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Serodiagnosis of human paragonimiasis by ELISA-inhibition test using monoclonal antibodies

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Abstract: ELISA-inhibition test using *Paragonimus westermani* specific monoclonal antibody (Mab) was investigated to improve the diagnostic specificity of paragonimiasis. By cell fusion, one hybridoma clone secreting anti-*P. westermani* specific Mab was selected (Pwa-14), which reacted on bands of 28 kDa, 42.5 kDa, 89 kDa and 120.5 kDa. IFA showed Pwa-14 was located at the vitelline follicles. By micro-ELISA, 100% of 22 paragonimiasis cases were found positive, but 5 of 40 clonorchiasis cases (12.5%), 3 of 26 cysticercosis cases (7.7%) showed false positive. None of 10 sparganosis patients or 28 normal controls reacted positively. On the other hand, by ELISA-inhibition test using a *P. westermani* specific Mab, 100% of paragonimiasis cases were found positive, and there were no positive in cysticercosis, sparganosis cases or normal controls, except 2 (5.0%) false-positive sera of 40 clonorchiasis cases. The ELISA-inhibition test using a Mab showed higher specificity in comparison with micro-ELISA for serodignosis of human paragonimiasis.

Key words: Paragonimiasis, monoclonal antibody, serodiagnosis, ELISA-inhibition test

INTRODUCTION

Paragonimiasis is one of the important trematode infections of man. Although demonstration of parasite eggs in sputum or stool is a diagnostic for paragonimiasis, various immunodiagnostic tests are also widely used nowadays (Choi, 1990). Of them, the enzyme-linked immunosorbent assay(ELISA) has been widely applied because of its high sensitivity, specificity and feasibility (Cho *et al.*, 1981; Kim *et al.*, 1982; Soh *et al.*, 1985). However, improvement of specificity still remains to be solved in employing ELISA. In order to improve the specificity, a number of affinity-purified antigens with Mabs, instead of crude extract, have been used in various parasitic infections (De Felice *et al.*, 1986; Kim *et al.*, 1986; Yong *et al.*, 1990), but their availability is limited.

The combined application of Mab and ELISA techniques has the potential for improving the immunological detection of parasitic diseases (Mitchell *et al.*, 1983; Yong *et al.*, 1991; Liu *et al.*, 1992). This paper describes an attempt to utilize the Mab in a ELISA-inhibition test for improving the specificity of micro-ELISA in diagnosis of paragonimiasis.

MATERIALS AND METHODS

1. Preparation of Parasite Extracts

P. westermani metacercariae were collected from naturally infected crayfish (*Cambaroides*

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similis) caught from Bogil Island, Chollanam-do. Adult worms were obtained from the lungs of an experimentally infected cat. The worms were homogenized in 0.01 M Tris-HCl buffer (pH 7.2) at 4°C for 30 minutes. The supernatant was obtained by centrifugation at 4°C, 15,000 g for 1 hour, and used as crude extract. Protein concentration was determined by the method of Lowry *et al.* (1951).

Extracts of *Clonorchis sinensis*, *Taenia solium* metacestodes, sparganum were prepared as similar as described above. Adult *C. sinensis* worms were harvested from the bile duct of a rabbit after infection with *C. sinensis* metacercariae collected from infected fresh-water fish, *Pseudorasbora parva*. The scoleces of *T. solium* metacestodes were obtained from a naturally infected pig. Sparganum were obtained from infected snakes, *Natrix tigrina lateralis*.

2. Sera

Twenty-two sera of paragonimiasis and 10 sera of sparganosis were obtained from patients who visited the Severance Hospital, Yonsei University during previous 4 years, or detected at survey in endemic areas (i.e. Bogil Island on paragonimiasisis) (Soh *et al.*, 1985). Twenty-eight sera of cysticercosis were obtained from inhabitants on Cheju Island with diagnosed parasitologically or from the Hospital as above with diagnosed serologically. Forty sera of *C. sinensis* infected humans were obtained from survey in endemic areas along Nakdong-river. Twenty-eight normal control sera were obtained from healthy humans. They had no parasite eggs in their stools and no serum antibodies for paragonimiasis, clonorchiasis, cysticercosis or sparganosis. The sera had been stored in a deep freezer at -70°C until used.

3. Production of Mabs

Mabs were generated by fusion of P3× 63Ag8. V653 myeloma cells with spleen cells of mice immunized with water-soluble *P. westermani* adult worm crude extract with some modifications (Köhler and Milstein, 1975). Hybridomas producing antibodies were identified by ELISA. Selelcted hybridomas were cloned by limiting dilution. Hybridoma cell lines were expanded, and supernatants were obtained as described previously (Yong *et al.*, 1991).

4. Characterization of the Mabs

Specificity of the anti-*P. westermani* antibodies in the culture supernatant was tested for listed extracts as above by ELISA.

EITB was performed according to the procedures described by Tsang *et al.* (1983) after SDS-PAGE on 5-15% gels according to the procedure of Laemmli(1970). EITB observation of major antigens of *P. westermani* reacting with an infected human serum was used as a reference to select a Mab.

Isotyping was performed with an isotyping kit using specific goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA according to the method of manufacturer (Hyclon, U.S.A.).

To identify the locality of the Mab, immunofluorescence experiments were carried out on cryocut *P. westermani* adult worm as described previously (Yong *et al.*, 1991).

5. ELISA-inhibition Test

A modification of the method described by Liu *et al.* (1992) was used. Polystyrene plate (Costar, U.S.A.) were coated with 50 μl of *P. westermani* extract (2 μg/ml) in bicarbonate-carbonate buffer (pH 9.6) at 4°C overnight. After washing 3 times in 0.01 M PBS containing plus 0.05% Tween 20 (PBS/T, pH 7.4), the plates were blocked with 50 μl of 3% skim milk in PBS/T for 30 min at 37°C. The plates were washed as above, 50 μl of human sera diluted 1/50 in PBS/T containing 0.5% BSA were added and incubated for 30 min at 37°C. Washed again, 50 μl of Mab was applied for 30 min at 37°C. After washing, 50 μl of 1:4,000 diluted peroxidase conjugated rabbit anti-mouse immunoglobulin (Cappel) was reacted for 30 min at 37°C. Washed as above, 0.05% orthophenylenediamine and 0.06% hydrogen peroxide, diluted in 0.1 M phosphate-citrate buffer (pH 5.0), were added to each well. After incubation for 30 min at room temperature, the absorbances were read at 490 nm using a ELISA Reader (Dynatech MR300). Inhibition rate was calculated, *i.e.*,

Inhibition rate (%) =

$$\frac{\text{absorbance value of test serum + Mab}}{\text{absorbance value of Mab alone}}$$

The cut-off absorbance was established through the mean + 2 standard deviations of inhibition rate (%) of Mab by normal controls. The result of ELISA-inhibition test was compared with that of micro-ELISA.

RESULTS

A specific Mab, named as Pwa-14, was selected. It did not cross-react with any other parasite antigens tested, such as *C. sinensis*, *T. solium* metacestodes and sparganum. SDS-PAGE revealed protein band pattern of *P. westermani* extract (Fig. 1). Immunoblots reacted with Pwa-14, immune mouse serum and infected human serum, and the protein band stained with Amido-Black B were shown on Fig. 2. Antigenic determinants of 28 kDa, 42.5 kDa, 89 kDa and 120.5 kDa were found to be reacted with Pwa-14. The antigenic determinants reacted on selected Mab was in very good agreement with major antigenic bands in infection. That finding was clearly noted on Fig. 2. Its isotype was IgG1. On the sectioned fluke, antigenic substances reacted on Pwa-14 were found evidently in the vitelline follicles of the parasite (Fig. 3).

By micro-ELISA, 22 sera of paragonimiasis cases were all found to be positive, but 5 of 40 clonorchiasis cases (12.5%), 3 of 26 cysticercosis cases (7.7%) showed false reactions. None of 10 sera of sparganosis patients or 28 normal controls reacted positively (Fig. 4). By ELISA-inhibition test

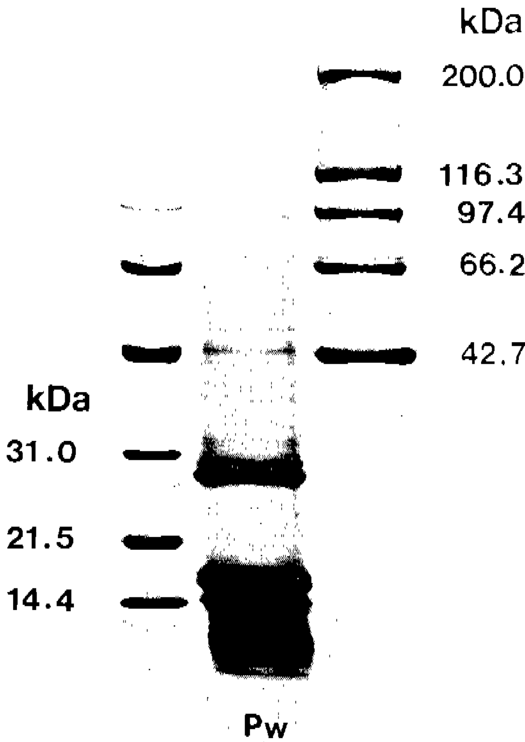

Fig. 1. SDS-PAGE finding of *P. westermani* adult worm crude extract (Pw) stained with Coomassie blue R-250.

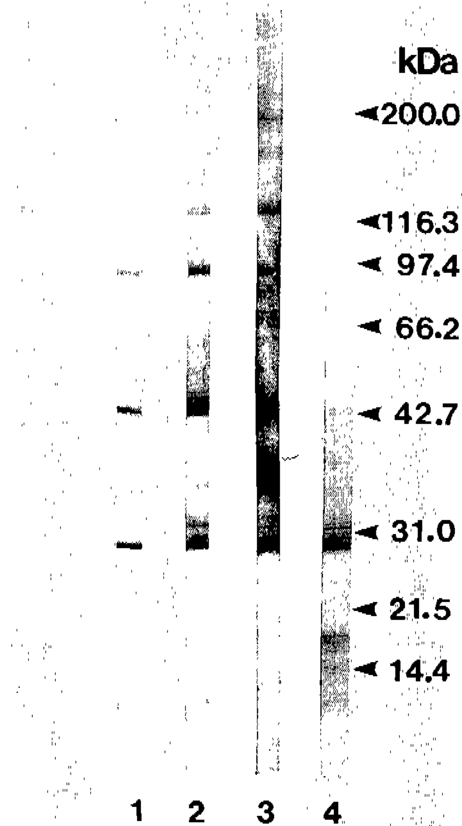

Fig. 2. Immunoblots reacted on: 1) Mab Pwa-14, 2) immune mouse serum, 3) infected human serum against *P. westermani* extract and 4) the protein band of *P. westermani* extract on a nitrocellulose paper, stained with Amido-Black B. Antigenic bands of 28 kDa, 42.5 kDa, 89 kDa and 120.5 kDa reacted on Pwa-14 were identified.

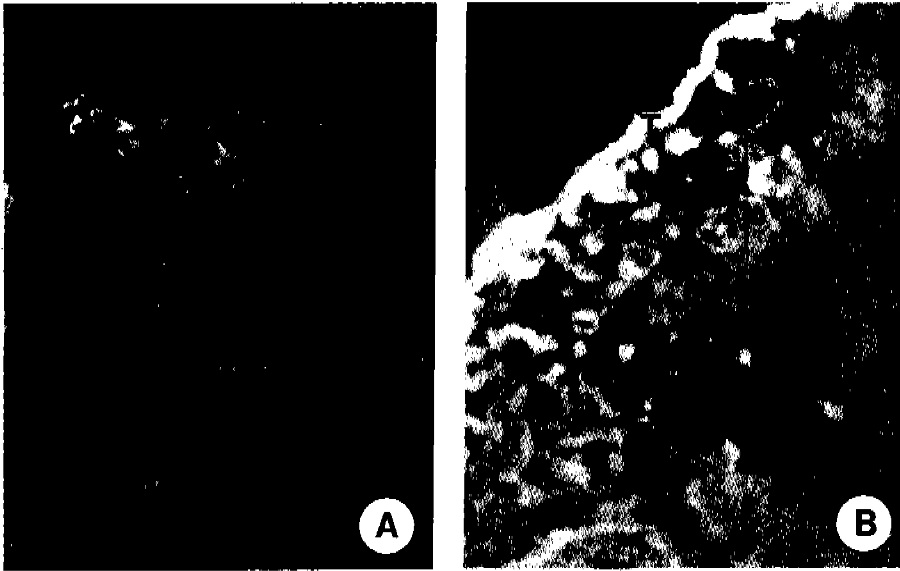

Fig. 3. Localization of Mab by indirect fluorescent antibody technique. Frozen sections of *P. westermani* adult worm was reacted with: (A) Pwa-14 Mab showing positive reactions at the vitelline follicles and (B) immune mouse serum as a positive control (× 100). (V: vitelline follicle, T: tegument).

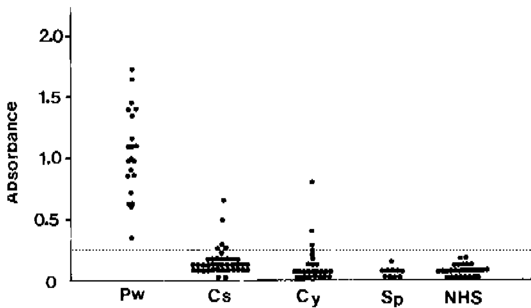

Fig. 4. Distribution of absorbance of human sera in paragonimiasis (Pw), clonorchiasis (Cs), cysticercosis (Cy), sparganosis (Sp) cases and normal controls (NHS) against *P. westermani* extract by micro-ELISA.

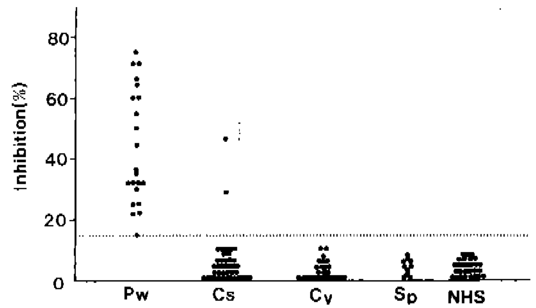

Fig. 5. Inhibition rate (%) of Mab by human sera in the ELISA-inhibition test using *P. westermani* specific Mab Pwa-14. The cut-off value is set at 15%.

using a Mab Pwa-14, 100% of paragonimiasis cases were found positive, and there were no false positive in cysticercosis, sparganosis cases or normal controls. But, 2 out of 40 (5%) clonorchiasis sera reacted positively in the ELISA-inhibition test using Pwa-14. The ELISA-inhibition test using a Mab showed higher specificity in comparison with a micro-ELISA for serodiagnosis of human paragonimiasis (Fig. 5).

DISCUSSION

P. westermani is one of the most important trematode parasites of man in Korea. In this study, Mabs were produced for improving specificity of the serodiagnosis of human paragonimiasis. A Mab which reacted specifically on *P. westermani* extract was selected and applied for the immunodiagnosis of paragonimiasis.

The antigenic determinants reacted on a selected Mab was in good agreement with

several major antigenic bands in infection as identified by EITB in this study. This finding indicate that each reacting antigenic band has the same antigenic determinant recognized by the Mab. The source of antigenic substances reacted with Pwa-14 was noted only in the vitelline follicles of a parasite, and agreed partially with a report of Rim *et al.* (1992) that in adult *P. westermani*, the cytoplasm between granules in the vitelline gland were one of the major sources of antigens by using an infected cat serum and immunogold labelling.

Several research groups reported Mabs directed against antigens of *P. westermani* in order to characterize the antigens (Sugiyama *et al.*, 1988; Kang *et al.*, 1991; Lee *et al.*, 1991; Zihao *et al.*, 1991). Sugiyama *et al.* (1988) investigated stage-specificity of the antigen using Mabs against adult antigen of *P. westermani*, and Kang *et al.* (1991) employed Mabs to investigate the nature of 2 component proteins in crude saline extract of adult *P. westermani*. Lee *et al.* (1991) reported Mabs produced against excretory-secretory antigens in order to improve the specificity of the immunodiagnostic test, and Zihao (1991) reported Mabs reacting on periodate-sensitive carbohydrate epitopes of the metacercarial stage-specific antigens. The Mab used in this study seemed to react on a different antigenic determinant from Mabs previously reported by other researchers, because the reacted antigen had a different locality and molecular weight from others as identified by IFA and EITB. It is noteworthy that the PFCK-136 Mab was reported by Kang *et al.* (1991) to react on 23, 46, 92 kDa determinants originated from eggs of the worm, and the determinants had been reported to have reacted strongly on human paragonimiasis sera (Kim *et al.*, 1988). Although the molecular weight of the determinants recognized by the Mab employed in this study was different as 28, 42.5, 89, 120.5 kDa, the reacting pattern on EITB was somewhat similar in the aspect of reacting several major bands recognized by the infected human serum.

Paragonimiasis could be diagnosed by sputum or stool examination. Detection of eggs, however, is very difficult in extra-pulmonary paragonimiasis, or from children who could not expectorate sputum. Sputum or stool examination requires a lot of time and labor, and the sensitivity is low. Detection of circulating antigens did not seem to be practically useful (Yong *et al.*, 1987). Therefore, immunodiagnosis of paragonimiasis, especially to detect specific antibodies has been used widely (Cho and Soh, 1976; Cho *et al.*, 1981 & 1983; Knobloch and Lederer, 1983). But, 12.5% of clonorchiasis cases, 7.7% of cysticercosis in this study and 11.1% of clonorchiasis cases (Kim *et al.*, 1982) showed false positive reactions. Trying to solve this problem, purified antigen using a Mab has been employed for immunodiagnosis in other parasitoses (Kim *et al.*, 1986; Yong *et al.*, 1990). When the purified antigen was used, the specificity was reported to have improved, but the sensitivity decreased much. If the purified antigen could not provide a good sensitivity for immunodiagnosis, it is probably useless because purifying procedure is usually very difficult and inefficient.

Mab based ELISA-inhibition test or competitive ELISA has also been applied for diagnosis of other parasitic diseases for improving specificity of the conventional immunodiagnostic test (Mitchell *et al.*, 1983; Jaffe and McMahon-Pratt, 1987; Cabrera *et al.*, 1989; Yong *et al.*, 1991; Liu *et al.*, 1992), and all of them reported to have good sensitivity and specificity. In this study, ELISA-inhibition test using *P. westermani* specific Mab was applied in the diagnosis of paragonimiasis. The results showed that the sensitivity of the ELISA-inhibition test was the same as the micro-ELISA, while the specificity improved. It did not cross-react with the sera of normal controls or those of other parasitic infections, such as clonorchiasis, sparganosis and cysticercosis. A few sera from clonorchiasis cases, however, showed positive reactions in spite of highly specific Mab against *P. westermani*. This will deserve further studies. The ELISA-inhibition test using a *P. westermani* specific Mab could be used satisfactorilly to differentiate human paragonimiasis from other parasitic infections.

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REFERENCES

Cabrera Z, Parkhouse RME, Forsyth K, Gomez-Priego A, Pabon R, Yarzabal L (1989) Specific detection of human antibodies to *Onchocerca volvulus*. *Trop Med Parasit* **40**(4): 454-459.

Cho KM, Soh CT (1976) Indirect fluorescent antibody test for the serodiagnosis of paragonimiasis and clonorchiasis. *Yonsei Rep Trop Med* **7:** 26-39.

Cho SY, Hong ST, Rho YH, Choi S, Han YC (1981) Application of micro-ELISA in serodiagnosis of human paragonimiasis. *Korean J Parasit* **19:** 151-156.

Cho SY, Lee DK, Kang SY, Kim SI (1983) An epidemiological study of human paragonimiasis by means of micro-ELISA. *Korean J Parasit* **21:** 246-256.

Choi DW (1990) *Paragonimus* and paragonimiasis in Korea. *Korean J Parasit* **28**(suppl) : 79-102.

De Felice G, Pini C, Afferni C, Vicari G (1986) Purification and partial characterization of the major antigen of *Echinococcus granulosus* (antigen 5) with monoclonal antibodies. *Mol Biochem Parasitol* **20:** 133-142.

Jaffe CL, McMahon-Pratt D (1987) Serodiagnostic assay for visceral leishmaniasis employing monoclonal antibodies. *Trans Roy Soc Trop Med Hyg* **81:** 587-594.

Joo KH, Ahn H, Chung MS, Rim HJ (1989a) Demonstration of species-specific and cross reactive components of *Paragonimus westermani* crude adult worm antigen by EITB. *Korean J Parasit* **27:** 9-14.

Joo KH, Hong SC, Chung MS, Rim HJ (1989b) Analysis of antigenic specificities of *Paragonimus westermani* developmental stages using immunoblot technique. *Korean J Parasit* **27:** 1-7 (in Korean).

Kang SY, Kong Y, Cho SY (1991) Component proteins in crude extract of adult *Paragonimus westermani* purified by immunoaffinity chromatography using monoclonal antibodies. *Korean J Parasit* **29:** 363-369.

Kim DC, Lee OY, Lee JS, Ahn SH (1982) Studies on the control of paragonimiasis II. Immunodiagnosis of human paragonimiasis by ELISA using secretory-excretory antigen. *Rep NIH Korea* **19:** 109-114 (in Korean).

Kim SH, Kong Y, Kim SI, Kang SY, Cho SY (1988) Immunoblot observation of antigenic protein fractions in *Paragonimus westermani* reacting with human patients sera. *Korean J Parasit* **26:** 239-243.

Kim SI, Kang SY, Cho SY, Hwang ES, Cha CY (1986) Purification of cystic fluid antigen of *Taenia solium* metacestodes by affinity chromatography using monoclonal antibody and its antigenic characterization. *Korean J Parasit* **24:** 145-158.

Knobloch J, Lederer I (1983) Immunodiagnosis of human paragonimiasis by an enzyme immunoassay. *Tropenmed Parasit* **34**(1): 21-23.

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256:** 495-497.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 681-685.

Lee OY, Lee JS, Kim TS, In TS, Choi KH, Kim DC (1991) Studies on the immunodiagnosis of paragonimiasis. *Rep NIH Korea* **28:** 122-131 (in Korean).

Liu D, Lightowlers MW, Rickard MD (1992) Evaluation of a monoclonal antibody-based competition ELISA for the diagnosis of human hydatidosis. *Parasitology* **104:** 357-361.

Lowry OH, Rosebrough NJ, Lewis-Farr A, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265-275.

Mitchell GF, Premier RR, Garcia EG, *et al* (1983) Hybridoma antibody-based competitive ELISA in *Schistosoma japonicum* infection. *Am J Trop Med Hyg* **32:** 114-117.

Rim HJ, Kim SJ, Sun IJ, Lee JS (1992) Antigenic localities in the tissues of *Paragonimus westermani* by development of stages using immunogold labelling method. *Korean J Parasit* **30:** 1-14.

Slemenda SB, Maddison SE, Jong EC, Moore DD (1988) Diagnosis of paragonimiasis by immunoblot. *Am J Trop Med Hyg* **39:** 469-471.

Soh CT, Min DY, Ryu JS, Yong TS (1985) Study on the reproducibility of ELISA technique for

the diagnosis of clonorchiasis and paragonimiasis. *Yonsei Rep Trop Med* **16:** 1-10.

Sugiyama H, Hinoue H, Katahira J, *et al* (1988) Production of monoclonal antibody to characterize the antigen of *Paragonimus westermani*. *Parasitol Res* **75:** 144-147.

Tsang VCW, Peralta JM, Simons AR (1983) Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. *Methods Enzymol* **92:** 377-391.

Yong TS, Im KI, Chung PR (1991) Analysis of *Clonorchis sinensis* antigens and diagnosis of clonorchiasis using monoclonal antibodies. *Korean J Parasit* **29:** 293-310.

Yong TS, Im KI, Lee OY, Kim DC (1990) Evaluation of *Clonorchis sinensis* specific antigen purified by affinity chromatography using monoclonal antibodies for serodiagnosis of human clonorchiasis. *Yonsei Rep Trop Med* **21:** 25-32.

Yong TS, Kim TS, Lee JS, Lee OY, Kim DC (1987) Detection of circulating antigens in rats experimentally infected with *Paragonimus westermani* by ELISA. *Korean J Parasit* **25:** 141-148.

Zihao Z, Yiping S, Piessens W (1991) Characterization of stage-specific antigens of *Paragonimus westermani*. *Am J Trop Med Hyg* **44:** 108-115.

=국문초록=

단클론항체를 이용한 폐흡충증의 면역진단

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폐흡충증의 진단을 위하여 근자에 효소면역측정법(ELISA)이 널리 쓰이고 있다. 이 논문에서는 폐흡충 항원에 대한 단클론항체를 제조하고 이를 효소면역억제측정법(ELISA-inhibition test)에 응용하여 효소면역측정법의 특이도를 더욱 높이고자 하였다. 폐흡충 성충의 수용성 항원으로 면역한 BALB/c 마우스의 비장세포와 형질세포종세포를 융합하여 림프잡종세포를 만들고 이 중에서 폐흡충 항원에 대한 특이 단클론항체를 생성하는 하나의 림프잡종세포를 선택하였다. 이는 Pwa-14라고 이름지워졌는데, EITB상 28 kDa, 42.5 kDa, 89 kDa, 120.5 kDa 항원대에 반응하였으며, 간접면역항체법으로 반응하는 항원의 위치가 난황선임을 확인하였다. 폐흡충 항원을 사용한 통상적인 효소면역측정법에 폐흡충 감염자 22명의 혈청은 모두 양성 반응을 보였으며, 간흡충 감염자 40명 중 5명(12.5%), 유구낭충 감염자 26명 중 3명(7.7%)은 양성을 나타내 교차 반응을 보였다. 이때, 스파르가눔 감염자 10명 및 정상 대조군에서는 양성 반응을 보이는 혈청이 없었다. 특이 단세포군항체를 이용한 효소면역억제측정법으로 측정한 결과, 폐흡충 감염자는 모두 양성반응을 보였으나, 유구낭충, 스파르가눔 감염자의 혈청은 교차 반응을 보이지 않았다. 이상의 결과로 보아 폐흡충의 면역 진단 시, 폐흡충 특이 단클론항체를 이용한 효소면역억제측정법은 통상적인 효소면역측정법에 비하여 더 높은 특이도를 보임을 알 수 있었다.

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