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Immunoelectrophoretic analysis of major component proteins in cystic fluid of *Taenia solium* metacestodes*

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Abstract: When cystic fluid of *Taenia solium* metacestodes (CF) was filtrated through Sephacryl S-300 Superfine, major proteins were in fractions III and IV. Major protein in fraction III was Band C protein of 150 kDa and that in fraction IV was Band N protein (Choi *et al.*, 1990). When CF was electrophoresed in 0.9% agarose gel and reacted with anti-CF rabbit serum (RACF), two main bands, a long outer and a short inner band, were precipitated, together with 8 minor bands. RACF reacted with fraction III forming the long outer band whereas RACF formed the short inner band with fraction IV in immunoelectrophoresis (IEP). The long outer precipitin band of CF fraction III was similar to antigen B in hydatid fluid (HF) of Oriol *et al.* (1971), while the short inner band of CF fraction IV was similar to HF antigen 5 of Capron *et al.* (1967). When HF was reacted with RACF, the short inner band was immunoprecipitated without forming the long outer band. Common antigenicity between CF and HF seemed to exist in fraction IV rather than in fraction III of CF. Patient sera of neurocysticercosis reacted more frequently with fraction III than with fraction IV.

Key words: *Taenia solium*, *Cysticercus cellulosae*, *Echinococcus granulosus*, cystic fluid, hydatid fluid, component protein, immunoelectrophoresis

INTRODUCTION

In serological diagnosis of cysticercosis, CF has been recognized to be more sensitive and specific than parenchymal extract (Choi *et al.*, 1986; Larralde *et al.*, 1986; Bailey *et al.*, 1988; Hayunga *et al.*, 1991). As much as 90% of neurocysticercosis can be diagnosed as specific antibody positive when both serum and CSF are tested by ELISA (Cho *et al.*, 1986). Patients infected with completely calcified or with a few worms exhibited low specific antibody levels below the cut-off absorbance (Chang *et al.*, 1988). Even though sensitive antigen of CF is used, cross-reactions with sera of sparganosis, paragonimiasis and hydatidosis are elicited (Cho *et al.*, 1986; Schantz and Gottstein, 1986; Kong *et al.*, 1989). To overcome non-specific cross-reactions, especially those with hydatidosis, the nature of antigenic proteins in CF and HF should be elucidated.

The major component proteins in HF, antigen 5 and antigen B, have been well studied (Capron *et al.*, 1967; Oriol *et al.*, 1971; Schantz and Gottstein, 1986; Shepherd and McManus, 1987). But the composition and properties of antigenic

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proteins in CF have only been limitedly reported. By non-denaturing disc-PAGE, Choi *et al.* (1986) observed that CF was composed of at least 5 protein bands. Of them, the Band C protein was purified by immuoaffinity chromatography (Kim *et al.*, 1986). Molecular mass of the Band C protein was 150 kDa and composed of 3 subunits of 15, 10 and 7 kDa either in reducing SDS-PAGE (Cho *et al.*, 1988) or in non-reducing SDS-PAGE (Choi *et al.*, 1990). This protein was heat resistant. In spite of the physical similarity with antigen B in HF, Band C protein in CF did not react crossly with hydatidosis sera when observed by SDS-PAGE/immunoblot (Maddison *et al.*, 1989; Kong *et al.*, 1989; Tsang *et al.*, 1989).

Another major protein in CF was recognized by gel filtration through Sephacryl S-300 Superfine (Choi *et al.*, 1990). Out of 6 protein fractions, the third fraction was composed mainly of the Band C protein of 150 kDa whereas the fourth fraction was composed mainly of a protein previously unrecorded. In disc-PAGE, the protein band was dispersed broadly. The protein was thus designated as Band N protein. Fraction IV was separated into 64, 21, 18 and 15 kDa subunits in reducing SDS-PAGE and 64, 46~44 and 26~21 kDa in non-reducing SDS-PAGE (Choi *et al.*, 1990).

On the nature of antigenic component proteins in CF, more studies are necessary not only to understand the cross-reactivities with other parasitic diseases but also to find biochemical and immunological properties of major antigenic proteins. In this study, we examined precipitin band patterns of CF and its major fractions in IEP, to characterize the major component proteins in CF, using an immunized serum and patients sera.

MATERIALS AND METHODS

1. Cystic fluid and parenchymal extract of *T. solium* metacestodes and other antigens

Cystic fluid(CF) and parenchymal extract were prepared as Choi *et al.* (1986) described. Unruptured cysts of *T. solium* metacestodes were harvested from muscles of naturally infected pigs. After washing with physiological saline 3 times, cysts were punctured individually to collect CF. Uncontaminated CF was centrifuged at 10,000 *g* for an hour. For preparation of parenchymal extract (CP), empty cysts, from which CF had been removed, were homogenized with physiological saline using teflon-pestle tissue homogenizer. The emulsion was stored overnight and centrifuged at 10,000 *g* for an hour. The resulting supernatant was regarded as the parenchymal extract. Protein content of CF was 5.3 mg/ml and that of CP was 3.8 mg/ml when measured by the method of Lowry *et al.* (1951). All procedures from cyst collection to extract preparation were done at 4°C. They were stored at −70°C until used.

Hydatid fluid(HF) was secured in surgery, from a Korean patient of imported hepatic hydatidosis (Huh *et al.*, 1988). Sparganum antigen used in the absorption test (see below) was prepared as Kim *et al.* (1984) described.

2. Fractionation of CF by gel filtration

CF was fractionated as described by Choi *et al.* (1990) using 1.6(ϕ)×70 cm long Sephacryl S-300 Superfine. The flow rate was 20 ml/cm^2·hour. Eluent was 0.15 M phosphate buffered saline (PBS) containing 0.001% merthiolate. Eluents of 35 drops (1.75 ml) were allocated in 80 tubes using Multirac 2111 Fraction Collector (LKB). Eluents were divided into 7 fractions according to absorbance at 280 nm. Each fraction was dialyzed against Tris-HCl buffer (5 mM, pH 7.4) and lyophilized.

3. Antisera

(1) Rabbit antiserum against CF(RACF): Two rabbits were injected subcutaneously with 100 μg of CF mixed in Freund's complete adjuvant. CF mixed in Freund's incomplete adjuvant was boostered twice in 2-week interval. Four days before the bleeding by heart puncture, 10 μg CF was injected intravenous route. Antibody levels in antiserum was measured by ELISA.

(2) Human sera of neurocysticercosis: A

total of 12 human cysticercosis sera was used in IEP against CF, its fractions and CP. The sera were selected out of antibody positive patients of neurocysticercosis. Their anti-CF antibody (IgG) levels ranged from absorbance 1.52 to 0.81 when measured by ELISA (Cho *et al.*, 1986).

4. ELISA, disc-PAGE and SDS-PAGE/immunoblot

ELISA methods described by Choi *et al.* (1986) were followed. The antigens in protein concentration of 2.5 μg in carbonate buffer (pH 9.6) were coated on EIA plate (Costar, U.S.A.). RACF or neurocysticercosis patients sera diluted in 1 : 100 in PBS were reacted for 2 hours at 36°C. 1 : 1,000 diluted peroxidase-conjugated anti-rabbit or anti-human IgG goat IgG (heavy- and light-chain specific, Cappel, U.S.A.) were reacted. The color was developed with 1% *o*-phenylenediamine chromogen solution. Absorbance were read at 490 nm using ELISA reader (BioRad, M3550, U.S.A.).

For disc-PAGE of CF and its fractions, methods described by Choi *et al.* (1990) were used using 8% column gels.

In SDS-PAGE of CF and its fractions, 3% stacking gel and 10~15% separating gel were used in Laemmli system (1970). For reducing SDS-PAGE, CF was mixed with the same amount of the sample buffer containing 10% 2-mercaptoethanol and 0.4% SDS. After heating the mixture at 95°C for 5 minutes, electrophoresis was carried out. In non-reducing SDS-PAGE, the sample buffer without 2-mercaptoethanol was mixed with the samples. The samples were not boiled. Molecular mass marker proteins of Pharmacia Co. were used. After the SDS-PAGE, immunoblot was undertaken according to Tsang *et al.* (1983). The electrophoretically separated proteins in the gel were transferred to nitrocellulose paper (Bio-Rad, U.S.A.) by electrophoresis at 100 V for 2 hours at 4°C. 1 : 5,000 diluted RACF was reacted for 4 hours. Peroxidase-conjugated anti-rabbit IgG was reacted in 1 : 1,000 dilution. The reacteds band were stained with 0.02% (W/V) 3,3′-diaminobenzidine solution.

5. Immunoelectrophoresis (IEP)

IEP was undertaken on slide glasses of 9.5×8.4 cm (Pharmacia Co.) covered with 18 ml of 0.9% UltraPure agarose (BRL, U.S.A.) in borate buffer (μ=0.05, pH 8.6) (the LKB Manual, 1986). The wells were filled with 25 μl of concentrated CF (25 mg/ml of protein concentration), fraction III (11.4 mg/ml), fraction IV (9.8 mg/ml) or concentrated CP (25 mg/ml), respectively. After the electrophoresis at 20 mA constant current for 4 hours, troughs were cut out and filled with 2 fold diluted RACF or 2 fold concentrated patient sera. The slides were incubated overnight in humid chambers at 36°C. The troughs were washed with PBS for a week, dried and stained with 0.1% (W/V) amido black B (in 45% methanol and 10% acetic acid solution).

For absorption test, RACF (100 μl) was mixed in the same amount of crude sparganum extract (protein content; 5.4 mg/ml) and was used in troughs for CF or its fractions (Fig. 4). When tested with patient sera, absorption test was not done (Figs. 5 & 6).

RESULTS

1. Fractionation of CF

Fig. 1 showed the gel filtration result of CF through Sephacryl S-300 Superfine. This is a same result with that reported by Choi *et al.* (1990). CF was separated into 7 fractions according to absorbance peaks at 280 nm. As Choi *et al.* (1990) did, the crude CF, fraction III and fraction IV of CF were examined by disc-PAGE (Fig. 2), and SDS-PAGE/immunoblot (Fig. 3).

Fraction III was composed mainly of Band C protein with another distinct bands of U and B (Fig. 2). In SDS-PAGE/immunoblot (Fig. 3), subunits of 66, 22, 15, 10 and 7 kDa polypeptides either in reducing or non-reducing SDS-PAGE were reacted with RACF. On the other hand, the major proteins in fraction IV were Bands N and D (Fig. 2), which were composed

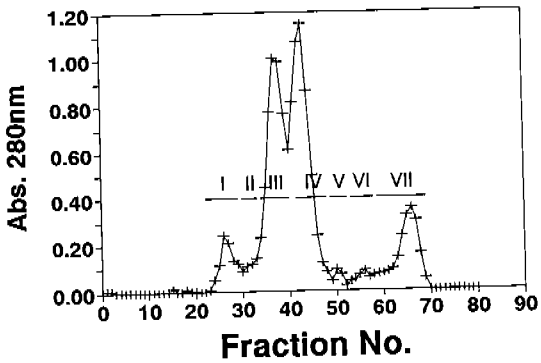

Fig. 1. Elution profile of CF through Sephacryl S-300 Superfine. The major fractions were fraction III and fraction IV.

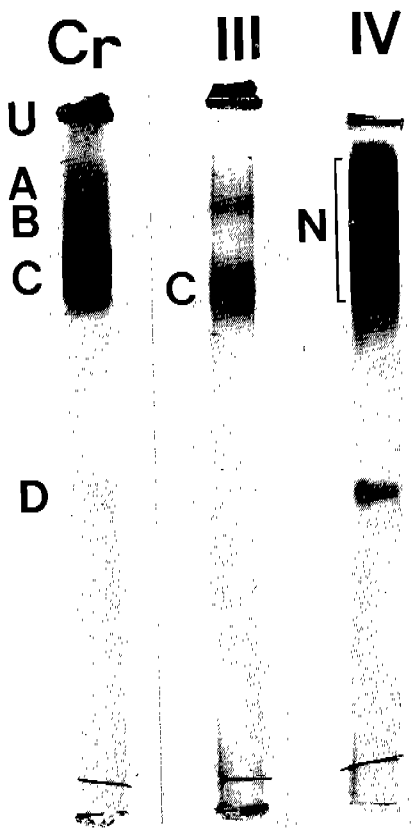

Fig. 2. Disc-PAGE findings of CF and its fractions in 8% gel. CF: Crude cystic fluid of *T. solium* metacestodes, III: fraction III, IV: fraction IV of CF. Letters at left border stand for the designated band names.

of 22, 18 and 15 kDa subunits in reducing condition and 44~46 and 21~26 kDa subunits in non-reducing condition (Fig. 3).

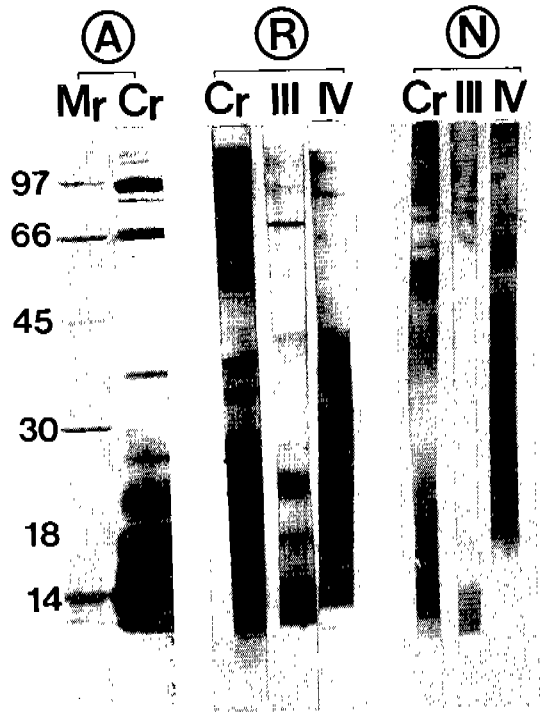

Fig. 3. SDS-PAGE/immunoblot findings of CF and its fractions using RACF as antibody. A: Molecular mass markers (Mr) and CF transfer blotted to nitrocellulose paper by electrophoresis and stained by amido black B (Cr). R: proteins in cystic fluid (Cr), Fraction III (III) and Fraction IV (IV) was separated by reducing SDS-PAGE and immunoblotted with RACF. N: CF, fraction III and IV were separated by non-reducing SDS-PAGE and immunoblotted with RACF.

2. IEP findings

(1) Reactions of RACF with CF and its fractions: Rabbit antiserum immunized with CF (RACF), showed at least 6 (before absorption) or 5 (after absorption test) precipitin bands when reacted with concentrated CF (Fig. 4). Of the precipitin bands, 2 were major bands; a long and outer precipitin band and a short and inner band. When RACF was reacted with fraction III of CF, at least 4 (before absorption) or 3 (after absorption) bands were recognized. The long, outer precipitin band was conspicuously formed with fraction III while short, inner band was formed less densely and more faintly than that with CF (Fr III, Fig. 4). RACF

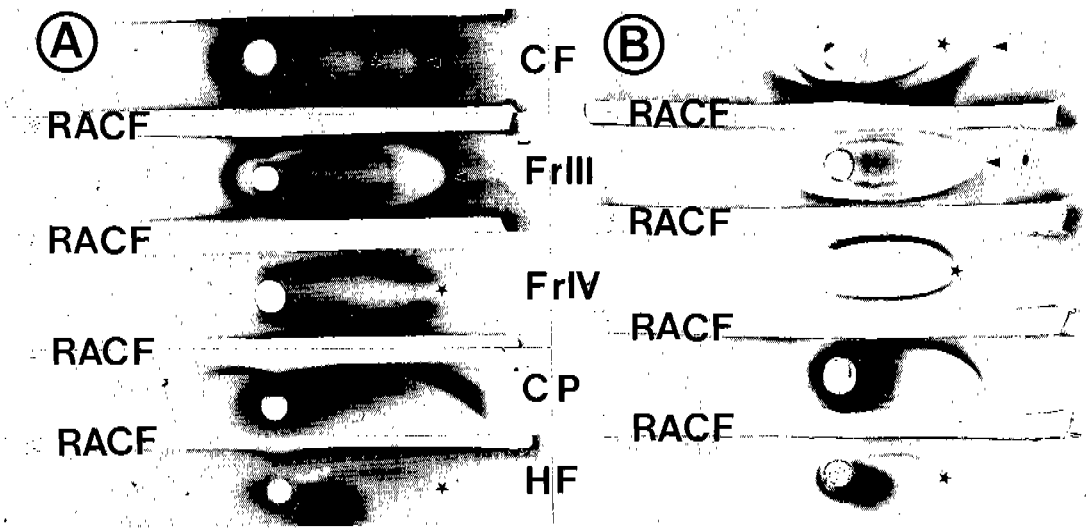

Fig. 4. Immunoelectrophoresis findings of CF and its fractions using RACF before absorption (A) or after absorption test (B). Troughs were filled with RACF. Antigens in the wells were shown as: CF, crude cystic fluid; Fr III, fraction III; Fr IV, fraction IV; CP, parenchymal extract of *T. solium* metacestodes; HF, hydatid fluid. ◀, Long outer precipitin bands; ★, short inner bands.

reacted with fraction IV proteins as shown in Fig. 4. Before absorption, fraction IV formed at least 3 bands while a single band was precipitated after the absorption. The long outer precipitin band was not formed but the short, inner band was precipitated.

Parenchymal extract of *T. solium* metacestode (CP) was reacting with RACF as shown in Fig. 4. Before absorption test, at least 11 bands were recognized; after absorption, 3 bands were formed. Either before or after the absorption, the pattern of precipitin bands was different from that with CF. HF reacted with RACF forming a short inner band (HF in Fig. 4).

(2) **Reactions of neurocysticercosis sera with CF and CP:** Eight sera of neurocysticercosis patients with absorbance (abs.) range of 0.81~1.52 by ELISA, which were markedly high antibody levels in view of our laboratory, were selected and concentrated 2 folds by dialysis-lyophilization. Reactions with concentrated CF antigen by IEP (Fig. 5) showed that all of 8 patient sera showed different degree of precipitin bands formation. The number of bands ranged from 1 to 7 by patient.

When the same sera were reacted with CP, 7 of 8 patients sera formed precipitin bands (Fig. 5) forming 1 to 9 precipitin bands in each patient.

(3) **Reactions of neurocysticercosis sera with CF fractions:** As shown in Fig. 6, 4 neurocysticercosis sera with high abs. of antibody by ELISA were reacted with concentrated antigens of fraction III and fraction IV separately, to compare the relative frequency of precipitin band formation. Fraction III formed 1~4 bands with all of the 4 sera. But fraction IV showed precipitin band formation in 2 out of 4 sera forming 2 or 3 bands.

DISCUSSION

Major proteins in CF were analysed immunoelectrophoretically using the fractionated proteins such as fraction III and fraction IV. As shown in Fig. 4, the major proteins in the fraction III formed the long outer precipitin band and major protein in fraction IV made the short inner band.

When fraction III of CF was reacted with

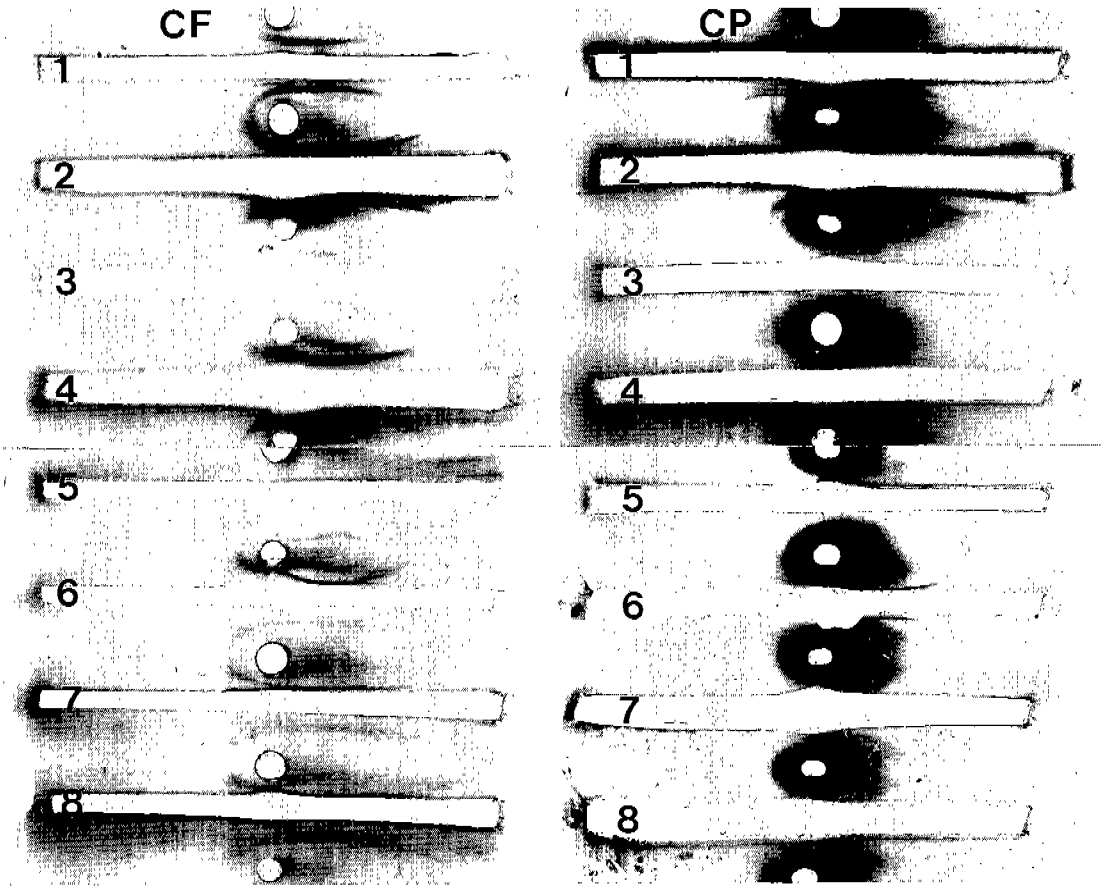

Fig. 5. Comparative findings of immunoelectrophoresis using CF and CP as antigens and reacted with patient sera of neurocysticercosis. Numbers at troughs mean different patient sera.

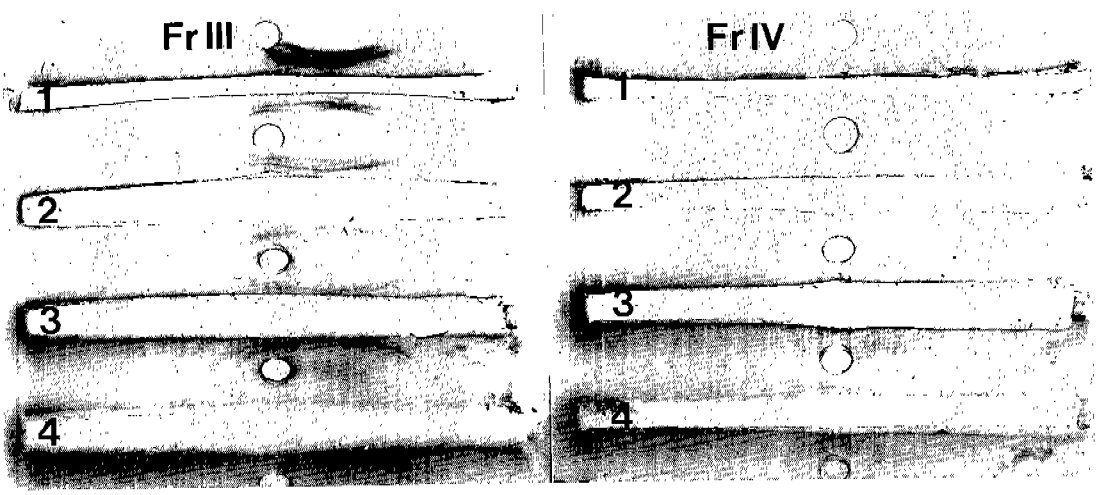

Fig. 6. Comparative findings of immunoelectrophoresis using Fraction III and Fraction IV as antigen and reacted with patient sera of neurocysticercosis. Numbers at troughs mean different patient sera.

RACF, there were 2 additional precipitin bands in IEP other than the long outer band(before and after absorption test). These minor bands were made because fraction III of CF was not a pure protein as shown in Figs. 2 and 3 but contaminated with other proteins. Because 150 kDa protein was the major component proteins in the fraction III, it made evidently the long outer precipitin band with RACF.

It has long been known that antigen B in HF is also a 150 kDa protein, which made, in IEP, the long outer precipitin band with its antisera (Oriol *et al.*, 1971). Antigen B in HF, localized at the tegument of protoscolices, is divided into 15, 10 and 8 kDa subunits when observed by SDS-PAGE (Harrison and Parkhouse, 1985). In spite of the physicochemical similarities between antigen B of in HF and 150 kDa protein in CF (Cho *et al.*, 1988), these antigens did not show cross-reactivities with the reciprocal sera when observed by SDS-PAGE/immunoblot (Maddison *et al.*, 1989; Kong *et al.*, 1989). In this study too, the long outer band was not made between RACF and HF (Fig. 4).

Component proteins in fraction IV formed at least 2 precipitin bands with RACF. Of them, the short inner precipitin band was formed by the major component protein in the fraction IV of CF, Band N protein in disc-PAGE (Figs. 2 & 4). The Band N protein in CF has not yet been purified. Therefore its physicochemical properties can not be definitely stated. However, as Choi *et al.*(1990) showed, Band N protein was estimated to have a molecular mass of about 65 kDa and its subunits were 22, 18, 15 kDa in reducing SDS-PAGE and 44~46 kDa and 22~26 kDa in non-reducing SDS-PAGE(Fig. 3).

Common antigenicity between the major protein of fraction IV in CF and antigen 5 in HF is strongly suggested in this study. Precipitin band of CF fraction IV was placed at similar location of antigen 5 (Capron *et al.*, 1968; Oriol *et al.*, 1971). As repeatedly reported by Gottstein *et al.*(1986), Maddison *et al.* (1989) and Kong *et al.*(1989), SDS-PAGE/immunoblot findings of CF with hydatidosis sera showed the positive reactions at subunits of the fraction IV protein in CF. In addition, RACF reacted with HF at the short inner precipitin band of antigen 5(Fig. 4).

Antigen 5 in HF is a famous protein of high antigenicity. This lipoprotein of 420 kDa is known to be composed of 67 kDa subunits in non-reducing SDS-PAGE, and dissociated into 47 and 27 kDa subunits in reducing SDS-PAGE (Piantelli *et al.*, 1977). It localized at the brood capsule wall, the germinal membranes and subtegumental cells (Yarzabal *et al.*, 1976).

IEP has long been applied in antibody tests for the diagnosis of neurocysticercosis(Flisser *et al.*, 1980), using crude extract of parenchymal tissue of *T. solium* metacestodes. Of the 8 bands precipitated with patients sera, antigen B of 95 kDa was the most frequently recognized. This important antigen is totally different from antigen B of Oriol *et al.*(1971) in HF, though their nomenclatural identity. This antigen B of Flisser *et al.*(1980), the best known antigen in *Taenia solium* metacestodes, was purified by affinity chromatography using a ligand of collagen(Laclette *et al.*, 1990). Laclette *et al.*(1991) reported also that antigen B had almost homologous sequence of amino acids with paramyosin in *Schistosoma mansoni*. In our results, 5 of 8 patients sera showed precipitin band of antigen B of Flisser *et al.*(1980) when CP was used in IEP.

Diagnostic sensitivity of IEP in neurocysticercosis, using either CF or CP, can not correctly be compared with those of ELISA because the tested sera were selected. The tested sera showed very high antibody levels out of the patients' sera of varying antibody levels. However, as an antibody test, IEP was definitely inferior to ELISA because it took 1 week for a correct interpretation instead of a day in ELISA. In addition, the amount of CF necessary in a single test of IEP, corresponded with that in 1,000 tests of ELISA. In spite of such drawbacks as a diagnostic test, IEP can differentiate the antigenic proteins, in different way from SDS-PAGE/immunoblot or immunoprecipitation. This

study made the major protein compositions of CF clear by observing their findings in disc-PAGE, reducing and non-reducing SDS-PAGE and IEP, and by comparing their relations of the findings in each method of analysis.

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=국문초록=

면역전기영동법에 의한 유구낭미충 낭액의 구성 단백질 분석

중앙대학교 의과대학 기생충학교실 및 조선대학교 의과대학 기생충학교실*
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혈청이나 뇌척수액에서 특이항체를 증명하여 유구낭미충증을 진단함에 있어 유구낭미충의 낭액이 가장 민감하고도 특이한 항원임은 이미 알려졌다. 낭액을 항원으로 사용하여도 다른 기생충증과 빈도는 낮지만 교차반응이 발생하므로 교차반응을 일으키는 구성단백질에 대하여 더 관찰할 필요가 있다. 이 연구에서는 유구낭미충 낭액을 Sephacryl S-300 Superfine 젤로 분획하고, 낭액 및 그 분획이 낭액을 면역시켜 만든 토끼 항혈청(RACF)과 반응하는 양상을 면역전기영동법으로 관찰하여 낭액의 구성 단백질의 성질을 분석하고자 하였다. 유구낭미충 낭액을 젤여과법으로 분획하면 제 3 분획과 제 4 분획에 단백질이 가장 많이 포함되었다. 제 3 분획은 disc-PAGE 상 분자량 150 kDa인 것으로 이미 알려진 Band C가 주(主) 구성 단백질이었다. 제 4 분획은 분자량이 약 65 kDa일 것으로 추정되는 Band N 단백질이 주 구성단백질이었다. 낭액에 대한 토끼 항혈청을 낭액과 면역전기영동법으로 반응시키면 주 침강대 2개와 작은 침강대 7~8개를 형성하였는데 낭액의 제 3 분획은 주 침강대 중 밖에 위치한 긴 침강대를 형성한 반면, 제 4 분획은 주 침강대 중 안쪽으로 위치한 짧은 침강대를 형성하였다. 유구낭미충과 혈청학적 교차반응을 가장 많이 일으키는 포충(包蟲, hydatid cyst)의 낭액과 RACF(유구낭미충 낭액에 대한 토끼 항혈청)을 면역전기영동법으로 반응시킨 바, 제 4 분획이 반응한 위치에서 침강대를 형성하여 제 4 분획의 주 구성 단백질이 포충증과의 혈청학적 교차반응의 원인인 것으로 추정하였다. 유구낭미충증으로 이미 진단한 환자의 혈청 중 효소면역측정법으로 항체가가 매우 높았던 혈청 8개를 선택하여 유구낭미충 낭액 및 유구낭미충 충체 추출액과 각각 면역전기영동법으로 반응시킨 바 각각 7명에서 침강대를 형성하였고, 제 3 분획 및 제 4 분획으로 면역전기영동법으로 반응시킨 환자혈청 4개 중에서는 제 3 분획에서는 4개 모두가 침강대를 형성한 반면 제 4 분획에서는 침강대가 혈청 2개에서 형성되었다.

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