CHARACTERIZATION OF ANTIGENS OF *PARAGONIMUS MIYAZAKII* BY ELISA

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INTRODUCTION

Infection with *P. miyazakii* induces a humoral (antibody) response in the host animal. Based on this, the diagnosis of paragonimiasis by the identification or determination of *P. miyazakii*-specific antibody can be made via double immunodiffusion or various immunoassays. However there is little information about the nature of *P. miyazakii* antigens. By use of agar double diffusion tests, Tada (1967) reported the number of precipitin bands appearing during the course of an infection in the sera of rats infected with *P. miyazakii*, or immunized with *P. miyazakii* extract.

The presence of specific *P. miyazakii* antigens was demonstrated by Tsuji *et al.*, (1967) in immunoelectrophoresis sera from immunized rabbits with *P. miyazakii* extract. For a more precise study, a more sensitive method of antigen detection is required as the immuno-diffusion test needs much antigen and serum for even single tests. For this purpose, we established a sandwich enzyme-linked immunosorbent assay as our method of choice.

The cross-reactivity of ELISA with other parasite antigens was examined. This sensitive ELISA was used for detection of antigen to gain information about molecular weight and isoelectric point of ES and adult worm of *P. miyazakii*.

MATERIALS AND METHODS

Preparation of antigen

P. miyazakii adult worms were collected from the lungs, livers and abdominal cavities of dogs and rats which had been infected with metacercariae collected from freshwater crabs, *Geothelphusa dehaani*, collected at Yatsu in Kuga-cho, Yamaguchi prefecture (Takemoto, 1985).

Preparation of the crude antigen of *P. miyazakii* adult worms was reported in a previous paper (Takemoto, 1985). Briefiy, after homogenizing worms in 0.1%NaC1, the homogenate was freezed, and was finely crushed to ice powder. This process of freezing and crushing was repeated over six times. After desalting by dialysis and removal of insoluble substances by centrifugation, the extract was freeze-dried.

Crude antigens of other parasites, *Paragonimus westermani*, *Fasciola hepatica*, *Fishoederius elongatus*, *Taenia saginata*, *Schistosoma mansoni* and *S. japonicum*, were prepared in the same manner.

Thirty five adult worms were cultured in sterile Tyrode solution for 9 days. Every two days, the culture medium was removed, transferred to visking tube and dialyzed against tap water overnight. The swollen visking tube was placed in an air stream, and the concentrated solution then lyophilized and stored at −20 °C until required. The total dry weight of the recovered excretory-secretory substance (ES) was 70.6 mg.

Immunoelectrophoresis

Techniques of immunoelectrophoresis used have been described by Grabar and Williams (1953) and Tsuji (1974).

Briefly, 0.1% extracts of the adult worms was subjected to electrophoresis in 0.9% agarose in veronal buffered saline (pH8.2, ionic strength 0.05) for 5 hours at 20 ± 2 V/8 cm. After the electrophoresis was terminated, the immune serum concentrated to 1/3 of the original volume was filled to the trough and incubated in a moist chamber at 4 °C for 3 days. When the bands of precipitation developed sufficiently, the plates were washed for 3 days in a physiological solution mixed with a veronal buffered saline, which was changed daily. The plates were then dried and stained.

Procedure of ELISA

The procedure of ELISA is summarized in Table 1. The procedure for the recovery of IgG from infected rat sera is outlined in Table 2. The fixed lot of crude purified antigen was used as the standard throughout. All ELISA readings are indicated as equivalent concentration to the standard antigen. The calibration curve of every assay consisted of at least three dilutions of the standard antigen. In one series (Fig. 3), the values of ELISA are recorded as absorbance at 490 nm (A490). Buffer A was 0.01 M phosphate buffer, pH 7.0, and contained 0.1 M NaCl and 0.1% BSA. The preparation of Fab'−HRP is summarized in Table 3. All assays were performed in duplicate. With our ELISA, we were able to detect 42 ng of the standard Pm antigen.

Table 1

Procedure of ELISA.

Nunc-Immuno Plate 1 (96F)
- coated with IgG (10 μg/ml in 0.15M PBS pH7.0), 4 °c, 18−24hr
- washed with buffer A
- antigen solution diluted with buffer A, 4 °c, 1hr
- washed with buffer A
- Fab'-HRP diluted with buffer A, 4 °c, 1hr
- washed with buffer A
- reacted with substrate (freshly prepared 0.02% H_2O_2 −0.4% O-phenylenediamine in Mclavain buffer pH 5.0), room temprature, 1hr, in the dark
- reaction stopped with 8N H_2SO_4
- optical density measured at 490nm

Table 2

Preparation of IgG.

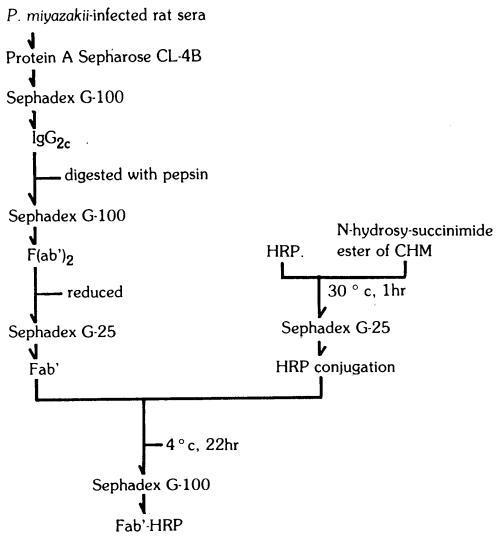
P. miyazakii infected rat sera
↓
Precipitate with 40% $(NH_4)_2SO_4$
↓
DEAE-cellulose column chromatography
↓
Sephadex G-100 gel filtration
↓
P. miyazakii bound sepharose CL-4B affinity chromatography
↓
Purified IgG

Protein concentration

Protein concentration was estimated by

Table 3

Preparation of Fab'-HRP.



measuring absorbance at 280 nm. In addition, protein concentration was determined by the method of Lowry *et al.*, (1951), with bovine serum albumin (BSA) as the reference.

Materials

As reference proteins for the estimation of molecular weights, authentic cytochrome C, myoglobin, chymotrypsinogen A, egg albumin and blue dextran were used.

Isoelectric focussing

Isoelectric focussing was performed in an LKB produkter with Ampholine carrier ampholites in the pH range 3.5–5.0. Electrophoresis was done at 10 °C for about 50 hours at 500–800 V.

RESULTS

Precipitin bands in immunoelectrophoresis

The changes in immunoelectrophoretic precipitin bands in rat sera during the course of *P. miyazakii* infection is shown in Fig. 1. The number of precipitin bands gradually increased during the 3rd to the 12th week.

Cross-reactivity of ELISA with other parasites (Fig. 2)

The cross-reactivity of the ELISA was examined by replacing *P. miyazakii* antigen with the antigens of *P. westermani*, *F. hepatica*, *F. elongatus*, *T. saginata*, *S. mansoni* and *S. japonicum*. As a reference, the OD of 50 μg/ml of *P. miyazakii* antigen at A490 was assigned the value of 100%. Cross-reactivity with the above listed helminths was

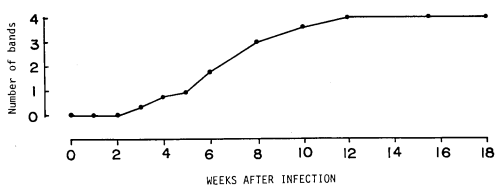


Fig. 1—Changes in number of precipitin bands after immunoelectrophoresis of sera of rat infected with *P. miyazakii*.

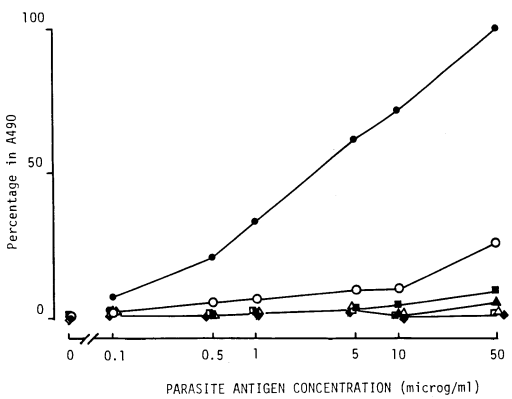


Fig. 2—Cross-reactivity of *P. miyazakii* antigen with other parasites. (●) *P. miyazakii* (○) *P. westermani* (■) *T. saginata* (▲) *F. hepatica* (△) *F. elongatus* (□) *S. japonicum* (◆) *S. mansoni*

slight or negligible. The antigen of *P. westermani* showed a higher degree of cross-reactivity. When A490 in each concentration of *P. miyazakii* was 100%, the cross-reactivity was below 30% even in every concentration of *P. westermani*.

Comparative antigenicity

After dilution shown in Fig. 3, the A490 of ES and adult worm antigen was parallel with each other. ES antigen had about five times as high affinity to antibody as adult wormn antigen had.

Molecular weights

Seventy mg (dry weight) of ES antigen was dissolved in 0.5 mM PBS, pH7.0, and insoluble ingredients were removed by centrifugation. About 50 mg (dry weight) of adult worm antigen was completely dissolved in the same buffer as the ES antigen. Both antigens were applied to Sephadex G−100 gel column. Gel filtration was performed 3 times with the adult worm antigen. Each chromatogram of the adult worm and ES antigens had one major peak in ELISA. Reference proteins also were chromatographed by the same column. The approximate molecular weight was the same, 36.000, for both antigens (Fig. 4).

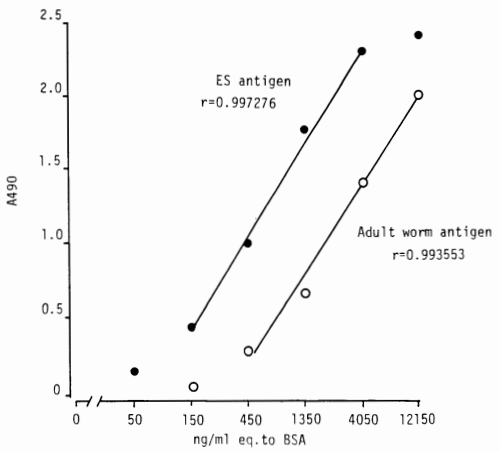


Fig. 3—Comparative antgenicity of ES and adult worm antigens of *P. miyazakii*.

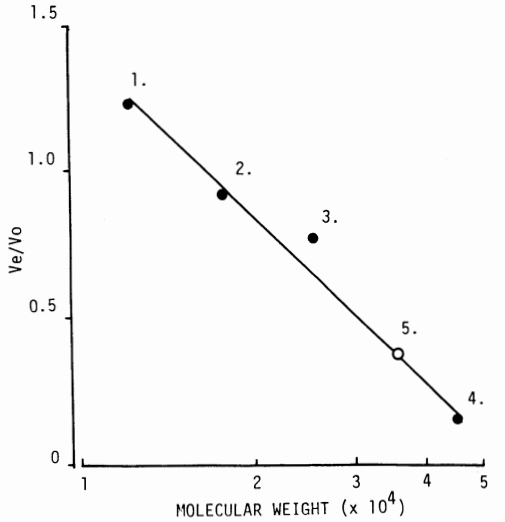


Fig. 4—Molecular weight of antigens of *P. miyazakii* estimated by sephadex G−100 gel filtration. Reference proteins: 1. Cytochrome C, 2. Mioglobin, 3. Chymotrypsinogen A, 4. Egg albumin, and five shows ES and adult worm antigens.

Isoelectric points

The ELISA peaks mentioned above were used for determination of isoelectric points. There were numerous isoelectric peaks for both antigens (Fig. 5). With adult worm crude antigen, the majority of antigenic fractions were eluted in a pH range of 4.4−4.7, with major peaks at pI4.6 and 4.7 The ES antigen showed four peaks in a pH range of 4.1−4.6, with much overlap to those seen in the adult worm antigen.

DISCUSSION

In the preceeding paper we reported that our established ELISA could detect Pm antigen with high sensitivity and linearlity

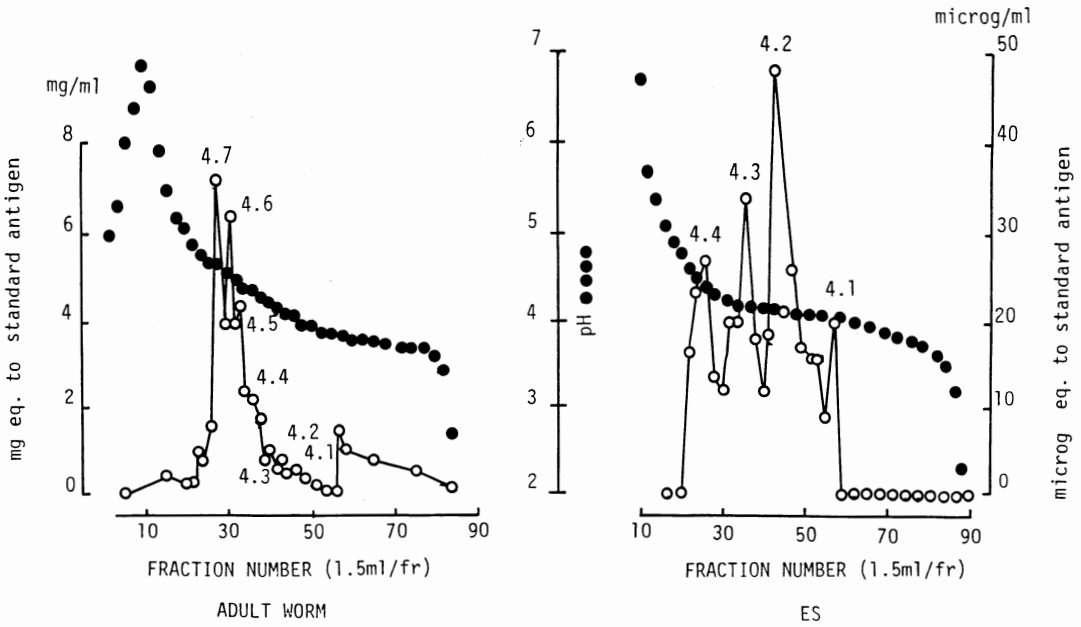


Fig. 5–Analysis of isoelectric points with adult worm and ES antigens.

(Takemoto, 1985). Secondly, cross reactivity was below 30% with *P. westermani*, the same genus as *P. miyazakii* and negligible with other parasites (Fig. 2). This result ascertained that our ELISA catched *P. miyazakii* specific antigen. The IgG used in ELISA was partially purified by *P. miyazakii* bound sepharose 4B affinity chromatography (Table 2). Probably well specificity of ELISA causes the success of separation of IgG, which has high specificity to *P. miyazakii* antigen.

Property of *P. miyazakii* antigen was investigated. The molecular weights of ES antigen and adult worm were the same (36.000). Their isoelectric points showed multiple forms (Fig. 5). Adult worm had seven peaks and most antigenic activity concentrated at pI 4.6 and 4.7. ES had four peaks with similar antigenic activity. The result of the isoelectric focussing suggests that the number of antigenic substances is seven at least in adult worm and four at least in ES. Worm contituents circulating in blood after infection are probably ES or necrotic substances. Therefore ES antigen was expected to have higher antigenic activity than adult worm. When ES antigenic activity was compared with adult worm by protein concentration and reactivity of ELISA. ES antigenic activity showed 5 times higher than adult worm (Fig. 3). This result supports that infected rat, whose serum was used in ELISA, had produced more IgG to ES than to adult worm. Again as the antigenic substances of adult worm at pI 4.6 and 4.7 had similar molecular weight as ES, these may be the precursor of ES within adult worm, which are processed before excreting or secreting.

Our animal of choice in this study was a rat. However, if characterization in specific antigenicity to human sera infected with *P. miyazakii* develops, infection with *P. miyazakii* will diagnose serologically before production of antibody.

We have pointed out the presence of three specific bands between *P. miyazakii* extract and the sera of rabbits immunized with *P. miyazakii* and absorbed with *P. westermani* extract (Tsuji *et al.*, 1967). Previously, our experiments utilized the sera of rabbits immunized with *P. miyazakii* adult worm antigen. In the present study, we used the sera of infected rats. Therefore, it is very likely that neither antigen nor antibody are the same as previously reported (Tsuji *et al.*, 1967).

Tada (1967) has reported that the number of precipitin bands obtained by the double diffusion test during the course of infection gradually increased to 5 or 6 bands by day 70, then diminished to 2 bands on the 90th and 150th day. In our method (Fig. 1), however, the number of precipitin bands was maximum 12 weeks post infection, then plateaued. The discrepancy between our results may be due to different antigenic preparations and to different analytical methods. Tada used antigen homogenized for 5 min at 0 °C and analyzed by the double diffusion test, while our antigen was homogenized, lyophilized, crashed to powder over six times and analyzed by immunoelectrophoresis.

SUMMARY

The characterization of adult worm and excretory-secretory (ES) antigens of *P. miyazakii* was performed with a sandwich exzyme-linked immunosorbent assay (ELISA) IgG and Fab' fragments were prepared from *P. miyazakii*-infected rat sera. The former was purified by affinity column chromatography. The latter was prepared from IgG2c by passage through a Protein A affinity column, then conjugated to horseradish peroxidase. Cross-reactivity of the ELISA with *Fasciola hepatica*, *Fischoederius elongatus*. *Taenia saginata*. *Schistosoma mansoni* and *S. Japonicum* was negligible. With *Paragonimus westermani*, the same genus as *P. miyazakii*, cross-reactivity was below 30%.

The molecular weight of both antigens was comparable (36.000) as determined by Sephadox G−100gel filtration. Isoelectric points in both antigens had many peaks in a pH range of 3.9−4.7. In adult worm, pI of the major peaks were 4.6 and 4.7 and minor part of peaks had the common isoelectric points with four ES peaks in a pH range of 4.1−4.6.

Antigenicity per protein concentration of both antigens was examined by the ELISA. The ES antigen was about five times as active antigenically as adult worm antigen. These results suggest that ES is one of the major antigens inducing antibody synthesis in rats infected with *P. miyazakii*, and that other antigens may be present within the adult worm.

REFERENCES

GRABAR, P. and WILLIAMS, C.A., (1953). Méthode permettant l'étude conjugée des propriétés étectrophorétiques et immunochimiques d'un mélange de proteines. Application au sérum sanguin. *Biochim. Biophys. Acta.*, *10* : 193.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, RJ.,(1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem. 193* : 265.

TADA, I.,(1967). Physiological and serological studies of *Paragonimus miyazakii* infection in rat. *J. Parasitol. 53* : 292.

TAKEMOTO, Y., (1985). Preparation of Fab'-horseradish peroxidase conjugate from sera infected with *Paragonimus miyazakii* and its use for ELISA. *Med. J. Hiroshima Univ. 33* : 1065.

TSUJI, M., YOKOGAWA, M., CAPRON, A. and BIGUET, J.,(1967). Studies on the comparison of antigenic structure of three species of *Paragonimus (P. westermani, P. miyazakii* and *P. ohirai)* in immunoelectrophoresis. *Jpn. J. Parasitol. 16 (suppl)* : 541.

TSUJI, M. (1974). On the immunoelectrophoresis for helminthological researches. *Jpn. J. Parasitol., 23* : 335.