ウェステルマン肺吸虫・幼虫期虫体の抗原解析

誌名	Japanese journal of veterinary science
ISSN	00215295
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巻/号	50巻1号
掲載ページ	p. 169-174
発行年月	1988年2月

Antigenic Characteristics of Larval *Paragonimus westermani*

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(Received 29 June 1987/Accepted 19 October 1987)

ABSTRACT. The characteristics of the antigens in larval flukes of *Paragonimus westermani* were examined. The agar gel double-diffusion test and competitive enzyme-linked immunosorbent assay, using the antiserum against the antigen extracted from larval flukes, showed cross-reactivity between the larval and adult antigens. In immunoblotting analysis, there was a decrease in reactivity of the antiserum absorbed with the adult fluke antigen against the band with a molecular weight of 26,000. However, the bands corresponding to 34,000 or higher molecular weights were still detected. The immunoperoxidase-staining technique revealed that the antigens reacting to the unabsorbed and absorbed antisera were both located on the surface of the gut epithelium and in the luminal contents of larval flukes. These results suggest that the extract of larval *P. westermani* possesses at least two antigens, originating probably from the gut, one being common to the adult and the other characteristic of the larva.—KEY WORDS: ELISA, immunoblotting, immunoperoxidase, lung fluke, *Paragonimus westermani*.

Jpn. J. Vet. Sci. 50(1): 169–174, 1988

Paragonimus westermani, a lung fluke, is one of the most important trematodes affecting man and animals in certain areas in the world, especially in Asia. Paragonimiasis westermani occurs by feeding crabs harboring the metacercariae of this fluke. In the definitive hosts, the larval flukes are released from the cysts of metacercariae into the intestinal lumen. The flukes migrate to the peritoneal cavity through the intestinal wall and lodge in the skeletal muscles. They return to the peritoneal cavity and enter the pleural cavity through the diaphragm. Most of worms eventually invade the lungs and mature there to adults forming parasitic cysts [9]. This cycle from metacercariae to adults takes about 10 weeks in cats [6, 11].

The infection with this lung fluke induces antibody formation in the hosts. We have examined the serum antibody response in experimentally infected cats by enzyme-linked immunosorbent assay (ELISA) [12]. Antibody against the antigen extracted from adult flukes was first detected in 3 weeks after infection though the titer was low. The early immune response seemed to be elicited by antigens of immature flukes such as infective larvae in the cysts of the metacercariae. The larval antigen, however, has been characterized only in *P. ohirai* [4, 5].

In the present study, attempts were made to characterize the antigens in larval *P. westermani* by the agar gel double-diffusion test, competitive ELISA and immunoblotting analysis. In addition, the location of the antigens on the larvae was examined using the indirect immunoperoxidase-staining technique.

MATERIALS AND METHODS

Preparation of antigen: Larval flukes of *P. westermani* (diploid type) were obtained by the excystation method [15] from the metacercariae harvested from the crabs, *Geothelphusa dehaani,* which were collected

in Mie Prefecture, Japan. Adult *P. westermani* were obtained from the lungs of the cats sacrificed 140 days after oral infection with 20 metacercariae of the fluke. The antigens were prepared by the method of Sugiyama *et al.* [13]. Protein contents were determined by the method of Lowry *et al.* [8].

Preparation of antiserum: A rabbit weighing about 3 kg was primed subcutaneously with 1 m*l* portion of a larval fluke antigen solution (10 μg protein/m*l*) emulsified in the same volume of Freund's complete adjuvant (Wako Pure Chemical Industries, Osaka). Two subsequent injections of the same antigen solution emulsified in the same volume of Freund's incomplete adjuvant (Wako) were given subcutaneously at one-week intervals. Three weeks after the third injection, the final injection of the antigen solution without adjuvant was given intravenously. The animal was bled one week after the final injection. The serum was stored in aliquots at −80°C until used. The agar gel double-diffusion test was performed by the method of Asao *et al.* [1].

Absorption of antiserum: The antiserum against the larval fluke antigen was absorbed with the adult antigen by the method of Bennett *et al.* [2] with a slight modification. In brief, the undiluted or diluted antiserum was mixed with an equal volume of the undiluted (2 mg/m*l*) or serially diluted antigen solution. The mixture was incubated for 2 hr at 37°C in a water bath-shaker and kept for 12 hr at 4°C. After centrifugation for 5 min at 10,000 ×g and 4°C, the supernatant was used as the absorbed antiserum.

Competitive enzyme-linked immunosorbent assay (Competitive ELISA): A 0.1-m*l* portion of a larval antigen solution or an adult antigen solution diluted to 5 μg/m*l* in 0.05 M carbonate buffer, pH 9.6, was added to each well of a 96-well Titertek immunoassay plate (Flow Laboratories, Amsterdam, the Netherlands). After 3 hr at 37°C, the wells were washed five times with 0.008 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS)-0.05% Tween 20 (Tween). The anti-larval serum was diluted 400-fold in PBS-Tween-1% bovine serum albumin (BSA, Sigma Chemicals Co., St. Louis, Missouri, USA). The antigen solution was 10-fold diluted serially from 100 to 0.01 μg/m*l* with 0.02 M veronal-buffered saline, pH 7.4. The equivalent mixture of the antiserum and antigen solution was prepared as described above and a 0.1-m*l* portion of the mixture was added to each well. After 1 hr at 37°C, the wells were washed with PBS-Tween and 0.1 m*l* of peroxidase-conjugated goat anti-rabbit immunoglobulin (containing IgA, IgG and IgM; Cappel Laboratories, Cochranville, Pennsylvania, USA), diluted 1,000-fold in PBS-Tween-1% BSA, was added to each well. After 1 hr, the wells were washed and 0.2 m*l* of a substrate solution (0.072% 5-aminosalicylic acid and 0.005% H_2O_2 in distilled water) was added to each well. After 1 hr at 37°C, the developed color was read with a microplate photometer (Bio-Rad Laboratories, Richmond, California, USA) at 450 nm. The well containing the mixture of normal rabbit serum and an antigen solution was used as a control.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting: SDS-PAGE was carried out in a 15% gel by the method of Laemmli [7]. The antigen solution was treated with SDS in the presence of 0.1 M dithiothreitol for 3 min at 100°C. Ten μg protein of the antigen was applied to each lane. After electrophoresis, the gel was stained with Coomassie Brilliantblue R 250 (E. Merck, Darmstadt, West Germany) and treated with a silver stain kit (Daiichi Pharmaceutical Co., Tokyo). The protein bands were each estimated for the molecular weight from the mobility relative to those of mar-

ker proteins (Pharmacia Fine Chemicals, Uppsala, Sweden). For the immunoblotting method [14], the antigens resolved by SDS-PAGE were transferred electrophoretically to nitrocellulose sheets (TM-2; Toyo Roshi, Tokyo). After blotting, the sheets were soaked in PBS-3% BSA solution containing the unabsorbed or absorbed antiserum diluted 200-fold. The absorbed antiserum was prepared with an equivalent mixture of the undiluted antiserum and an undiluted adult antigen solution (2 mg/m*l*). The sheets were treated for 30 min with peroxidase-conjugated anti-rabbit immunoglobulin (containing IgA, IgG and IgM) diluted 1,000-fold in PBS-3% BSA for 30 min. After washing, bound peroxidase was allowed to act on a substrate solution (0.05% 3,3'-diaminobenzidine and 0.003% H_2O_2 in PBS).

Location of antigens in adult and larval flukes: Sections of flukes were processed by the indirect immunoperoxidase-staining technique [3]. In brief, both adult and larval flukes were fixed in 10% phosphate-buffered formalin, dehydrated through a graded series of ethanol, embedded in paraffin, and sectioned at 4-μm thick. The de-paraffined sections were incubated in 0.03% H_2O_2 in methanol for 20 min, washed in distilled water, incubated in 0.05% trypsin (Sigma) in 0.05 M Tris-HCl buffer, pH 7.6, for 10 min, rinsed in PBS-Tween and exposed to the unabsorbed or absorbed antiserum diluted 200-fold in PBS-Tween-1% BSA. The absorbed antiserum was prepared with an equivalent mixture of the undiluted antiserum and an undiluted adult antigen solution (2 mg/m*l*). After 30 min, the specimens were washed with PBS-Tween and treated with peroxidase-conjugated goat anti-rabbit immunoglobulin (containing IgA, IgG and IgM) diluted 1,000-fold in PBS-Tween-1% BSA. After 30 min, they were washed and incubated with a substrate solution (0.02% 3,3'-diaminobenzidine and 0.005% H_2O_2 in 0.05 M Tris-HCl buffer, pH 7.6). After about 5 min, they were washed and counterstained lightly with a kernechtrot solution.

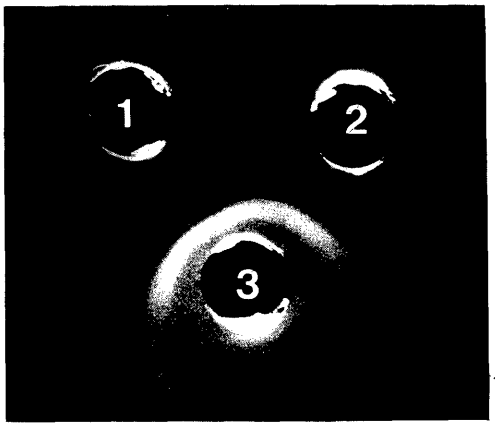

Fig. 1. Agar gel double-diffusion test. 1, larval antigen; 2, adult antigen; 3, anti-larval serum.

RESULTS

Characterization of larval antigen: In agar gel double-diffusion tests, the larval antigen formed two precipitin lines against anti-larval serum; one of them coalesced with the line formed between the adult antigen and anti-larval serum (Fig. 1). In competitive ELISA, the reactivity of anti-larval antibody to larval antigen was depressed by the increasing concentration of adult antigen, though the reactivity of the antibody was inhibited more effectively by larval antigen (Fig. 2).

Identification of antigen by immunoblotting: In SDS-PAGE with the larval antigen, at least 20 bands with molecular weights ranging from about 10,000 to 100,000 or higher were detected (Fig. 3, lane 1). Immunoblotting analysis showed that the anti-larval serum reacted with the bands with molecular weights ranging from about 26,000 to 100,000 or higher (Fig. 3, lane 2). Even when the anti-larval serum absorbed with the adult antigen was used, the bands

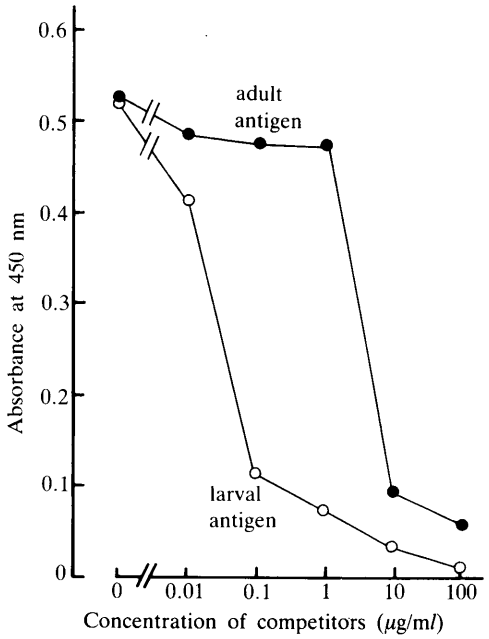

Fig. 2. Competitive ELISA titers of anti-larval serum against larval antigen. A larval (○) and an adult (●) antigen solutions, ranging from 0.01 to 100 μg/ml, were used as competitors.

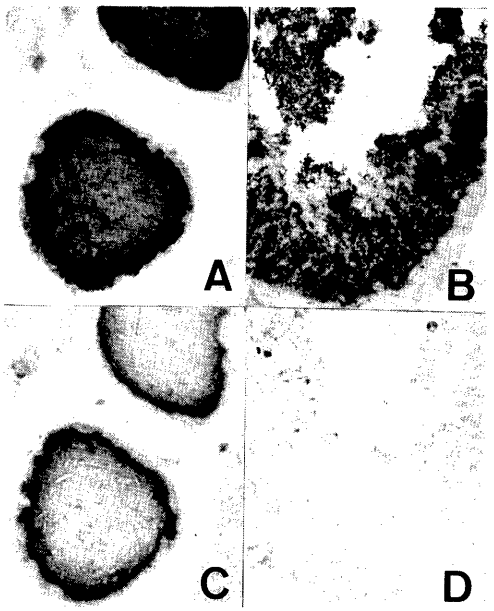

Fig. 4. Location of antigens on sectioned *P. westermani*. High power magnification of the gut tract of larval (A and C, ×500) and adult (B and D, ×200) flukes. A and B: treated with anti-larval serum; and C and D: with anti-larval serum absorbed with adult antigen.

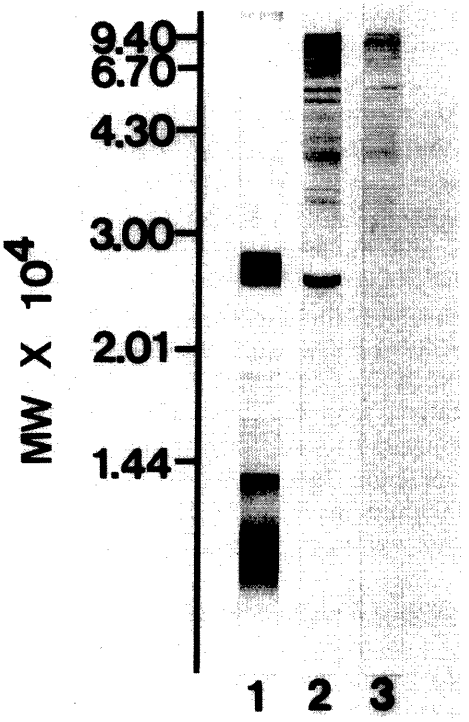

Fig. 3. SDS-PAGE and immunoblotting analysis of larval *P. westermani* antigen. The gel was stained with Coomassie Brilliantblue R 250 and silver (lane 1), treated with anti-larval serum (lane 2), and anti-larval serum absorbed with adult antigen (lane 3). Locations of the molecular marker proteins are shown to the left. The markers from the top were: phosphorylase b (MW 94,000), bovine serum albumin (MW 67,000), ovalbumin (MW 43,000), carbonic anhydrase (MW 30,000), soybean trypsin inhibitor (MW 20,100), and α-lactalbumin (MW 14,400).

of molecular weights higher than about 34,000 were still detected. The band with a molecular weight of about 26,000, which reacted most strongly with unabsorbed anti-larval serum, was no longer detected (Fig. 3, lane 3).

Location of antigen in flukes: In the sectioned larval flukes, the substances reacting with anti-larval serum were located on the surface of the gut epithelium and in the luminal contents (Fig. 4, A). A positive reaction in the adult flukes was also observed on the gut epithelium and in the luminal contents (Fig. 4, B). When the

anti-larval serum absorbed with the adult antigen was applied, a positive reaction was seen on the surface of the gut epithelium and in the luminal contents of the larval flukes (Fig. 4, C), whereas no positive staining was observed in the adult flukes (Fig. 4, D).

DISCUSSION

The previous study [13] with the sera of *P. westermani*-infected cats and rats suggested that the antigenic substances in adult flukes parasitizing in the lungs of cats were the same or at least cross-reactive to those in immature flukes parasitizing in the skeletal muscles of rats. In the present study, we characterized the antigen in an immature *P. westermani* using the extract from larval flukes which parasitized in freshwater crabs. The agar gel double-diffusion tests showed that one of the two lines formed between anti-larval serum and larval antigen was identical to that formed between anti-larval serum and adult antigen. Competitive ELISA also revealed immunological cross-reactivity between the antigens of the adult fluke and larva. In immunoblotting analysis, the reactivity of anti-larval serum to the band with a molecular weight of 26,000 was found to be depressed by absorption with adult antigen. These results indicate that the adult antigen contains a protein immunologically relating to that with a molecular weight of 26,000 in the larval extract.

The immunoperoxidase-staining of the sectioned flukes provided the evidence that the antigens recognized by the anti-larval serum were located on the surface of the gut epithelium and in the luminal contents of both adult and larval flukes. These results are in accordance with our previous finding that a cross-reactive antigen was located on the guts of different developmental stages [13].

In immunoblotting, anti-larval serum absorbed with adult antigen still reacted with the bands with molecular weights of 34,000 or higher. Furthermore, the absorbed antiserum was found to bind with the substance on the surface of the gut epithelium and in the luminal contents of the larval flukes. These findings suggest that the bands with molecular weights of 34,000 or higher, probably being substance(s) from the gut of the larva, are larval-specific. In *P. ohirai*, it has been stated that immature flukes had distinct antigenicities from those of adult ones [10] and antigens of larval flukes were located in the gut [4].

The present results show that the extract of larval *P. westermani* possesses at least two antigens, one of which is common to the adult and the other is characteristic of the larva. Applicability of this larval antigen for diagnosis of paragonimiasis westermani, especially for detecting paratenic hosts, is being assessed in this laboratory.

ACKNOWLEDGEMENTS. We express our gratitude to Dr. Shunji Kozaki of this University for his valuable discussions and critical reading of the manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research (No. 62760253) from the Ministry of Education, Science and Culture of Japan.

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要　約

ウェステルマン肺吸虫・幼虫期虫体の抗原解析：杉山　広・堀内貞治・冨村　保（大阪府立大学農学部家畜病理学教室）—— ウェステルマン肺吸虫の脱嚢幼虫を対象として，幼虫期虫体の抗原解析を試みた．ウサギ免疫血清を用いたゲル内沈降反応および酵素抗体法により，幼虫抽出粗抗原中には成虫抗原と免疫学的に共通する物質が含まれることが示された．免疫血清を成虫抗原で吸収し，幼虫抽出粗抗原との反応におよぼす影響を immunoblotting 法により調べると，吸収により分子量26,000の物質との反応は消失したが，34,000以上の物質との反応は大きく変化せず，幼虫抽出粗抗原中には幼虫期に特異的な物質も含まれていることが示された．抗原の局在部位を免疫染色により調べたところ，免疫血清は，成虫抗原による吸収の有無にかかわらず，幼虫の腸管上皮細胞およびその内容物と反応した．以上の成績から，ウェステルマン肺吸虫の幼虫期虫体は，成虫との共通抗原物質のほか，幼虫期に特異的な抗原物質を含み，いずれも虫体の腸管由来であることが示唆された．