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Immunochemical Characterization of Major Antigenic Components of *Paragonimus westermani* (Triploid type) Adult Worms

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Abstract

When a crude extract of *Paragonimus westermani* adult worms was chromatographed through a Sephacryl S-300 gel column, two major antigenic components, a high molecular weight component termed PW-1 (m.w. 440,000) and a low molecular weight component termed PW-2 (m.w. 13,000), were identified by IgG-ELISA and double diffusion using pooled serum from chronically infected cats as a probe. In double diffusion, PW-1 produced one sharp precipitin band, whereas PW-2 produced a broad band which crossed against PW-1 band. In polyacrylamide gel electrophoresis (PAGE), PW-1 gave a single band close to the cathodic end, while PW-2 gave one major and several minor bands with relatively anodic mobility. After crossed immunoelectrophoresis, both PW-1 and PW-2 gave 2 or 3 different precipitin arcs against pooled sera from chronically infected cats. From the results of the double diffusion and ELISA inhibition test, PW-1 seemed to be derived from eggs, whereas PW-2 from excretory-secretory (ES) products. In the chronological study using periodically bled serum from infected cats, anti-PW-1 or anti-egg antibodies became detectable at 10 weeks, whereas anti-PW-2 or anti-ES antibodies became detectable as early as 4 weeks after infection. Both anti-PW-1 and anti-PW-2 antibodies increased progressively with time. Thus, the eggs and ES, both of which are produced and released from adult worms, seem to be the most potent immunogen to the host infected with *P. westermani*.

Key words: *Paragonimus westermani*, antigen, gel filtration, IgG-ELISA, immunodiffusion, cat

Introduction

Soluble extracts of *Paragonimus westermani* adult worms has been used as the antigen source for immunodiagnosis. The crude extracts of adult worms of *Paragonimus* species contain various different antigenic components, when they were examined by double diffusion in agar or by immunoelectrophoresis (Biguet et al., 1965; Yogore et al., 1965; Seed et al., 1966; Tada, 1967; Katamine et al., 1968; Capron et al., 1969). Sawada et al. (1964a) reported that, by using starch zone electrophoresis of the crude extract of *P. westermani* adult worms, a cathodic fraction had higher precipitin titers than a nonmigrating or anodic fraction. They also reported that anodic fraction provoked the most intense skin reaction in the proven cases of paragonimiasis (Sawada et al., 1964b). Up to now, many attempts have been done to sort out appropriate antigens for intradermal test, complement fixation or other serological tests in the diagnosis of paragonimiasis (Yokogawa et al., 1955; Sadun et al., 1959; Takano, 1960; Ishii and Morisawa, 1961; Sawada et al., 1964a, b; Nomoto, 1967). However, except for the classical studies of Sawada et al. (1964a), not much efforts have been made to isolate or characterize the major antigenic components of *Paragonimus* adult worms recognized by precipitating antibodies which were produced during the course of infection. In the present study, therefore, major antigenic components recognized by IgG-ELISA were isolated from *P. westermani* adult worms, and their immunochemical properties and their origin was examined.

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Materials and Methods

Preparation of antigens: Adult worms of *Paragonimus westermani* were obtained from the lungs of experimentally infected cats. Adult worms were defatted with cold acetone and anhydrous ether, homogenized in physiological saline, and then extracted for 48 hr at 4°C. After centrifugation for 60 min at 20,000 g, the supernatant was used as the crude adult worm extract (PW). In addition, the cyst fluid in which the adult worms are bathed and the eggs in the fluid were harvested for the preparation of excretory-secretory (ES) product and crude egg extract, respectively. Egg extract was prepared by the same methods for adult worms. The protein concentration was measured by the method of Lowry et al. (1951) and was adequately adjusted for serological tests.

Gel chromatography: Fractionation of PW was carried out by gel filtration at 5°C through Sephacryl S-300 (Pharmacia, Sweden) in a 1.6 x 70 cm column. The column was equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl. Two ml (34 mg protein) of PW was applied on the S-300 column and eluted at a flow rate of 20 ml/h with the same buffer. Two ml fractions were collected and the elution profile was monitored by an absorbance at 280 nm. Ferritin (440,000), bovine serum albumin (67,000) and cytochrome-c (12,300) were used as molecular weight markers.

Serum sample: Twenty two cats were infected with 35 metacercaria each. Five out of 22 cats were bled every second week. All cats including periodically bled 5 cats, were finally exsanguinated under Ketamin-HCl (10 mg/kg body weight) anesthesia at 20–24 weeks after the infection. A part of serum from each cat was pooled and served as serum from chronically infected cats. Periodically bled serum samples were also pooled and used for the time course study.

Enzyme-linked immunosorbent assay (ELISA) and inhibition test: Antigenic activity of gel-chromatographed fraction was measured by ELISA as described previously (Matsuda et al., 1981; Imai, 1987). Briefly, the wells of micro-ELISA plate (Dynatech, M-129A) were coated by 0.1 ml of each fraction diluted 1:1000. After washing three times with saline containing 0.1% Tween 20, 0.1 ml of pooled cat serum (1:200 dilution) was added to the well. After incubation, the wells were washed and a 1:3000 dilution of goat anti-cat IgG conjugated with peroxidase was added. The reaction was developed for 45 min using ABTS: (2-2'-azino-di(3-ethylbenzthiazoline sulfonic acid)) (Sigma, St. Louis, MO, USA) as the substrate. The final reaction was read by OD 405 nm using an ELISA reader (Multiskan, Flow Labo, Rockville, MD, USA).

Inhibition test was performed according to the method of Abdel-Hafez et al. (1983). Each well of micro-ELISA plates was coated with 0.1 ml of fractionated antigen preparations (PW-1, 2.5 μg/ml; PW-2, 20 μg/ml in protein). One day prior to the assay, various concentrations of blocking antigens (0.5 to 200 μg/ml in protein) were added in 1:1 ratio (v:v) to chronically infected cat serum which was diluted 1:100 with Tris-HCl buffer (pH 7.4) containing 10% horse serum. Subsequent procedures were the same as those for ELISA. Percent inhibition was calculated from the formula: {OD(ICS-Ag)-OD(ICS+Ag)}/OD(ICS-Ag) x 100, where ICS is the infected cat serum, and Ag is the blocking antigen sample. The optical density (OD) of control well containing normal cat serum was subtracted from the ICS sample prior to the above calculation.

Double diffusion (DD): Double diffusion was performed in 1% agarose gel containing 0.01 M phosphate buffered saline (PBS), pH 7.2. After the reaction for 48 hr, the plates were rinsed, dried and stained with 0.5% Coomassie Brilliant Blue R-250. The antigen preparations were applied at the concentration of 2–5 mg protein per ml for the test.

Crossed immunoelectrophoresis: Crossed immunoelectrophoresis was performed according to the methods of Weeke (1973) except that polyacrylamide gel electrophoresis (PAGE) was employed for the first dimension. Briefly, the fractionated antigen solutions (PW-1 and PW-2, 120 μg protein each) was first electro-

phoresed in 7.5% polyacrylamide gel slab (100 x 100 x 1.0 mm) using Tris-glycine buffer, pH 8.3, at 1 mA/cm until a bromophenol blue marker has moved 75 mm. After electrophoresis, antigen-containing gel slab was cut (84 x 20 x 1 mm) and transferred on the cathodic side of the second plate (84 x 94 mm), and then 9 ml of antibody-containing (8% v/v) agarose (1%) was poured on the remaining part of the plate. The second dimension electrophoresis was run for 20 hr, 0.5 mA/cm at 10°C using barbital buffer, pH 8.6, ionic strength 0.02. After the second dimension electrophoresis, the gel plate was kept at 4°C overnight. The plate was then washed, dried and stained with 0.5% Coomassie Brilliant Blue R-250.

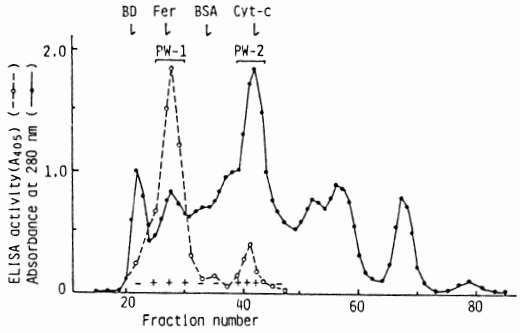

Fig. 1. Gel filtration pattern of adult worm extract of *P. westermani* on a Sephacryl S-300 column. Absorbance at 280 nm (●—●); IgG-ELISA activity (o---o); Double diffusion (DD) test (+++). The two main peaks of both ELISA and DD positive fractions were termed PW-1 and PW-2, respectively. Blue dextran (BD), ferritin (Fer), bovine serum albumin (BSA) and cytochrome c (Cyt-c) were used as the markers.

Results

Crude extract of *P. westermani* adult worms was chromatographed on a Sephacryl S-300 column and each fraction (undiluted sample for DD; 1:1000 diluted sample for ELISA) was assayed by double diffusion (DD) and IgG-ELISA using pooled serum from chronically infected cats. Antigen-containing fractions were recognized as two different peaks coincidentally by both methods, one at the elution position of ferritin (m.w. 440,000) and the other at the elution position of cytochrome c (m.w. 12,300), (Fig. 1). These two peaks were separately pooled, concentrated, and rechromatographed on the same column. After rechromatography, a high molecular weight component, termed PW-1, and a low molecular weight component, termed PW-2, were collected and served for further analysis.

Antigenic properties of PW-1 and PW-2 were examined by DD-test using pooled serum from chronically infected cats. This serum gave two distinct precipitin bands against crude worm extract (PW), a sharp band relatively close to serum-containing well and the other broad band close to antigen-containing well (Fig. 2). PW-1 gave a sharp precipitin band against the same serum, which completely fused with the similar sharp band formed between PW and the serum (Fig. 2). PW-2 gave a broad band which fused with the broad band formed between PW and the serum (Fig. 2). When antigenic specificity of PW-1 and PW-2 was examined by DD-test, the sharp band of PW-1 completely crossed against the broad band of PW-2 (Fig. 2).

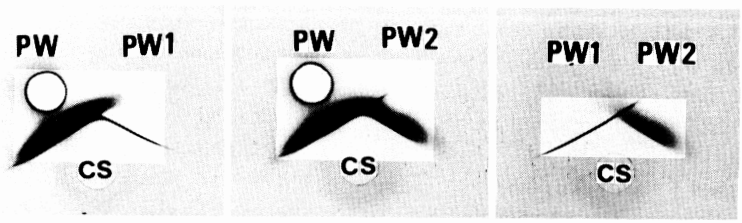

Fig. 2. Double diffusion analysis of the crude worm extract (PW) and the fractionated antigens (PW-1 and PW-2) against pooled serum from chronically infected cats (CS).

After crossed immunoelectrophoresis (Fig. 3) against pooled serum from chronically infected cat, PW-1 gave two closely associated, distinct precipitin arcs in the cathodic end, while PW-2 gave at least two weak arcs with relatively anodic mobility.

Since both ES and eggs are released from adult worms and retained in and around worm cysts, they are likely to be major antigenic substances. Therefore, antigenicity of PW-1 and PW-2 was compared to that of ES or egg extract by DD-test using pooled serum from chronically infected cats. When antigenicity of PW-1 and egg extract was compared, a completely fused sharp single band was observed (Fig. 4). The combination of PW-2 and ES also gave a completely fused broad band against the same cat serum (Fig. 4). To confirm further the antigenic homology between PW-1 and egg extract or between PW-2 and ES, ELISA-inhibition test was performed. The results are summarized in Fig. 5. The anti-PW-1 activity in the serum from chronically infected cats was inhibited by the homologous antigen or egg extract but not by PW-2 or ES. Similarly, the anti-PW-2 activity was, although incompletely within the dose range tested, inhibited in a dose dependent manner by PW-2 or ES but not by PW-1 or egg extract.

Since PW-1 and PW-2 were identified as fundamentally different products or constituents of adult worms of *P. westermani*, kinetic changes of the antibody responses against these two antigen fractions, as well as that against egg extract or ES, were examined by DD-test using experimentally infected cats (Fig. 6). When cat sera obtained at two week

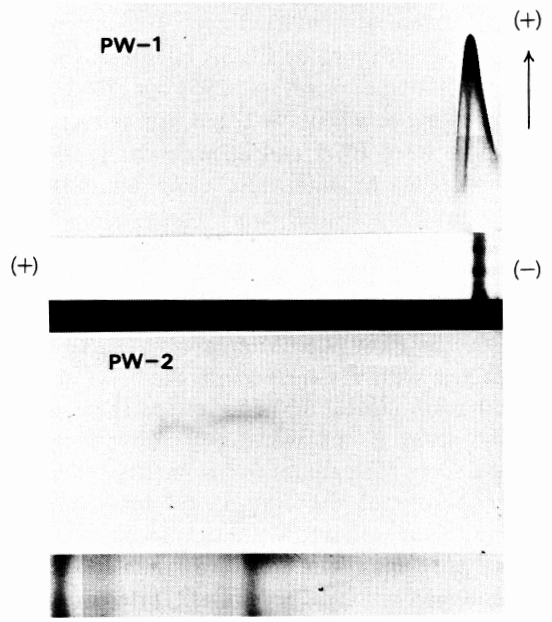

Fig. 3. Crossed immunoelectrophoresis of PW-1 (top) and PW-2 (bottom) against serum from chronically infected cats. Running condition; First dimension (strip): 7.5% polyacrylamide gel, Tris-glycine buffer (pH 8.3), 1 mA/cm for 3 hr. Second dimension (slab): 1% agarose gel containing 8% (v/v) serum from infected cats, barbital buffer (pH 8.6), 0.5 mA/cm for 20 hr at 10°C. The gel plates were stained with 0.5% Coomassie brilliant blue R-250.

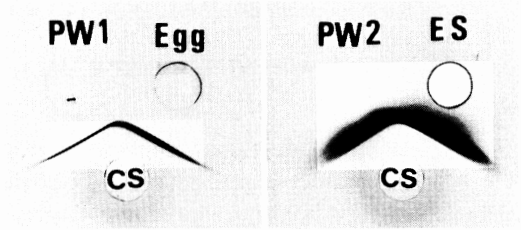

Fig. 4. Double diffusion test showing antigenic homologies between PW-1 and egg extract (Egg), or between PW-2 and excretory secretory product (ES) using serum from chronically infected cats (CS) as a probe.

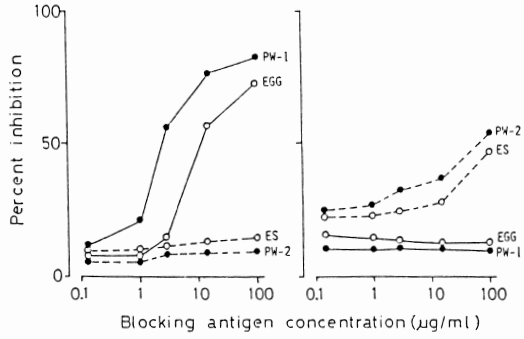

Fig. 5. ELISA inhibition test. Serum from chronically infected cats (1:100 dilution) was pre-incubated with various concentrations (0.25 to 100 μg/ml) of four different blocking antigens (PW-1, PW-2, egg extract, and ES product), and then antibody titers against PW-1 (left) or PW-2 (right) were measured by IgG-ELISA.

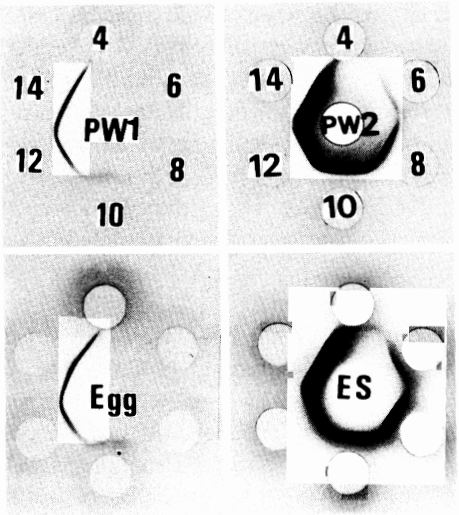

Fig. 6. Double diffusion test showing the kinetic appearance of antibody in periodically bled sera from infected cats. PW-1, PW-2, egg and ES were used as test antigens. Figures in the outer well indicates the time (weeks) after the infection.

interval after the infection were tested against PW-1 or egg extract, a precipitin band became detectable at 10 weeks after the infection and the intensity of the reaction gradually increased with time. On the other hand, precipitin reaction against PW-2 or ES became detectable at 4 weeks after the infection and again the intensity of the reaction gradually increased with time.

Discussion

The results reported here show that the crude extract of *P. westermani* adult worms contain at least two major soluble antigenic components, both of which were detected by IgG-ELISA and double diffusion in agarose. They were separated by gel chromatography through a Sephacryl S-300 column. A high molecular weight (approx. 440,000) component, which is quantitatively dominant in the crude extract, was termed PW-1 and a lesser component with a low molecular weight (approx. 13,000) was termed PW-2. By polyacrylamide gel electrophoresis (PAGE), PW-1 gave a strongly stained single band close to the cathodic end, while PW-2 gave one major and several minor bands with relatively anodic mobility. Thus, not only their molecular weight but also electrophoretic mobility is different between PW-1 and PW-2. When the antigenic homogeneity as well as the antigenic intensity of PW-1 and PW-2 were examined by crossed immuno-electrophoresis, PW-1 produced strongly stained, partially fused double arcs close to the cathodic end, whereas PW-2 produced several faint arcs with relatively anodic mobility. These results were consistent with those of Sawada et al. (1964a), in that a cathodic fraction prepared by starch block electrophoresis had higher precipitin titer than nonmigrating or anodic fraction. The antigenicity of PW-1 and PW-2 is different because they formed completely crossed precipitin bands by double diffusion test using serum from chronically infected cats. This difference was also confirmed by ELISA inhibition test.

As for the origin of PW-1 or PW-2, the homology between PW-1 and egg extract and also between PW-2 and ES were demonstrated by double diffusion test using serum from chronically infected cats. These homologies were confirmed further by ELISA inhibition test. Thus, the eggs and ES, both of which are produced and released from adult worms, seem to be the most potent immunogen to the host infected with *P. westermani.* Since the major histhopathological changes of paragonimiasis westermani is the worm cyst formation and egg-associated granulomatous lesions found in the vicinity of the cysts in the lungs (Yokogawa, 1965), these two antigenic components may play important roles to stimulate host's immunopathological responses. Different from our results, Sugiyama *et al.* (1987) reported that, by using immunohistochemical techniques, only the intestinal epithelial cells and gut contents of *P. westermani* adult worms were recognized by the serum from chronically infected cats. However, they only examined formalin-fixed, paraffin embedded tissue sections. Thus, the antigenicity of eggs may be destroyed during processing of histological

specimen. Related to this point, strong antigenicity of *P. ohirai* eggs have been demonstrated by Ohara *et al.* (1985) using frozen sections of adult worms. In contrast to the conflicting results of the antigenicity of eggs, the presence of precipitinogens in ES and their identity to some of the antigenic components of whole worm extract have already been reported by using double diffusion and immunoelectrophoresis (Yogore *et al.*, 1965). The origin of ES should be determined in future.

In the chronological study using periodically bled serum from infected cats, the specific antibody against PW-1, or against egg extract, became detectable at 10 weeks after infection when the adult worms started to lay eggs (Takizawa, 1964). In contrast, anti-PW-2, or anti-ES antibody, became detectable as early as 4 weeks after infection. These results suggest that ES antigen is produced and released by even immature young worms. Thus, these partially purified PW-1/egg and PW-2/ES antigens seem to be useful for the chronological diagnosis of human paragonimiasis cases.

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