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Variation of antigenic proteins of eggs and developmental stages of *Paragonimus westermani*

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Abstract: Diagnosis of early paragonimiasis is difficult because parasitological evidence is not easily obtained. Antibody tests have been proposed as a good substitute for classical diagnostic techniques. Using the crude extracts of *Paragonimus westermani* eggs, metacercariae, 4- and 7-week juveniles, and 16-week adults as antigens, we observed the early antibody responses. Sera were obtained from 4 experimental cats, fed 50 metacercariae each, at intervals until 13 weeks post-infection. Antibody (IgG) responses were identified by ELISA using extracts of 4-week juveniles, followed by those of 7- and 16-week worms. Antibody responses were minimal against the metacercarial extracts. Antibodies to *P. westermani* egg extracts were elevated after 10 weeks post-infection. In immunoblot analysis, more than nine protein bands in 4-week juveniles reacted with the early infection sera. Antigenic proteins in adult worms were different from those of juveniles. After four weeks of infection, 32 and 35 kDa bands in the adult extracts were increasingly reactive. Egg specific proteins at 28, 46 and 94 kDa were reactive only after 10 weeks. Antigenic components reacting to the early infection sera changed during the maturation stages of *P. westermani*; almost all juvenile antigens were replaced by adult antigen components.

Key words: *Paragonimus westermani*, paragonimiasis, antigenic variation, early infection

INTRODUCTION

Human paragonimiasis is an acute and chronic infection affecting the lungs and other organs (Toscano *et al.*, 1995; WHO Study Group, 1995). Parasitological diagnosis is made by the detection of eggs either in sputum/stool, or in tissue biopsy. In the early or late chronic infections or in the cases with extrapulmonary lesions only, parasitological diagnosis is insensitive because eggs are rarely detected. In these situations, sensitive antibody tests can be utilized to diagnose *Paragonimus* infection in patients because antibody levels return to normal after successful chemotherapies (Yokogawa *et al.*, 1962; Cho *et al.*, 1989).

Crude extracts of adult *P. westermani* have been used as a diagnostic antigen in antibody tests. Specific antigenic components of

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extracts or excretory-secretory products of *Paragonimus* species have been reported (Sugiyama *et al.*, 1987;Kim *et al.*, 1988; Slemenda *et al.*, 1988; Joo *et al.*, 1989; Indrawati *et al.*, 1991; Maleewong *et al.*, 1992). While component proteins in the extracts of *P. westermani* are specific for different developmental stages when observed by non-denaturing electrophoresis (Huer *et al.*, 1985), the antigenic component proteins in the developmental stages show minimal differences when analyzed by SDS-PAGE or immunoblot (Joo *et al.*, 1989).

In this study, we observed the changing patterns of antibody levels and reacting components employing crude extracts of *P. westermani* in early experimental paragonimiasis to determine the presence of a useful antigen(s) for diagnosis of early paragonimiasis.

MATERIALS AND METHODS

Parasite and antigens

Five different crude extracts of *P. westermani* were prepared from their eggs, metacercariae, 4-week and 7-week old juveniles, and 16-week old adults. The egg extracts were prepared as described by Kang *et al.* (1995). The metacercariae, from naturally infected freshwater crayfish, were washed with sterile physiological saline and homogenized. Juveniles and adult worms were collected at 4, 7 and 16 weeks after experimental infections of four domestic cats which were fed 50 metacercariae each. The crude saline extracts were prepared as described by Cho *et al.* (1989).

Serum samples

Sequential sera samples of the four experimental cats were secured before the infection and at 1, 2, 3, 4, 6, 8, 10 and 13 weeks after infection, respectively.

ELISA

Antibody tests by ELISA were conducted as described elsewhere (Cho *et al.*, 1989). Microtiter plates (Costar, USA) were coated overnight at 4°C with 200 μl of each antigen (diluted at 2.5 μg/ml of protein content in carbonate buffer, 0.05 M, pH 9.6). Individual cat serum was diluted at 1:200 in phosphate buffer containing 0.05% Tween 20 (PBS/T, pH 7.4) and reacted at 37°C for 2 hours. Peroxidase-conjugated anti-cat IgG (whole molecule, Cappel, West Chester, PA, USA) was diluted at 1:1,000 in PBS/T and incubated at 37°C for 2 hours, sequentially. The color reaction was developed by 0.05% (w/v) *o*-phenylene diamine containing 0.03% (v/v) H_2O_2, and stopped by adding 25 μl of 8 N H_2SO_4. Absorbance was read at 490 nm using Microplate Reader M-3550 (Bio-Rad, Richmond, CA, USA).

SDS-PAGE/Immunoblot

SDS-PAGE was carried out in 7.5-15% gradient polyacrylamide gel and stained with Coomassie blue for analysis.

Immunoblot was undertaken using commercially available precast 4-20% gels (no. 01-102, 106, -020 and -026, SDS-PAGE Mini; TEFCO, Tokyo, Japan). After electrophoresis of the crude extracts of eggs, 4-week juveniles and 16-week adults, the separated proteins were transfer-blotted to polyvinylidene difluoride membranes. The membranes were probed with 1:400 diluted, experimental cat sera collected before infection, and at 1, 2, 3, 4, 6, 8, 10 and 13 weeks after infection. The sera from the four experimental cats were pooled at the same age of infection. After reacting with 1:1,000 diluted peroxidase conjugated anti-cat IgG (whole molecule, Cappel, West Chester, PA, USA), the reactions were developed using 4-chloro-1-naphthol chromogen.

RESULTS

Antibody levels by ELISA

Antibody levels began to elevate in 3 weeks after the infection (Fig. 1). Out of five antigens examined, the antibody levels against the extracts of 4-week juveniles were elevated the highest forming a sigmoidal curve. The antibody responses against 7- and 16-week worm extracts increased linearly until 13 weeks post-infection. The antibody levels to metacercarial extracts were elevated at low level until 8 weeks, then formed a flat plateau.

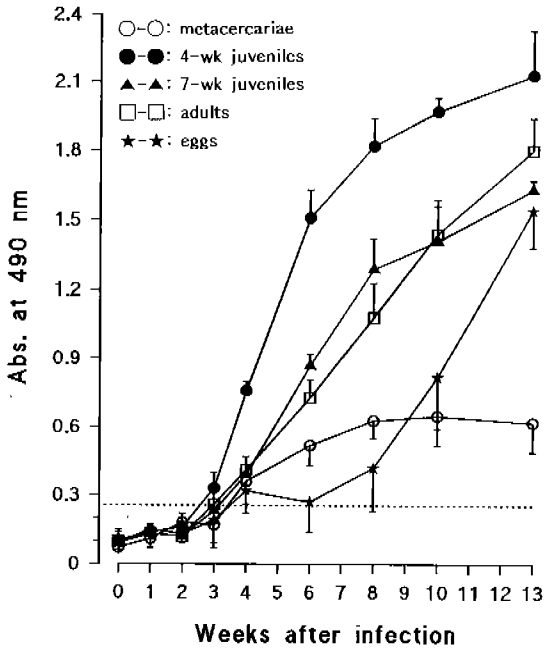

Fig. 1. Antibody levels in the sera of early experimental cat paragonimiasis (mean – S.D., n = 4) measured against five different antigenic preparations of *P. westermani*.

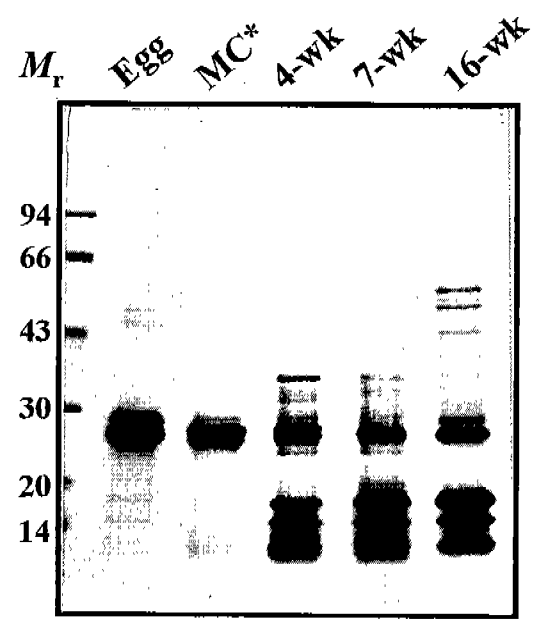

Fig. 2. Analysis of the crude extracts of different stages of *P. westermani* as seen by SDS-PAGE (Coomassie blue stained). M_r, molecular weight in kDa; MC*, metacercariae.

The antibody levels to egg extracts were the lowest until 8 weeks, however, they began to elevate linearly after 10 weeks of the infection.

SDS-PAGE analysis of the crude extracts of *P. westermani*

Protein components of each extract are shown in Fig. 2. Major components at 27 and 28 kDa bands were recognized in the extracts of eggs, metacercariae and 4-week juveniles, whereas the band at 27 kDa was a major one in extracts of 7-week juveniles and 16-week adults.

In the extracts of 4-, 7-week juveniles and 16-week adults, 2 major protein bands at 15 and 17 kDa, were exhibited together with another one below 10 kDa. Molecular weight of the major band below 10 kDa was not the same for extracts of 4-week juveniles and those of older worms. These major bands were shown as minor bands in the extracts of eggs and metacercariae. In all the extracts, many proteins were recognized as minor bands in the range of 30-94 kDa.

Antigenic components of *P. westermani* in its developmental stage

Preinfection sera did not react with any component in the extracts of eggs, 4-week juveniles and 16-week adults (Fig. 3). Egg components began to react with the experimental sera at 8 weeks post-infection. Egg specific antigens at 28, 46 and 94 kDa exhibited positive reactions with the sera collected later than 10 weeks post-infection.

Extracts of 4-week juveniles began to react with 1 week infection sera producing a 31 kDa component and one below 10 kDa which increased in reactivity until 13 weeks post-infection. The 29 kDa component in the same extracts began to react with infection sera at 4 weeks, was highly reactive at 6-8 weeks post-infection and began to decrease thereafter. The six antigenic bands between 10-25 kDa reacted with infection sera at six weeks; thereafter the reaction became faint.

The antigenicity of the 16-week adult extracts is shown in lanes 16-wk of Fig. 3. Two major bands at 32 and 35 kDa began to be reactive with the sera of 4-week infections and gradually increased its reactivity until 13

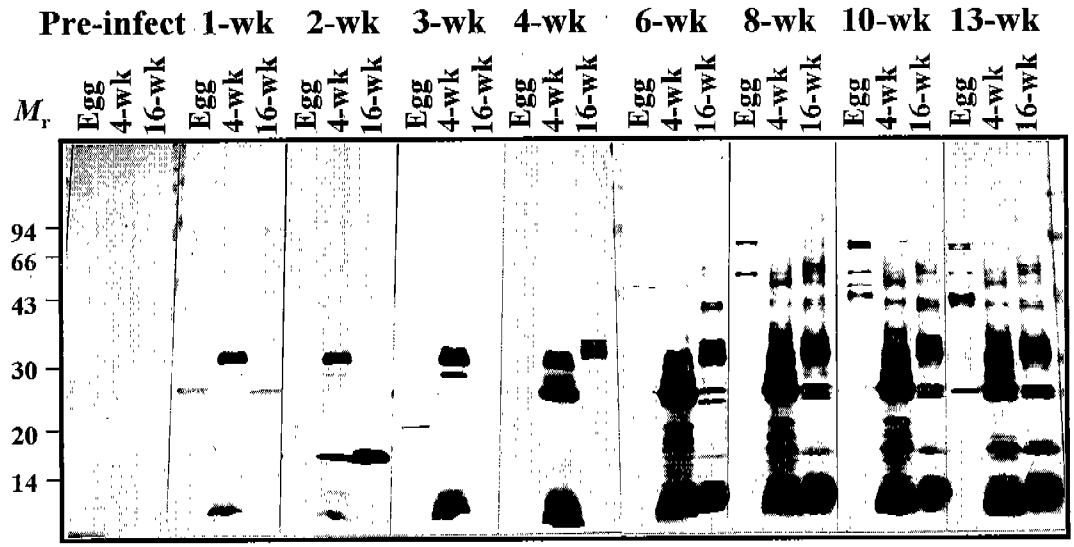

Fig. 3. Immunoblot analysis of the crude extracts of eggs (lanes Egg), 4-week old juveniles (lanes 4-wk) and 16-week old adults (lanes 16-wk) using the cat sera collected until 13-week of infection. Top indicates infection age of the examined sera. M_r, molecular weight in kDa. The experimental cat sera (n = 4) were pooled, diluted at 1:400 in Tris-HCl containing 2% casein (w/v) and probed overnight. A 1:1,000 diluted peroxidase conjugated anti-cat IgG was used as a secondary antibody.

weeks. Two antigenic bands at 27 and 28 kDa reacted after 6-week post-infection together with three antigenic bands below 15 kDa.

DISCUSSION

Unlike similarities of major proteins between the extracts of juvenile and adult *P. westermani*, as analyzed by SDS-PAGE (Fig. 2), antigenic bands in the adult extracts were clearly different from those of the juveniles. Few proteins of the same molecular weight, existed between them, reacted commonly with the early infection sera. This indicates that during maturation, the antigenic components are replaced by antigenically different proteins.

Egg extracts of *P. westermani*, which revealed 27, 28, 46 and 94 kDa bands as major components, have been known to be highly antigenic (Kang *et al.*, 1991; Kong *et al.*, 1992). In this longitudinal experiment, high levels of antibodies to the egg extracts were exhibited by ELISA later than 10 weeks post-infection (Fig. 1). Also, egg components were reactive by immunoblot analysis, with sera collected after 10 weeks (Fig. 3). These observations support the results of this study since juvenile *P. westermani* matures to egg-laiden adults in eight weeks (Im *et al.*, 1993).

Antibody responses against the metacercarial extracts were relatively low when compared to those of older worms. Low antigenicity of the metacercarial extracts may result from a simple composition of major antigenic proteins (Fig. 2). However, it may also reflect antigenic variation which occurs during the maturation stage.

Between the extracts of 4- and 16-week worms, marked differences in component proteins were exhibited in non-denaturing PAGE (Huer *et al.*, 1985). However, except for the egg protein bands, the subunits were similar to each other when analyzed by SDS-PAGE (Fig. 2). Unlike the subunit similarities, antigenic proteins in the adult extracts were different from those of the juveniles. Because few proteins were investigated in their biological roles or molecular information, it is too early to indicate the nature of the antigenic variations.

Extracts of 4-week juveniles were the most antigenic of those examined, reacting highest with the early infection sera. More than nine protein bands in a range below 10 kDa to 31 kDa demonstrated antigenicity. However, some of these components such as 14 and 17 kDa

were found to be cross-reactive with other parasitic diseases and with normal sera. Moreover, the 17 and 31 kDa bands exhibited cross reactions with human fascioliasis (Kong *et al.*, unpublished data). The 31 kDa antigenic band is non-specific because it reacts positively even in the first week of infection. Specific antibody reactions to the 18-25 and 29 kDa bands in the 4-week juveniles began to appear in 4 weeks, but were faint in sera 13 weeks post-infection. These bands are possible diagnostic antigen candidates since they are specific to the early paragonimiasis.

The 32 and 35 kDa bands in the adult extracts were also reactive at four weeks post-infecton. These antigenic proteins are potential diagnostic characters for early human paragonimiasis. Future work should be directed to determine the nature of these antigenic components and their level of specificities. These antigenic proteins were repeatedly recognized as important diagnostic antigens for paragonimiasis for different species of *Paragonimus* (Itoh and Sato, 1988; Kim *et al.*, 1988; Indrawati *et al.*, 1991; Kong *et al.*, 1992; Maleeworng *et al.*, 1992).

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=초록=

폐흡충 발육 단계에 따른 항원 단백질의 변화

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고양이 4마리에 폐흡충 피낭유충을 50마리씩 감염시키고, 1주부터 13주까지 감염단계별 혈청을 채취하고 폐흡충 충란, 피낭유충, 4주, 7주 및 16주 충체 추출물 항원에 대한 항체 반응을 관찰하였다. 초기 항체 반응은 감염 3주 후부터 관찰되었다. 항원별로는 4주 충체 항원에 대한 항체가가 가장 먼저, 높게 증가하였고, 7주, 16주 충체 항원에 대한 반응이 그 다음으로 높았다. 충란 항원에 대한 항체 반응은 10주 이후 증가하였다. 피낭유충 충체 항원에 대한 반응은 관찰한 초기 감염 전체 기간에 걸쳐 비교적 낮았다. Immunoblot을 실시한 결과, 충란 항원 특이 28, 46, 94 kDa 항원대에 대한 반응은 10주 이후부터 관찰할 수 있었다. 4주 충체 항원은 10-25, 29, 31 kDa 등이 감염 초기부터 항원성을 나타내고 있었고, 그 중에는 감염 13주 혈청에 대하여 이미 반응이 약하게 된 항원대도 있었고 다른 기생충 질환 혈청과 교차반응을 나타내는 것이 알려진 항원도 있었다. 16주 성충 충체 항원의 32, 35 kDa 항원대는 감염 4주 후부터 특이한 반응을 나타내고 있었다. 이상의 결과, 감염 경과에 따라 폐흡충 항원단백질의 항원 결정기도 성충의 것으로 바뀌는 것을 알 수 있었고 성충 충체항원의 32 및 35 kDa 단백질은 4주 이후 초기 폐흡충증도 진단할 수 있는 항원 성분으로 생각하였다.

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