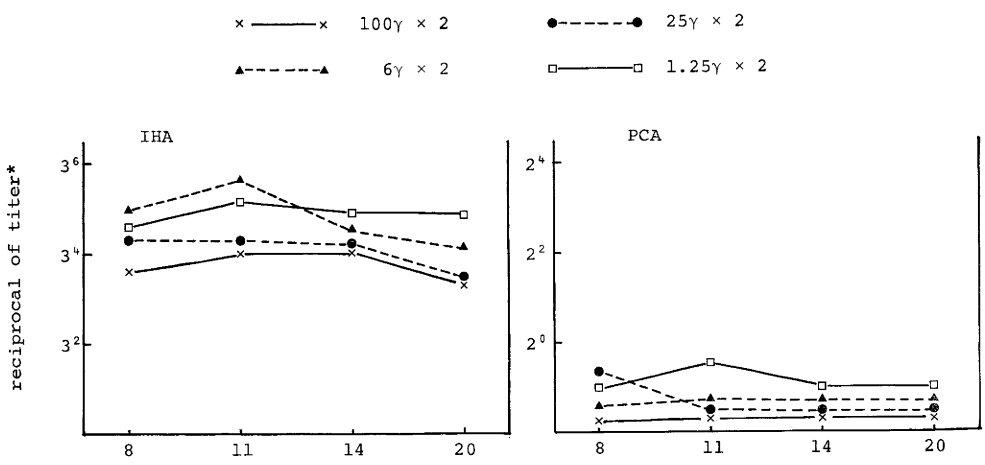
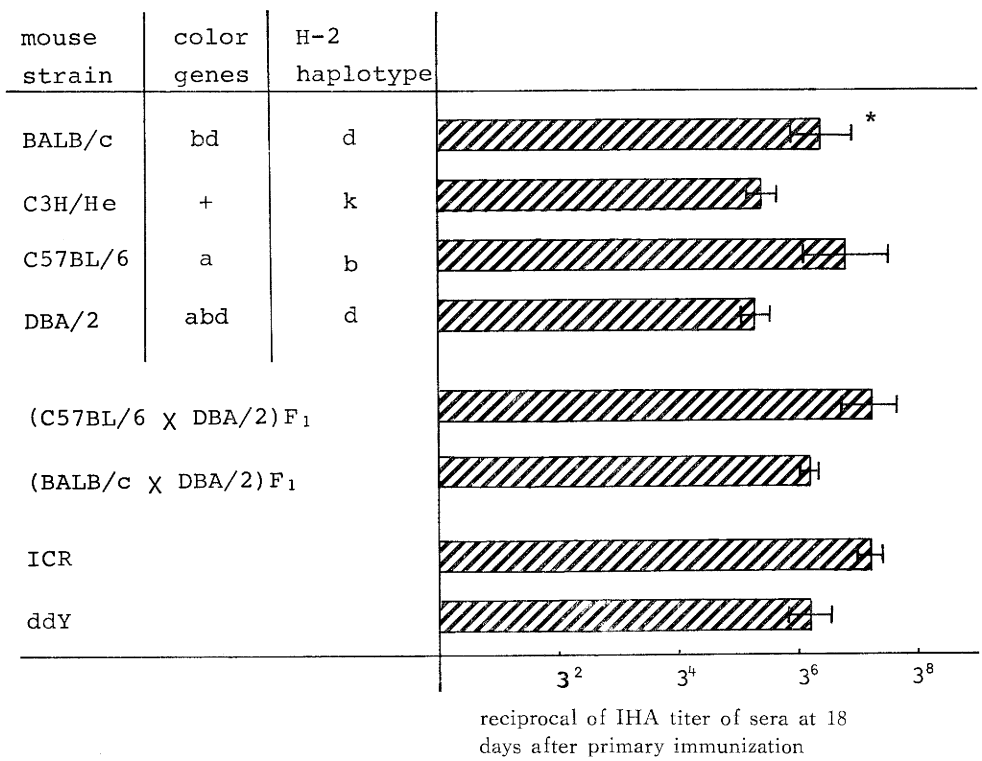


Fig. 5. Relationship between antigen dose and production of IHA and PCA antibodies in the mice immunized with partially purified IgG-inducing antigen (Frac. D-a) plus aluminum hydroxide adjuvant



*mean value with S.E. of five mice immunized with 1.25γ×2 of antigen

Fig 6. IHA titers of different strains of the mice immunized with Frac. D-a plus Freund's complete adjuvant

Mice of ddY strain were also immunized with Frac. D-a. In spite of the use of aluminum hydroxide as adjuvant, no response of PCA antibody was observed at all, whereas IHA antibody of relatively high titers was observed with doses up to $100\gamma \times 2$ from $1.25\gamma \times 2$, as shown in Fig. 5. Then antibody production against Frac. D-a was compared among the various strains of mice. Two outbred mouse strains (ddY and ICR), four inbred strains (BALB/c, C3H/He, C57BL/6 and DBA/2) and two of their hybrids (C57BL/6 × DBA/2) F_1 and (BALB/c × DBA/2)F_1, were immunized with 1.25γ of Frac. D-a and boosted at 5 days. Their serum IHA and PCA titer was assayed at 18 days after primary antigen challenge. As shown in Fig. 6, almost no difference in IHA antibody production among mice strains was observed, and the mice produced almost no PCA antibody.

DISCUSSION

A feature of acquired resistance noted in several filarial infections is the absence of circulating microfilariae in hosts harboring viable adult worm burdens (Weiss, 1970, Wong, Suter, Rhode and Guest, 1973). Such infection is called as occult, latent or amicrofilaremic filariasis and the etiology of this disease is regarded as the same as that of the part of tropical eosinophilia (Joe, 1962, Beaver, 1970). However, considerable confusion continues to exist over the clinical entity of tropical eosinophilia. For example, it is not yet determined whether the entity is caused by single or by multiple etiologic agents (Neva et al., 1975).

With a view to studying the pathological causes of the amicrofilaremic filariasis, Neilson (1975, 1978) carried out experiments to analyse the antigenic character of *Dipetalonema vitae*. He succeeded in inducing golden hamsters in amicrofilaremic state by the repeated infections with *D. vitae*, but could not find which antigens induced such situation in the host.

In order to know the host-parasite relationship in the filarial infections, we have separated the IgG-inducing antigen from allergen fractions in *Dirofilaria immitis* adult worms, and clarified some physicochemical properties of the antigen. On the other hand, we could obtained the allergen in the highly pure form, as already reported (Fujita et al., 1979). The allergen was composed of one kind of protein and existed as a small compact molecule which was very stable to denaturation, as shown by its resistance to chymotrypsin, trypsin or pepsin, periodate oxidation and agents such as heat, vibration and acid or alkali.

In the present investigation, IgG-inducing antigen was purified in relative pure form and its physicochemical properties were compared with those of allergen. Although Frac. D-a had still two kinds of proteins, it could induce IgG antibody formation in rats as well as in mice, and was incapable of inducing IgE antibody in the hosts, in spite of the use of aluminum hydroxide as adjuvant. The partially purified IgG-inducing

antigen was resistant to various kinds of proteases and periodate oxidation but very sensitive to pronase digestion as the allergen did so.

Other physicochemical properties of the IgG-inducing antigen was also similar to those of allergen. Further comparison between IgG-inducing antigen and allergen is necessary to study. The localization of the allergen and IgG-inducing antigen in the adult Dirofilaria worm is now studying in our laboratory. It should be worthwhile to investigate what important parts these antigens played in the host-parasite relationship in the filarial infection.

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犬糸状虫由来 IgG 誘導抗原の精製とその物理・化学的特徴

藤田紘一郎，月館説子（長崎大学医学部医動物学教室），池田照明（金沢医科大学医動物学教室）

われわれは，すでに犬糸状虫成虫体よりアレルゲンを単一の糖蛋白として精製し，その物理・化学的特徴について報告した．今回，宿主にIgG抗体を誘導する物質を犬糸状虫体より分離・精製し，精製アレルゲンの免疫学的および物理化学的諸性質と比較検討した．

犬糸状虫由来のIgG誘導抗原は虫体内に多種類存在したが，そのうち最も効果的に宿主にIgG抗体を誘導する物質は，分子量約60万，糖約20%を有する物質で，熱・酸・アルカリおよび振盪などに対して，またトリプシンやキモトリプシン，過ヨード酸や核酸処理などの物理化学的処理に対して極めて強い抵抗性を示した．また，この物質は特別な酵素活性をもたなかった．抗原活性部位は，アレルゲンと同様蛋白部分にあった．この抗原を種々の濃度でラットおよび8系統のマウスに免疫すると，IgG抗体のみ誘導し，IgE抗体の産生は見なかった．なお，この抗原はまだ，2種類の蛋白の混成であることがディスク電気泳動で明らかにされた．この抗原で誘導された抗体は主としてIgG抗体であることが，2-ME 処理後の免疫血清，Sephadex G-200 ゲル沪過の第2ピークおよびゾーン電気泳動後の DEAE-Cellulose 分画にそれぞれ活性が集中する事実によって確認された．

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