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Isolation and Properties of IgG-Inducing Antigens from *Dirofilaria immitis**

KOICHIRO FUJITA** and SETSUKO TSUKIDATE
Department of Hygiene, Juntendo University, School of Medicine, Tokyo, Japan

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Introduction

In a previous paper (Fujita, 1975), allergen in *Dirofilaria immitis* was separated from the IgG-inducing antigens and some of the properties of the allergen was examined. The allergen had a low carbohydrate content (1.5%) and its molecular weight was 20,000. Allergenic activity was located in protein part of the allergen. IgG-inducing antigens, however, should be also studied in order to know the immunological effector mechanism occurred in parasitic infections. Namely, IgG-inducing antigens as well as allergen were considered to play a significant role in the ultimate host-parasite relationship (Hogarth-Scott, 1973), and a study of parasite antigens should yield information relevant to the fundamental understanding of this relationship.

Additionally, IgG-inducing antigens are the principal components involved in the immuno-diffusion test reaction (Takaoka *et al.*, 1973), flocculation test (Kagan *et al.*, 1963), hemagglutination test (Tanaka *et al.*, 1968b), and complement fixation test reaction (Ridley, 1956; Tanaka *et al.*, 1969) widely used in the field both for epidemiological surveys (Fujita *et al.*, 1970; Tanaka *et al.*, 1970) and for the individual diagnosis of disease (Kagan, 1953). Antigen responsible for hemagglutination test in cotton rat filariasis has partially been characterized by us (Tanaka *et al.*, 1968a). This antigen was of protein nature and had a molecular weight of approximately 140,000. Ishii (1970) reported the antigenicity of excretory and secretory products of the cotton rat filaria. But, it was not yet determined whether these antigens were the same as IgG-inducing antigens or not.

The work described in this paper was carried out to investigate the separation and some properties of IgG-inducing antigens from *Dirofilaria immitis*.

Materials and Methods

The parasite and differential centrifugation of antigens: Parasites were harvested from dogs infected with *Dirofilaria immitis* in Tokyo area, washed thoroughly in saline and lyophilized. Lyophilized worms were stored at −20 C until required for use. Worms were homogenized with glass and teflon homogenizers, disrupted by sonication (Insonator, 200 M, Kubota, Tokyo) at 140 Watt for 5 min and extracted in phosphate buffered saline (PBS) at pH 7.2 for 2 days in a refrigerator. The emulsion was centrifuged at 13,000 g for 20 min at 0 C to remove relatively large particles, such as cell debris, nuclei and mitochondria. The 13,000 g supernatant was designated as CE and used as a starting material. A "microsomal fraction" was obtained by centrifugation of CE at 100,000 g for 60 min.

Animals and immunization: White, male, random-bred rabbits weighing 2,000 g and Wistar strain of both sexes weighing 120 to

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** Present address: Department of Medical Zoology, Kanazawa Medical University, Uchinada, Ishikawa-ken, Japan

200 g were used throughout the experiment. Rabbits were injected intramuscularly with 10 mg of CE emulsified in Freund's complete adjuvant at one-week intervals and blood was taken one week after the 5th injection. The sera were stored at −20 C and used as positive controls in the hemagglutination test (titer $1:2^{16}$). Rats were injected subcutaneously into the foot pads with CE or one of its fractions in quantities ranging from 0.004 to 1.5 mg protein previously mixed with 10^{10} of killed *Bordetella pertussis* organisms. The animals were bled 14 days after injection and the sera were stored at −20 C.

It was reported in the previous paper (Fujita, 1975) that rats produced homocytotropic antibody (Hc) most when 1.5 mg of CE was used for immunization, but when they were immunized with its fractions, they produced Hc antibody with highest titer in quantities ranging from 0.025 to 0.4 mg. Therfore, 0.4 mg of antigen concentration was employed in separation of IgG-inducing antigen from allergen.

Passive cutaneous anaphylaxis (PCA), indirect hemagglutination test (HA) and estimation of protein and carbohydrate were carried out according to the previous paper (Fujita, 1975).

Preparation of fraction D and E: Five milliliters of CE containing 100 mg of protein were applied to a 1,0×20 cm Diethylaminoethyl (DEAE) Sephadex A-50 (Pharmacia, Uppsala). Elution was carried out at room temperature with 24 to 30 ml of 0.015 M phosphate buffer pH 7.2, containing stepwise increasing concentrations sodium chloride at (1) 0 M (starting buffer) (2) 0.125 M (3) 0.25 M (4) 0.5 M and (5) 1 M. The effluent was monitored by a Toyo double beam UV analyzer (Toyo Uvicon, 540M. Toyo Kagaku, Tokyo). Three-milliliters fractions were collected at a flow rate of 0.2 ml per min and five protein peaks were obtained and designated as Frac. A, B, C, D and E in order of appearance.

Gel filtration on Sephadex G-200: Sephadex G-200 (Pharmacia, Uppsala) was swollen in distilled water for 3 days at room temperature and equilibrated in PBS at pH 7.2. Fifteen-milliliters of Frac. D or E containing 15 to 20 mg of protein were applied to double columns of Sephadex G-200 (1.6×180 cm). Three ml fractions were eluted with PBS. Five peaks were obtained from the gel filtration of Frac. D as well as E and designated as Frac. D-a, D-b, D-c, D-d and D-e, and E-a, E-b, E-c, E-d and E-e in order of appearance respectively. The protein peaks obtained were pooled separately, concentrated and dialyzed with PBS and stored at 4°C until tested.

A diagram of the fractionation procedures is shown in Fig. 1.

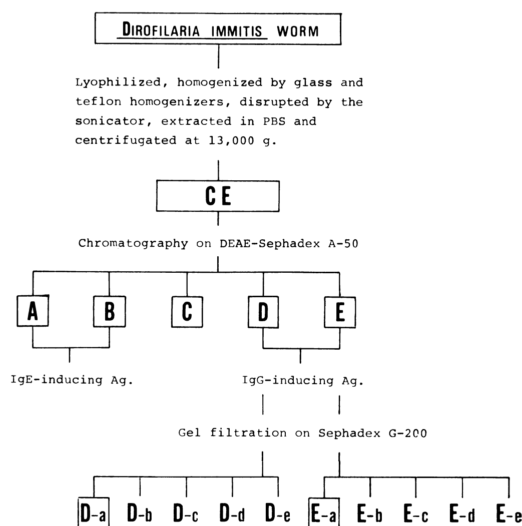

Fig. 1 Flow chart of techniques employed.

Polyacrylamide gel electrophoresis: Disc electrophoresis was carried out with two kinds of polyacrylamide gels; separating gel and stacking gel according to Davis (1964). Separating gel and stacking gel contained 7.5% and 2.5% polyacrylamide respectively in Tris-Hcl buffer pH 8.0, and 0.2 ml of stacking gel was layered on the top of separating gel in 0.5×7.0 cm tube. Samples of 0.1 ml containing 50 μg of protein mixed with 0.1 ml of 40% sucrose was placed on stacking gel. Electrophoresis was carried out in 7 cm long tubes for 90 min by applying a current of 5 mA per tube. The tubes

were run for 10 min after the tracking dye reached the bottom of the tube. The gels were stained for 1 hr in 0.02% Coomassie brilliant blue solution and destained in 7.5% acetic acid −5% methanol solution.

Molecular weight estimation: Molecular weight of Frac. D-a and E-a was estimated by Sephadex G-200 gel filtration. The elution volume expressed as Ve of a given molecular species was plotted against its molecular weight. Horse apo-ferritin (Apo, M. W: 4.8×10^5) and rat macro globulin (IgM, M. W: 8.0×10^5) were used as markers.

Tryptic digestion of Frac. D-a and E-a: A solution containing 0.1% trypsin (Schwarz Mann Co., New York) in PBS pH 7.2 was used as protease solution (Fujita *et al.*, 1969). Soybean trypsin inhibitor (Schwarz Mann Co., New York) in PBS at pH 7.2 was used to interrupt progress of digestion; 0.5 ml of Frac. D-a or E-a containing 0.4 mg protein and 0.1 ml of protease solution were incubated in a water bath at 37 C for 2 hr and mixed with 0.1 ml of 0.5% trypsin inhibitor solution.

Pronase digestion and periodate oxidation: As protease solution, 0.2% pronase (Kaken Kagaku Co., Tokyo) in PBS pH 7.2 was used and incubated with 0.5 ml of Frac. D-a or E-a at 37 C for 24 hr. Periodate oxidation was carried out with sodium metaperiodate (Koso Chemical Co., Tokyo) in 0.05 M sodium acetate buffer at pH 4.5 and 4 C in the dark (Spiro, 1964). The protein concentration was 1.5 mg in 1 ml and molar ratios of periodate to protein 100 : 1 to 200 : 1 were employed. The oxidation was stopped after 5 hours by the addition of 0.5 ml of ethylene glycol.

Results

Separation of IgG-inducing antigens.

1) Fractionation by differential centrifugation: In an attempt to prepare IgG-inducing antigens which could stimulate HA antibody effectively, differential centrifugation was applied at the first step of purification. Three rats per group were immunized with the supernatant fraction of 13,000 g, that of 100,000 g, with the precipitate fraction of 13,000 g or that of 100,000 g. The results showed that HA antibody as well as Hc appeared only in rats immunized with the supernatant fractions. Therefore, 13,000 g supernatant fraction (CE) was used for further purification.

2) Chromatography of CE on DEAE-Sephadex A-50: IgG-inducing antigens were separated from allergen by chromatography on DEAE-Sephadex A-50. Five milliliters of CE containing 100 mg of protein was fractionated and five peaks were obtained as described in Materials and Methods. Four rats per group were immunized with each fraction and HA activity as well as PCA was determined. The results showed that only fraction D and E elicited positive HA reaction but never elicited positive PCA reaction as shown in Table 1. On the other hand, fraction B induced only Hc antibody but not HA antibody. Disc electrophoretic patterns of these fractions were examined and shown in Fig. 2. It was known from disc electrophoretic patterns that all fractions were composed of many kinds of proteins. But, it was very interesting that the electrophretic pattern of Frac. D was very similar to that of Frac. E and pattern of Frac. A also similar to that of Frac. B respectively.

Purification of IgG-inducing antigen.

The preceding date indicate that Frac. D as well as Frac. E induced only HA antibody in rats, and they were composed of many kinds of proteins. In order to know the

Table 1 HA and Hc antibody titers in rats immunized with fractions from chromatography on DEAE-Sephadex A-50

Immunized with	Reciprocal* of	
	HA titer	PCA titer
Fraction A	$2^{3,8}\pm 2^{0,42}$**	$2^{4,0}\pm 2^{0,58}$
B	0 ± 0	$2^{4,9}\pm 2^{0,87}$
C	0 ± 0	$2^{0,3}\pm 2^{0,25}$
D	$2^{3,6}\pm 2^{0,37}$	0 ± 0
E	$2^{4,0}\pm 2^{0,41}$	0 ± 0

* mean value with four immunized rats
** mean±S.E.

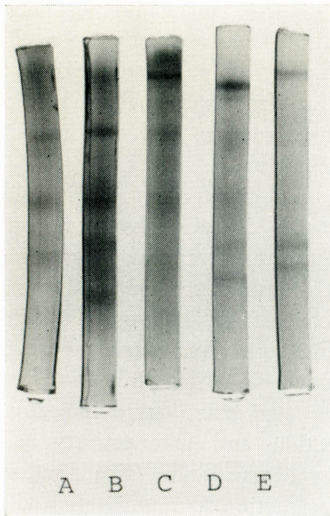

Fig. 2 Disc electrophoresis patterns of fractions obtained from CE of *D. immitis* by chromatography on DEAE-Sephadex A-50.

nature of IgG-inducing antigens, further purification of Frac. D and E were carried out by gel filtration on Sephadex G-200. As shown in Fig. 3, Frac. D was further separated into five peaks as described in Materials and Methods. Frac. D-a, D-b and D-c had the ability to elicite HA antibody but never elicite Hc antibody at least in rats. Namely, in rats, Frac. D was considered to be composed of only IgG-inducing antigens. Disc electrophoretic patterns of these fractions were shown in Fig. 4. It was shown that common proteins were existed among Frac. D-a, D-b and D-c. Frac. D-a which was most effective to induce IgG had two main protein bands common with Frac. D-b.

Frac. E was also further separated into

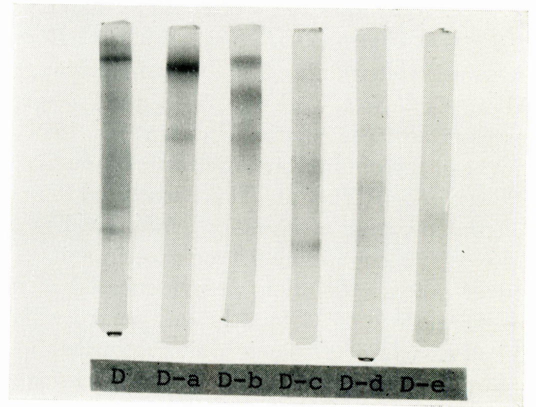

Fig. 4 Disc electrophoresis patterns of fractions obtained from Frac. D by gel filtration through Sephadex G-200.

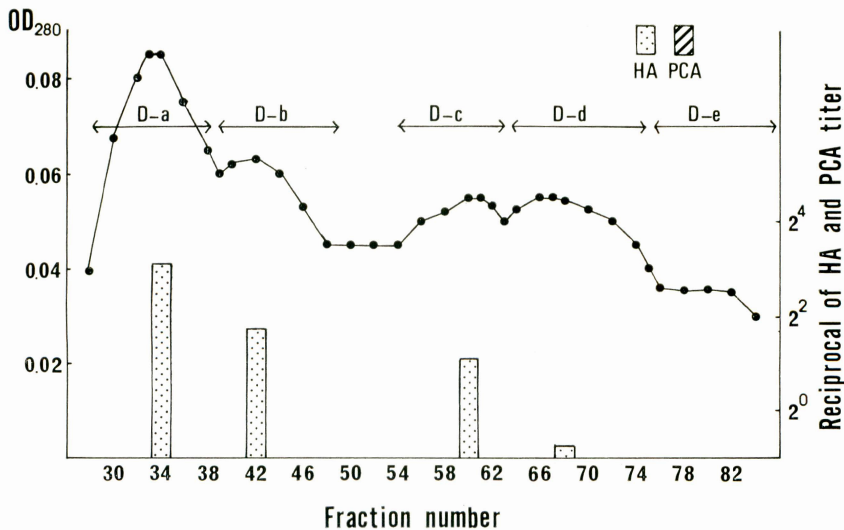

Fig. 3 Purification of IgG-inducing antigens in Frac. D by gel filtration through Sephadex G-200.

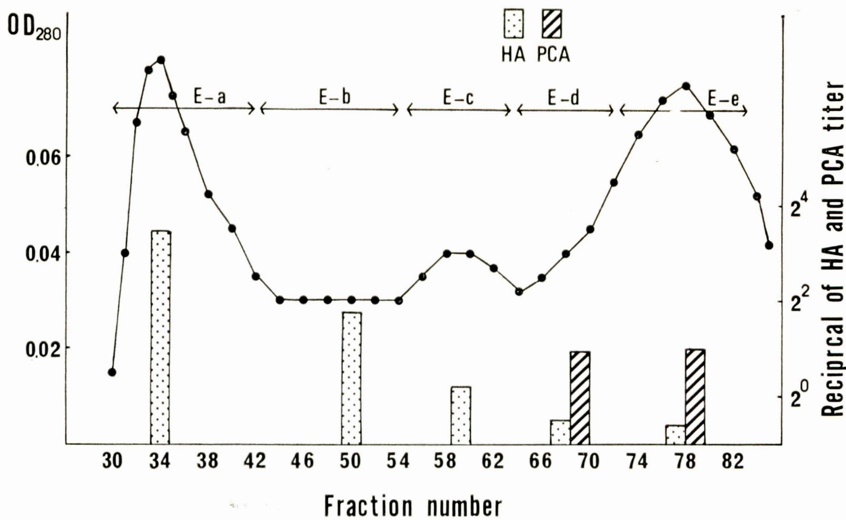

Fig. 5 Purification of IgG-inducing antigens in Frac. E by gel filtration through Sephadex G-200.

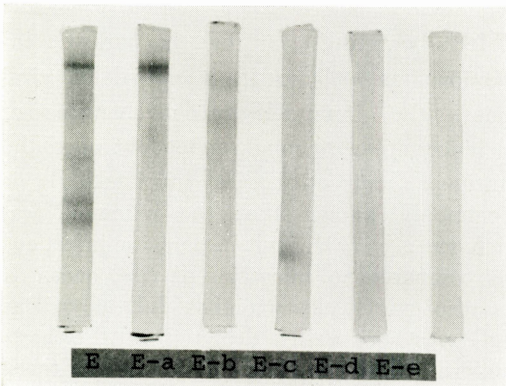

Fig. 6 Disc electrophoresis patterns of fractions obtained from Frac. E by gel filtration through Sephadex G-200.

five peaks by gel filtration on Sephadex G-200 as described in Materials and Methods. Frac. E-a as well as E-b and E-c produced HA antibody only, but Frac. E-d and E-e produced Hc antibody predominantly with low titers of HA antibody (Fig. 5). In this case, Frac. E-a was the most reactive antigen which induced IgG. Disc electrophoretic patterns of these fractions were shown in Fig. 6. As shown in Fig. 6, the pattern of Frac. E-a was similar to that of Frac. D-a ; Frac. E-a was considered to be the same substance with Frac. D-a. Frac. D-a as well as E-a still had two kinds of proteins, but it was not yet known which protein induced IgG more effectively. It was shown by electrophoretic patterns that Frac. E-b was a little different from Frac. D-b, but shared at least two kinds of common proteins with Frac. D-b. In this case also, it should be studied which protein induced IgG effectively.

Some physicochemical properties of the partially purified IgG-inducing antigens.

1) *Estimation of the molecular weight of Frac. D-a and E-a.* Since disc electrophoresis showed that Frac. D-a and E-a which were considered to be the most purified antigens inducing IgG were the same substance, the molecular weight of these fractions were estimated on a calibrated Sephadex G-200 column. From the correlation between the elution volume and the molecular weight of reference proteins, the molecular weights of Frac. D-a and E-a were estimated to be also the same, being approximately 600,000 (Fig. 7).

2) *Carbohydrate contents of fractions obtained by Sephadex G-200.* The carbohydrate contents in percentage of total protein in the fractions obtained by Sephadex G-200

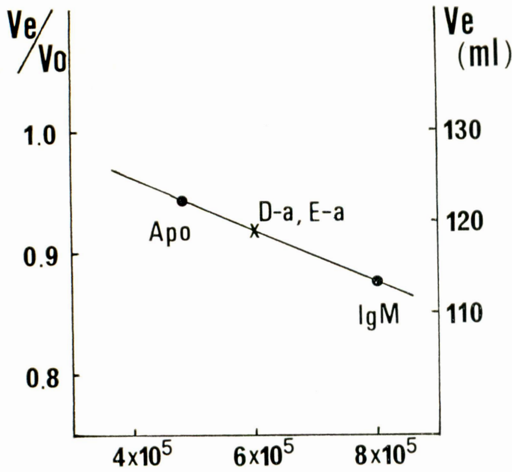

Fig. 7 Sephadex G-200 calibration curve for molecular weight determination of IgG-inducing antigen.

Ve : elution volume Vo : void volume
Apo : horse apo-ferritin
IgM : rat macro globulin

Table 2 Carbohydrate contents of fractions obtained from gel filtration through Sephadex G-200

Fraction	%*	Fraction	%*
D	4.2	E	5.2
D—a	20.4	E—a	20.2
D—b	8.8	E—b	16.5
D—c	16.8	E—c	7.9
D—d	4.5	E—d	11.5
D—e	9.2	E—e	3.1

* carbohydrate (mg/ml)/protein (mg/ml) × 100

are given in Table 2. Frac. D-a had carbohydrate in 20.4% which was quite similar to that of Frac. E-a (20.2%).

3) *Effect of cleavage of peptide bond, pronase digestion and periodate oxidation of Frac. D-a and E-a on HA activity.* In order to see some chemical natures of IgG-inducing antigens, Frac. D-a and E-a were digested with trypsin and pronase and were oxidated with periodate. As shown in Table 3, antigens were resistant to trypsin digestion and periodate oxidation but very

Table 3 Effect of cleavage of peptide bond, pronase digestion and periodate oxidation of IgG-inducing antigens on HA activity

Treatment with		Reciprocal of HA titer of the rats immunized with	
		Frac. D-a	Frac. E-a
Control		$2^{3.5}$	2^{5}
Trypsin		2^{3}	2^{3}
Pronase		0	0
Periodate oxidation	(64.mg)	$2^{4.5}$	2^{4}
	(19.2mg)	2^{3}	2^{3}

sensitive to pronase digestion. The results lead to the conclusion that the determinant group of antigen is suggested to be protein but not carbohydrate in nature.

Discussion

It is becoming clear that in parasitic infections, as well as in microbial or viral ones, the immunological mechanisms of defence are very much dependent upon the antigens and antibodies involved. It is commonly known that the immunological defence is very difficult in parasitic infections as compared to microbial or viral ones. It is also widely known that IgE as well as IgG was very easily detected in the hosts suffered from parasitic infections (Andrews, 1962). It is occurred very frequently that antibodies produced by the host are directed against antigens whose combination with antibody does not effect the normal metabolism or proliferation of the parasite. In the case that these antigens are immunodominant, it may be possible that whole response of the host is of antibodies completely ineffective against the invader. Such a situation could represent an advantage to the invader. It must be also kept in mind that the nature of the antibodies directed against antigens in the host was very important because each class of antibodies plays different roles in host-parasite relationship. These considerations have prompted us to undertake a detailed study of

the antigens of *Dirofilaria immitis*.

IgG-inducing antigens in *D. immitis* have been able to be separated from allergen as already reported previously (Fujita, 1975). The present investigation has lead to the isolation of IgG-inducing antigens in a relatively high state of purity as detarmined by disc electrophoresis. The partially purified IgG-inducing antigen was resistant to trypsin digestion and periodate oxidation but very sensitive to pronase digestion, and contained 20% of carbohydrate but its determinant group was protein in nature. Molecular weight of this antigen was ca. 600,000. In a preceding paper (Fujita, 1975), immunogenicity with respect to Hc antibody was studied. As compared to the IgG-inducing antigen, the allergen has small molecular weight (ca. 20,000) and relatively small contents of carbohydrate (2%). Physicochemical nature of the allergen, however, was very similar to that of the IgG-inducing antigen (Fujita and Tsukidate, unpublished data). It would be worthwhile to investigate what important parts these antigens played in the host-parasite relationship.

Ishii *et al.* (1969) revealed by the fluorescent antiboy technique that antigens were located in the sub-cuticle area of the adult cotton rat filaria. No investigation, however, has been made to differentiate the location of antigen responsible for IgG induction from that of allergen responsible for IgE induction. The question remains to be solved whether IgG-inducing antigen is located in the important parts of parasite in which various kinds of metabolisms of parasite are being carried out. It should be examined whether this antigen are necessary for proliferation of parasite or not.

Further purification of the IgG-inducing antigen and its location in parasite are currently being studied in our laboratory.

Summary

IgG-inducing antigens in crude extract of *Dirofilaria immitis* were partially purified and separated from allergen (IgE-inducing antigen) by a combination of DEAE-Sephadex A-50 chromatography and Sephadex G-200 gel filtration. The molecular weight of the purified preparation (Frac. D-a or E-a) was estimated to be approximately 600,000 by gel filtration. It was resistant to tryptic digestion and periodate oxidation but very sensitive to pronase digestion. The carbohydrate content of the preparation was relatively high, about 20%, but its determinant group was protein in nature. The preparation has still two protein bands by disc electrophoresis.

Rats immunized with the IgG-inducing antigen developed only a hemagglutination antibody and no homocytotropic antibody.

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犬糸状虫より分離された IgG 誘導抗原の精製とその生化学的特徴

藤田紘一郎　月館説子
（順天堂大学医学部衛生学教室）

寄生虫感染においては役割を異にする種々の抗体が産生される．寄生虫虫体内にはそれらの抗体を誘導するそれぞれの抗原が存在することが考えられる．これらの抗原を明らかにし，host-parasite relationship における役割を明らかにすることは重要である．我々は先にラットにおいて IgE を誘導する抗原（アレルゲン）を犬糸状虫（*Dirofilaria immitis*）成虫体より分離し，その生化学的特徴を明らかにした．

今回は感染防御の面に何らかの形で関与すると考えられる IgG 誘導抗原を成虫粗抗原より分離したのでその生化学的特徴と併せて報告する．

ラットにおいて IgG を誘導する抗原は DEAE-Sephadex A-50 によるクロマトグラフィーと Sephadex G-200 によるゲル濾過の組み合せにより犬糸状虫虫体粗抗原より分離された．精製された抗原，すなわち分画 D-a 及び E-a は共に分子量約 60 万で 20％の糖を含んでいたが，活性部位は蛋白部分にあつた．トリプシンや過ヨード酸には抵抗性を示したがプロナーゼには完全に失活した．しかし，これらの抗原はディスク電気泳動で調べるとなお2本の蛋白の混成物であつて，このいずれが IgG を誘導するか，また虫体内のどの部位に存在するかは不明である．

この分画 D-a 及び E-a の両抗原 0.004 mg から 1.5 mg までの蛋白濃度でラットを2回免疫すると IgG のみ誘導され，IgE の産生をみなかつた．

以上の結果は IgG をラットに誘導する抗原と IgE を誘導する抗原が寄生虫体に別々に存在し，それらの抗原は感染時にはそれぞれ異なつた役割を果たすものと思われる．