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Toxocara canis: Immunogenic Sources of *Toxocara canis* in Infected Rats

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Introduction

Although the importance of protective immunity to parasitic infections in man and animals has been emphasized, the nature and origin of the antigens which stimulate host immune response have not been sufficiently clarified.

Recent advances in the culture methods of the helminth parasites have enhanced an understanding of the immunogenic property of larval excretions/secretions (LES) in helminth infection (Kobayashi *et al.*, 1968; Ogilvie and Worms, 1976; Soulsby, 1977).

In *Toxocara canis* infection, it has been shown that antibodies directed to LES could be detected in infected rabbits, man and pigs from which the sera reacted with LES *in situ* at the pore of living larvae (Olson, 1960; Hogarth-Scott, 1966; Stevenson and Jacobs, 1977). *T. canis* LES collected by an *in vitro* culture method has been demonstrated to be potentially a specific antigen in haemagglutination/and a soluble antigen in fluorescent antibody tests to diagnose visceral larva migrans (de Savigny and Tizard, 1977). More recently, LES antigen has been used in highly sensitive immunological method such as enzyme immunoassay (Glickman *et al.*, 1978; de Savigny *et al.*, 1980) or radioimmunoassay (Smith *et al.*, 1980) for serodiagnosis of toxocariasis.

However, the nature and potency of LES as immunogen has not been extensively investigated in comparison with other antigenic preparations such as somatic extracts of worms which have been widely used for the serodiagnosis of visceral larva migrans.

The present investigation was undertaken to clarify the relative potency of LES as antigens for the detection of antibodies elicited in *T. canis* infected rats.

Materials and Methods

Rats. Male outbred Wistar rats were used for infection and as recipients of passive cutaneous anaphylaxis (PCA) tests. The body weight of the rats ranged from 240 to 260 g for the infection and 180 to 200 g for PCA tests.

Antigen preparations. Adult excretions/secretions (AES): Fifty fresh adult male and female worms of *T. canis* collected from puppies were washed with a large

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amount of sterilized saline thoroughly and then incubated in 100 ml of phosphate buffered saline (PBS) containing penicillin (100 U/ml) and streptomycin (250 μg/ml) at 30 C for 18 h. Every 3 h, the incubated medium was changed with fresh one and spent all cultured mediums were pooled. Then, the medium was concentrated by pressured dialysis using cellulose tube. Larval excretions/secretions (LES): Eggs collected from the uteri of adult worms were incubated in 0.5% formalin for 30 to 40 days at 30 C. The fully embryonated eggs were de-shelled by the incubation in 50% sodium hypochloride solution for 10 min, then repeatedly washed with sterilized water (Kondo *et al.* 1981). To obtain the second stage larvae, the de-shelled eggs were mechanically ruptured using a loosely fitted Teflon homogenizer. The larvae were cultured by the method of de Savigny (1975) and 5×10^4 larvae/ml of Eagle's minimum essential medium were incubated at 37 C. The larvae were transferred to fresh medium every week and spent cultured mediums were pooled and stored at −20 C until further treatment. The pooled and stored mediums were concentrated by pressured dialysis using a cellulose tube. Adult extracts (AEX) and larval extracts (LEX): Live adult worms or larvae were cut into small pieces and homogenized with 10 volumes of PBS with a Teflon homogenizer. The homogenate was sonicated by a sonicater (UP-200 P, Tomy Seiko Co. Tokyo) operating at 20 KHz for 5 min, and centrifugated for 30 min at 10,000 g. The supernatant was dialysed against PBS. The determination of protein and carbohydrate: The amounts of protein and carbohydrate in each antigen preparation were determined according to the methods of Hartree (1972) and of Roe (1955), respectively.

Collection of serum. Three groups of 5 rats each were orally infected with 50, 500 and 5,000 of fully embryonated eggs according to the procedure of Oshima (1961) and Kondo *et al.* (1981). At selected times after infection, a blood sample was collected from the tail vein. The blood was allowed to clot at room temperature and separated serum was stored at −20 C until used for antibody titrations. A serum obtained from rat 6 weeks after infection with 5,000 eggs was used as standard antiserum.

Skin test. Direct skin test was carried out according to the method of Collins and Ivey (1975). Briefly, 6 weeks after infection with 25 eggs, each rat was intradermally injected with 0.1 ml of antigen adjusted to 5 μg of protein/ml. Immediately after antigen injection, 1.0 ml of 0.5% Evans blue in saline was intravenously injected. Thirty minutes later, the rat was killed and diameter of skin lesion stained with the dye was measured. Statistical evaluation was made by paired-sample *t* test.

Precipitation test in gel. Double diffusion by Ouchterlony using 1.4% agarose gel equilibrated with veronal buffer (PH 8.6, μ=0.1) was used. Immunoelectrophoresis (IFP) using the same agarose gel and veronal buffer (PH 8.6, μ=0.07) as electrode buffer run at constant voltage of 150 V for 60 min. For the quantitation of precipitating antibodies, reversed single radial immunodiffusion (RSRID) was performed according to Milford-Ward (1977). Briefly, 1.2% agarose impregnated with the LES (final concentration of protein was 4.5 μg/ml) was poured into a mold of U-frame plastic spacer sandwiched between two glass plates. Wells of 2 mm diameter were punched in the gel layer with a borer. Each well was filled with 3 μl of test serum. After precipitin was formed, the gel was washed with saline and dried, then stained with Coomassie brilliant blue. The diameter of the precipitin ring was measured and the area of the ring was calculated. The quantity of antibody was expressed as a ratio of the area formed on the same plate

with the standard antiserum.

Complement fixation test (CF test). CF test was carried out by 50% haemolysis method (Kabat and Mayer, 1961) using a box titration.

Passive cutaneous anaphylaxis (PCA). Homologous PCA for reagin-like antibody was performed according to the method of Tada and Okumura (1971). The titer of PCA was expressed as the reciprocal of the highest dilution which showed a blue reaction more than 5 mm in diameter.

Acrylamide gel electrophoresis. Slab electrophoresis in 7.5% polyacrylamide gel at PH 8.6 was carried out using a ST-1060. SD aparatus (Atto, Tokyo). Samples containing 80 μg of protein were layered on the top of the gel. The gel was stained for protein with Coomassie brilliant blue. The periodic acid-Schiff (PAS) stain was employed for the location of glycoproteins or the other components with high carbohydrate content according to Zacharious *et al.* (1969).

Results

Comparison of antigenic potency

Skin test: Mean lesion sizes provoked by 0.5 μg protein of four kinds of antigen were compared with each other (Table 1). LES yielded the largest lesion. The preparations of AEX and AES yielded larger lesions than the saline control, but the sizes were significantly smaller than those yielded with the larval preparations. The order of the lesion size was LES, LEX, AEX and AES. It is reasonable to assume that the allergen which provoked skin lesions in infected rats is possibly present in all preparations of *T. canis*, but LES is obviously the most potential allergen.

Table 1 Skin test with four kinds of antigen preparations from *T. canis*

Antigens	Lesion size *(mm²)	P value †
LEX	155.5±33.1	
		P<0.01
LES	185.7±48.7	
		P<0.01
AEX	119.6±27.8	
		P<0.001
AES	51.3±10.0	
		P<0.01
Saline	31.7±13.0	

* Mean number of sizes (±SD) of 10 rats.
† Paired-sample *t*-test is used.

Table 2 Minimum amounts of protein and carbodydrate to elicit CF test with four kinds of antigens from *T. canis* and maximum antibody titers

Antigens	Protein (μg/ml)	Carbohydrate (μg/ml)	Antibody titers
LEX	13.0	3.1	2^8
LES	20.0	65.0	2^7
AEX	145.3	74.5	2^3
AES	550.0	387.4	2^2

CF test: The maximum antibody titers referred to the highest serum dilution and minimum amount of protein or carbohydrate to elicit complement fixation were shown in Table 2. The titers with two larval preparations, LES and LEX, were significantly higher than those with adult preparations, AEX and AES. Minimum

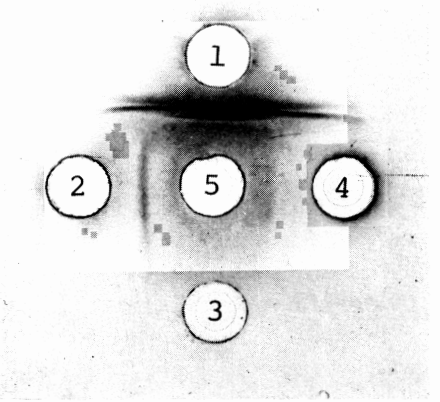

Fig. 1 Ouchterlony gel diffusion: the reaction of *T. canis* antigens, AEX, AES, LEX and LES against standard antiserum which was obtained from rat six weeks after infection with 5,000 eggs. 1=LES, 2=LEX, 3=AES, 4=AEX, 5=antiserum.

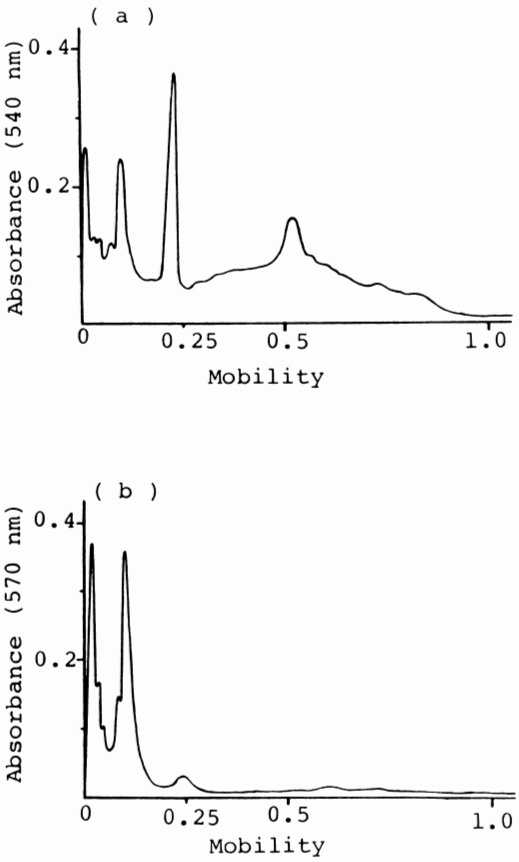

Fig. 2 Analytical slab gel electrophoresis of LES run on 7.5% acrylamide gels. a: Pattern of absorbance of Coomassie brilliant blue stained gel applied 50 μl LES (80 μg protein). b: Pattern of absorbance of PAS stained gel applied 50 μl LES (260 μg carbohydrate).

amount of protein or carbohydrate contents of LEX antigen to fix 50% complement in reaction with an optimum diluted antiserum, was the lowest, although the difference of protein content between LES and LEX was small. Carbohydrate contents of LES was 5 to 15-fold higher than that of the other antigen preparations.

Ouchterlony gel diffusion: As shown in Fig. 1, LES formed four precipitin lines with standard antiserum, one of them was intense and the others were less. LEX and AEX formed a precipitin line which fused

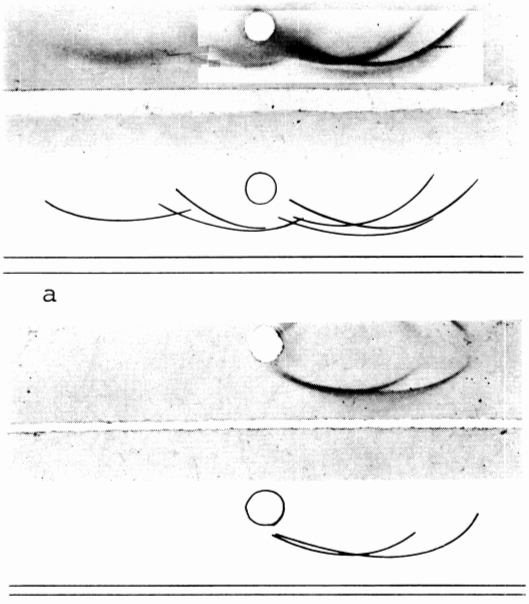

Fig. 3 Immunoelectrophoresis of LES. a: At least seven precipitin arcs were formed with the serum obtained from rat six weeks after *T. canis* 5,000 eggs infection. b: Two of them were also stained by PAS.

with one of less intense precipitin lines with LES, but AES did not form any precipitin line with standard antiserum. The results implied that LES, LEX and AEX preparations shared at least one common antigenic component but the most potent antibody was provoked by LES specific antigen.

Electrophoretic analysis of LES

The above results showed that LES preparation possesed highly potent antigenicity but there were some heterogenous components as shown by Ouchterlony double diffusion. The further analysis of antigenic components of LES was done by acrylamide gel electrophoresis and immunoelectrophoresis. As shown in Fig. 2a, seven bands were densitometrically detected by protein staining after electrophoresis of LES in acrylamide gel. Six out of seven bands were also stained with PAS

(Fig. 2b). Immunoelectrophoresis of LES showed six precipitin arcs with standard antiserum (Fig. 3a) and at least two of them were also stained with PAS (Fig. 3b). This indicates that LES contains at least six antigenic components and some of them are probably glycoprotein.

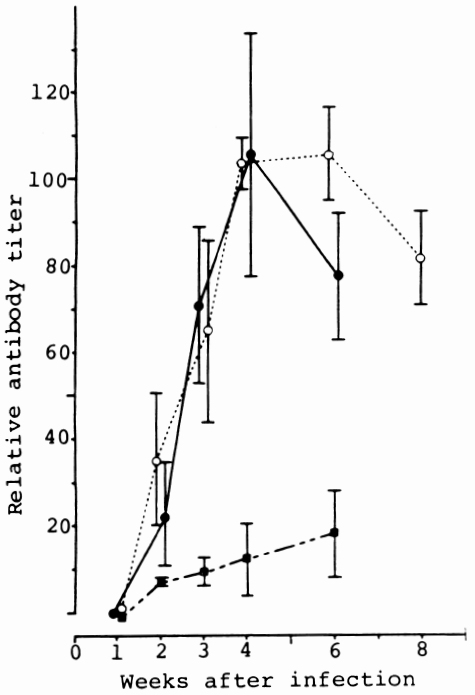

Fig. 4 Relative antibody titers to LES in rats sera assesed by RSRID following infection with 50 (■), 500 (●) and 5,000 (○) *T. canis* eggs. Antibody titer was expressed by relative titer to standard antiserum which was obtained from 5,000 eggs infected rat six weeks after infection. Each point and bar indicate mean of five rats and SD.

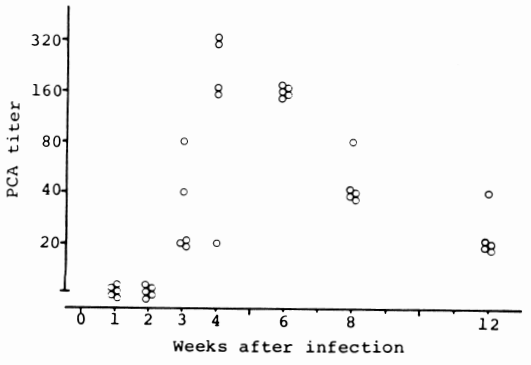

Fig. 5 Transition of PCA titers in rats followed with LES after infection with 5,000 *T. canis* eggs.

Kinetics of antibody production directed to LES

The kinetics of antibody titers to LES after infection was measured by reversed single radial immunodiffusion. The antibody titers after infection with 50, 500 and 5,000 eggs were shown in Fig. 4. The antibodies were detected from two weeks after infection and the titers gradually increased until four weeks. As shown in Fig. 5, reagin-like antibody measured by PCA was detected with LES from three weeks after infection with 5,000 eggs. The kinetics of the production of the antibody coincided with that of precipitating antibody.

Discussion

It has been reported that the excretions/secretions of second stage larvae of *T. canis* are potent immunogens. Antibodies were detected by *in vitro* incubation of living larvae with infected rabbit, human and pig sera (Olson, 1960; Hogarth-Scott, 1966; Stevenson and Jacobs, 1977). De Savigny (1975) first reported an *in vitro* culture method to obtain LES antigen of *T. canis* and de Savigny and Tizard (1977) showed the antibody by haemagglutinating and fluorescent antibody methods in experimentally infected rabbits and human patients. Sugane *et al.* (1981) reported that LES had PAS stainable antigenic substance by DISC electrophoresis. Our present experiment confirmed that LES of *T. canis* is antigenic in infected rats, and the antibody was shown by several different immunological methods. As pointed out by the previous authors, our results also suggested that LES was generally the most potent immunogen in non-specific host. This high potency of LES to the host may

be possibly connected with continual exposure since infected larvae live in tissues for a long time without further maturation (Beaver, 1969).

As to polysaccharides which have been thought to be the principal allergen source in helminth infection (Pepys, 1979), the present study showed that LES contained about 5 to 15-fold carbohydrate as compared with the other antigen preparations such as LEX, AEX and AES. Therefore, a high immunogenic potency of LES may be attributed to the amount of carbohydrate. This fact is also supported by the strong precipitin arcs stainable with PAS by immunoelectrophoresis.

LES prepared by *in vitro* cultivation of larvae of *T. canis* might be more sensitive in the immunodiagnosis of visceral larva migrans, since antibody could be detected in animals infected with 25 or 50 eggs. This fact coincided with the results of a study with 5 eggs/kg body weight by de Savigny and Tizard (1977). LES of *T. canis* was thought to a genus specific but not species specific antigen (Hogarth-Scott, 1966; de Savigny and Tizard, 1977; Stevenson and Jacobs, 1977) and weak cross reaction with *Ascaris suum* infected serum was demonstrated by a paper radioimmunosorbent test (Smith *et al.,* 1980). AEX and LEX of *T. canis* react with antibodies to other genera of helminths (Bisseru and Woodruff, 1968; Aljevori and Ivey, 1970) and natural antibody in healthy human sera (Hogarth-Scott, 1968). Although the specificity of antigen preparations used in this experiment has not been determined, it was found that LES contains several antigenic components and some of them were shown to be common in all antigenic preparations except for AES. It is possibly to be assumed that some components may be species specific. However, cross reaction among the genus *Toxocara* antigens seems to provide some merits for serological screening of toxocariasis in human population (Hogarth-Scott and Feery, 1976) because *T. cati* is also another causative agent of visceral larva migrans in man (Woodruff, 1970).

Another interesting feature of LES is to know whether or not it has activity as "functional antigen" capable of inducing protective immunity to the host (Soulsby, 1963). Protective immunity provoked by LES has been reported in other helminth infections, such as *A. suum* (Stromberg and Soulsby, 1977), *Taenia saginata* (Ricard and Adolph, 1976) and *Fasciola hepatica* (Rajasekariah *et al.,* 1979). We have preliminary results that a protective immunity was provoked in mice by injection of *T. canis* LES mixed with Freund's complete adjuvant (Unpublished data).

Summary

Four kinds of antigen preparations from *T. canis,* AEX, AES, LEX and LES, were compared for their antigenic potency by means of skin test, CF test and precipitation test in gel. The strongest antigenicity was found in LES by both skin test and precipitation test, and in CF test, LEX and LES showed stronger antigenicity than AES and AEX. It was also confirmed that LES induced reagin-like antibody. Analyses by immunoelectrophoresis and acrylamide gel electrophoresis revealed that LES was composed of at least six antigenic components and some of them were stainable by PAS.

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犬蛔虫感染ラットの免疫原について

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犬蛔虫感染ラットに産生される血清抗体の免疫原を知る目的で，幼虫および成虫の虫体抽出物 (EX)，代謝産物 (ES) を抗原とし，沈降反応 (Ouchterlony, IEP, RSRID)，皮内反応および補体結合反応 (box titration) により検討した．感染6週目 (虫卵5,000個経口投与) の血清の Ouchterlony 法による沈降反応では，成虫 ES を除いた各抗原に共通な1本の沈降線が認められた．さらに，幼虫 ES では特異的と思われる3本の沈降線が認められた．幼虫 ES は皮内反応において他抗原よりも有意に強く反応し，CF 反応では幼虫 EX との間にはほとんど差がみられないものの，成虫抗原と比べ有意に強い反応性を示した．このことから，幼虫 ES が非固有宿主において強い免疫原となっていると考えられる．幼虫 ES はアクリルアミドゲル電気泳動法では7本の蛋白バンドが認められたが，うち6本は PAS 陽性を示した．また免疫電気泳動法では感染6週目の血清との間に6本の沈降線が認められたが，このうち2本は PAS 陽性を示した．これらのことから，幼虫 ES は複数の糖蛋白質から構成されているものと思われる．