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Analysis of *Clonorchis sinensis* antigens and diagnosis of clonorchiasis using monoclonal antibodies*

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Abstracts: *Clonorchis sinensis* is a common parasite of man in Korea. Researches on the specific antigens of *C. sinensis* would be valuable not only because those elucidate the molecular characteristics of this fluke but also because it is applicable to immunodiagnosis. Although many monoclonal antibodies have been used in the field of parasite immunology, few articles on monoclonal antibodies against *C. sinensis* have been published so far. The aim of this study was to analyze *C. sinensis* antigens recognized by monoclonal antibodies, and to set up ELISA-inhibition test using *C. sinensis* specific monoclonal antibodies for improved specificity of immunodiagnostic tests.

By fusion between spleen cells of the mice immunized with *C. sinensis* water-soluble crude adult worm antigens and plasmacytoma cells of mouse origin, 29 hybridoma clones secreting anti-*C. sinensis* monoclonal antibodies were made, and 8 clones among those were found specific. After cell cloning, isotypes of 6 selected specific monoclonal antibodies were determined to be IgG1, IgG2b and IgA. Four exposed antigenic determinants of natural infection were recognized by different specific monoclonal antibodies. By enzyme-immunoelectrotransfer blot, 10 KD, 34 KD antigenic determinants were found to be reacted with CsHyb 0714-20, CsHyb 0605-10 monoclonal antibodies, respectively. The antigenic determinant recognized by CsHyb 0714-20 monoclonal antibody was revealed to be located at the surface and parenchyme of a parasite by indirect immunofluorescent antibody technique, and those reacted with CsHyb 0605-10, CsHyb 0714-25 monoclonal antibodies were found at the parenchyme and intestine. The antigenic determinant reacted with CsHyb 0605-23 monoclonal antibody was found mainly around the uterine eggs. Four antigenic determinants recognized by specific monoclonal antibodies were all found to be present in the early eluted fractions of *C. sinensis* antigens separated by Sephadex G-200 gel filtration.

By conventional ELISA, 75% of clonorchiasis cases were found positive, but 7.1% of normal controls and 37.5% of paragonimiasis cases showed false positives. However, by ELISA-inhibition test using *C. sinensis* specific monoclonal antibody (CsHyb 0605-23), 77.1% of clonorchiasis cases were found positive, and there were no false positives in normal controls or paragonimiasis cases, indicating 100% specificity. The ELISA-inhibition test using monoclonal antibodies was found to have same sensitivity and definitely high specificity in comparison with conventional ELISA for serodiagnosis of human clonorchiasis.

Key words: *Clonorchis sinensis*, monoclonal antibodies, diagnosis, ELISA-inhibition test

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INTRODUCTION

Clonorchis sinensis is a common parasite of man, of which geographical distribution is largely confined to Eastern Asia including Japan, China, Taiwan, northern Vietnam and southern Korea. In Korea, it has been well-known that the human infection of *C. sinensis* is widely spread along the great rivers and streams (Soh *et al.*, 1976; Seo *et al.*, 1981).

There has been many publications on the epidemiology and pathogenesis of clonorchiasis (Rim, 1986), and also the morphology and biology of *C. sinensis* (Inatomi *et al.*, 1968; Jeong *et al.*, 1978, 1980a&b). Last few decades, however, continuous efforts has been made on immunodiagnosis or serodiagnosis for clonorchiasis along with the immunologic studies on the parasite. Various immunodiagnostic tests have been applied to the diagnosis of clonorchiasis successively. Intradermal test has been commonly used for epidemiologic studies, but sometimes for diagnosis at the hospital (Sawada *et al.*, 1964; Ahn *et al.*, 1975). Gel diffusion (Sun and Gibson, 1969; Lee, 1975), complement fixation test (Sadun *et al.*, 1959) and immunofluorescent test (Im, 1974; Choi, 1975; Kwon *et al.*, 1984) were also applied successfully. Recently several research groups have reported the enzyme-linked immunosorbent assay (ELISA) to be excellent because of high sensitivity and feasibility (Lee *et al.*, 1981; Yang *et al.*, 1983; Jin *et al.*, 1983; Soh *et al.*, 1985; Lee and Ahn, 1987).

Pathological changes of the bile duct and surrounding liver tissues could be occurred by *C. sinensis* infection, such as desquamation, proliferation, glandular change and metaplasia of the cholangial epithelium. Infection of *C. sinensis* might be an important factor in the formation of gall stones and cholangial cancer (Marcial-Rojas, 1971; Rim, 1986). During infection, antigenic materials of the worm trespass the cholangial epithelium, and sensitize the immune system of an infected host. Therefore researches on the antigens of *C. sinensis* must be valuable since it can elucidate the pathogenesis of clonorchiasis (Min and Soh, 1983).

Generally, antigens of the parasite are so complicated that it is not easy to analyze them. But recent developments of biotechnology have made parasite antigen analysis possible to some extent. Especially the monoclonal antibody having high affinity for a specified antigenic determinant has been applied to the immunologic studies and immunodiagnosis on tropical parasitic diseases of man (Köhler and Milstein, 1975; Goding, 1983; Weir *et al.*, 1986). In Korea, a few articles on monoclonal antibodies against parasite antigens have been published so far (Kim *et al.*, 1986; Ryu, 1988; Cho *et al.*, 1990), and it is difficult to find publications on the monoclonals directed against *C. sinensis* antigens.

The aim of this study was to analyze *C. sinensis* antigens recognized by monoclonal antibodies and to set up ELISA-inhibition test using *C. sinensis* specific monoclonal antibodies for improvement of the specificity of immunodiagnostic tests.

MATERIALS AND METHODS

Preparation of Antigens

1. Antigens of *C. sinensis*

C. sinensis metacercariae were collected from naturally infected *Pseudorasbora parva*. Infected fish were minsed with a waring blender, and digested with artificial gastric juice for 2 hours at 37°C. After washing 5 times with normal saline, the metacercariae were collected under a stereomicroscope. These metacercariae were orally infected to experimental rabbits. Eight weeks later, the adult worms were obtained from the bile ducts of the sacrificed hosts. Adult worms were homogenized with a teflon homogenizer in 0.01 M tris-HCl buffer (pH 7.2) at 4°C for 30 minutes. The supernatant was obtained after centrifugation at 4°C, 15,000g for 1 hour. The supernatant was used as crude antigens throughout this experiment. Protein concentration was

determined by the method of Lowry *et al.*, 1951.

2. Antigens of the other parasites

Antigens were prepared as same as the above. Antigens of the following parasites were used.

1) Adult worms of *Paragonimus westermani*: Metacercariae were obtained from the crayfish (*Cambaroides similis*) caught from Bogil Island, Chollanam-do. Adult worms were collected from the lungs of a dog 12 weeks after it is infected by *P. westermani* metacercariae.

2) Cystic fluid of hydatid cyst: It was obtained from the lungs of an infected man.

3) Sparganum: It was obtained from the subcutaneous tissue of an infected man.

4) Proglottides of *Diphyllobothrium latum*: Naturally expelled proglottides from an infected man were used.

5) Proglottides of *Hymenolepis diminuta*: These were obtained from the intestine of a naturally infected field mice (*Apodemus agrarius*).

6) Proglottides of *Hymenolepis nana*: These were obtained from the intestine of a naturally infected field mice (*Crocidura lasiura*).

7) *Cysticercus cellulosae*: It was obtained from the muscles of a naturally infected domestic pig in Cheju Island.

8) Adult worms of *Ascaris suum*: Those were obtained from the intestines of a pig slaughtered in Seoul.

Cell Fusion and Production of Monoclonal Antibodies

1. Immunization

Six-week old BALB/c mice were immunized with water-soluble crude *C. sinensis* adult worm antigens. They were injected intraperitoneally (i.p.) with 100 μg of antigens mixed in Freund's complete adjuvant at first. Six weeks after the first immunization, 100 μg of antigens mixed in Freund's incomplete adjuvant were i.p. injected. Four weeks after the second immunization, third immunization was conducted with 100 μg of antigen intravenously (Fig. 1) (Kim *et al.*, 1986).

2. Cell fusion

Three days after third immunization, the spleen of a mouse was removed aseptically, and

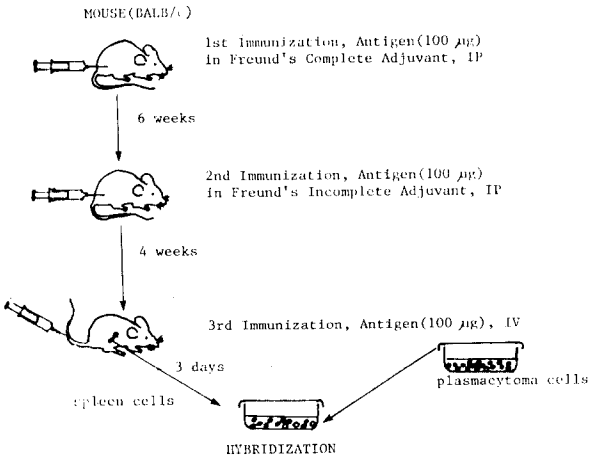

Fig. 1. Immunization schedule for BALB/c mice with *C. sinensis* water-soluble crude adult worm antigens.

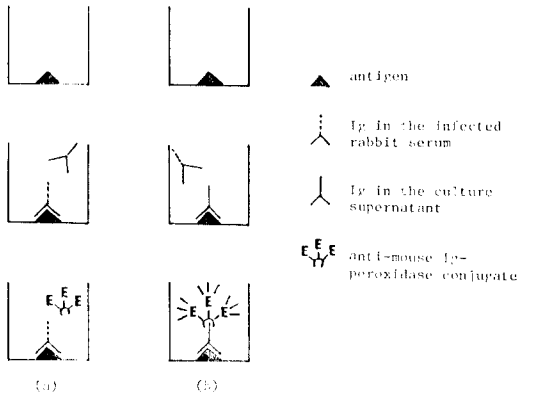

Fig. 2. Test for exposed antigenic determinants of natural infection by ELISA-inhibition test: (a) exposed antigenic determinant of natural infection, (b) non-exposed antigenic determinant of natural infection.

spleen cells were obtained. Red blood cells were hemolyzed by short treatment (about 7 seconds) with distilled water. A total of 10^8 spleen cells were prepared. The mouse plasmacytoma cell line (P_3—X63—Ag 8. V653) maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), penicillin 100 IU/ml and streptomycin 100 μg/ml was used for hybridization.

Hybridization was performed according to the method of Köhler and Milstein (1975) with some modifications. Briefly, 10^8 spleen cells and 2×10^7 plasmacytoma cells were washed 3 times

with serum free RPMI 1640, and mixed. One ml of 50% polyethyleneglycol 4,000 (Merck) was added drop by drop for 1 minute at 37°C. Twenty ml of RPMI 1640 was added during next 5 minutes. After hybridization, cells were dispensed to 96 well culture plates in RPMI 1640 with 20% FCS. Next day HAT medium (RPMI 1640 containing hypoxanthine 13.6 μg/ml, aminopterin 0.174 μg/ml, thymidine 3.87 μg/ml and FCS 20%) was added to the wells. Thereafter HAT medium was changed every other day, and growing hybridomas were checked with inverted microscope.

3. Selection of antibody secreting hybridoma clones

Enzyme-linked immunosorbent assay (ELISA) was used to select hybridomas secreting antibodies directed against *C. sinensis* antigens. Crude water-soluble adult worm antigens of *C. sinensis* (5 μg protein/ml) were coated on wells in polystyrene plate (Nunc) by overnight incubation at 4°C. After washing 3 times in 0.9% saline with 0.05% Tween 20, blocking was performed using 3% skim milk (Difco) in phosphate-buffered saline (PBS). Wells were washed 3 times, and culture supernatant was incubated for 2 hours at 37°C. 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 0.1 M phosphate-citrate buffer (pH 5.0) for 30 minutes at room temperature. The reaction was stopped by adding 50 μl/well of 2 N H_2SO_4. The optical density was read at 490nm using ELISA Reader (Dynatech MR 300).

Characterization of Monoclonal Antibodies

1. Specificity of anti-*C. sinensis* antibodies in culture supernatant

Specificity of the anti-*C. sinensis* antibodies in the culture supernatant was tested for 9 kinds of antigens described as above including *C. sinensis* antigens by ELISA.

2. Cell cloning & isotyping

The specific antibody-secreting hybridoma clones were selected, and moved to 24 well plates. For cloning, cells were diluted in HT media so as the number to be 0.1~0.5/well. They were cultured for 10~14 days, and reselected. This procedure was repeated once again. Thereafter, isotyping was performed with an isotyping kit according to the method of manufacturer (Hyclon). Briefly, goat anti-mouse immunoglobulin was coated on the ELISA plate. After reacting culture supernatant, subclass specific rabbit anti-mouse immunoglobulin (IgG1, IgG2a, IgG2b, IgG3, IgM and IgA) was reacted. Thereafter substrate, chromogens and stopping solution were incubated successively as described as above for the ELISA.

Characterization of *C. sinensis* Antigens

1. Test for the exposed antigens during natural infection reacted with monoclonal antibodies

The concept of the ELISA-inhibition test was applied to determine the antigens reacting with monoclonal antibodies exposed during natural infection. Coating of *C. sinensis* antigens and blocking were performed as above. Then, 20 μl of undiluted *C. sinensis* infected rabbit serum and 80 μl of culture supernatant were put into a same well, and incubated simultaneously for 1 hour at 37°C. At the same time, culture supernatant alone was incubated in an adjacent well. After washing, 1:4,000 diluted peroxidase conjugated goat anti-mouse immunoglobulin (Cappel) was reacted for 1 hour at 37°C. Remaining procedures were performed as same as above. Then, the ELISA titers of these 2 wells were compared each other. Inhibition rate was calculated, using the following formula;

$$\text{Inhibition rate}(\%) = \left(1 - \frac{\text{Abs.sample}}{\text{Abs.cont'l}}\right) \times 100$$

Abs.sample : Absorbance in case of co-incubation of infected rabbit serum and monoclonal antibody

Abs.cont'l : Absorbance in case of the supernatant incubation alone

When the titer of co-incubated wells were inhibited not so much (less than 50%) in com-

parison with that of culture supernatant incubating alone, it was determined to be a non-exposed or weakly exposed antigenic determinant during natural infection.

2. Enzyme-linked immunoelectrotransfer blot (EITB)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the procedure of Laemmli (1970). Vertical electrophoresis system of 17×12 cm was used (Manhattan Co., Korea). Separating gel of 9cm long and 1.5 mm thick was prepared in 0.37 M tris-HCl buffer (pH 8.8) containing 0.1% SDS. Linear gradient gel of 5~20% were made. Stacking gel of about 1cm long was prepared with 3% polyacrylamide in 0.125 M tris-HCl buffer (pH 6.8) containing 0.1% SDS. Crude water-soluble *C. sinensis* antigens in the same amount of sample buffer (0.125 M tris-HCl buffer, pH 6.8 containing 4% SDS, 20% glycerol, 10% mercaptoethanol and 0.001% bromophenol blue) were treated at 95°C for 3 minutes. Fifty μl of antigens (containing about 100 μg of protein) were applied to each well. Samples were electrophoresed at 20 mA for about 5 hours. Protein bands were stained with 0.125% Coomassie brilliant blue R-250 containing 50% methanol, 10% acetic acid, and destained with 7% methanol and 10% acetic acid solution.

EITB was performed according to the procedures described by Towbin *et al.* (1979) and Tsang *et al.* (1983). Protein fractions in the electrophoresed gel were transferred to nitrocellulose (NC) paper (Bio-Rad) in transfer buffer (0.02 M tris, 0.15 M glycine and 20% methanol). Electrotransfer was carried out at 50 volts, 4°C for 16 hours. After electrotransfer, the NC paper was washed on shaker 3 times with PBS (pH 7.4) containing 0.5% Tween 20 (PBS/T) each for 10 minutes. Blocking was followed with 3% skim milk in PBS/T for 1 hour at 37°C. After washing with PBS/T 3 times, 1:100 diluted rabbit test sera were applied on shaker at room temperature for 2 hours. After the wash as above, 1:2,000 diluted peroxidase conjugated anti-rabbit IgG was reacted for 1 hour at room temperature. After washing again, substrate (50 mg of diaminobenzidine, 10 μl of 30% H_2O_2 and 100 ml of distilled water) was reacted until enough visualization of the band pattern about for 10 minutes. The reaction was stopped by washing the NC paper with distilled water.

3. Locations of the antigens inside a parasite

Locations of the antigens reacted with monoclonal antibodies were revealed by cryocutting of the adult *C. sinensis* worm and indirect immunofluorescent microscopy.

A live *C. sinensis* adult worm collected from a rabbit bile duct was washed several times with PBS, pH 7.2, and freezed quickly to −20°C in the chamber of Minitome Cryocutter (IEC). The frozen worm was embedded using Tissue-Tek II O.C.T. compound, then sectioned into 7 μm thick slices. The sectioned worm slices were laid on the slides, and air-dried. After washing with 0.01 M PBS, pH 7.2 for 10 minutes, worm sections were incubated with 1:100 diluted immune mouse serum or undiluted culture supernatant for 1 hour at 37°C. After washing, 1:40 diluted fluorescent isothiocyanate (FITC) conjugated anti-mouse IgG containing 0.5% bovine serum albumin was reacted for 1 hour at 37°C. After washing again, slides were put into 0.5% Evans blue solution for 5 minutes at room temperature for reduction of non-specific reactions. The slides were washed, air-dried, and observed using a fluorescent microscope (Leitz Dialux 20).

4. Antigenic fractions separated by Sephadex G-200 gel filtration

C. sinensis antigens were ultracentrifuged at 105,000g at 4°C for 1 hour using an Ultracentrifuge (Beckman). The supernatant was separated by Sephadex G-200 gel filtration, and the fractions obtained by gel filtration were reacted against monoclonal antibodies.

Freeze-dried Sephadex G-200 gel was put into distilled water, and cooked for 5 hours in a double boiler to swell up. The gel was poured into a glass column (16×16 mm) up to 85 cm high. The gel was washed with tris-HCl buffer

(pH 7.2) at a flow rate of 6 ml/hour for about 24 hours to reach an equilibrium. This column was moved into a Minicoldlab (LKB) at 4°C. Void volume was checked using Blue dextran before application of the sample. Standard proteins for gel filtration (carbonic anhydrase, 29 KD; bovine serum albumin, 66 KD; γ-globulin, 150 KD) were used to calculate the molecular weight of proteins in the sample. The sample (*C. sinensis* antigens 20 mg/4ml) was applied, and monitored using an UV monitor at 206nm. Each fraction (6 ml) was collected, and the buffer was flowed through the gel continuously until the absorbance was dropped down to zero. The reactivity of monoclonal antibodies directed against each antigenic fraction was investigated by ELISA. Each antigenic fraction was coated on wells at 5 μg/ml, respectively, and remaining procedures were as same as described above.

Diagnosis of Human Clonorchiasis by ELISA-inhibition Test Using Monoclonal Antibodies

ELISA-inhibition test was set up by using a specific monoclonal antibody, and the result was compared with that of conventional ELISA (Fig. 3). For the conventional ELISA, all of the procedures were performed as above, except that human serum samples were 1:200 diluted, and 1:2,000 diluted peroxidase conjugated goat anti-human IgG (Cappel) was used.

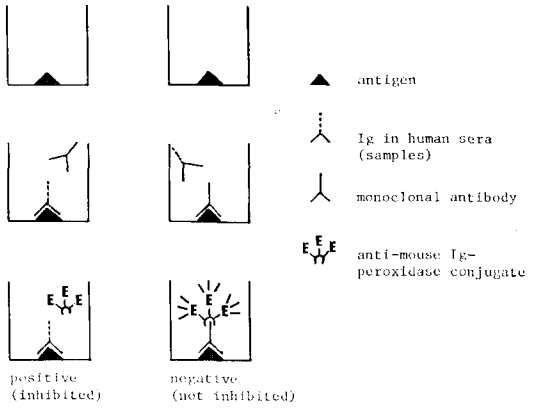

Fig. 3. ELISA-inhibition test using specific monoclonal antibody to detect anti-*C. sinensis* antibodies in the human sera.

Following were the procedure of the ELISA-inhibition test.

At first, antigens were coated, blocked and washed as described above. Twenty μl of 1:4 diluted human serum samples and 80 μl of undiluted culture supernatant containing highly specific anti-*C. sinensis* monoclonal antibody were co-incubated in the same well. After washing, 100μl of 1:4,000 diluted peroxidase conjugated rabbit anti-mouse immunoglobulin was reacted for 1 hour at 37°C. The remaining procedures were as same as above.

In this experiment, 48 sera of clonorchiasis, 16 sera of paragonimiasis and those of normal controls were used. Sera of clonorchiasis were obtained from infected humans living in an endemic area (Chinyang-gun, Kyongsangnam-do), and those of paragonimiasis were obtained from infected patients who visited the Severance Hospital, Yonsei University or Hanyang University Hospital. All of them were confirmed cases by the parasitological method. Normal control sera were obtained from humans who had no parasite eggs in their stool specimens. All sera had been stored in a deep freezer at −70°C until use.

RESULTS

Cell Fusion

Immunized BALB/c mice showed high serum antibody titers(>1:50,000) investigated by ELISA on the day of hybridization.

A total of 4 cell fusion was performed, cell suspensions were dispensed in three 96 well plates each time. Fusion rates were very different during each hybridization from 30% to 95%, and growing hybridomas were found from a total of 720 wells. Hybridomas secreting anti-*C.*

Table 1. Efficiency of making hybridomas secreting anti-*C. sinensis* antibodies

Wells	Number
1) with dispensed cells	1152
2) having hybridomas	720
3) having hybridomas secreting anti-*C. sinensis* antibodies	29

sinensis antibodies were investigated by ELISA. When 1:400 diluted immunized mouse serum was used, the absorbance of ELISA was 1.2, and a total 29 wells (absorbance>0.3) were fonnd to be positive. Those were about 4% of a total number of wells with hybridomas (Table 1).

Table 2. Specificity of anti-*C. sinensis* antibodies secreted by 29 hybridoma clones by ELISA

Clones	Reactivities to antigens of								
	Cs	Pw	Hn	Hd	Cys	Hyd	Dl	Spa	As
1)	6	0	0	0	0	0	0	0	0
2)	6	0	0	0	0	0	0	0	0
3)	6	0	0	0	0	0	0	0	0
4)	6	0	0	0	0	0	0	0	0
5)	6	0	0	0	0	0	0	0	0
6)	6	0	0	0	0	0	0	0	0
7)	6	0	0	0	0	0	0	0	0
8)	6	0	0	0	0	0	0	0	0
9)	6	7	6	6	5	6	7	5	5
10)	6	7	1	0	0	0	2	0	7
11)	6	6	1	0	0	0	2	0	6
12)	6	5	4	5	3	5	6	4	5
13)	6	3	2	3	2	1	3	1	1
14)	6	2	3	0	0	0	3	2	3
15)	6	2	2	3	0	0	3	1	1
16)	6	2	2	1	0	0	1	1	2
17)	6	2	2	0	0	0	3	2	3
18)	6	1	1	4	0	1	3	1	1
19)	6	1	1	0	1	0	1	1	1
20)	6	1	0	0	1	0	0	0	0
21)	6	0	4	2	0	0	1	1	1
22)	6	0	3	0	3	0	1	2	1
23)	6	0	3	2	7	0	1	3	1
24)	6	0	2	1	6	0	0	2	0
25)	6	0	2	0	7	0	0	2	0
26)	6	0	1	0	1	0	0	0	0
27)	6	0	1	0	0	0	1	0	0
28)	6	0	0	1	1	0	0	0	0
29)	6	0	0	1	0	0	0	0	0

* Score 0: Abs. 0.00~0.19 ** Cs : *C. sinensis*
1: 0.20~0.39 Pw : *Paragonimus westermani*
2: 0.40~0.59 Hn : *Hymenolepis nana*
3: 0.60~0.79 Hd : *Hymenolepis diminuta*
4: 0.80~0.99 Cys : *Cysticercus cellulosae*
5: 1.00~1.19 Hyd : hydatid cyst
6: 1.20~1.39 Dl : *Diphyllobothrium latum*
7: 1.40~1.60 Spa : sparganum
As : *Ascaris suum*

Characteristics of Monoclonal Antibodies

1. Specificity of monoclonal antibodies

Fused cells grown in 29 selected wells were moved into 24 well plates in HT media, and expanded their cell numbers. The specificity of culture supernatant was tested by ELISA. The absorbance was converted into a numerical value in order to make the reactivity of monoclonals easily understandable. Only 8 wells (27.6%) were found to be containing specific antibodies directed against *C. sinensis* antigens. Other 21 wells were found to have cross-reacting antibodies directed against various parasite antigens tested in this experiment (Table 2).

2. Cell cloning and isotype

Six kinds of hybridomas were successfully cloned. They were named as CsHyb 0714-20, CsHyb 0605-10, CsHyb 0605-23, CsHyb 0714-25, CsHyb 0714-9 and CsHyb 0714-11. Isotype of CsHyb 0605-10 and CsHyb 0605-23 were IgG2b and IgA, respectively. Other 4 monoclonals were all found to be IgG1 (Table 3).

Table 3. Isotypes of anti-*C. sinensis* monoclonal antibodies assayed by ELISA*

Hybridoma clones	Isotypes
CsHyb 0714-20	IgG1
CsHyb 0605-10	IgG2b
CsHyb 0605-23	IgA
CsHyb 0714-25	IgG1
CsHyb 0714-9	IgG1
CsHyb 0714-11	IgG1

*Hyclon isotyping kit was used.

Characteristics of the Antigens

1. Exposed antigens during natural infection

Among 6 different hybridoma clones, ELISA titer of the supernatant of 4 clones were inhibited significantly (>50%) in case of co-incubation with infected rabbit serum in comparison with that in the case of the supernatant incuba-

Table 4. Evaluation of antigenic determinants recognized by monoclonal antibodies for exposed antigens of natural infection

Monoclonal antibodies	Infected rabbit serum (+)	Infected rabbit serum (−)	Inhibition rate (%)
CsHyb 0714-20	0.37	0.87	57.5*
CsHyb 0605-10	0.13	0.75	82.7*
CsHyb 0605-23	0.09	0.64	86.0*
CsHyb 0714-25	0.08	0.58	86.2*
CsHyb 0714-9	1.20	1.32	9.1
CsHyb 0714-11	0.48	0.57	15.8

*Exposed antigenic determinants of natural infection

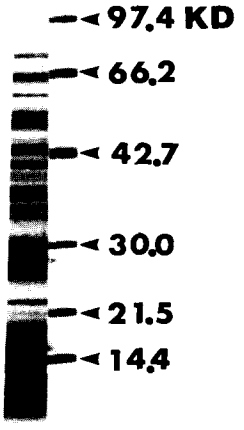

Fig. 4. SDS-PAGE finding of *C. sinensis* water-soluble crude adult worm antigens.

tion alone. So these 4 hybridoma clones were determined to be producing monoclonal antibodies against exposed antigenic determinants of natural infection. The other 2 clones were determined to be secreting monoclonal antibodies against nonexposed or weakly exposed antigens (Table 4).

2. EITB

The protein band pattern of *C. sinensis* antigens is shown on Fig. 4. Following SDS-PAGE, proteins were electrotransferred onto a NC paper, and reacted with an immune mouse serum or 4

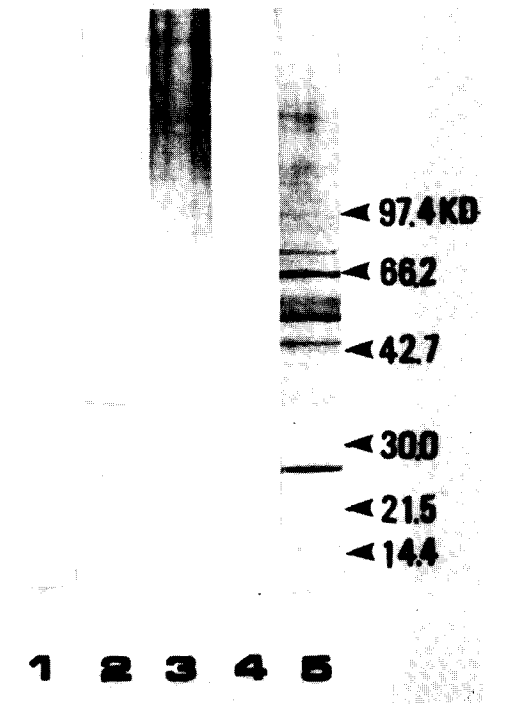

Fig. 5. Enzyme-immunoelectrotransfer blot pattern of *C. sinensis* antigen fractions against monoclonal antibodies:
(1) CsHyb 0714-20, (2) CsHyb 0605-10,
(3) CsHyb 0605-23, (4) CsHyb 0714-25,
(5) immune mouse serum.

kinds of monoclonals. Ten KD and 34 KD antigenic determinants were found to be reacting with CsHyb 0714-20 and CsHyb 0605-10 monoclonal antibodies, respectively. But, the antigenic determinants reacting with the other 2 monoclonal antibodies could not to be recognized by EITB (Fig. 5).

3. Location of the antigens inside a parasite

By indirect immunofluorescent microscopy, antigens reacted with CsHyb 0714-20 monoclonal antibody were recognized on the surface, suckers and parenchyme of a worm. Those reacted with CsHyb 0605-10 or CsHyb 0714-25 monoclonal antibodies were found from the intestine and parenchyme. However, antigens reacted with CsHyb 0605-23 monoclonal antibody were distributed especially around the uterine eggs. Those

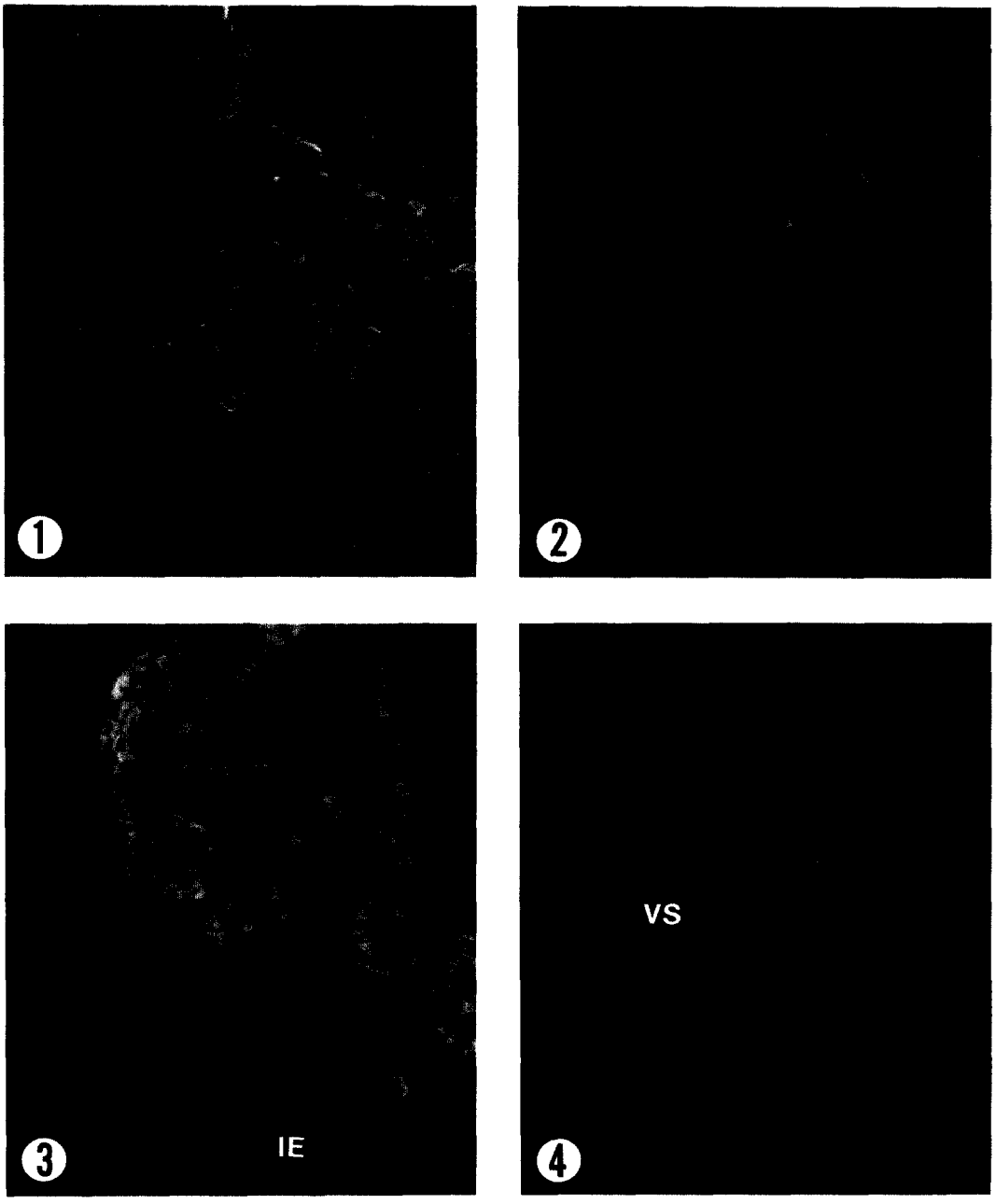

Fig. 6. Distribution of *C. sinensis* antigens reacted with monoclonal antibodies by indirect fluorescent antibody technique. Frozen sections of *C. sinensis* adult worm was reacted with:

1) immune mouse serum as a positive control,
2) normal mouse serum as a negative control,
3) CsHyb 0605-23 monoclonal antibody showing positive reactions strongly around the uterine eggs, intestinal epithelium(IE) reacting rather weakly. But the worm surface showed no fluorescence at all,
4) CsHyb 0714-20 monoclonal antibody showing strong positive reactions on the surface, ventral sucker(VS) and parenchyme.

Table 5. Distribution of antigens reacted with monoclonal antibodies

Organs of *C. sinensis*	Hybridoma clones (CsHyb-)				Immune
	0714-20	0605-10	0605-23	0714-25	serum
Tegument	++	−	−	−	++
Suckers	++	−	−	−	++
Intestinal epithelium	+	+	+	+	++
Intestinal content	−	+	+	+	++
Uterus & Eggs	+	−	++	−	++
Testis	−	−	−	−	+
Seminal receptacle	−	−	−	−	+
Vitellaria	−	−	−	−	+
Parenchyme	++	+	+	+	++

were also found in the intestine and parenchyme (Fig. 6, Table 5).

4. Antigenic fraction by Sephadex G-200 gel filtration

First fraction of the antigen appeared 10 hours after application of the sample, so the void volume was 60 ml. During 33 hours after the beginning of gel filtration, 23 fractions were collected (6 ml/hour) (Fig. 7). Protein concentration of each fraction were determined by the method of Lowry *et al.*, 1951, and ELISA was performed. Reacting pattern of 4 different monoclonal antibodies and immune mouse serum against each antigenic fraction was shown on Table 6. Four antigenic determinants recognized by specific monoclonal antibodies were all found to be present in the early eluted fractions of *C. sinensis* antigens separated by Sephadex G-200 gel filtration, which might be considered to have high molecular weight (Table 6).

Diagnosis of Human Clonorchiasis by ELISA-inhibition Test Using Monoclonal Antibodies

By conventional ELISA, the mean absorbance of 28 normal controls was 0.13, and standard deviation (S.D.) was 0.10, so the cut-off value (mean+2×S.D.) was determined to be 0.33. Of 48 clonorchiasis cases, and 36 cases (75.0%) were found to be positives. Two among 28 cases (7.1%) of normal controls and 37.5% (6 among 16 cases) of paragonimiasis cases showed false positive reactions. Mean±S.D. were 0.47±0.21 in clonorchiasis cases, 0.13±0.10 in normal controls and 0.30±0.22 in paragonimiasis cases

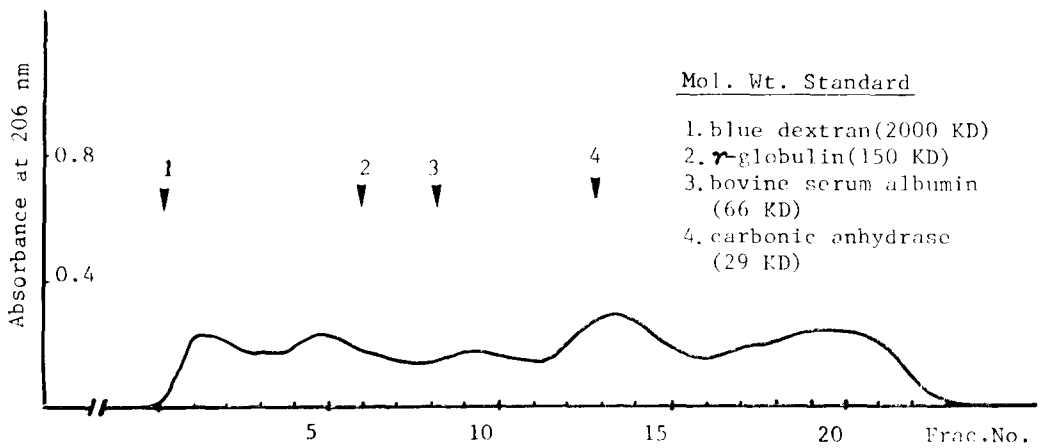

Fig. 7. Elution profile of *C. sinensis* water-soluble crude adult worm antigens separated by Sephadex G-200 gel filtration.

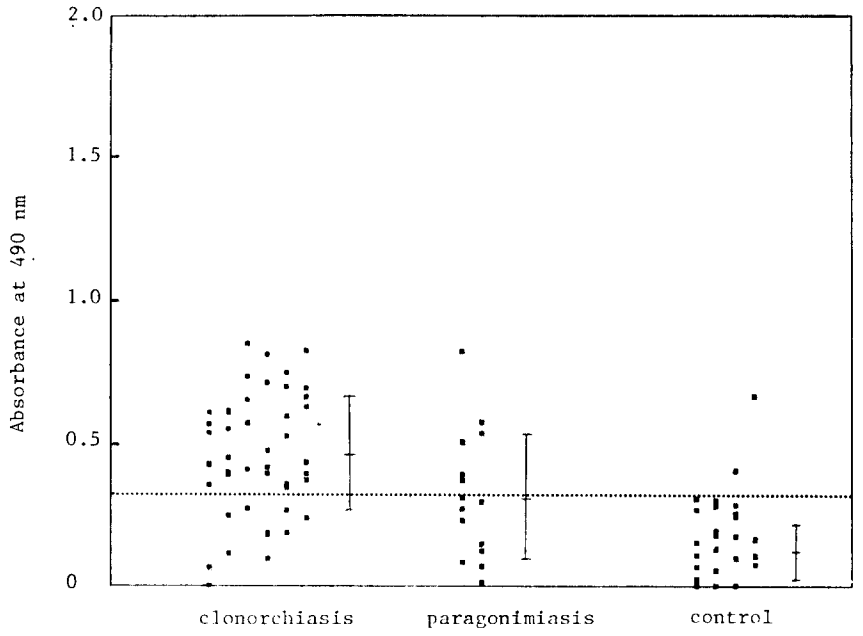

Fig. 8. Distribution of absorbance values of the sera in clonorchiasis and paragonimiasis cases against *C. sinensis* antigens by conventional ELISA.

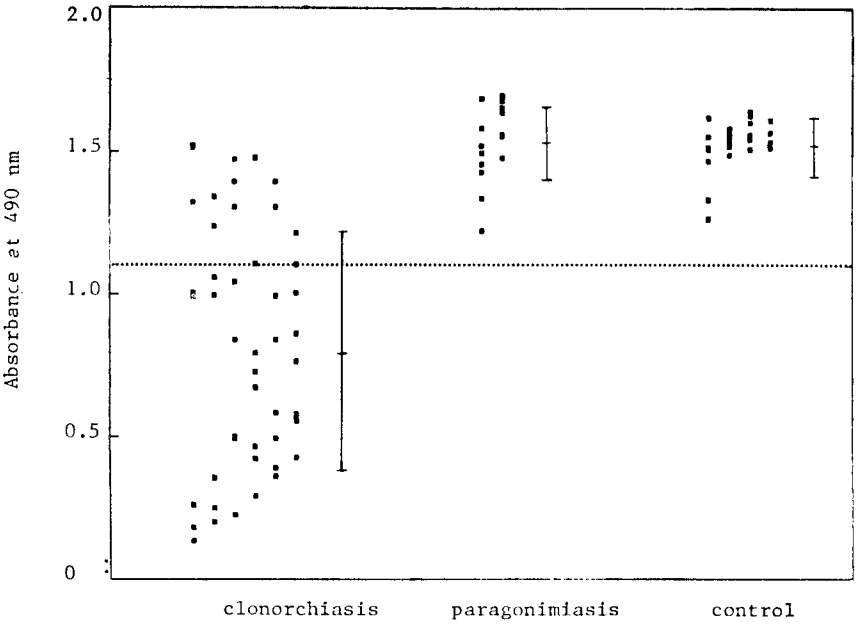

Fig. 9. Distribution of absorbance values of the sera in clonorchiasis and paragonimiasis cases against *C. sinensis* antigens by ELISA-inhibition test using specific monoclonal antibody ("CsHyb 0605-23").

Table 6. The fractions of *C. sinensis* antigens separated by Sephadex G-200 gel filtration reacted with monoclonal antibodies by ELISA

Fraction	Hybridoma clones (CsHyb-)				Immune serum
	0714-20	0605-10	0605-23	0714-25	
1	2.92	2.92	2.92	2.23	2.95
2	2.92	2.80	2.25	2.08	3.00
3	2.62	2.50	2.80	2.08	3.00
4	1.65	0.96	1.51	1.13	2.50
5	1.08	0.20	0.35	0.37	1.84
6	1.48	<	<	0.14	1.99
7	0.13	<	<	<	1.23
8	0.26	<	<	<	1.18
9	<	<	<	<	2.42
10	<	<	<	<	0.54
11	<	<	<	<	0.46
12	<	<	<	<	1.40
13	<	<	<	<	2.37
14	<	<	<	<	2.41
15	<	<	<	<	2.33
16	<	<	<	<	1.60
17	<	<	<	<	1.21
18	<	<	<	<	0.50
19	<	<	<	<	0.38
20	<	<	<	<	0.37
21	<	<	<	<	0.27
22	<	<	<	<	0.27
23	<	<	<	<	0.32

< : Absorbance is lower than 0.10

(Fig. 8).

Among 4 kinds of specific monoclonal antibodies, CsHyb 0605-23 was the most valuable monoclonal antibody in the ELISA-inhibition test for the diagnosis of clonorchiasis. Optimum dilution of human sera was predetermined to be 1:4. In the ELISA-inhibition test using CsHyb 0605-23 monoclonal antibody and 1:4 diluted human sera, the mean absorbance of normal controls was 1.53. The absorbance level below 1.10 was considered as positive, and 77.1% (37 among 48 cases) of clonorchiasis cases were determined to be positive. There were no false positives in normal controls or paragonimiasis cases, indicating 100% specificity (Fig. 9). The ELISA-inhibition test using specific monoclonal antibodies was found to have same sensitivity and definitely high specificity in comparison with conventional ELISA.

DISCUSSION

C. sinensis is one of the most important trematode parasites of man in Korea. In this study, monoclonal antibodies were produced and applied to the analysis of *C. sinensis* antigens and the immunodiagnosis of human clonorchiasis.

At first, specificities of monoclonal antibodies were tested by using various parasites' antigens by the ELISA. Each monoclonal antibody directed against different *C. sinensis* antigens reacted with other parasites' antigens. Some reacted strongly, and others, weakly. These findings indicated concretely that there are common antigens between *C. sinensis* antigens and those of other parasites. Existence of common antigens has been described between different parasite species (Schantz *et al.*, 1980; Kim *et al.*, 1986). The common antigen is one of the main causes of cross-reactions in the immunodiagnosis. There have been many reports saying that cross-reacting antigenic compositions exist between *C. sinensis* antigens and several parasites' antigens, such as *Paragonimus westermani* (Cho and Soh, 1974; Kim *et al.*, 1987), *Opisthorchis viverrini* (Sirisinha *et al.*, 1990), *Schistosoma* spp. (Mott, 1987) and *Cysticercus cellulosae* (Cho *et al.*, 1986) *etc.* It is thought that almost all parasitic species would have common antigens between different species. Concerning this, reduction of the cross-reactivity in the immunodiagnosis is very important. In this experiment, antigens of other parasites were used to test the specificity for certain. As a result, 8 among 29 hybridoma clones were found to be producing specific antibodies directed against only *C. sinensis* antigens, so hybridomas producing specific antibodies against any parasite species are not so difficult to obtain. However, 2 hybridoma clones could not be used for further experiment since those were not recovered after cryopreservation.

In this experiment, ELISA-inhibition test was conducted to reveal whether or not the antigenic

determinant recognized by each monoclonal antibody was an exposed antigenic determinant during natural infection. If the ELISA titer of a monoclonal antibody was not inhibited by the infected rabbit serum, the monoclonal antibody was determined to be produced against non-exposed antigenic determinant of *C. sinensis* crude antigens. Two among 6 clones corresponded to this. In this experiment, 50% of inhibition rate was considered to be a cut-off value, because the hybridoma clones were intended to be included as many as possible. However, the inhibition rate of determining exposed or non-exposed antigenic determinants should be reevaluated in the future, and further developed ideas are required to solve this problem.

The molecular weight of 2 kinds of antigens against monoclonals were revealed by EITB. The other 2 antigens may be revealed by immunoprecipitation technique *etc.* in the future. On the other hand, the EITB has been applied several times for the diagnosis of clonorchiasis recently (Joo *et al.*, 1986; Nam and Lee, 1989). The antigens of specificity in the EITB, however, did not correspond to the antigens recognized by monoclonals made in this study. These antigens recognized in this study were very limited parts of *C. sinensis* antigens, more studies on the biochemical nature of *C. sinensis* antigens should be conducted in the future.

By immunofluorescent microscopy, the localities of the antigens were studied. The antigen reacted with CsHyb 0605-10 monoclonal antibody was distributed all over the worm parenchyme, so a certain antigenic determinant could be distributed to very wide portions or the organs of a worm. The antigen reacted with CsHyb 0605-23 monoclonal antibody was distributed mainly around the uterine eggs. The eggs of *C. sinensis* were reported not to have antigenicity at all times (Sun, 1969; Kwon *et al.*, 1984). Since the eggs of *C. sinensis* passed out with the feces through the bile duct and intestines, and there is no holes on the egg shells unlike *Schistosome* eggs, antigenicity of the eggs has to be very weak. Although the eggs do not have any antigenicity, it is thought to be that the antigenic materials must be distributed around the uterine eggs. Especially, it is worth while to note that the monoclonal antibody reacted with this antigen was found to be very useful in the ELISA-inhibition test for the diagnosis of clonorchiasis. The other 2 monoclonal antibodies were found to have weak reactions against their antigens on the intestinal epithelium observed with immunofluorescent microscope, though those were strongly reacted in the ELISA. Immunogold labelling technique may be used in the future to obtain a better resolution (Sung *et al.*, 1988).

The antigens reacted with 4 monoclonals used in the study were all found to be present in the early obtained fractions separated by Sephadex G-200 gel filtration. It is interesting to note that the other monoclonals which reacted with later obtained fractions or with all of the fractions could be found, although those were not included in the result of this study. This chromatography technique was conducted to reveal what antigenic fractions separated by gel filtration contained the antigenic determinants reacted with monoclonals in the untreated conditions, that is being not influenced by any treatment, such as detergents or heating during SDS-PAGE. It might be one of the useful methods to test the reactivity of the monoclonals against the antigens.

Many studies have already been carried out on other parasitic antigens such as *Schistosoma* antigens (Mott, 1987). Although purification of the allergenic component of *C. sinensis* antigens was tried in the past (Min *et al.*, 1980), it is very difficult to find out the publications on the analysis of *C. sinensis* antigens. In the future, more antigens of *C. sinensis* should be purified, and characterized by biochemical or immunological methods.

Diagnosis of clonorchiasis has been thought to be relatively easy by stool exam. But, it is not the best method. Collecting stool specimens from individuals is not so easy. If there were many samples to be tested, or no detectable eggs

during early infectious stage, or the bile duct were obstructed due to inflammation or other causes, the stool exam would not be a better method of diagnosis than the immunodiagnosis.

Generally, sensitivity and specificity are the two most important points in the immunodiagnosis. Concerning specificity, immunodiagnosis of parasitic diseases has the same problem mainly because of the common antigen. *C. sinensis* and *P. westermani* are closely related than the other tissue-invading parasites endemic in Korea, so they must have a lot of common antigenic determinants. Cross reactions in the skin test have been very well known (Ahn *et al.*, 1975). Although no cross reaction between two antigens was reported by immunofluorescent test (Im, 1974), most of the reports recognized that there was a cross reaction. It is worth while to note that 16.6% of paragonimiasis cases showed postiive reactions against *C. sinensis* antigen (Cho and Soh, 1974; Choi, 1975). Cho and Soh(1976) reported that the cross reaction was decreased so much by absorption of the antigens. But, it is very complicated to perform practically, and a lot of the antigen consumed. Indirect hemagglutination test was applied to be good enough for diagnosis of clonorchiasis (Kim *et al.*, 1987). EITB was also applied for diagnosis of clonorchiasis (Hur *at al.*, 1988), but it is not so practically useful for the routine diagnosis for various reasons such as strictly standardized conditions of a laboratory.

Now the ELISA would be the best method among various immunodiagnostic techniques since it is very easy to perform, and has a high sensitivity. In Korea, ELISA was used for the diagnosis of parasitic infection for the first time by Lee *et al.*, 1981, and it was also for the diagnosis of human clonorchiasis. Thereafter several reports have been followed (Yang *et al.*, 1983; Hahm *et al.*, 1984; Han *et al.*, 1986; Lee *et al.*, 1988). Although it has been recognized that there are some differences of the results, the sensitivity was considered to be high, from 78.2% to 93.0%, and the specificity was also high, so there were almost no false positives in the normal control group. But, 18.2% of paragonimiasis cases showed false positive reactions directed against *C. sinensis* antigens (Kim *et al.*, 1987). In other parasitoses, purified antigen using monoclonal antibody has been applied for the diagnosis (Kim *et al.*, 1986; Al-Yaman and Knobloch, 1989). In those cases, the specificity was found to be very good, but the sensitivity was reported to be decreased much. If the purified antigen has a defect in the sensitivity for diagnosis, it is probably useless because purification procedure seems to be very difficult and inefficient.

ELISA-inhibition test has been applied for the diagnosis of other diseases (Klaster *et al.*, 1985; Cabrera *et al.*, 1989; Whang, 1990). In this study, ELISA-inhibition test using *C. sinensis* specific murine monoclonal antibodies was applied to increase the specificity of the ELISA in the diagnosis of clonorchiasis. The results showed that the sensitivity of the ELISA-inhibition test was as same as the conventional ELISA, and the specificity of the test increased greatly. Moreover, individual serum samples showed almost the same level of titers by the ELISA and ELISA-inhibition test. The antigenic determinant recognized by this monoclonal antibody must be one of the major and exposed antigens of *C. sinensis* during natural infection.

However, sensitivity or absorbance value of the test was thought to be relatively low in both types of the ELISA, probably due to long duration of about 10 years of storage of the sera with accidental thawing occasionally. If the test were performed just after the collection of the sera, the sensitivity should be higher than that of this experiment. The specificity of ELISA-inhibition test was high enough not to cross react with the sera of normal controls or those of lung fluke infection. There is no *Opisthorchis viverrini* infections in Korea, the positive result of ELISA-inhibition test in this experiment is surely due to infection of *C. sinensis*. Although it was not included in the result of this experiment, not even a little percentage of the non-infected controls showed positive titers in con-

ventional ELISA. Those sera turned out to be negative in the ELISA-inhibition test using *C. sinensis* specific murine monoclonal antibody. Cabrera *et al.* (1989) reported that conventional ELISA for the diagnosis of onchocerciasis using *Onchocerca volvulus* antigen extracted in PBS showed 98% in sensitivity but only 6% in specificity because of the cross reaction with sera of *Wuchereria bancrofti*, *Mansonella ozzardi* or other intestinal parasitic infections. He also reported that by the ELISA-inhibition test using murine monoclonal antibody, the specificity reached 94%.

ELISA-inhibition test using *C. sinensis* specific murine monoclonal antibody can be performed easily, taking less time and having high specificity as well as high sensitivity. Great amount of the monoclonal antibody can be obtained easily from the ascites of a mouse inoculated with hybridomas intraperitoneally. Time or effort for purification of the antigen is not needed. ELISA-inhibition test can practically be applied for immunodiagnosis of clonorchiasis, and for diagnosis of other parasitic infections widely in the future.

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=국문초록=

단세포군 항체를 이용한 간흡충 항원의 분석 및 간흡충증의 진단

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우리 나라의 중요한 인체 기생충인 간흡충(*Clonorchis sinensis*)을 연구함에 있어, 단세포군 항체제조법을 이용하여 보았다. 간흡충의 성충 조(粗)항원으로 면역한 마우스의 비장 림프구와 형질세포종(plasmacytoma) 세포를 융합하여 간흡충의 항원에 대한 단세포군 항체를 분비하는 융합 세포를 만들어, 간흡충 항원에 대한 특이 단세포군 항체를 얻은 후, 이의 특성 및 반응하는 항원의 특성을 분석하고, 아울러 ELISA 억제 검사법을 이용하여 면역 진단법 상 특이도의 개선을 모색하였다.

그 결과, 간흡충 항원에 대한 항체를 분비하는 총 29개의 융합세포군을 확인하였는데, 이 중 8개는 다른 기생충 항원에 대해 교차 반응을 나타내지 않는 높은 특이성을 지니고 있었다. 클로닝 후 선택된 6종류의 단세포군 항체는 Ig G1이 넷이었고, 나머지는 Ig G2b 및 Ig A로 나타났다. 위와 같이 선택된 단세포군 항체 중 4개만이 자연 감염 시에도 표현되는 항원 결정기에 대하여 생성된 것으로 판단되었다. 효소 면역 전기영동 블로팅으로 분자량 10KD, 34 KD 항원은 각각 CsHyb 0714-20 및 CsHyb 0605-10 단세포군 항체와 항원항체 반응을 나타냄을 확인하였다. 간접형광항체법을 이용하여 각 항원결정기의 충체 내 위치를 관찰한 결과, CsHyb 0714-20 단세포군 항체에 대한 항원은 충체의 표면 및 실질 대부분에 분포하였으며, CsHyb 0605-10 및 CsHyb 0714-25 단세포군 항체에 대한 항원은 충체의 실질 및 장관의 상피세포에 분포하고 있었다. 한편 CsHyb 0605-23 단세포군 항체에 대한 항원은 주로 자궁내 충란 주위에 많이 분포하고 있었다. 단세포군 항체와 반응하는 Sephadex G200 젤 여과 항원 분획을 검색한 결과, 검색한 항원 결정기는 모두 전반적으로 빨리 젤 여과를 통하여 나온 분획에 속하여 있는 것을 알 수 있었다.

한편 이 단세포군 항체를 인체 간흡충증의 진단에 응용하여 그 특이도를 개선하고자 하였다. 통상적인 방법의 ELISA로 시행한 항체가로는 간흡충 감염자의 75%가 양성 범위에 들었으며, 정상 대조군의 7.1%, 폐흡충 감염자의 37.5%가 위양성 반응을 보였다. 반면 CsHyb 0605-23 단세포군 항체를 함께 사용하여 ELISA 억제 검사를 시행한 결과는 간흡충 감염자의 77.1%가 양성으로 판정되었으며, 정상 대조군 및 폐흡충 감염자에서는 양성 반응을 찾아 볼 수 없어서 100%의 특이도를 나타내었다. 따라서 이러한 단세포군 항체를 이용한 ELISA 억제검사는 통상적인 ELISA 검사에 비하여 같은 정도의 민감도를 유지하면서도 매우 높은 특이도를 가지는 것으로 판단되었다.

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