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Immunoblot observation of antigenic protein fractions in *Paragonimus westermani* reacting with human patients sera

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Abstract: In order to observe the antigenic fractions in saline extract of adult *Paragonimus westermani*, proteins in the crude extract were separated by sodium dodecyl sulfate-polyacylamide gel electrophoresis(SDS-PAGE) in reducing conditions. The separated protein fractions were transferred to nitrocellulose paper on which 20 sera from human paragonimiasis were reacted and immunoblotted. Out of 15 stained protein bands in SDS-PAGE, 7 reacted with infected sera while 8 did not. Additionally, 7 unstained protein bands in SDS-PAGE reacted with the sera. Of 14 reacted bands, 30 kilodalton(kDa) band was the most frequently reacted (95%) and was a strong antigen. Protein bands of 23 and 46 kDa were also strong antigens. Bands of over 150 kDa, 120 kDa, 92 kDa, 86 kDa, 74 kDa, 62 kDa, 51 kDa, 32 kDa, 28 kDa, 16.5 kDa and 15.5 kDa were also reactive but their frequencies of the reaction were variable.

Key words: *Paragonimus westermani*, human paragonimiasis, antigenic proteins, SDS-PAGE/immunoblot

INTRODUCTION

Since 1970s the incidence of human paragonimiasis has been lowered throughout Korea. And introduction of praziquantel for paragonimiasis made its treatment even more effective and simple (Rim *et al.*, 1981). These desirable development provided unexpected difficulty because correct diagnosis of uncommonly occurring human paragonimiasis were at least delayed or even neglected. Diagnostic priority of paragonimiasis was lowered in lung diseases in general. In addition, sensitivity of egg detection and specificity of intradermal test were concomitantly lowered (Cho *et al.*, 1983). In this context, detection of specific antibody in patient sera is now replacing classical approaches to its diagnosis. Many serodiagnostic techniques such as complement fixation test, double diffusion, counterimmunoelectrophoresis, indirect fluorescent antibody test, and enzyme-linked immunosorbent assay(ELISA) have been applied in paragonimiasis. Of them, ELISA is now widely used in detecting the specific IgG antibody in sera (Cho *et al.*, 1981 & 1983; Lee and Choi, 1983; Yokogawa *et al.*, 1983; Kojima *et al.*, 1983).

In serodiagnosis by ELISA, cross reactions may occur in other parasitic diseases (Cho *et al.*, 1981). Therefore sensitive and specific protein components for diagnostic purpose have been searched for. Choi *et al.* (1986) reported that highly antigenic proteins in *P. westermani* were 196~270kDa and 30 kDa when analysed by SDS-PAGE. By immunoaffinity chromatography of the crude extract using infected cat sera as ligand, Lee and Chang (1986) reported that

protein bands of 36.4, 34.7, 27.6 and 11.5 kDa were isolated. By SDS-PAGE/immunoblot, Chung *et al.* (1987) reported that 181, 73, 63, 45, 31~27, 21, 18~16, and 15 kDa protein bands in *P. westermani* extract were reacted with a certain number of patients sera.

In this study, the antigenic protein fractions of *P. westermani* were observed by SDS-PAGE and immunoblot using sera from confirmed paragonimiasis patients.

MATERIALS AND METHODS

1. Antigen

Saline extract of 13-week old adult *P. westermani* was used as antigen. The crude extract was prepared as described by Cho *et al.* (1981).

2. Human sera

A total of 20 sera from confirmed human paragonimiasis patients was used. The patients were a part of those referred from teaching hospitals in Seoul to Department of Parasitology, Chung-Ang University from September 1982 to January 1988. By ELISA all patients showed specific IgG antibody levels above 0.50 (positive criterion; abs. 0.25 or above). Four of 20 patients were confirmed as paragonimiasis by egg detection. The sera were frozen at −40°C and thawed simultaneously when tested.

3. SDS-PAGE

Methods of Laemmli (1970) were applied. Chemicals including marker proteins were obtained from Sigma Chemicals(USA). Vertical electrophoresis system of 17×12 cm was used. Linear gradient gel of 10~15% polyacrylamide was prepared to resolve proteins (Cho *et al.*, 1987). Saline extract of *P. westermani* (1.0 mg/ml of protein as measured by Lowry *et al.*, 1951) was heat-treated at 95°C for 5 minutes with the same amount of sample buffer (containing reducing agent, 10% 2-mercaptoethanol). A total of 250 μl of the saline extract was applied to a wide well in stacking gel and electrophoresed at 30 mA for 3~4 hours to reach dye front of bromphenol blue to buttom of separating gel. Protein bands were stained in 0.125% Coomassie brilliant blue R-250 solution.

4. Immunoblot

Methods of Tsang *et al.* (1983) were adopted. Resolved protein bands in gel were transferred to nitrocellulose paper by electrophoresis (at 100v for 2 hours). The paper was divided longitudinally into 5 mm wide pieces. The paper pieces were washed 3 times with phosphate buffered saline (pH 7.4)/0.5% Tween 20 each for 5 minutes. Then 5 ml of 1 : 100 diluted patient serum was reacted to each piece; after washing, 5 ml of 1 : 2,000 diluted conjugate (peroxidase-conjugated antihuman IgG, Cappel, USA) was reacted for 1 hour. After washing, the reaction sites were coloured by reacting substrate (50 mg of 3, 3′-diaminobenzidine, 10 μl of 30% H_2O_2 and 100 ml of distilled water) for 15 minutes. The reaction was stopped by washing paper with distilled water.

RESULTS

1. Protein bands separated in SDS-PAGE

As shown in Fig. 1, saline extract of *P. westermani* was resolved into 15 protein bands

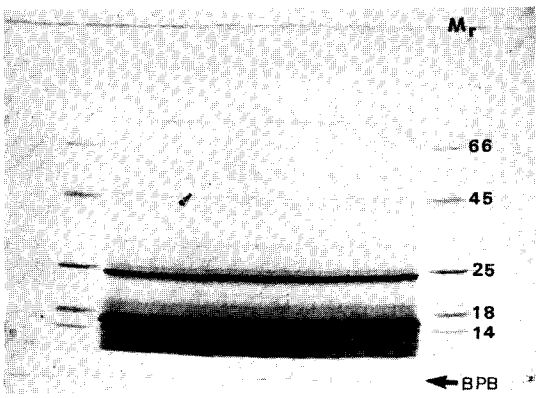

Fig. 1. Findings of SDS-PAGE of saline extract of adult *P. westermani*. SDS-PAGE was done in 10~15% linear gradient gel. Antigen was heat-treated with sample buffer containing 10% 2-mercaptoethanol. Coomassie brilliant blue R-250 stained.
M_r: Molecular weight of marker proteins in kDa.
BPB: Dye front of bromphenol blue.

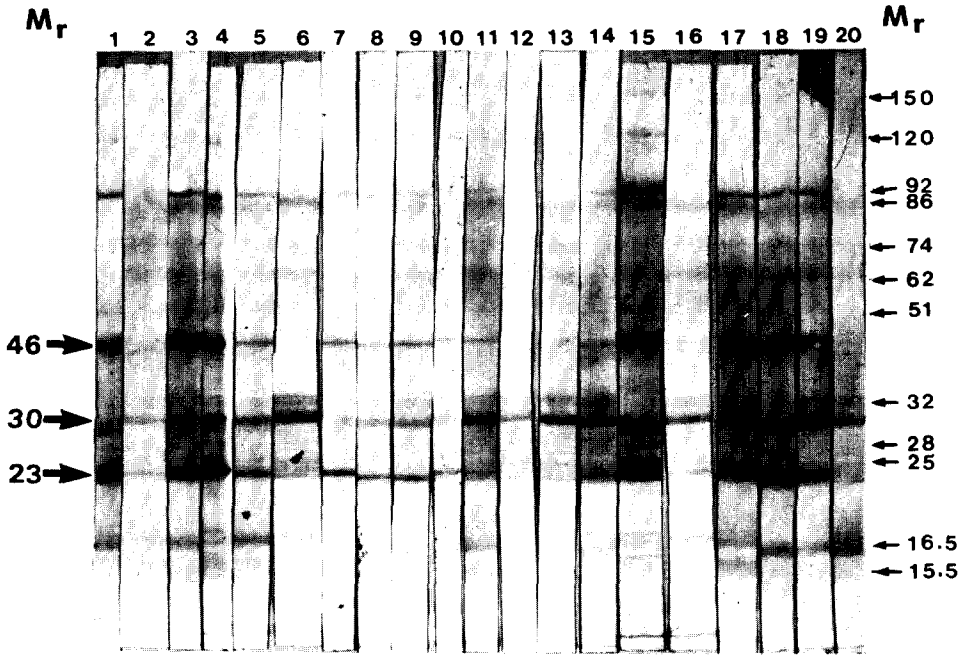

Fig. 2. SDS-PAGE/immunoblot findings in 20 active human paragonimiasis patients. M_r: Molecular weight in kDa.

in reducing SDS-PAGE when stained with Coomassie brilliant blue R-250. The bands were 80, 51, 46, 45, 30, 25, 23, 21, 19, 16.5, 15.5, 14.3, 13, 12, and 10 kDa respectively. Of them, 23, 16.5, 14.3, 13, 12 and 10 kDa were major bands. The remaining bands were stained faintly.

2. Protein bands reacted with patients sera

As shown in Fig. 2, serum from each patient of paragonimiasis reacted in different combination of protein bands. A total of 15 bands was reacted to polyclonal antibodies in patients sera. Of them, 3 bands of 46, 30 and 23 kDa were major reacting bands. Out of them, band of 46 kDa showed reaction in 14 patients (70%), 30 kDa in 19 patients(95%) and 23 kDa in 16 patients (80%). Patients sera which reacted to 46 kDa exhibited concomitant reaction to 23 kDa. But strong reaction at 30 kDa did not necessarily associate with reaction to either 23 or 46 kDa bands.

Of other reacting bands, band of over 150 kDa was reacting in 2 patients(10%), band of 120 kDa was in 4 patients (20%), 92 kDa in 13 patients (65%), 86 kDa in 12 patients (60%), 74 kDa in 9 patients (45%), 62 kDa in 15 patients (75%), 51 kDa in 7 patients (35%), 32 kDa in 12 patients(60%), 28 kDa in 4 patients (20%), 25 kDa in 1 patient (5%), 16.5 kDa in 15 patients(75%), 15.5 kDa in 6 patients(30%). These bands were either faintly stained or unstained ones in Coomassie staining (bands of over 150, 120, 92, 86, 74, 62, 32, 28 kDa) or weak reactions in immunoblot (bands of 51, 25, 16.5, and 15.5 kDa).

Out of stained bands, 80, 45, 25, 19, 14.3, 13, 12 and 10 kDa bands were not reacting with any patient serum.

DISCUSSION

By Coomassie staining of SDS-PAGE, 15 protein bands were identified in saline extract of *P. westermani*. After immunoblot, however, as many as 7 bands were additionally reacting

with human sera. And 8 stained bands were non-reacting. Therefore, out of a total of 22 bands in the crude extract, 15 bands were reacting with patients sera.

Of the bands, 30 kDa band was evidently the strongest antigen both in number of reacting patients sera and in their reaction intensity. The 30 kDa band was faintly stained in SDS-PAGE but its relative antigenicity was evidently strong. Protein bands of 23 and 46 kDa were also strong antigen in intensity of immunoblot. Interestingly, the reaction intensity was higher in 23 kDa than in 46 kDa. Furthermore, in SDS-PAGE (Fig. 1), band 23 kDa was major band while 46 kDa was faintly stained. These relations suggested that these bands were subunits of an original protein.

Protein bands of 92, 86, 74, 62, 32, and 16.5 kDa were also reacted with patients sera frequently except the above 3 bands. Out of these proteins, band of 16.5 kDa only was stained in SDS-PAGE. Therefore, the significance of these weakly reacted protein bands is hardly explainable at the present moment.

Our results were coincided with that of Choi *et al.* (1986) in which 30 kDa protein was reported to be strongly sensitive antigen. Another protein in their report, 220 kDa was not recognized in the present study. Molecular weights of the reacting bands in our study were not exactly the same as those reported in Lee and Chang(1986) and Chung *et al.* (1987) but reactions in bands of similar molecular weight were observed. Standardized measurement of molecular weight in antigenic proteins are necessary in this respect.

Kim *et al.* (1983) reported that secretory-excretory antigen from adult *P. westermani* was more antigenic than crude extract when analysed with infected human sera. Sugiyama *et al.* (1987) reported that infected rats with *P. westermani* produced specific antibody against 27 kDa protein band. The protein was localized at intestinal epithelium of the juvenile worms when indirect immunoperoxidase staining was done. These results suggested that intestinal enzymes from the worm are actually strong antigen. In this respect, Nam *et al.* (1987) reported that cysteine protease of *P. westermani* was 70~80 kDa protein when purified through Sephadex G-100. Similar study on cysteinyl proteinase of *P. westermani* by Song *et al.* (1987) reported that proteins of molecular weight 70 kDa, 35 kDa and 21 kDa had peak enzyme activities. Again, in terms of molecular weight of enzymes, the results are not exactly identical but confusing. But it deserves further studies on the relations between parasite enzymes and their role as antigens.

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=국문초록=

폐흡충 항원단백질에 대한 폐흡충증 환자 혈청의 반응 양상

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폐흡충의 생리식염수 추출액을 항원으로 효소면역측정법을 실시하여 특이 IgG항체 활성도를 측정하면 폐흡충증 현증 환자를 민감하고도 특이하게 감별할 수 있다. 진단에 사용하는 생리식염수 추출액은 조항원(粗抗原)이므로 그 구성단백질중 환자 혈청내 특이항체와 반응하는 단백질항원이 어느 것인지에 대해서는 아직도 논란의 대상이 되고 있다. 조항원 중 특이항원을 정확히 판단하면 드물게 나타나는 교차반응의 기전을 이해하는데에도 도움이 될 뿐만 아니라 종특이 단일 단백질 항원을 제작할 수 있게 하는 데에도 기초가 된다.

이 연구는 폐흡충 성충의 생리식염수 추출액 중 폐흡충증 환자 혈청과 반응하는 단백질 분획을 관찰하기 위하여 실시하였다. 개에 실험적으로 감염시킨지 13주째에 얻은 폐흡충 성충의 생리 식염수 추출액을 10~15% linear gradient gel에서 환원조건하의 SDS-PAGE를 실시하였다. Coomassie 염색결과 단백질 대(帶) 15개를 판별할 수 있었고 그 분자량은 80, 51, 46, 45, 30, 25, 23, 21, 19, 16.5, 15.5, 14.3, 13, 12 및 10 kDa이었다. 그중 23, 16.5, 14.3, 13, 12 및 10 kDa가 진하게 염색되는 주(主) 단백질대이었다.

활동성 폐흡충증으로 확진한 환자 20명의 혈청을 효소면역전기영동이적법(immunoblot)으로 분리된 단백질대에 반응시켰을 때에 46, 30 및 23 kDa 단백질에 가장 가장 강하게 반응하였다. 그 중에서도 30 kDa는 가장 많은 환자 혈청과 반응하였고 가장 강한 반응이 나타났다. 그리고 46 kDa 및 23 kDa에서도 강한 반응이 있었다.

이상의 단백질대 3개 이외에도 150 kDa 이상, 120 kDa, 92 kDa, 86 kDa, 74 kDa, 62 kDa, 51 kDa, 32 kDa, 28 kDa, 25 kDa, 16.5 kDa 및 15.5 kDa 단백질대에서도 반응이 있었다.