Yonsei Medical Jouranl
Vol. 35, No. 1, 1994

Serodiagnosis of Human Sparganosis by a Monoclonal Antibody-Based Competition ELISA

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Competition ELISA test using sparganum specific monoclonal antibodies (Mab) was investigated to improve the diagnostic specificity of sparganosis. By cell fusion, one hybridoma clone secreting anti-sparganum specific Mab was selected (Sp-20), which reacted on bands of 32 kDa and 38 kDa. Sp-20 reacted on calcium corpuscles on IFA. By micro-ELISA, 16 of 17 sparganosis cases (95%) were found positive, but 1 of 18 clonorchiasis cases (5%), 4 of 16 cysticercosis cases (25%) and 2 of 16 normal controls (11%) showed false positive reactions. On the other hand, by competition ELISA using a sparganum specific Mab (Sp-20), 16 out of 17 (95%) of sparganosis cases were found positive, but 2 of 18 clonorchiasis cases (10%), 2 of 16 cysticercosis cases (12%), 3 of 16 paragonimiasis cases (18%) and 1 of 16 normal controls (6%) showed false positive reactions.

Key Words: Sparganosis, monoclonal antibody, serodiagnosis, competition ELISA

Sparganosis is a tissue-invading disease caused by plerocercoid of *Spirometra mansoni*. Confirmative diagnosis of sparganosis is made possible by surgical removal from the lesion. Serodiagnostic tests, however, have been used widely. Of them, the enzyme-linked immunosorbent assay (ELISA) has widely been applied because of its high sensitivity, specificity and feasibility (Kim *et al.* 1982). However, the problem of improvement of specificity still remains to be solved in employing ELISA. In order to improve the specificity, a number of affinity-purified antigens with monoclonal antibodies (Mab), instead of the crude extract, have been used for serologic diagnosis of 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 competition ELISA for improving the specificity of micro-ELISA in the diagnosis of sparganosis.

Received September 16, 1993

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This study was supported by the Research Grant from the Korea Science and Engineering Foundation (1992).

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MATERIALS AND METHODS

Preparation of parasite extracts

Spargana were collected from naturally infected snakes (*Rhabdophis tigrina*) caught on Jiri Mountain, Kyungsangnam-do. The worms were homogenized in a 0.01 M Tris-HCl buffer (pH 7.2) at 4℃ for 30 minutes. The super-

natant was obtained by centrifugation at 4℃, 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 adult *Clonorchis sinensis* and *Taenia solium* metacestodes were prepared as described above. Adult *C. sinensis* worms were harvested from the bile duct of an experimental rabbit after infection with *C. sinensis* metacercariae collected from infected fresh-water fish, *Pseudorasbora parva*. The scolices of *T. solium* metacestodes were obtained from a naturally infected pig.

Sera

Nineteen sera of sparganosis were obtained from patients who visited the Severance Hospital, Yonsei University during the previous 4 years. The diagnosis was confirmed by excision of the worm out of the tissue. Sixteen sera of cysticercosis were collected from inhabitants in Cheju Island who were diagnosed parasitologically or from patients of the Severance Hospital with serological diagnosis. Twenty sera of *C. sinensis* infected humans confirmed by egg detection were obtained during the field survey in endemic areas along Nakdong-river. Sixteen 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 spar-ganosis. The sera had been stored in a deep freezer at −70℃ until used.

Production of Monoclonal antibody

Mabs were generated by the fusion of P3X63Ag8.V653 myeloma cells with spleen cells of mice immunized with water-soluble sparganum crude extract with some modifications (Köhler and Milstein, 1975). Hybridomas producing antibodies were identified by ELISA. Selected hybridoma cells were cloned by limiting dilution. Cloned hybridoma cell lines were expanded, and supernants were obtained as described previously (Yong *et al.* 1991).

Micro-ELISA

Micro-ELISA was used to select hybridomas secreting antibodies against sparganum antigens and to measure antibody levels in human sera. Crude water-soluble antigens of sparganum (5 μg protein/ml) were coated on 96-well plates (Costar). After overnight incubation at 4℃ the plates were washed 3 times in 0.9% saline with 0.05% Tween 20 and blocking was performed using 3% skim milk in PBS. Wells were washed 3 times and the culture supernatant was incubated for 1 hour at 37℃. Wells were washed again and incubated with 1:2,000 diluted peroxidase conjugated anti-mouse immunoglobulin (Cappel). After washing, wells were developed using 100 μl/well of 0.05% orthophenylenediamine and 0.006% H_2O_2 in 0.1M phosphate-citrate buffer (pH 5.0) for 30 minutes at room temperature. The reaction was stopped by adding 50 μl/well of 2N H_2SO_4. The optical density was read at 490 nm using the ELISA Reader (Dynatech MR 300).

Characterization of the Monoclonal antibody

Specificity of the anti-sparganum monoclonal antibodies in the culture supernatant was tested for listed extracts as above by ELISA.

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

Competition ELISA

A modification of the method described by Liu *et al.* (1992) was used. Polystyrene plates (Costar, U.S.A.) were coated with 50 μl of sparganum extract (12 μg/ml) in a bicarbonate-carbonate buffer (pH 9.6) at 4℃ 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℃. 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℃. After washing again, 50 μl of Mab was applied for 30 min at 37℃. After washing, 50 μl of 1:4,000 diluted peroxidase conjugated

rabbit anti-mouse immunoglobulin (Cappel) was reacted for 30 min at 37℃. Washed as above, 0.05% orthophenylenediamine and 0.06 % hydrogen peroxide, diluted in 0.1M 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 an ELISA Reader (Dynatech MR300).

The cut-off absorbance was established through the mean + 2 standard deviations of absorbance. The result of competition ELISA was compared with that of micro-ELISA.

RESULTS

A specific Mab, named Sp-20, was selected. It did not cross-react with any other parasite antigens tested by micro-ELISA, such as *C. sinensis*, *T. solium* metacestodes and *P. westermani* (Absorbance: Sparganum 0.721, PW 0.181, Cs 0.150, Cys 0.165). SDS-PAGE revealed the protein band pattern of the sparganum crude extract (Fig. 1). Immunoblots reacted on Sp-20 and immune mouse serum, and the protein bands were shown in Fig. 2. Antigenic determinants of 32 kDa and 38 kDa were found to react on Sp-20. The antigenic determinants reacted on selected Mab was in very good agreement with major antigenic bands in infection. That finding was clearly noted in Fig. 2. On the sectioned worm, antigenic substances reacted on Sp-20 were found in the calcium corpuscles of the parasite (Fig. 3).

By micro-ELISA, 16 of 17 sparganosis cases

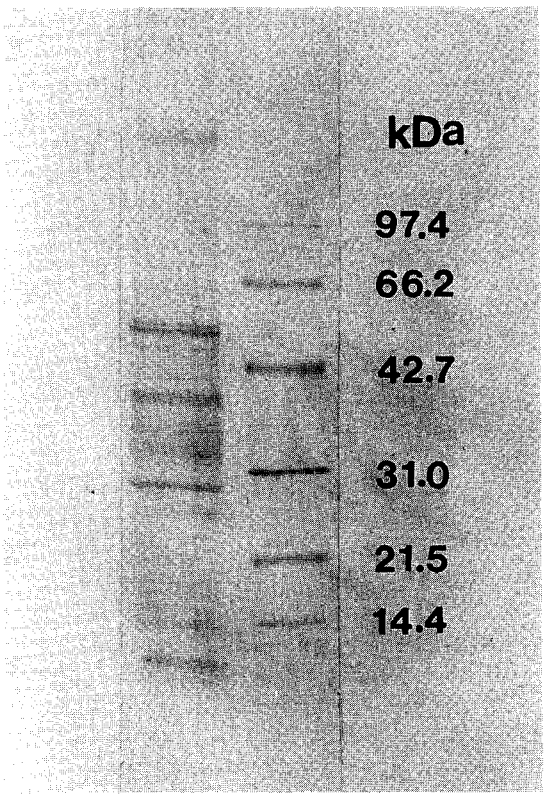

Fig. 1. *SDS-PAGE finding of sparganum crude extract after electroblotted to nitrocellulose paper.*

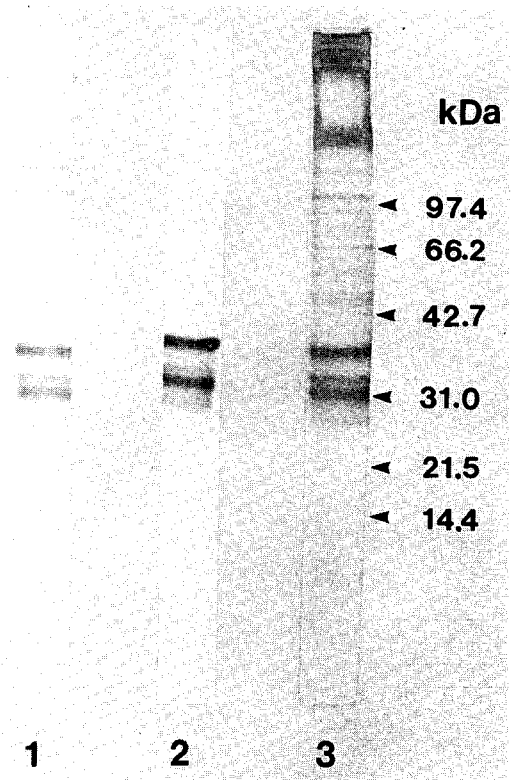

Fig. 2. *SDS-PAGE/Immunoblots reacted on: 1) Mab Sp-10, 2) Mab Sp-20, 3) immune mouse serum. Antigenic bands of 32 kDa and 38 kDa reacted on Sp-20 were identified.*

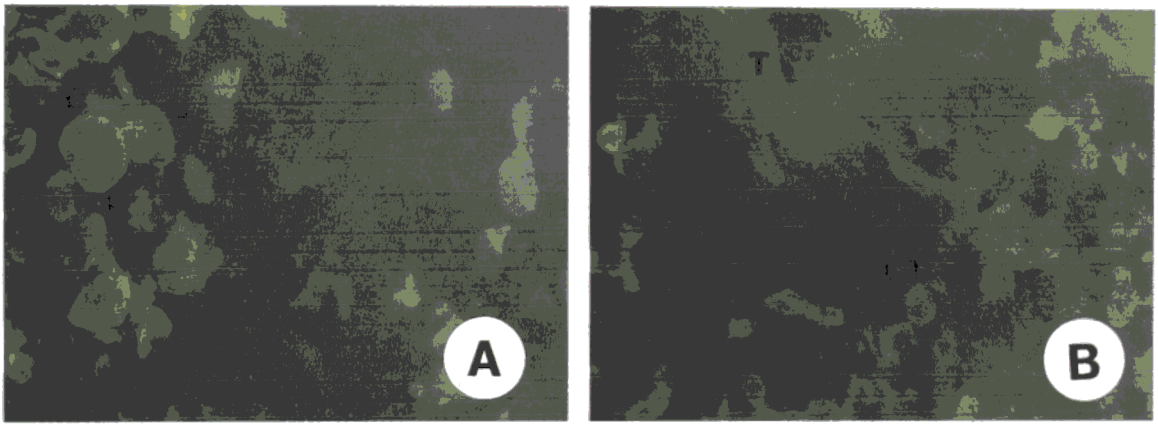

Fig. 3. *Localizaztion of Mab by indirect fluorescence antibody technique. Frozen sections of sparganum were reacted on: (A) Sp-20 Mab showing positive reactions at the calcium corpuscles (×250) and (B) immune mouse serum as a positive control (×100). (C: calcium corpuscle, T: tegument, CT: connective tissue)*

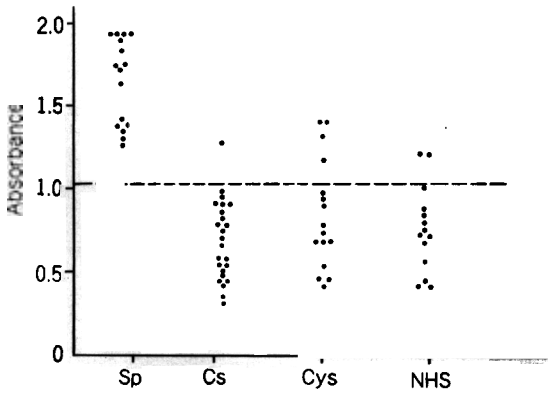

Fig. 4. *Distribution of absorbance of human sera in sparganosis (Sp), clonorchiasis (Cs), cysticercosis (Cy) and normal controls (NHS) against sparganum extract by micro-ELISA.*

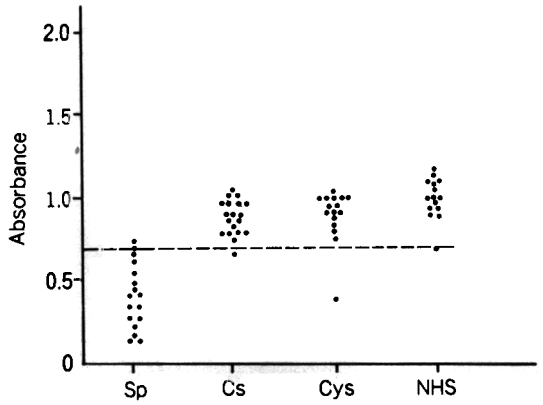

Fig. 5. *Distribution of absorbance of human sera in sparganosis (SP), clonorchiasis (Cs), cysticercosis (Cy) and normal controls (NHS) against sparganum extract by competition ELISA using a specific monoclonal antibody (Sp-20).*

(95%) were found positive, but 1 of 18 clonorchiasis cases (5%), 4 of 16 cysticercosis cases (25%) and 2 of 16 normal controls (11%) showed false positive reactions(Fig. 4). On the other hand, by the competition ELISA test using a sparganum specific Mab (Sp-20), 16 out of 17 (95%) sparganosis cases were found positive, but 2 of 18 clonorchiasis cases (10%), 2 of 16 cysticercosis cases (12%), 3 of 16 paragonimiasis cases (18%) and 1 of 16 normal controls (6%) showed false positive reactions (Fig.5). The competition ELISA using a Mab showed a slightly higher specificity in comparision with a micro-ELISA for serodiagnosis of human sparganosis(86% vs 94%).

DISCUSSION

Sparganum is one of the most important

cestode parasites of man in Korea. In this study, Mabs were produced for analysing sparganum antigen and the immunodiagnosis of human sparganosis. The specifity of the Mab was tested against various parasites' antigens. As might be expected, common antigens were found. One hybridoma clone producing a specific Mab was selected for this experiment, and the others were discarded.

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 indicates that each reacting antigenic band has the same antigenic determinant recognized by the Mab.

Cho *et al.* (1990) reported a Mab against antigenic proteins of sparganum in order to characterize the antigens. They purified antigenic proteins(36 and 29kDa) of sparganum by immunoaffinity chromatography using the monoclonal antibody. Kong *et al.* (1991) purified the same proteins by gelatin-affinity chromatography.

The Mab used in this study seemed to have reacted on a different antigenic determinant from Mabs previously reported by other researchers, because the reacted antigen had a different molecular weight from others as identified by EITB.

It is difficult to make a diagnosis of sparganosis because it doesn't produce eggs and it has no predilection site like paragonimiasis or clonorchiasis. A confirmative diagnosis can be made only by excision of the worm out of the infected tissue. But this diagnostico-therapeutic method has a very limited range of application. Therefore, the application of immunodiagnosis, especially to detect specific antibodies, to sparganosis is very useful and important. As we have seen in this study, the problem of cross reaction with other parasites still remains to be solved. A higher frequency of cross reaction with *T. solium* metacestodes was observed compared to other worms. It is presumed to be a result of the common antigenic components which they share as larval cestodes. In order 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 less useful than the crude antigen because the purifying procedure is usually very difficult and inefficient.

A Mab based ELISA-inhibiton 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 better sensitivity and specificity. In this study, the competition ELISA test using sparganum specific Mab was applied in the diagnosis of sparganosis. The results showed that the sensitivity of the competition ELISA was the same as that of the micro-ELISA, while the specificity has improved. The competition ELISA using a sparganum specific Mab could be used satisfactorily to differentiate human sparganosis from other parasitic infections.

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