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Detection of circulating antigens in rats experimentally infected with *Paragonimus westermani* by ELISA

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INTRODUCTION

Paragonimus westermani is one of the common flukes of man in Korea. The diagnosis of paragonimiasis has mainly depended on microscopic examinations to find out characteristic eggs in the stool or sputum. Because of its low sensitivity, however, immunodiagnostic techniques have been applied, such as skin test (Kim and Yang, 1964; Kang *et al.*, 1964; Ahn *et al.*, 1966), gel diffusion (Lee *et al.*, 1970), indirect fluorescence antibody test (Choi *et al.*, 1975) and enzyme-linked immunosorbent assay (ELISA) (Cho *et al.*, 1981; Kim *et al.*, 1982; Soh *et al.*, 1985; Lee and Chang, 1986). All these tests were used to measure specific antibodies against *P. westermani.*

The detection of antigens would be more promising in the diagnosis of active parasitic infection than antibodies. There have been reports on the detection of circulating antigens in the sera of schistosomiasis, amoebiasis and filariasis patients (Reddy *et al.*, 1984).

In paragonimiasis it has been a question whether circulating antigens are detectable in the blood of the host infected with this parasite. The present study thus was designed to detect circulating antigens in rats experimentally infected with *P. westermani*, as a basic step for the understanding of the immunopathology and for the immunodiagnosis of paragonimiasis.

MATERIALS AND METHODS

1. Experimental infection and collection of sera

The metacercariae of *P. westermani* were isolated from the crayfish which had been collected at Bogildo, Wando-gun, Cholla Nam-do, Korea, in 1987. A total of 22 albino rats (200 g, female) was fed each with 25 metacercariae.

They were divided into 2 groups.

Blood samples were taken, 2 times a week until 4 weeks after infection, and once a week thereafter until 12 weeks after infection, alternately from these 2 groups of rats by puncture of retroorbital venous plexuses.

2. Isolation of specific IgG against *P. westermani*

The serum obtained from a cat infected with *P. westermani* was used.

1) Isolation of IgG from the serum:

The isolation was done as described by Johnstone and Thorpe (1982). The procedures were as follows:

Solid ammonium sulfate 2g was dissolved in 10 ml of cat serum and incubated for 30 minutes at 25°C. After centrifugation at 3,000 g for 30 minutes, the precipitate was dissolved in water up to 5 ml. One g of ammonium sulfate was added and repeated as above. Dialysis was done against 0.07M sodium phosphate buffer (pH 6.3) overnight at 4°C. Anion-exchange chromatography with Trisacryl-DEAE (LKB) was done for further purification of IgG. Ion

exchanger was fully equilibrated with the buffer described as above. Dialysed samples were applied to the column and eluted with the same buffer. More purified IgG, eluted with the starting buffer, was taken.

2) Purification of specific IgG against *P. westermani* by affinity chromatography:

The affinity chromatography was done according to the manual of "Affinity Chromatography, principles & methods" (Phamacia Fine Chemicals).

Freeze-dried CNBr-activated Sepharose 4B (Pharmacia) was washed and swollen with 1 mM HCl on a sintered glass filter(G3). Somatic antigens of adult *P. westermani* extracted in PBS were dialysed against coupling buffer-$NaHCO_3$ buffer (0.1M, pH 8.3) containing NaCl(0.5M). It was mixed with a gel suspension and stirred gently for 2 hours at room temperature. To block remaining active groups, the coupled gel was transferred to a buffer with blocking agent, 0.2M glycine(pH 8.0), for 2 hours at room temperature. To remove the uncoupled protein, the gel was washed alternately 4 or 5 times with an acetate buffer (0.1 M, pH 4.0) containing NaCl (0.5M) and a coupling buffer. The gel was packed into a column and washed with a coupling buffer overnight at 4°C. Sample (IgG isolated from *P. westermani* infected cat serum as above) was applied to the column (20 ml/hour). To wash away unbound substances, the gel was washed with a coupling buffer overnight. Bound substances to the gel were eluted with 0.1M glycine buffer, pH 2.5 and adjusted to pH 8.0 with 1M Tris solution immediately. Purified anti-*P. westermani* cat IgG by affinity chromatography was concentrated and frozen for further study.

3. Conjugation of peroxidase with IgG by periodate oxidation method (Voller *et al.*, 1979)

Two mg of horseradish peroxidase (RZ 2.7) (Sigma) was dissolved in 0.5 ml of water and 0.1 ml of freshly made 0.1M $NaIO_4$ was added. The mixture was stirred for 20 minutes at room temperature. Dialysis was done against 1 mM sodium acetate buffer(pH 4.4) overnight at 4°C. Ten μl of 0.2M carbonate buffer(pH 9.5) and 4 mg of purified anti-*P. westermani* IgG dissolved in 0.5 ml of 0.01M carbonate buffer (pH 9.5) were added respectively. After stirring for 2 hours at room temperature, 0.05 ml of freshly made sodium borohydride solution(4 mg/ml in water) was added and allowed to stand for 2 hours at 4°C. Equal amount of saturated ammonium sulfate solution was added and centrifuged. The precipitate was washed twice with half saturated ammonium sulfate solution and dialyzed against PBS overnight at 4°C. Bovine serum albumin(BSA) was added to 1%. The conjugate was then filtered through a millipore filter(0.2 μm) and an equal amount of glycerol was added.

4. Enzyme-immunoassays

1) Detection of circulating antigens:

So-called "double antibody sandwich ELISA method" was used. Purified anti-*P. westermani* antibodies(5 μg protein/ml) in 0.05M carbonate-bicarbonate buffer(pH 9.6) were coated on wells in polyvinyl chloride plate(Titertek), 100 μl/well, by overnight incubation at 4°C. After wash 3 times in 0.9% saline with 0.05% Tween 20 added, rat test sera, 100 μl/well, optimally diluted (1 : 10) in diluent buffer(PBS with 0.5% BSA, 0.05% Tween 20 added), were incubated for 1 hour at 37°C. Wells were washed 3 times as above and incubated 100 μl/well for 1 hour at 37°C with an optimal dilution of peroxidase conjugated purified anti-*P. westermani* IgG (1 : 4,000). After the wash, wells were developed using 100 μl/well of 0.05% OPD and 0.006% hydrogen peroxide in 0.1M phosphate-citrate buffer(pH 5.0) as the chromogen for 30 minutes at room temperature in the dark. The reaction was stopped by adding 50 μl/well of 2N H_2SO_4. The optical density was read at 490 nm using ELISA Reader(Dynatech).

2) Detection of circulating antibodies(IgG):

Excretory-secretory antigens of *P. westermani* (5 μg protein/ml) were coated on wells in polystyrene plate(Dynatech), 100 μl/well, by overnight incubation at 4°C. After wash 3 times

as above, rat test sera(1 : 200) were incubated, 100 μl/well, for 1 hour at 37°C. Wells were washed 3 times and incubated for 1 hour at 37°C with 1 : 4,000 diluted peroxidase conjugated anti-rat IgG(Cappel), 100 μl/well. After the wash, wells were developed, stopped and read as above.

5. Recovery of *P. westermani* immature worms from sacrificed rats

At 12 weeks after infection, 10 rats were selected at random and sacrificed to find immature worms in the tissues of rats.

The immature worms in the lungs and pleural cavities were collected. They were confirmed with a dissecting microscope. To detect immature worms distributed in muscles, all of the muscles were sliced in about 3 mm thickness. For the liberation of worms, the muscles were soaked in normal saline and incubated for about 5 hours at 37°C with intermittent shaking. The contents were then passed through the sieve of 1.5 mm meshes and the sediments were washed 5 times with normal saline. The precipitate was thoroughly examined with a dissecting microscope for the immature worms.

RESULTS

1. Circulating antigen levels

The results of ELISA were presented by optical density(O.D.) (Fig. 1). The mean O.D. of 22 sera obtained from rats before infection, as a control, was 0.04(S.D.=0.04). After infection, mean(S.D.) O.D. values were changed serially: 0.03(0.01) at 0.5 week(3 days), 0.55 (0.50) at 1 week, 0.69(0.45) at 1.5 week, 0.20 (0.19) at 2 weeks and 0.13(0.10) at 2.5 weeks. Thereafter O.D. values returned to the level before infection. The mean O.D. value of control reactions plus 3 S.D. amounted to 0.16 ($\bar{x}$=0.04, S.D.=0.04). When O.D. value of 0.16 was considered as the cut-off value, those higher than this value were observed only in sera obtained between 1 week and 2.5 weeks after infection.

Instead of test sera, serially diluted *P. westermani* excretory-secretory antigens (ESA) were used to draw a standard curve and the linear

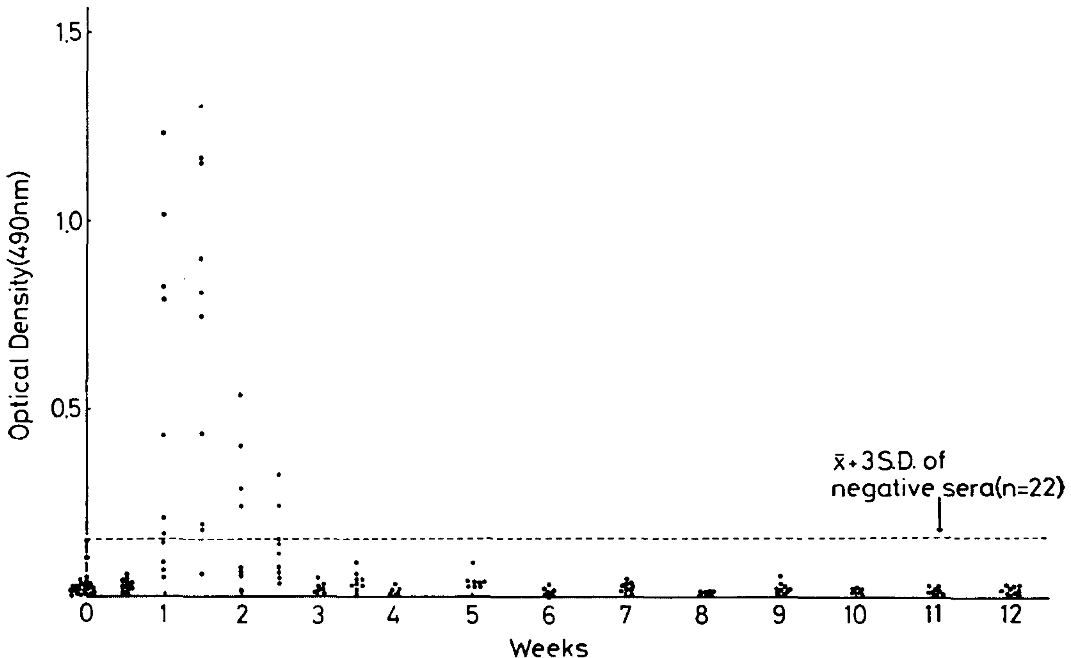

Fig. 1. Changing pattern of circulating antigen levels presented by O.D. in albino rats each infected with 25 metacercariae of *Paragonimus westermani*.

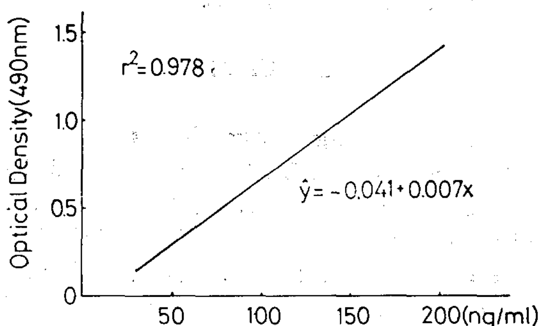

Fig. 2. The linear regression line of standard curve, representing circulating antigen levels, using *P. westermani* excretory-secretory antigens diluted in PBS, instead of rat test sera.

regression line was plotted (Fig. 2). Because test sera were used as 10 times diluted, the actual concentration of circulating antigens in the test sera would be estimated 10 times higher than plotted ESA concentrations.

2. Circulating antibody levels

O.D. values increased gradually until the end of this study (12 weeks after infection) (Fig. 3). Great differences were observed among individual O.D. values. It was difficult to correlate increasing pattern of O.D. values and recovered worm numbers from a rat sacrificed at 12 weeks after infection individually.

Table 1. Recovery of immature worms at 12 weeks after infection from 10 rats each infected with 25 metacercariae of *P. westermani*

Rat No.	No. of worms in		Total(%*)
	Lungs & Pleural cavity	Muscles	
1	3	11	14 (56)
2	11	2	13 (52)
3	3	7	10 (40)
4	3	5	8 (32)
5	1	6	7 (28)
6	1	6	7 (28)
7	0	7	7 (28)
8	0	6	6 (24)
9	0	6	6 (24)
10	0	6	6 (24)
Average	2.2(8.8)	6.2(24.8)	8.4(33.6)

* Recovery rate

3. Recovery of immature worms from tissues of rats(Fig. 4, 5)

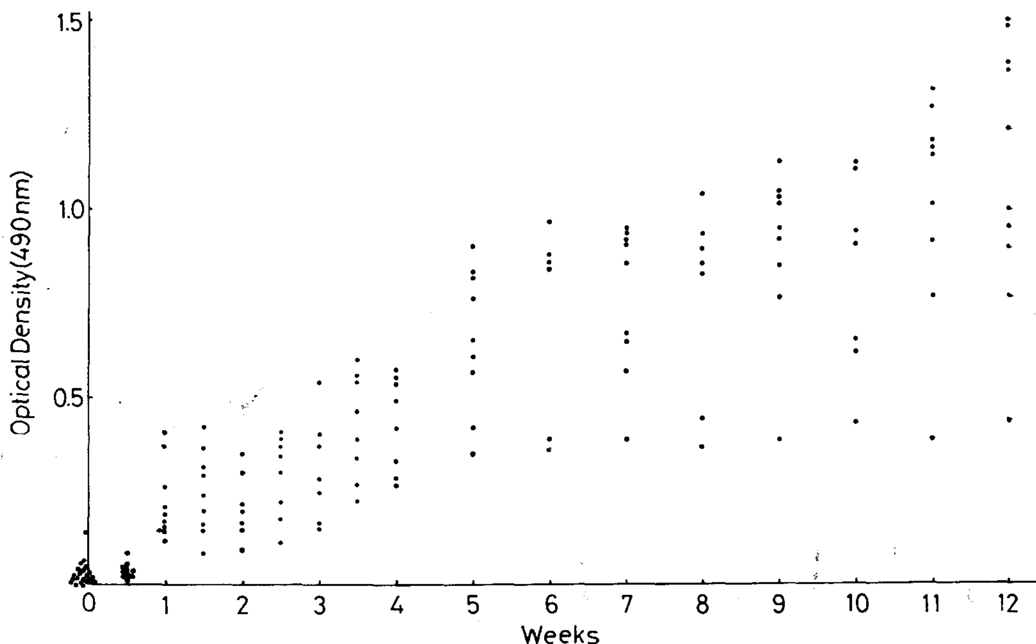

Fig. 3. Changing pattern of circulating specific IgG levels presented by O.D. in albino rats each infected with 25 metacercariae of *P. westermani*.

Fig. 4. An immature worm of *P. westermani* recovered from the lungs of a rat at 12 weeks after infection.

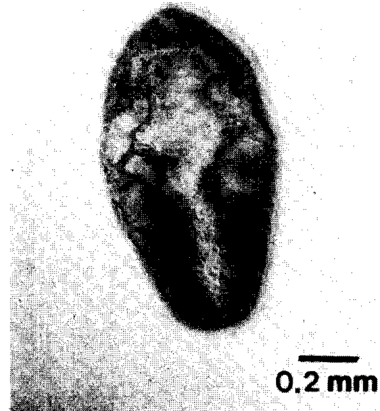

Fig. 5. An immature worm of *P. westermani* recovered from the muscles of a rat at 12 weeks after infection.

The immature worms recovered in the lungs and pleural cavities were somewhat grown to measure 4～6×2～3 mm and pinkish in color. But all worms were immature and had no eggs in the uterus. In the lungs they were found to be paired or single in each cyst. But the worms in the pleural cavities had no cyst.

The immature worms recovered from the muscle were tiny (2～3×1 mm) and light pinkish.

Average number of recovered worms per rat was 8.4 (2.2 from the lungs and pleural cavities, and 6.2 from muscles) in a total of 10 rats each fed with 25 metacercariae (Table 1).

DISCUSSION

It has been known that the level of circulating antibodies detected in the serum has some problems as an ideal parameter for immunodiagnosis because of the delay in their appearance and the persistence in the circulation even after cure. More emphasis has been thus put on the detection of circulating antigens. In fact, the antigens are usually present in the circulation before the appearance of specific antibodies and do not persist long in the circulation after the disappearance of parasites (Ouaissi *et al.*, 1984).

Circulating antigens have been detected in various helminthic infections of man, such as schistosomiasis (Santoro *et al.*, 1979), onchocerciasis (Ouaissi *et al.*, 1984), lymphatic filariasis (Reddy *et al.*, 1984), cystic echinococcosis (Gottstein, 1984; Craig, 1986) and visceral larva migrans (Bowman *et al.*, 1987). Some of very sensitive techniques, such as enzyme-immunoassay or radioimmunoassay have been used to detect either antibodies or antigens in the circulation.

Various techniques have been used for the immunodiagnosis of paragonimiasis, including ELISA. Almost all these techniques, however, have been used to detect specific antibodies, rarely for antigens at all. A trial was conducted in this study to detect circulating antigens in the sera of *P. westermani* infected rats.

The results obtained in the present study clearly showed that circulating antigens of *P. westermani* were detectable in the early stage of paragonimiasis by ELISA. The peak was observed at 7 to 10 days after infection. The serial pattern of O.D. levels, representing the concentration of circulating free antigens in the

sera, showed that they were decreased concurrently with the rise of specific antibody levels. Circulating antigens were thought to be trapped by specific antibodies produced, unavailable for the detection in this assay for free antigens. Because *P. westermani* do not multiply in the tissue of a rat, circulating antigens must be excretory-secretory antigens liberated from tissue invading immature worms.

Since the rat is not a final host of *P. westermani*, this pattern might not be the same as in the final host, such as dogs, cats or humans. It is necessary to study further in an infected final host. More experiments to reveal changing patterns of specific immune complexes in a final host or a paratenic host are also needed. Circulating antigens may be detected further in higher levels for prolonged duration if the antigens in the antigen-antibody complexes are freed artificially. Bowman *et al.* (1987) detected circulating antigens in antigen-antibody complexes in the sera of mice infected with *Toxocara canis.* Although free antigen levels decreased concurrently with rise of antibody levels, the complexed antigens could be detected at relatively high levels throughout the course of infection if the test sera were pretreated with EDTA and heat in their study.

Detected antigens were believed to be highly specific for *P. westermani* because antibodies purified by affinity chromatography were used in ELISA. But in some control sera the levels were shown to be slightly elevated, although they were within the range of negative value (below mean+3 S.D.). These results suggested that there may be some cross reactive components with antigens of *P. wetermani* in control sera of rats or in those with other infections or diseases. Actually there has been a report that high concentrations of *Ascaris suum* protein were observed in the sera of patients with inflammatory bowel diseases(Tanaka *et al.*, 1983). Hybridoma technology with the production of monoclonal antibodies could be used to minimize these problems and for the characterization of circulating antigens liberated in the sera.

It is also a question to be solved in the future whether or not a relatively large amount of secreted antigens detectable in the sera of the host is advantageous for the sake of the parasite as an immune evasion mechanism during tissue invasion.

In addition, many live immature worms(about 1/3 of the metacercariae fed) were recovered at 12 weeks after infection in this study. The immature worms recovered from muscles were known to be infective to a final host (Kim and Lee, 1970; Lee *et al.*, 1976).

SUMMARY

Circulating antigens in rats experimentally infected with *Paragonimus westermani* were examined by ELISA.

From a total of 22 albino rats, each fed with 25 metacercariae, blood samples were collected until 12 weeks after infection. The specific antibodies against *P. westermani* in the serum of an infected cat were purified by ammonium sulfate precipitation, DEAE anion-exchange chromatography and affinity chromatography serially. So-called double antibody sandwich ELISA method was used for the detection of circulating antigens.

The results were as follows: Mean value of O.D. in control sera was 0.04 (S.D.=0.04). After infection, mean O.D.(S.D.) values were changed serially: 0.03(0.01) at 0.5 week(3 days), 0.55(0.50) at 1 week, 0.69(0.45) at 1.5 week, 0.20(0.19) at 2 weeks and 0.13(0.10) at 2.5 weeks of infection. They returned, thereafter, to the level before infection. When 0.16(mean+3 S.D.) were considered as cut-off value, those higher than 0.16 were observed only in the sera collected between 1 and 2.5 weeks after infection.

Average 8.4 immature worms (2.2 from the lungs and pleural cavities; 6.2 from muscles) were recovered in a rat at 12 weeks after infection.

The fact that circulating antigens were not detected after 3 weeks of infection was considered

to be caused by the formation of antigen-antibody complexs.

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=국문초록=

ELISA법을 이용한 폐흡충 감염 백서의 혈청내 항원 검출

국립보건원 병독부 기생충과
용태순 · 김동수 · 이종수 · 이온영 · 김동찬

실험적으로 폐흡충을 감염시킨 백서의 혈청내 항원을 ELISA 법으로 측정하였다.

시험용 혈청은 폐흡충의 피낭유충을 마리당 25개씩 먹인 총 22마리의 백서에서 감염후 12주가 될 때까지 채혈하여 얻었다. 폐흡충에 대한 특이항체는 폐흡충 감염 고양이의 혈청으로부터 ammonium sulfate 침전과 anion-exchange chromatography를 이용, IgG를 분리한 후 affinity chromatography를 이용하여 순수분리하였다. 항원을 찾기 위한 ELISA는 소위 "double antibody sandwich method"를 사용하였다.

대조로서 사용한 감염전 혈청의 O.D.치 평균값은 0.04(표준편차 0.04)이었으며 감염후 O.D.치(표준편차)의 변화를 보면 0.5주(3일)는 0.03(0.01), 1주는 0.55(0.50), 1.5주는 0.69(0.45), 2주는 0.20(0.19), 2.5주는 0.13(0.10)이었고 감염 3주 이후는 대조 혈청의 값으로 떨어졌다. 대조혈청의 평균값+3× 표준편차, 즉 0.16 이하를 이 system에서의 비특이적 수치로 잡았을 때 이 이상의 수치를 나타낸 경우는 감염 후 1주부터 2.5주까지의 혈청에서만 관찰되었다.

한편 감염 12주 후에 총 10마리의 백서를 희생시켜 유충을 회수하여 보았다. 폐와 흉강에서는 평균 2.2마리, 근육에서는 평균 6.2마리의 유충을 회수할 수 있었다.

폐흡충 감염 백서에 있어 충체가 생산하는 항원은 감염 1주 후부터 혈청 내에서 검출되었다. 그러나 감염 3주 후부터는 검출되지 않았는데 이것은 항원-항체 복합체 형성에 의한 반응 저해 현상에 의한 것으로 생각되었다.