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Serodiagnosis of cysticercosis by ELISA-inhibition test using monoclonal antibodies

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Abstract: Monoclonal antibodies (Mabs) were produced against crude scolex extract of *T. solium* metacestodes, and applied to ELISA-inhibition test for improving the specificity of serodiagnosis of human cysticercosis. Four hybridomas secreting species-specific anti-cysticercal Mabs (Cya-1, Cya-7, Cya-28 and Cya-31) were selected. Each Mab reacted on antigenic components of 25.5 kDa (Cya-1), 28 kDa (Cya-7), 87.5 kDa (Cya-28), and 12.5 kDa (Cya-31). IFA showed that Cya-1 was located at the calcium corpuscles, and Cya-7 at the loose connective tissue of *T. solium* metacestode scolex. Cya-28 and Cya-31 reacted on the tegument of the scolex. By conventional ELISA, 23 out of 28 (82.1%) cysticercosis patients were found serologically positive, but 1 out of 9 (11.1%) sparganosis cases and 6 out of 31 (19.4%) paragonimiasis cases showed false positives. By ELISA-inhibition test using species-specific anti-cysticercal Mab Cya-7, 19 out of 28 (67.9%) cysticercosis cases were found serologically positive, but there were no false positives in other parasitic infections.

Key words: Cysticercosis, monoclonal antibody, serodiagnosis, ELISA-inhibition test, IFA

INTRODUCTION

Cysticercosis is a very important tissue-invading cestode infection of man. Demonstration of parasites from infected human beings is confirmative in diagnosis of cysticercosis. Serodiagnostic tests, however, have been widely used, and the cysticercosis ELISA has been developed (Arambulo *et al.*, 1978; Coker-Vann *et al.*, 1984; Cho *et al.*, 1986 & 1987; Kim and Yang, 1988; Diaz *et al.*, 1992). Although the cysticercosis ELISA is highly sensitive and specific, the cross-reactions with other parasitic infections have been reported not so infrequently (Gottstein *et al.*, 1987; Diaz *et al.*, 1992).

In order to improve the specificity of the immunological detection of helminthiasis, monoclonal antibodies (Mabs) and ELISA have been employed together (Mitchell *et al.*, 1983; Yong *et al.*, 1991; Liu *et al.*, 1992). This study aimed to utilize the species-specific anti-cysticercal Mab in a Mab-based ELISA-inhibition test for improving the specificity of conventional ELISA in serodiagnosis of human cysticercosis.

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MATERIALS AND METHODS

1. Preparation of parasite extracts

T. solium metacestodes were excised from the skeletal muscle of a naturally infected pig. Scolices of the metacestode were obtained, and homogenized in 0.01 M tris-HCl buffer (pH 7.2) at 4°C for 30 minutes. The supernatant was obtained after 15,000 g centrifugation of homogenate at 4°C for 1 hour, and it was used as crude extract. Protein concentration was determined by the method of Lowry *et al.*, 1951. Extracts of sparganum or *Paragonimus westermani* were prepared by the same method described as above. Sparganum was obtained from snakes, *Rhabdophis tigrina*. Metacercariae of *P. westermani* were collected from naturally infected crayfish (*Cambaroides similis)* caught in Chollanam-do. Adult worms of *P. westermani* were obtained from the lungs of a dog 8 weeks after experimental infection.

2. Sera

Twenty-eight sera of cysticercosis were obtained mostly from infected humans on Cheju Island in 1983. *T. solium* cysticerci were confirmed by excision biopsy from their skin nodules. Thirty-one sera of paragonimiasis and 9 sera from sparganosis cases were obtained from parasitologically confirmed patients, or whom were detected at survey on an endemic area for paragonimiasis (Soh *et al.*, 1985). Twenty normal control sera were obtained from humans who showed no parasite eggs in their stool specimens and no serum antibodies for *T. solium* metacestode, sparganum or *P. westermani* employing by ELISA. All of the sera were stored in a deep freezer at -70°C until used.

3. Production of Mabs

Hybridization was done by fusion of P3X63Ag8. V653 myeloma cells with spleen cells from mice immunized with crude scolex extract of *T. solium* metacestodes according to the method of Köhler and Milstein (1975) with some modifications. Hybridomas producing antibodies to the antigen were identified by ELISA. Selected 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-cysticercal Mabs in the culture supernatant was confirmed using the above 3 different parasite antigens by ELISA. Isotyping of Mabs was performed by using an isotyping kit using specific goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA according to the method of manufacturer (Hyclone). Enzyme-immunoelectrotransfer blot (EITB) was performed according to the procedure described by Tsang *et al.* (1983) with SDS-PAGE on 5-15% gels according to the procedure of Laemmli(1970). Localities of the Mabs were identified by IFA. Cryocut scolices of *T. solium* metacestode were used as antigens. Mabs were identified whether or not it was produced against exposing antigen in infection of pig by ELISA-inhibition test using an infected pig serum as described previously (Yong *et al.*, 1991). If Mabs were inhibited significantly in its ELISA titers by an infected pig serum than by normal control serum, those were determined to be produced against exposing antigenic determinants in infection of *T. solium* metacestode.

5. Diagnosis of human cysticercosis by ELISA-inhibition test

Using a selected Mab, ELISA-inhibition test was carried out for the serodiagnosis of cysticercosis. The method described by Liu *et al.* (1992) was used with some modifications. Polystyrene ELISA plates (Costar) were coated with 50 μl of crude scolex extract of *T. solium* metacestodes (protein content of 2 μg/ml) in bicarbonate-carbonate buffer, pH 9.6, at 4°C overnight. After 3 washings in 0.01 M PBS, pH 7.4, plus 0.05% Tween 20, the plates were blocked with 50 μl of 3% skim milk in PBS for 30 min at 37°C. The plates were washed as above, 50 μl of human sera diluted 1/25 in PBS containing 0.5% BSA were added and incubated for 30 min at 37°C. After washings, 50 μl of Mab was applied for 30 min at 37°C. After washings, 50 μl of 1:4,000 diluted peroxidase conjugated rabbit anti-mouse immunoglobulin (Cappel) was reacted for 30 min at 37°C. After washings, 0.05%

orthophenylenediamine solution with 0.06% hydrogen peroxide diluted in 0.1 M phosphate-citrate buffer, pH 5.0 was added to each well. After incubation for 30 min at room temperature, the plates were read at an absorbance of 490 nm using an ELISA Reader (Dynatech MR300). Inhibition rate was calculated by following manner:

$$\text{Inhibition rate (\%)} = \frac{\text{absorbance of test serum + Mab}}{\text{absorbance of Mab}}$$

The cut-off value of absorbance was the mean + 2 standard deviations of inhibition rate (%) of normal controls. The result of ELISA-inhibition test was compared with that of conventional ELISA.

RESULTS

Four species-specific anti-cysticercal Mabs, not cross-reacting with any other parasite antigens examined in this experiment, were obtained. The Mabs were named as Cya-1, Cya-7, Cya-28 and Cya-31. Isotypes of all these 4 different Mabs were identified to be IgG1.

SDS-PAGE revealed protein band pattern of crude scolex extract of *T. solium* metacestodes (Fig. 1). Immunoblots reacted on Cya-1, Cya-7, Cya-28, Cya-31 and immune mouse serum were shown on Fig. 2. Each Mab reacted on antigenic determinants of 25.5 kDa (Cya-1), 28 kDa (Cya-7), 87.5 kDa (Cya-28), and 12.5 kDa (Cya-31).

IFA showed that Cya-1 had very unique location at the calcium corpuscles. Cya-7 were located at the loose connective tissue of the scolex of *T. solium* metacestode. Cya-28 and Cya-31 reacted on the tegument of the scolex more strongly than the parenchyme (Fig. 3).

Cya-7 and Cya-28 were identified as Mabs

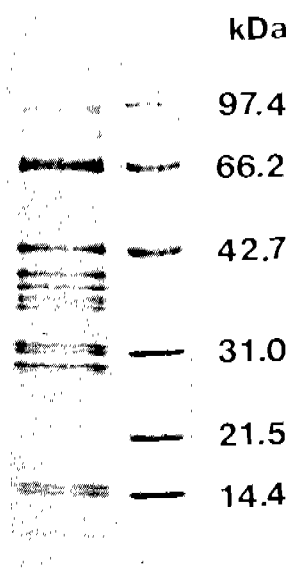

Fig. 1. SDS-PAGE pattern of crude scolex extract of *T. solium* metacestodes in 5-15% linear gradient gel, stained gel, stained with Coomassie brilliant blue R-250.

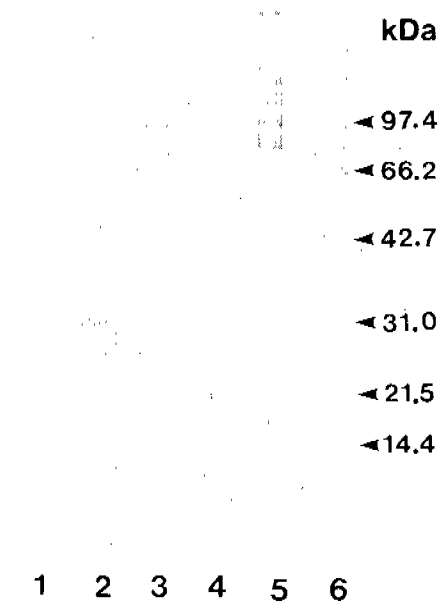

Fig. 2. Findings of immunoblots reacted on species-specific anti-cysticercal Mabs: 1) Cya-1 (25.5 kDa), 2) Cya-7 (28 kDa), 3) Cya-28 (87.5 kDa), 4) Cya-31 (12.5 kDa), 5) immune mouse serum and 6) the protein band pattern of crude scolex extract of *T. solium* metacestodes on a nitrocellulose paper stained with Amido-Black B.

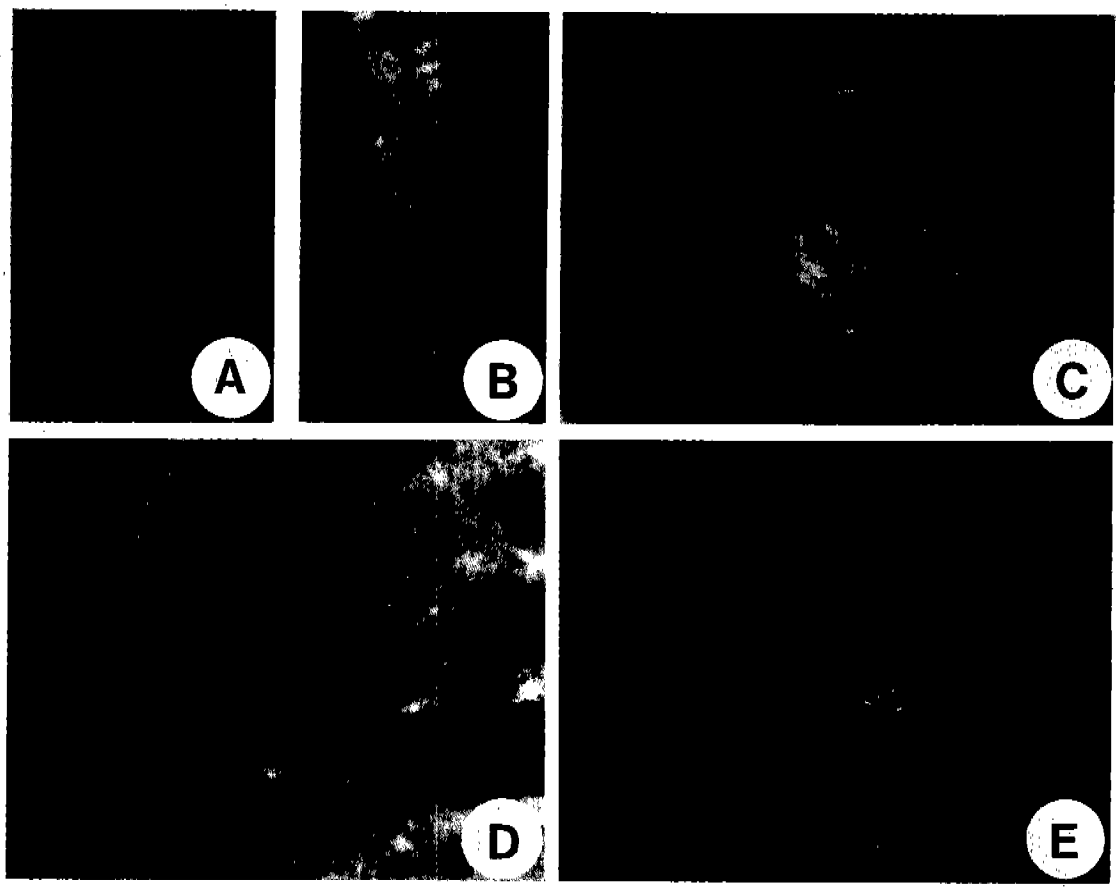

Fig. 3. Localization of Mabs by indirect fluorescent antibody technique. Frozen sections of scolices of *T. solium* metacestode were reacted on: (A) Cya-1 showing positive reactions at calcium corpuscles (× 100), (B) a magnification (× 200) of Fig. 1, (C) Cya-7 showing positive reactions at the loose connective tissue of a scolex (× 100), (D) Cya-31 showing positive reactions at the tegument of the parasite (× 100), and (E) immune mouse serum as a positive control. (▼: calcium corpuscle, C: loose connective tissue, T: tegument).

directed against exposing antigens in infection of pig by ELISA-inhibition test using an infected pig serum. Of these, Cya-7 was employed for serodiagnosis of human cysticercosis since it showed the most prominent reaction. About 60% (*i.e.*, absorbance 0.9 to 0.36) of absorbance was inhibited by using a cysticercotic pig serum.

Employing conventional ELISA, when the cut-off value was set at 0.24, twenty-three out of 28 (82.1%) cysticercosis patients were found positive, but 1 out of 9 (11.1%) sparganosis cases and 6 out of 31 (19.4%) paragonimiasis cases showed false positives. None of 20 normal controls reacted to be positive. Otherwise, by ELISA-inhibition test using species-specific anti-cysticercal Mab (Cya-7), 19 out of 28 (67.9%) cysticercosis cases were found positive. There were no false positives in sparganosis, paragonimiasis cases or normal controls.

DISCUSSION

In this study, Mabs were produced against crude scolex extract of *T. solium* metacestodes, and applied to ELISA-inhibition test for improving the specificity of serodiagnosis of human cysticercosis. By ELISA using heterologous parasite antigens the Mabs without showing high specificities against crude scolex extract of *T. solium* metacestodes

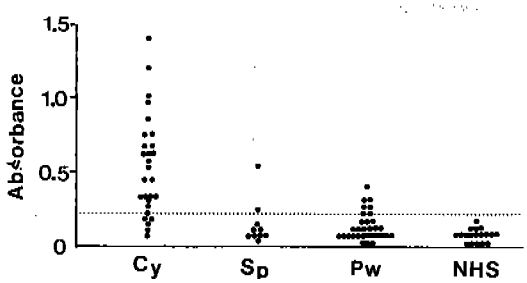

Fig. 4. Distribution of absorbance values of human sera in cysticercosis (Cy), sparganosis (Sp), paragonimiasis (Pw) cases and normal controls (NHS) against crude scolex extract of *T. solium* metacestodes by conventional ELISA. Twenty-three out of 28 (82.1%) cysticercosis patients were found positive, but 1 out of 9 (11.1%) sparganosis cases and 6 out of 31 (19.4%) paragonimiasis cases showed false positives.

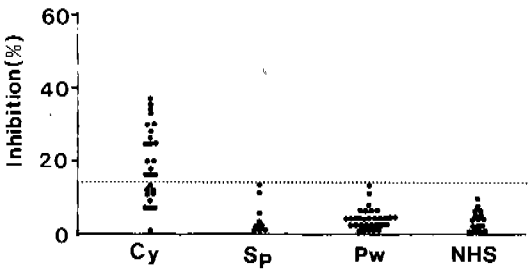

Fig. 5. Inhibition rate (%) of Mab by human sera in the ELISA-inhibition test using species-specific anti-cysticercal Mab (Cya-7) directed against an exposing antigen in infection for serodiagnosis of cysticercosis. The cut-off value is set at 12%. Nineteen out of 28 (67.9%) cysticercosis cases were found positive, and there were no false positives in other infections.

were discarded. In this study, heterologous antigens of only 3 parasite species were used since those should have been considered as the most important tissue-invading parasites in Korea, and differential diagnosis is frequently required.

The EITB have been applied for analysis of *T. solium* metacestode antigens, and reported to be very useful in serodiagnosis of cysticercosis (Cho *et al.*, 1987; Gottstein *et al.*, 1987; Larralde *et al.*, 1989; Tsang *et al.*, 1989; Diaz *et al.*, 1992). Several bands observed by EITB were reported to be very specific and discriminating, nevertheless the results reported by different research groups seemed not to be agreed well. Those were antigenic bands of 26, 8 kDa reported by Gottstein *et al.* (1987), 15, 10 and 7 kDa by Cho *et al.* (1987, 1988) and 108, 23 kDa by Larralde *et al.* (1989) *etc.* Although the antigenic band against each Mab of proven specificity easily recognized by EITB in this study, the result was not easy to interprete, or to compare with previous reports using infected human sera. The molecular weight (28 kDa) of the species-specific antigenic component recognized by the Mab was also different from previous reports. It deserves further studies whether this finding was derived from technical difference between laboratories or the antigenic components recognized by the Mab different from previous reports.

In the serodiagnosis of cysticercosis, detection of *T. solium* metacestode antigens from CSF or sera have been reported not so promising practically (Cho *et al.*, 1992; Diaz *et al.*, 1992), and detecting specific antibody seems to be more useful (Knobloch and Delgado, 1985; Cho *et al.*, 1986 & 1987; Espinoza *et al.*, 1986; Gottstein *et al.*, 1987; Larralde *et al.*, 1989 & 1990; Diaz *et al.*, 1992). But, cross-reactions in the ELISA for diagnosis of cysticercosis have not been so uncommonly reported. According to Cho *et al.* (1986), taeniasis (2/18), sparganosis (2/20), paragonimiasis (1/56), clonorchiasis (1/15) and fascioliasis (1/1) cases showed cross reactions. Kong *et al.* (1989) reported similar results with Cho *et al.* (1986) as above, including cross-reaction with hydatid disease (100%). Gottstein *et al.* (1987) reported that the specificity of ELISA for diagnosis of cysticercosis was calculated to be 51%, and most cross-reactions were observed with sera from patients with cystic (90%) and alveolar (95%) echinococcosis and filariasis (75-100%), depending on the species involved. Eom *et al.* (1988) reported the false positive rate against *T. solium* metacestode antigen in the ELISA, such as infection of *Taenia solium* (80%), *T. saginata* (42.9%). *Diphyllobothrium latum* (14.3%), *Paragonimus westermani* (9.1%) and *Clonorchis sinensis* (15.0%). Diaz *et al.* (1992) also reported it as 11% and 20% of sera from hydatid and *H. nana* patients, respectively.

Trying to solve this problem, *i.e.* to improve specificity of the test, purified antigen using Mabs has been employed in the immunodiagnosis for cysticercosis or other parasitoses. Although Nascimento *et al.* (1987) reported that the percent positive serum samples from cysticercosis patients were 100% by the ELISA with 3 different antigens purified by Mabs, immunodiagnostic tests based on the antigen purified by Mabs with a single specificity would be expected to suffer from sensitivity problems generally (De Felice *et al.*, 1986; Kim *et al.*, 1986). As an another way for improving specificity of the conventional immunodiagnostic test, Mab based ELISA-inhibition test or competitive ELISA has been applied for diagnosis of other parasitic diseases as this study (Mitchell *et al.*, 1983; Jaffe and McMahon-Pratt, 1987; Cabrera *et al.*, 1989; Yong *et al.*, 1991; Liu *et al.*, 1992). All of them reported good sensitivity and specificity of the Mab-based ELISA-inhibition test. In this study, ELISA-inhibition test using *T. solium* metacestode species specific Mab was applied in the diagnosis of cysticercosis. ELISA-inhibition test using an infected pig serum revealed that some specific Mabs were determined to be produced against exposing antigenic determinants in natural infection of *T. solium* metacestodes but others were not. The Mab, most remarkably inhibited in ELISA titer by an infected pig serum was selected, and applied for immunodiagnosis of human cysticercosis. This procedure would be essential since not all Mabs were produced against exposing antigens in infection. On the contrary, Mabs seemed to be produced more readily against non-exposing antigenic determinants in infection because the mice were immunized with crude scolex extract of *T. solium* metacestodes. It might be expected that major exposing antigens in infection are similar in pigs and in humans, although it deserves further studies whether the exposing antigenic components in pigs are the same as in humans.

The results showed that the sensitivity of the ELISA-inhibition test decreased than the conventional ELISA, but the specificity improved a lot, not to cross-react with the sera of normal controls or those of other important tissue-invading parasitic infections found in Korea, such as sparganosis and paragonimiasis. One of the reason of decreased sensitivity might be caused by using single antigenic determinant in the ELISA-inhibition test. In the future, Mabs directed against highly specific, major antigenic determinants and having a suitable physical character, *i.e.* the absorbance inhibited easily by the sera of infection are expected to be produced, and be applied without decreasing sensitivity in ELISA-inhibition test.

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=국문초록=

단세포군항체를 이용한 효소면역억제측정법에 의한 유구낭미충증의 혈청학적 진단

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유구낭미충 항원에 대한 단세포군항체를 제조하여 그 특성을 분석하고, 이를 효소면역억제측정법(ELISA-inhibition test)에 응용함으로써 통상적인 효소면역측정법(ELISA)의 특이도를 더욱 높이고자 하였다. 유구낭미충의 두절항원으로 면역한 BALB/c 마우스의 비장세포와 형질세포종세포를 융합하여 림프잡종세포를 만들고, 이 중에서 유구낭미충 항원에 대한 특이 단세포군항체를 생성하는 4개의 림프잡종세포군을 선택하였다. EITB상 Cya-1 단세포군항체는 25.5 kDa, Cya-7은 28 kDa, Cya-28은 87.5 kDa, Cya-31은 12.5 kDa 항원대에 각각 반응하는 것을 알 수 있었다. 간접면역형광항체법으로 Cya-1는 칼슘소체에, Cya-7은 실질조직에, Cya-28과 Cya-31은 충체 표피에 주된 반응을 나타내고 있었다. 유구낭미충 항원을 사용한 효소면역측정법으로는 유구낭미충 감염자 28명의 혈청 중 23명(82.1%)이 양성 반응을 보였으며, 폐흡충 감염자 31명 중 6명(19.4%), 스파르가눔 감염자 9명 중 1명(11.1%)이 교차 반응을 보였다. 반면, 특이 단세포군항체 Cya-7를 이용한 효소면역억제측정법으로는 유구낭미충 감염자는 28명 중 19명(67.9%)이 양성반응을 보였고, 다른 기생충 감염자 혈청과의 교차 반응은 관찰되지 않았다.

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