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Demonstration of species-specific and cross reactive components of *Paragonimus westermani* crude worm antigen by EITB

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Abstract: Enzyme-linked immunoelectrotransfer blot(EITB) using crude worm antigen of adult *Paragonimus westermani* was performed for human patients sera to identify the species-specific components. Crude antigen was obtained by homogenizing and centrifuging 24-week old adult worms at 10,000 rpm for 60 minutes in phosphate buffered saline (PBS, pH 7.2) containing phenyl methyl sulfonyl fluoride (PMSF). Gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) was performed and blotted electrophoretically onto a sheet of nitrocellulose paper. The sheet was cut into strips and exposed to sera diluted 1 : 200 with PBS. SDS-PAGE showed 26 protein bands ranging 229 to 10 kDa. Of them 229, 91, 60, 50, 35~31, 27, 25, 21, 17, 11 and 10 kDa components showed positive reaction with serum antibody of patients with *P. westermani*. Sera of patients infected with *Clonorchis sinensis* reacted with 35~31, 19, and 11 kDa bands. Human sera from cysticercosis and diphyllobothriasis cases showed non-specific cross reactions with 229, 35~31, 27, 25 and 17 kDa bands. Protein bands of 91, 60, 21 and 10 kDa showed strong positive reaction without cross reactions with sera from other helminthic infections.

Key words: *Paragonimus westermani*. western blot/EITB, immunodiagnosis, human paragonimiasis

INTRODUCTION

Detection of characteristic eggs in sputum is the most accurate tool for the diagnosis of human paragonimiasis. However, application of serological diagnostic method is inevitable because ectopic paragonimiasis occasionally occurs. In addition, low sensitivity of sputum examination and low specificity of intradermal test is another reason why serological diagnosis should be considered.

For immunological diagnosis of human paragonimiasis various serological techniques such as complement fixation test (Yokogawa *et al.*, 1962), immunodiffusion (Yogore *et al.*, 1969; Choi and Lee, 1981), indirect fluorescent antibody technique (Choi *et al.*, 1975; Cho and Soh, 1976), ELISA (Cho *et al.*, 1981; Kim *et al.*, 1982; Soh *et al.*, 1985; Lee and Chang, 1986) have been developed. Among them, ELISA is now widely used and considered as a choice of test applicable to paragonimiasis. But common antigen in crude extract of *P. westermani* makes cross reaction with antibody in sera of patients infected with taxonomically similar parasites (Hunter *et al.*, 1958; Sadun *et al.*, 1959; Hillyer *et al.*, 1983). In order to increase the sensitivity and specificities of serodiagnosis of paragonimiasis and to minimize the cross reactivity with other parasitic infections, efforts have been made to purify the antigens of *P. westermani*

(Sadun *et al.*, 1959; Kim *et al.*, 1983; Choi *et al.*, 1986).

Recent studies on purification of *P. westermani* antigen have shown that some fractionated antigens were more sensitive than the crude saline extracts but the specificity was not improved (Kim *et al.*, 1983). Furthermore, complete isolation of species-specific antigenic protein was practically difficult up to present because protein of the worm is much complicated in composition.

Enzyme-linked immunoelectrotransfer blot technique(EITB) has become a powerful tool for the immunochemical characterization of complex antigenic mixtures when evaluated using polyclonal and monoclonal antibodies (Tsang *et al.*, 1983). EITB combines the high resolving power of gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) and the high sensitivity of the enzyme-linked immunosorbent assay to produce an extremely powerful qualitative tool for studying antigen-antibody pairs. This procedure has a possibility of testing a serum against any number of antigens present in a preparation.

The present study was performed to determine the antigenic profile of crude *P. westermani* adult worm antigen which reacts with the antibody from patients infected with *P. westermani* based on SDS-PAGE and immunoblotting techniques. In addition, cross reactive components of antigenic fractions against sera of patients with clonorchiasis or cestode infections were demonstrated.

MATERIALS AND METHODS

1. Antigen

Saline extract of 24-week old *P. westermani* was used as antigen. Briefly, the worms were homogenized with PBS(pH 7.2) containing 0.05 mM PMSF(phenyl methyl sulfonyl fluoride). The homogenate was extracted at 4°C for 2 hrs and centrifuged at 10,000 rpm for 60 min. The supernatant was stored as small alliquots at −40°C until used. Protein content was 12.6 mg/ml quantitated by the Bradford technique.

2. Serum specimens

Infected sera were obtained from Korean patients of parasitologically confirmed (eggs observed in sputum) paragonimiasis. The serum was stored at −40°C until used. Other helminthic infection sera included samples from patients with following diseases; clonorchiasis, cysticercosis, taeniasis, and diphyllobothriasis. The sera were selected for their cross reactivities to *P. westermani* crude extract antigen.

Healthy controls were the sera from apparently healthy students of Korea University and staff members of laboratory who had no history of paragonimiasis, clonorchiasis, or tapeworm infection.

3. SDS-PAGE

SDS-PAGE was performed as described by Tsang *et al.*(1983) with slight modification. Buffers were similar to system J 4179 of Jovin *et al.*(1979) as modified by Neville *et al.*(1971). All SDS-PAGE chemicals including molecular weight markers were obtained from Bio-Rad Laboratories. Gels were casted in a Pharmacia GSC-2 slab gel casting apparatus and run in a 160×200×0.8 mm vertical slab system from Pharmacia Fine Chemicals. Antigen samples in K^+-free buffers were treated with a 2.5% final concentration of SDS. SDS(10%), 9 M urea, in 0.01 M Tris-HCl, pH 8.0 solution was used as the buffer.

A sufficient amount of the 10% SDS solution was added to protein to give a final concentration of 2.5% SDS and 0.2 μg/μl protein in the final treated sample. Tracking dye was added at 10 μl per 100 μl of treated sample. Samples were then heated at 65°C for 30 min in a water bath. The gel system contained a gradient resolving gel(3.0% to 20%) and a 3% stacking gel. SDS protein complexes were stacked onto the stacking gel at 10 mA and increased to 20 mA when all samples have entered the stacking gel. Electrophoresis was continued until the bromophenol blue dye reached the bottom of the resolving gel. Volume of sample application was 10 μl for each 5.0

mm width sample lane. SDS-PAGE gels were stained with the silver stain of Merril *et al.* (1981) with minor modifications.

4. Enzyme-linked immunoelectrotransfer blot (EITB)

Resolved protein fractions were transferred to nitrocellulose paper as described by Tsang *et al.* (1983).

Electrotransfer of SDS complex was performed for 1 hr at 250 Vdc constant (current 0.5~2.0 A) using transblot cell from Bio-Rad Laboratories. The nitrocellulose sheet was cut into 0.5 cm strips and exposed to diluted antibody solutions overnight with agitation on a rotary shaker using slotted incubation tray. Then, strips were exposed to peroxidase-conjugated anti-human IgG rabbit serum(Dako Corp.) at 1 : 2,000 dilution in PBS-0.3% Tween for 1.5 hrs. The processed strips were then exposed to the substrate solution(50 mg of 3,3′-diaminobenzidine, Sigma and 10 μl of H_2O_2 30% in 100 ml of PBS, pH 7.2) for 5~10 min until positive reaction bands appeared.

RESULTS

SDS-PAGE: Analysis of the crude extract from *P. westermani* adult worm disclosed a complex protein band pattern ranging from 229 to 10 kDa by extrapolation in relative molecular weight. As shown in Fig. 1, at least 26 bands were found. Among them, 229, 112, 91, 85, 67, 65, 60 kDa, 6 fine bands between 56 and 45 kDa, 41 kDa, 8 fine bands between 36 and 18 kDa, 17, 15, 11 and 10 kDa components were prominent bands.

EITB (Western blot): Nitrocellulose strips blotted with *P. westermani* adult worm antigens when incubated with the serum of 10 human cases infected with *P. westermani* showed multiple bands with molecular weight of 229, 91, 60, 50, 35~31, 27, 25, 21, 17, 11 and 10 kDa(Table 1 and Fig. 2). Three components of 112, 85 and 19 kDa reacted with serum antibody of 4 among 5 cases tested. The component 67 kDa reacted with sera of 3 cases only.

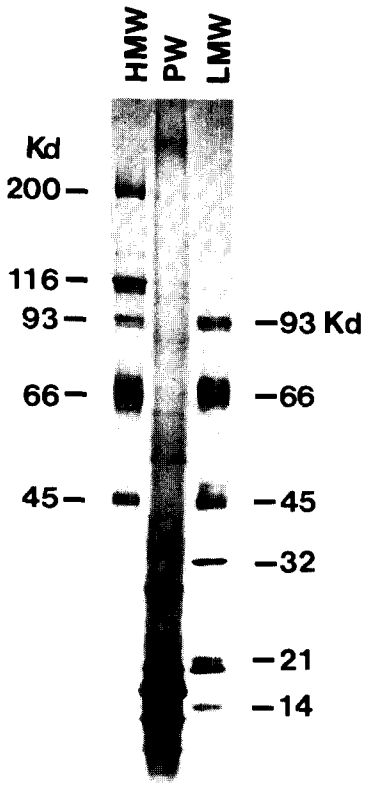

Fig. 1. Silver-stained SDS-PAGE of *P. westermani*. The two outside lanes(HMW, LMW) contained molecular weight protein standards given in kilodaltons.

When the strips were incubated with the sera of patients with *Clonorchis sinensis* infection which showed positive reaction to *P. westermani* antigen in ELISA, bands of molecular weight 19 kDa and 11 kDa, were seen in one case, and 17 kDa was seen in 2 cases (Table 1 and Fig. 2). Cross reactions by EITB were also observed with sera from patients with cysticercosis. Components of 229, 27, 25 and 17 kDa showed positive reaction with sera of cysticercosis patients. One patient serum infected with *Diphyllobothrium latum* recognized antigenic band of 17 kDa. One band with molecular weight of 50 kDa was obtained with normal healthy control sera.

As shown in Table 1, components of 91, 60,

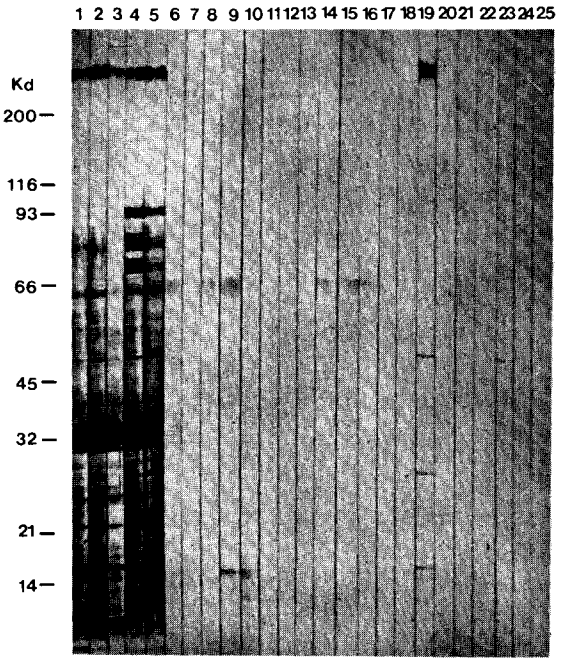

Fig. 2. Location of antibody binding bands of soluble extract of adult *P. westermani*. Serum from patients infected with following parasites were investigated. 1~5 : paragonimiasis, 6~10 : clonorchiasis, 11~13 : diphyllobothriasis, 14~16 : taeniasis, 17~19 : cysticercosis, 20~24 : non-infected sera, 25 : PBS.

21 and 10 kDa bands bound antibodies from all patients of *P. westermani* infection investigated and no cross reactions were observed.

DISCUSSION

Since 1981, ELISA has been used for the serodiagnosis of paragonimiasis. Many reports on the serodiagnosis of paragonimiasis revealed that the sensitivity of ELISA is superior to any other methods (Cho *et al.*, 1981; Kim *et al.*, 1982; Jin *et al.*, 1983). Antigens used in immunodiagnosis of paragonimiasis were usually crude soluble extracts of the adult worm (Hillyer *et al.*, 1983), and generally shared common antigen with other helminths. In Korea, cross reaction with *C. sinensis* was a problem for the serodiagnosis of paragonimiasis because the prevalence rate of *C. sinensis* infection is high (Kim *et al.*, 1982; Choi *et al.*, 1984). To solve this problem many efforts such as comparison of various antigens (Lee, 1986), use of egg antigen(Kim *et al.*, 1986), *etc.* has been performed. Lee and Chang (1986) made an affinity purified antigen, but it was impossible to obtain antigens completely free from cross reaction because of the complex protein composition of *P. westermani*.

In this paper, SDS-PAGE and EITB techniques were used to separate and identify antigenic parasite molecules binding to immunoglobulins of sera from patients with homologous and heterologous parasite infections.

Analysis of crude extract revealed a complex protein bands ranging from 229 to 10 kDa. Lee (1986), Lee and Chang(1986) reported that 22 bands were identified in *P. westermani* adult worm antigen by SDS-PAGE. The molecular weights of antigens were in the range of 66,100 to 10,000 Da and the main protein bands were distributed below 30,000 Da. Among the 22 protein bands identified, 27,600, 17,400~14,800, 11,500 Da bands were stained prominently. The results obtained were basically similar to those of the present study although the molecular weights of the bands were not exactly same.

Choi *et al.*(1986) reported that two fractions, molecular weight of 220 kDa and 30 kDa by SDS-PAGE were strongly sensitive antigen. The relationship between the molecular weight described by Choi *et al.* (1986) and in the present study is uncertain. However, although we accept that the bands of 220 kDa and 30 kDa were the identical ones to 229 kDa and 31~34 kDa bands of our study, these bands may not be species-specific ones (Table 1). As shown in Table 1, 229 kDa bands reacted with sera of cysticercosis patients and 31~35 kDa bands reacted with sera of patients with *C. sinensis*. As expected, cross reactions were observed with sera from patients infected with *C. sinensis*. Among the strongly sensitive bands that appeared in immunoblot and ELISA, only 4 bands were not reactive with sera from

patients with other helminthic infections investigated. In addition, these 4 components(91, 60, 21 and 10 kDa relative molecular weights) are strongly positive for 5 homologous sera investigated without exception. It seems that one of these 4 components can be used as a diagnostic band for the detection of antibody in patients with *P. westermani*.

Itoh and Sato (1988) reported that 27 kDa and 50 kDa components of *P. westermani* were major components detected by sera of *P. westermani*-infected hosts, and that 27 kDa component cross-reacted with *P. miyazakii*-infected sera, but the 50 kDa component was detected only by *P. westermani*-infected sera. Recently, Slemenda *et al.* (1988) reported that immunoblots with *P. westermani* antigen probed with pooled human sera of egg-proven *Paragonimus* infections revealed many bands including a significant antibody response to an approximately 8 kDa component. Based on the results of all serum samples tested, their immunoblot sensitivity was 96% and the specificity was 99%. Unfortunately, 8 kDa component was not identified in our blot. Anyway, it is possible that future investigation will show that one of the 4 components in our immunoblot can be used as a diagnostic band.

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=국문초록=

Immunoblot를 이용한 폐흡충 조항원의 특이 항원대의 증명

고려대학교 의과대학 기생충학교실 및 열대풍토병연구소

주경환 • 안 혁 • 정명숙 • 임한종

폐흡충증의 진단은 일반적으로 객담에서 충란을 검출하는 것이 가장 확실한 진단법으로 간주되고 있지만 이소기생의 경우는 물론, 본 기생충의 감염자 및 감염 강도의 감소로 인해 실제 폐에 기생한 경우에 있어서도 객담 검사의 민감도가 매우 낮아져 혈청학적 검사의 필요성이 점차 대두되고 있다. 따라서 식염수 추출액을 항원으로 하여 효소면역 측정법을 실시하는 방법으로 면역 진단을 실시하고 있으나 조항원의 단백 구성이 복잡하여 간흡충을 비롯한 몇몇 윤충류 감염자 혈청에서 교차 반응이 야기되고 있다. 그동안 정제 항원을 만들고자 하는 시도가 많았음에도 불구하고 특이 항원의 분리는 실제로 불가능하였다. 이와 같은 문제점을 보완하기 위하여 폐흡충 조항원을 SDS-PAGE로 전기영동한 다음 EITB를 이용하여 항원대별 항원성 및 특이성을 관찰하여 폐흡충 조항원의 폐흡충증 혈청에 대한 특이 반응대를 확인해 보고자 하였다.

실험에 사용된 항원은 실험적으로 개에 감염시켜 24주만에 얻은 폐흡충 성충의 식염수 추출액이며 3~20%의 linear gradient gel에서 SDS-PAGE하였다. Silver 염색한 결과 229 kDa~10 kDa 사이에서 26개의 항원대를 구성하고 있었으며 주요 항원대에 대하여 EITB를 실시한 결과 폐흡충 감염자 혈청은 229, 91, 60, 50, 35~31, 27, 25, 21, 17, 11 및 10 kDa의 분자량을 갖는 항원대와 반응하였다. 간흡충 감염자 혈청은 35~31, 19, 11 및 17 kDa의 항원대와 반응하였고 낭미충 감염자 혈청은 229, 35~31, 27, 25 및 17 kDa의 항원대와 반응하였다. 광절열두조충 1예도 17 kDa의 항원대와 반응하였다. 따라서 91, 60, 21 및 10 kDa의 항원대 중 일부가 폐흡충 조항원에 있어서 폐흡충에 대한 종특이 항원대로 생각되었으며 향후 immunoblot을 이용한 폐흡충증의 면역진단에 이용될 수 있는 항원대로 간주할 수 있었다.