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Studies on Visceral Larva Migrans: Detection of Anti-*Toxocara canis* IgG Antibodies by ELISA in Human and Rat Sera

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Abstract

An ELISA for the diagnosis of toxocaral migrans, using three kinds of *Toxocara canis* antigens (Adult extract: AX, Larval extract: LX, Embryonated egg extract: EX), was used to study sera from; a) patients with suspected clinical toxocariasis (n = 49); b) patients with other helminthic infections (n = 61); c) normal individuals (n = 21); and d) rats (n = 13) experimentally infected with eggs or larvae of *Toxocara canis.*

The mean ELISA titer of the patients with clinical toxocariasis was higher than that of patients with other helminthic infections, but there was some cross reactivity with them. On the reverse examinations with the various helminthic antigens and sera from patients with suspected toxocariasis, it was also found some cross reactivity.

Of the 49 suspected cases of toxocariasis, antibody to AX was detected in 25 cases (51%), to LX in 23 cases (47%) and to EX in 21 cases (43%). In general, the highest titers were obtained with the larval extract antigen.

In the *Toxocara*-infected rats, antibodies to *T.canis* were detected 1 week postinfection. Peak antibody titers occurred at 9 to 18 weeks and persisted for 1 year postinfection, after which titers began to decrease.

This study indicates that ELISA is potentially useful in the immuno-diagnosis of toxocariasis canis, but shows some cross-reactivity with other nematodes. As conclusion, the serological tests for toxocariasis should be conducted simultaneously with other techniques.

Key words: Toxocariasis, *Toxocara canis,* Larva migrans, ELISA

Introduction

Visceral larva migrans (VLM) is a clinical syndrome caused by the migration of nematode larvae through the tissues of abnormal hosts (Beaver *et al.*, 1952). The parasite responsible for most cases of VLM is the common roundworm of the dog, *Toxocara canis*, the larvae of which can invade almost any tissue in the human body. The major signs are hepatomegaly, persistent eosinophilia and hyperglobulinemia.

Typically, VLM occurs in children up to 6 years of age, which a history of pica, and exposure to a puppy. Recently, VLM has been reported in adults (Morris and Katerndahl, 1987; Glickman *et al.*, 1987), and in individuals following the ingestion of larvae in paratenic hosts (Ito *et al.*, 1986; Inoue, 1987). It seems likely, therefore, that *Toxocara* infections are more prevalent than commonly suspected.

As *Toxocara* larvae do not develop to adult stage in hosts other than the dog, a microscopic examination of the stool is useless for the diagnosis of VLM in humans. A definitive diagnosis is based upon the identification of larvae in tissue biopsies, but it generally is difficult to recover larvae. This has stimulated efforts to develop a valid and reliable sero-diagnostic test (Pattersons *et al.*, 1975; Cypess *et al.*, 1977; Glickman *et al.*, 1978; De Savigny *et al.*, 1979; Smith *et al.*, 1980; Brunello *et al.*, 1986). Recently, Inoue (1987) developed an ELISA for the detection of antibody to *T. canis* in rats using adult, larval and embryonated egg extract as antigens. The present study was designed to test the efficacy of the assay with

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human sera.

Materials and Methods

The study population consisted of 49 patients with suspected toxocariasis whose sera were sent to our Department for a serologic work up. Sera that reacted positively with *T.canis* adult antigen in Ouchterlony and immuno electrophoresis tests were selected for this ELISA study. Sixty one patients with other helminth parasites were diagnosed by positive Ouchterlony tests with antigens of *Toxocara cati* (Tct), *Anisakis* larvae (An), *Dirofilaria immitis* (Di), *Trichuris vulpis* (Tv), *Ascaris suum* (As), *Angiostrongylus cantonensis* (Ac), *Gnathostoma doloresi* (Gd), *Paragonimus westermani* (Pw), *P. miyasakii* (Pm), *Fasciola hepatica* (Fh), *Schistosoma japonicum* (Sj), *Taenia saginata* (Ts) and *Diphyllobothrium latum* (Dl). These 61 sera reacted with one antigen, and are considered single parasite infections. Known negative control (N.con) sera were obtained from 21 non-infected persons. The known positive control (P.con) sera were selected and pooled from 4 patients with clinical manifestations of toxocariasis, which was confirmed in our laboratory. In addition, two groups of 7 and 6 rats were experimentally infected with 2,200 larvae or embryonated eggs of *T.canis* according to the procedure of Inoue (1987).

Antigen preparation: *T.canis* (T.can) adult worms were collected from the intestinal tract of pups at autopsy, and placed in dishes containing Eagle's Minimum Essential Medium (MEM). Eggs deposited by adult worms collected and incubated in saline at 30°C until development to second-stage larvae within the egg. Larvae were obtained from these fully embryonated eggs according to Kondo *et al.* (1981).

T.canis antigens of embryonated eggs or larvae were prepared as described in detail elsewhere (Inoue, 1987). Other helminthic antigens were extracted by the method of Tsuji (1974). The protein content of the antigenic reagents was estimated by method of Lowry *et al.* (1951).

ELISA: ELISA was adapted to toxocariasis by using three *T.canis* antigens; adult (AX), larval (LX) and embryonated egg extract (EX). The ELISA was performed on microtiter plates (A/S Nunk, Denmark) using horseradish peroxidase-conjugated anti-human IgG (H + L) goat antibody (ICN Immuno-Biologicals, USA) and o-phenylenediamine (OPD). The concentrations of conjugate and substrate were: 1:800 diluted conjugate, 0.01% OPD with 0.003% H_2O_2 for the 1:100 serum dilutions and 1:400 conjugate, 0.05% OPD with 0.015% H_2O_2 for the 1:1,000 serum dilutions.

The inhibition test and ELISA for the human and the experimentally infected rat sera were carried out according to Inoue (1987). After the antigen-antibody reaction, the plates were washed 3 times with washing buffer (buffer A: 1/30 M phosphate buffer pH 7.2 — 0.15 M NaCl — 1% BSA — 0.05% Tween 20, without BSA). The results were determined photometrically at 490 nm by a microwellplate reader (Inter Med Immunoreader NJ-2000) and expressed as optical density (OD_{490}), or as mean OD_{490} of test sample/mean OD_{490} of normal control group (X/N). Wells incubated with only buffer A instead of serum sample were used as background controls.

Statistical analysis: Data were analyzed by F test for the variances, and statistical significance was evaluated by student's t test or by the Welch method.

Results

Determinations of optimal ELISA conditions

Checkerboard titrations, to obtain optimal antigen concentrations and serum dilutions, were performed using the pooled, known positive sera (P.con) and the three *T.canis* extract antigens. The highest ELISA readings were obtained with 10-80 μg/ml of AX, 20-40 μg/ml of LX, and 2.5-5 μg/ml of EX, with serum dilutions ranging from 1-8 $\times$ 10^{-2}, and an antigen dilution of 20 μg/ml as our standard.

From the results of the inhibition tests, antigen volume was calculated as 0.9 μg/ml in AX, 0.6 μg/ml in LX and 0.08 μg/ml in EX to achieve

50% inhibition.

Cross reactivity

The cross reactivity of *T. canis* by ELISA was examined by testing sera from 49 cases of suspected toxocariasis and 61 cases serologically diagnosed as a single infection with helminth. The sera of 21 normal, uninfected individuals (N.con) served as control.

An ELISA using 1:1,000 dilution of test serum detected more efficiently the antibody to *T.canis* than a 1:100 dilution because of less cross reactivity from results of our pre-examinations. Therefore, 1:1,000 dilution of each serum sample was used in this study. The titers of Gd and Fh sera were significantly high and those of An, Di, Tv, Ac, Pm and Sj sera were low when the AX antigen was used (Fig. 1). The Di, Ac, Pw, Pm and Sj were reacted with the LX and EX antigens showed significantly low titers (Figs. 2, 3).

Sera of 49 cases of clinical toxocariasis were tested with various helminthic antigens. The three *T.canis* antigens and antigens of Tct, An, As, Gd, Pw and Fh, which cross-reacted with *Toxocara* anti-sera in previous examinations (Figs. 1, 2, 3), were used to determine the extent of common antigenicity in the sera of toxocariasis patients. The mean ELISA values with the As antigen was significantly higher than that with *T.canis* antigen and other nematode antigens differing from Pw and Fh also showed high titers. There was reactivity against *T.canis* sera in some nematode antigens (Fig. 4).

Detection of IgG antibodies in sera of rats and patients infected with *T.canis*

The IgG antibody titers were followed in time course of infection in the sera of rats experimentally infected with *T.canis* embryonated eggs or larvae (ca. 2,200). The mean ELISA values were highest after 4 months (OD_{490}: 0.34—0.37, X/N: 14—15) using LX antigen (Fig. 5). Then, the titers persisted for a long time remained at 65% of the highest values after 12 months.

The IgG antibody titers of 49 patients with suspected toxocariasis are shown in Table 1. The mean ELISA values of patients were 0.151 (OD_{490}), 3.8 (X/N) to AX, 0.264 (OD_{490}), 15.5

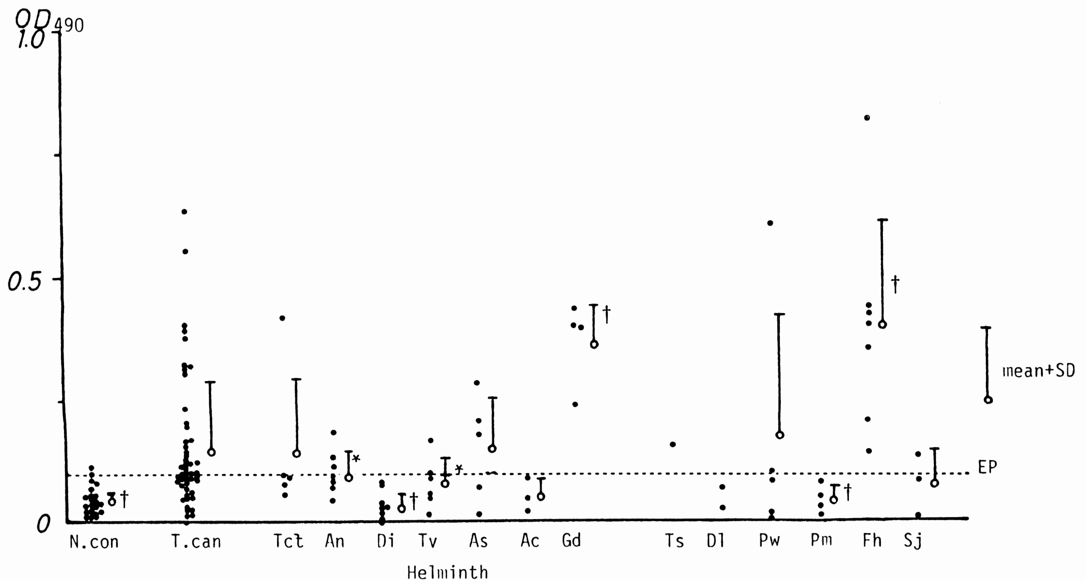

Fig. 1. Comparison of ELISA values to *T.canis* adult antigen in patients with clinical toxocariasis or other helminthic infections.
Serum dilution: 1:1,000, EP: End point of reaction (mean + 2SD of N.con sera), Significantly different from clinical toxocariasis (*P<0.05, †P<0.01)

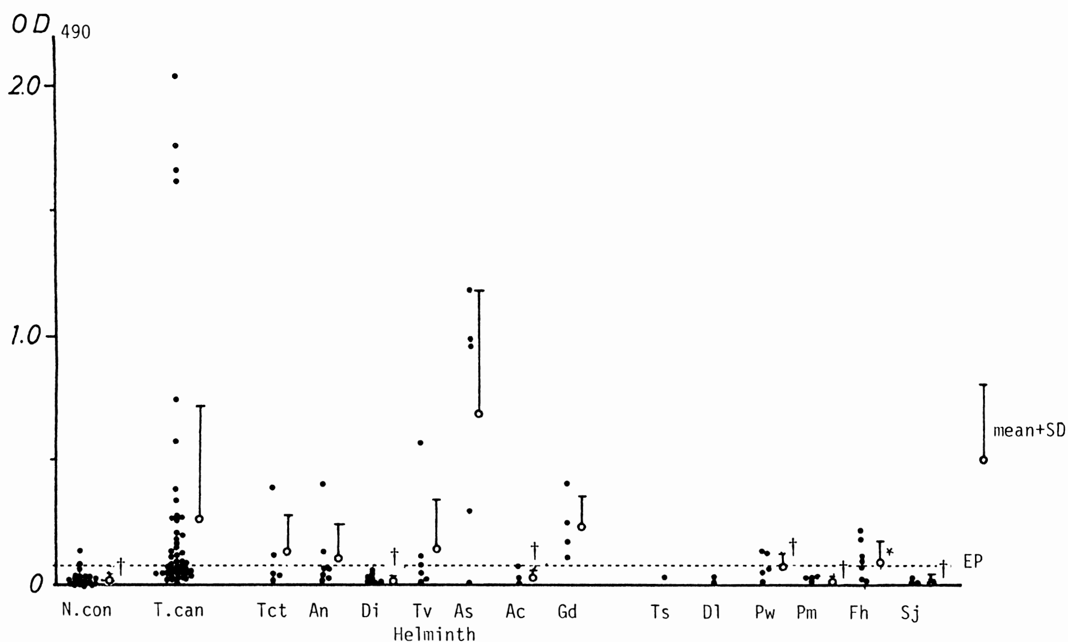

Fig. 2. Comparison of ELISA values to *T.canis* larval antigen in patients with clinical toxocariasis or other helminthic infections.
Serum dilution: 1:1,000, EP: End point of reaction (mean + 2SD of N.con sera), Significantly different from clinical toxocariasis (*P < 0.05, †P < 0.01)

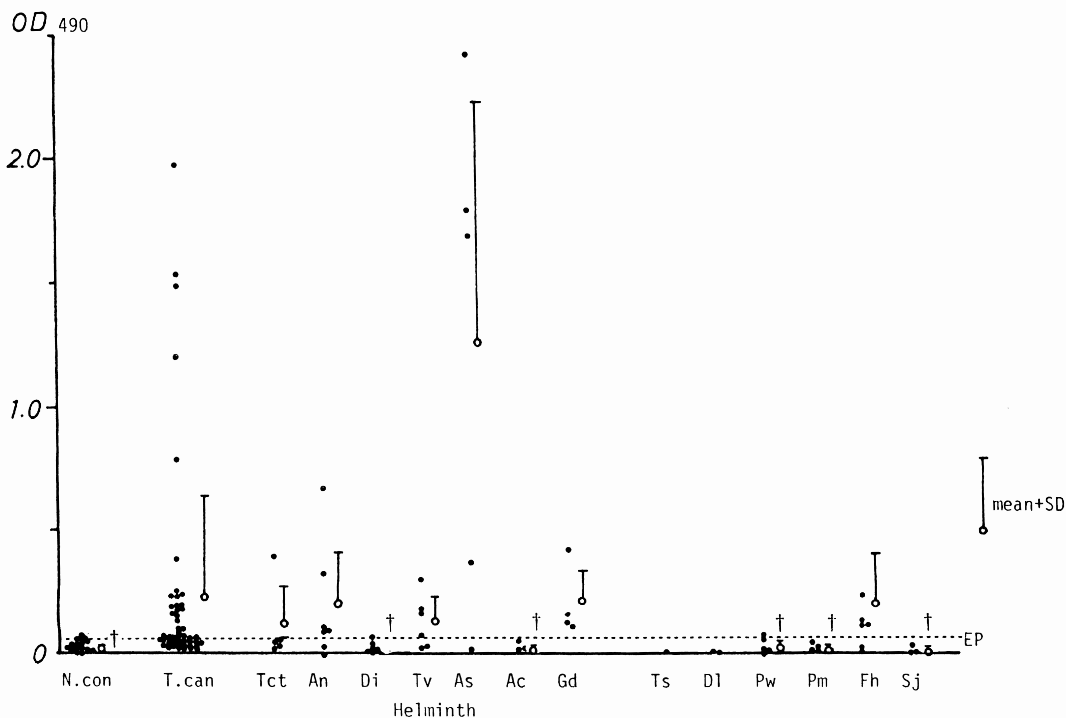

Fig. 3. Comparison of ELISA values to *T.canis* embryonated egg antigen in patients with clinical toxocariasis or other helminthic infections.
Serum dilution: 1:1,000, EP: End point of reaction (mean + 2SD of N.con sera), Significantly different from clinical toxocariasis (*P < 0.05, †P < 0.01)

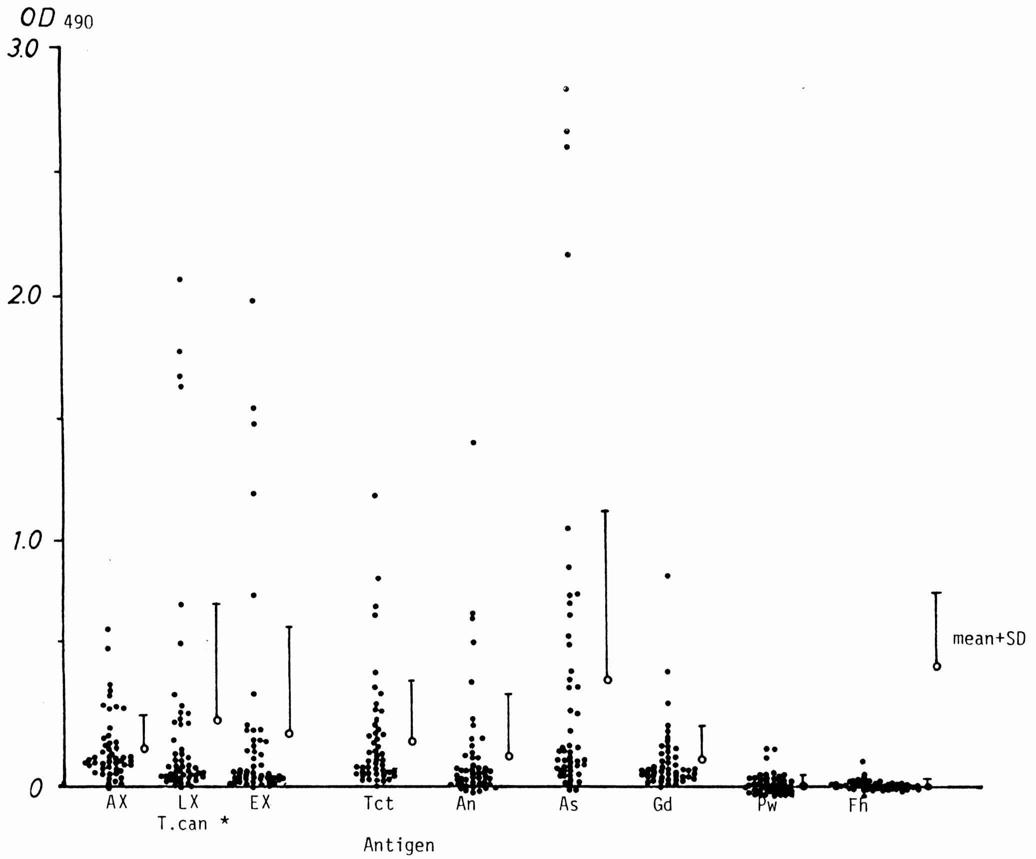

Fig. 4. Distribution of ELISA values with various helminthic antigens in 49 sera of toxocariasis.
* AX: Adult extract, LX: Larval extract, EX: Embryonated egg extract.

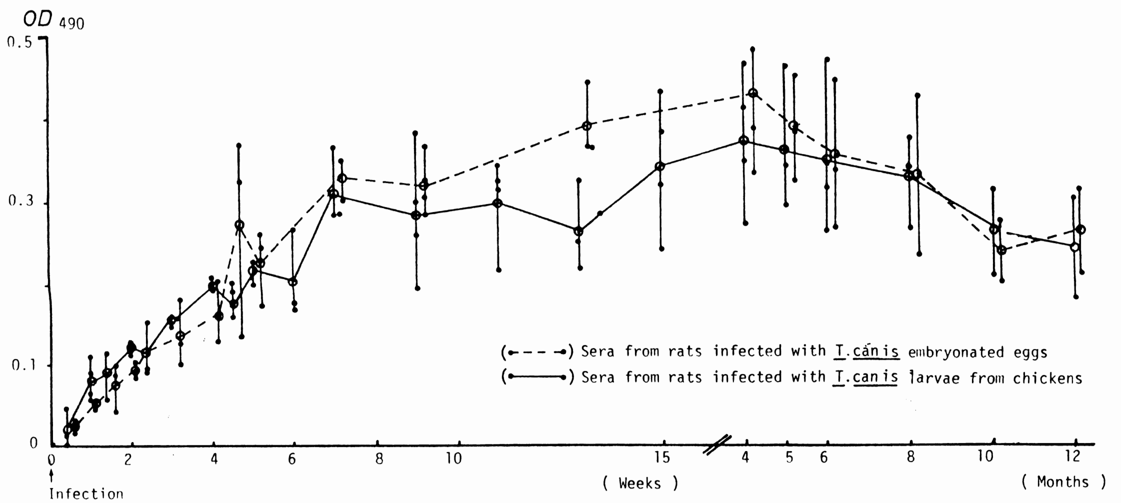

Fig. 5. Changes in ELISA reactivity of *T.canis*-infected rat sera to larval worm antigen. Mean ELISA values (o), Individual ELISA value (•).

Table 1 ELISA values in 49 cases of suspected toxocariasis

No. of sera	Adult antigen		Larval antigen		Embryonated egg antigen	
	OD*	X/N†	OD*	X/N†	OD*	X/N†
1	317	7.9	272	16.0	184	9.2
2	11	0.3	14	0.8	7	0.4
3	183	4.6	83	4.9	55	2.8
4	87	2.2	34	2.0	27	1.4
5	554	13.9	579	34.1	377	18.9
6	324	8.1	380	22.4	245	12.3
7	92	2.3	181	10.6	150	7.5
8	193	4.8	60	3.5	61	3.1
9	74	1.9	56	3.3	53	2.7
10	88	2.2	59	3.5	57	2.9
11	164	4.1	248	14.6	163	8.2
12	233	5.8	265	15.6	186	9.3
13	116	2.9	44	2.6	26	1.3
14	166	4.2	38	2.2	36	1.8
15	101	2.5	53	3.1	60	3.0
16	22	0.6	108	6.4	120	6.0
17	−5	−0.1	27	1.6	7	0.4
18	32	0.8	43	2.5	16	0.8
19	97	2.4	15	0.9	12	0.6
20	52	1.3	120	7.1	55	2.8
21	84	2.1	265	15.6	187	9.4
22	388	9.7	1,763	103.7	1,532	76.6
23	325	8.1	1,661	97.7	1,201	60.1
24	636	15.9	2,044	120.2	1,976	98.8
25	373	9.3	1,615	95.0	1,477	73.9
26	149	3.7	69	4.1	68	3.4
27	96	2.4	334	19.6	223	11.2
28	127	3.2	136	8.0	87	4.4
29	55	1.4	109	6.4	86	4.3
30	317	7.9	34	2.0	54	2.7
31	46	1.2	20	1.2	25	1.3
32	11	0.3	16	0.9	9	0.5
33	22	0.6	28	1.6	20	1.0
34	64	1.6	77	4.5	30	1.5
35	404	10.1	148	8.7	130	6.5
36	124	3.1	745	43.8	787	39.4
37	83	2.1	301	17.7	232	11.6
38	83	2.1	288	16.9	224	11.2
39	100	2.5	85	5.0	42	2.1
40	87	2.2	81	4.8	45	2.3
41	119	3.0	42	2.5	34	1.7
42	91	2.3	47	2.8	28	1.4
43	42	1.1	44	2.6	11	0.6
44	113	2.8	54	3.2	33	1.7
45	69	1.7	38	2.2	18	0.9
46	200	5.0	70	4.1	43	2.2
47	47	1.2	−5	−0.3	31	1.6
48	133	3.3	127	7.5	150	7.5
49	115	2.9	39	2.3	44	2.2
Mean	151	3.8	264	15.5	219	11.0
SD	139	3.5	479	28.2	427	21.4
EP	98	2.5	83	4.9	64	3.2

Serum dilution: 1:1,000 * O D: $OD_{490} \times 1,000$

† X/N: O D of test sample/mean O D of normal control group

EP: End point of reaction (mean + 2SD of N.con sera)

(X/N) to LX, and 0.219 (OD_{490}), 11.0 (X/N) to EX antigens, and the ELISA values (mean OD_{490} ± SD) of N.con sera were 0.040 ± 0.029, 0.017 ± 0.033, 0.020 ± 0.022, respectively. Of 49 patients sera suspected as toxocariasis, 25 cases to AX, 23 cases to LX and 21 cases to EX antigens were positive, when a cut-off point values of reactions was the mean +2 standard deviations of the N.con sera from the results of pre-examinations.

Discussion

The common symptoms of VLM by *T.canis* are cough, stridor, pale complexion, fever and pica, and the syndrome of eosinophilia, hyperglobulinemia and hepatomegalia is well known. The majority of VLM by *T.canis* is in children up to 6 years of age, although several occurrences in adults have been reported (Morris and Katerndahl, 1987; Glickman *et al.*, 1987). In sero-epidemiological surveys, Matsumura and Endo (1983) reported 3.1% positive in children and 3.7% in adults in Yamaguchi prefecture, Glickman and Schantz (1981) reported 1,900 cases over in 48 countries. Clemett *et al.* (1985) also reported 4 (4.4%) of 90 healthy individuals and 23 (25.6%) out of 90 hydatid control officers were shown to be positive in Newzealand, Brunello *et al.* (1986) reported 4.3% positive out of 605 healthy Italians by ELISA and Kondo (1988) reported 36 cases. Therefore, *Toxocara* infections are more numerous than commonly considered. It is well known that the infection route of *T.canis* to human is by ingestion of embryonated eggs from sandy gardens or in house dust. Recently, it was suggested that another route of infection would be by the ingestion of larvae in liver of paratenic hosts (Ito *et al.*, 1986; Inoue, 1987).

A reliable diagnosis of VLM depends on the recovery of the larvae by biopsy. The recovery of worms, however, is very difficult, therefore, there was a considerable amount of works on the serological diagnosis, such as Ouchterlony, IEP, IHA, IFA, RIA, ELISA has been reported (Patterson *et al.*, 1975; Cypess *et al.*, 1977; De savigny and Tizard, 1977; De savigny *et al.*, 1979; Glickman *et al.*, 1978; Smith *et al.*, 1980; Galant *et al.*, 1980; Kawamura, 1983; Clemett *et al.*, 1985; Brunello *et al.*, 1986).

The present study was designed to determine the efficacy of detection by ELISA. In a previous study (Inoue, 1987), it was recognized that of three kinds of antigens, larval, adult and embryonated egg extract, the larval antigen showed the highest ELISA titers with infected rat sera. The reactivity of ELISA with the above three antigens with human sera is different in individuals in OD observation, but the larval antigen showed the highest at X/N ratio.

Koizumi *et al.* (1983) indicated that ES antigen was important as immunogen in rats, and Kondo *et al.* (1984) reported that antibody titers increased 2 weeks after infection in rabbits in both of IFA and ELISA. In our experiences in rats, antibody titers showed elevated tendency from 9 to 18 weeks after infection with antigen of either embryonated eggs or larvae.

It seems that the ELISA is a useful method for the diagnosis of toxocariasis, but still remains cross reactivity with other nematodes. This phenomenon of cross reactivity is also recognized with the sera of patients infected with ascariasis, anisakiasis, crohn's disease by RIA (Tanaka *et al.*, 1983; Kawamura, 1983), collagen disease by ELISA (Cypess *et al.*, 1977). In reversal examinations with *Ascaris* antigen, high cross reaction was observed by precipitin test and by RIA (Patterson *et al.*, 1975). Therefore, it appears difficult to differentiate the cross reactivity between *T.canis* and *Ascaris suum*. Further studies are expected to clear the reason why cross reactions occurs and vary dependent on experimental conditions. Another interesting phenomenon is why antibody production is different by the migrating regions of larvae, in the liver or ocular. By the reports of De Savigny and Tizard (1977), 23 (85.2%) out of 27 VLM cases showed an eosinophilia greater than 20%, and 30 (25.4%) out of 118 did that less than 20%, but only 4 (16.0%) of 25 were positive in ocular larvae migrans (OLM). Shield (1984) and Brunello *et al.* (1986) also reported that the

antibody titers of cases of VLM were higher than in OLM cases, and the serological reactions were closely associated with the survival of the worm. Individual variations among humans must also be considered. Our results indicate that ELISA is a useful method for screening VLM by monitoring antibody titers, and for the diagnosis, but it should be conducted simultaneously combined with other serological techniques.

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