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Immunoblot patterns of clonorchiasis

Sung-Tae HONG[1]*, Weon-Gyu KHO[2], Mejeong LEE[1],
Jong-Soo LEE[3] and Soon-Hyung LEE[1]

Department of Parasitology and Institute of Endemic Diseases[1], Seoul National University College of Medicine, Seoul, 110-799, Department of Parasitology[2], Inje University College of Medicine, Pusan, 614-735, Department of Medical Zoology[3], National Institute of Health, Seoul, 122-020, Korea

Abstract: *Clonorchis sinensis* is a liver fluke which is the most prevalent helminth of humans in Korea. The better diagnostic measure of clonorchiasis is required for its nationwide control program. The present study observed antigenic bands of *C. sinensis* and reacting immunoglobulins in serum of infected residents. Adult *C. sinensis* were recovered from experimentally infected rabbits and soluble crude extract of the worms was used as the antigen after supplementation of E-64, a cysteine proteinase inhibitor. SDS-PAGE of the crude antigen resolved more than 20 protein bands between 200 and 14 kDa. The sera of infected humans collected at an endemic village showed specific IgG and IgE antibodies but little IgM and IgA antibodies. The protein bands of 94, 80, 72, 68, 52, 47, 43, 37, 34, and 28-25 kDa strongly reacted with serum Ig(GMA) or IgG antibody and 64, 62, 52, 47, 44, 34, 28, and 26 kDa bands reacted with serum IgE antibody. However, the 94, 80, 72, 68, 64, 62, 52, 47, and 40 kDa bands of *C. sinensis* antigen were found non-specific. The protein bands of 43, 34, and 28-25 kDa of *C. sinensis* are primary target molecules of further analysis.

Key words: *Clonorchis sinensis*, antigen, IgG antibody, IgE antibody, E-64

INTRODUCTION

Clonorchis sinensis is a fluke of humans which dwells in the bile duct. When the fluke infects humans, the bile duct is severely dilated and the ductal wall is thickened due to mucosal hyperplasia and fibrosis. As the infection becomes chronic and heavier in the intensity, complications develop and clonorchiasis becomes a medical problem (Rim, 1986).

In Korea, the egg positive rate of *C. sinensis* was 2.2% among whole population (MHW & KAH, 1992). This rate is surprisingly high compared to that of other helminths, most of which had been highly transmitted but now are successfully controlled in Korea. The egg positive rate of *C. sinensis* was 4.6% in 1971, 1.8% in 1976, 2.6% in 1981, 2.7% in 1986, and 2.2% in 1992. At present it is still estimated that one million people are infected over the country (Seo *et al.*, 1981). Although praziquantel, the best anti-*C. sinensis* medication, is produced in Korea and supplied free to infected cases through health centers throughout the country, control of clonorchiasis is still far from satisfaction (Rim, 1986).

The most difficult step in control of clonorchiasis is case detection in the field. This is because voluntary cooperation of the

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*Corresponding author

subjected residents to the screening examination becomes less attractive. Also routine stool examination, which is the standard diagnosis of clonorchiasis, is becoming less sensitive because of low intensity of infection. Furthermore, fee for man-power is rapidly increasing and the cost effectiveness is becoming worse year by year. In this context, serodiagnosis may replace the stool examination if sensitive and specific antigen of *C. sinensis* is determined and well characterized.

Out of many protein bands of *C. sinensis*, 13 and 12.5 kDa bands were proposed as the promising antigen of diagnostic value in an experiment of rabbit clonorchiasis (Kim, 1994). Yong *et al.* (1996) suggested that ELISA inhibition test with a monoclonal antibody against a polysaccharide antigen of *C. sinensis* was a new diagnosis candidate. However, rather basic knowledge about serological reaction to antigenic molecules in human clonorchiasis is essential for development of the better diagnosis. The present study adopted immunoblotting of infected human sera with antigen of *C. sinensis*.

MATERIALS AND METHODS

1. Preparation of *C. sinensis* antigen

Metacercariae of *C. sinensis* were collected from digested freshwater fish, which were obtained at the Nakdong-gang, Kyongnam, Korea. The metacercariae were orally fed to rabbits and adult flukes were recovered from their liver 8 weeks after the infection. The flukes were chopped and homogenized in ice. The soluble extract of the flukes was used after high speed centrifugation. Protein concentration of the crude antigen was 4.667 mg/ml.

2. Supplementation of proteinase inhibitors

One serine proteinase inhibitor, PMSF (phenylmethanesulphonyl fluoride, Sigma), and one cysteine proteinase inhibitor, E-64 (L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane), were supplemented to crude extract of *C. sinensis*. PMSF 1 mM and E-64 10 μM were used as the standard supplement. Both inhibitors were supplemented before homogenization and/or before boiling of the crude autigen.

3. SDS-PAGE

After boiling for 5 minutes, 7.5 μl (35 μg protein) of the crude antigen was loaded into each well in 12.5% separating gels with the 3% stacking gel. The protein bands were separated through the constant current of 20 mA in the Hoefer electrophoresis system (U.S.A.). The gel was stained with Coomassie R-250 and observed for the protein bands.

4. Immunoblotting

The protein bands on gels were transferred to the PVDF membrane (Millipore Co.) by constant current of 5 mA for two hours. The membrane was cut into 15 strips and its surface was blocked by 3% skim milk (Difco Lab.). After washing with PBST (0.1% tween 20 in phosphate buffered saline), serum (usually 1:100 dilution) was applied to the strips for 2 hours. The secondary antibody was the diluted peroxidase conjugated goat antibody to human IgG, IgE, IgM, and IgA antibodies (Cappel Co.). The color was developed after reaction with the substrate, 0.6% chloronaphtol, 0.2% diaminobenzidine, and 0.02% H_2O_2.

5. ELISA

ELISA was executed as usual. The antigen was diluted 1:100, sera were diluted 1:80, and the conjugate was 1:1000 diluted. Diaminobenzidine was the substrate for color reaction and absorbance was read at 490 nm.

6. Sera of infected humans

The human sera were collected at Sangju-gun, Kyongbuk, Korea. Total 49 egg positive cases and 24 egg negative cases were included. They were also screened by intradermal test (IDT) (Table 1).

RESULTS

1. Protein bands of *C. sinensis* crude antigen by supplemented proteinase inhibitors

The present system resolved more than 20 protein bands from the crude antigen of *C.*

Table 1. Prevalence of clonorchiasis in residents of a village, Sangju-gun, Kyongbuk, 1994

Examination methods	No. of examined			No.(%) of positive		
	Male	Female	Total	Male	Female	Total
Stool examination	31	42	73	26 (83.9)	23 (64.8)	49 (67.1)
Intradermal test	31	42	73	28 (90.3)	26 (61.9)	54 (74.0)
ELISA	31	42	73	30 (96.8)	29 (69.0)	59 (80.8)

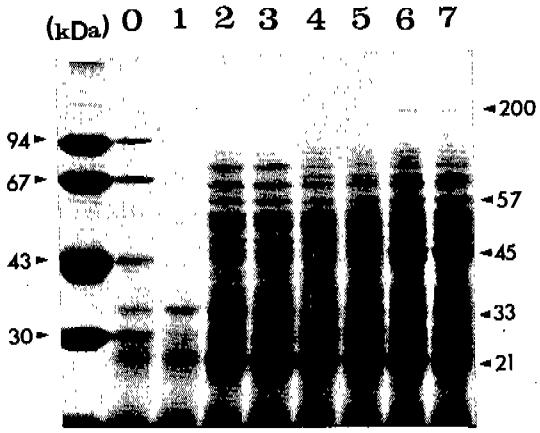

Fig. 1. The influence of endogenous proteinase on electrophoresis patterns of *C. sinensis* crude antigen. The lysis of protein bands was prevented by supplementation of a cysteine proteinase inhibitor, E-64, before homogenizing the worms and before boiling the samples. The crude antigen was treated with 2 inhibitors in several combinations. About 50 μg of each sample was loaded and separated in a 10% acrylamide gel using SDS-discontinuous buffer system. Protein bands were stained with Coomassie blue. Lanes 0-3, inhibitors were not supplemented before homogenizing; lane 0, no supplements. this lane was contaminated with molecular weight marker (SM).; lanes 1-3, inhibitors were added only before boiling; lane 4-7, PMSF and E-64 were treated before homogenizing; lanes 5-7, inhibitors were treated also before boiling; lanes 1 & 5, PMSF (1 mM); lanes 2 & 6, E-64 (10 μm); lanes 3 & 7, PMSF and E-64.

sinensis. The bands were in range from 200 to 14 kDa, and the bands over 60 kDa were degraded in simple crude antigen or in PMSF supplemented one (Fig. 1). Only the high molecular bands remained in E-64 supplemented crude antigen. There was no difference between the two supplementation timings, at homogenization or homogenization and boiling (Fig. 1). The crude antigen supplemented with E-64 before homogen-ization was used as the standard antigen for immunoblotting in this study.

2. Examination of *C. sinensis* infection among residents of a village at Sangju-gun

One village of Sangju-gun, Kyongbuk at the upper reach of the Nakdong-gang (river) was selected and 73 residents of the village were examined (Table 1). The positive rate was 67.1% by stool examination, 74.0% by intradermal test, and 80.8% by ELISA. The proportion by infection intensity was 69.4% in light infection (less than EPG 1,000), 24.5% in moderate infection (EPG 1,000-9,900), and 6.1% in heavy infection (EPG over 10,000).

3. Intradermal test (IDT) and serum IgG antibody levels by ELISA

The intradermal test with VBS antigen (made in NIH Korea) revealed 74.0% positive rate among 73 subjected cases. Forty nine egg positive cases and 24 egg negative cases were screened by ELISA for anti-*C. sinensis* IgG antibody in serum. The egg positive cases showed their mean absorbance as 0.444 ± 0.226 and the egg negative cases showed 0.243 ± 0.157 (Fig. 2). The positive rate by ELISA was 80.8%. The subjected cases were grouped by results of the 3 examinations (Table 2).

4. Immunoblot patterns with Ig(GMA) antibodies (Fig. 3)

Immunoblotting with Ig(GMA) antibodies of all egg, IDT, and ELISA positive (P) cases reacted to 94, 80, 72, 68, 52, 47, 43, 40, 37, 34, and 28-25 kDa bands. The immunoblotting of egg negative but IDT and ELISA positive (IE) cases showed the reaction to bands of 80, 72, 68, and 52 kDa. The blotting of egg and IDT negative but ELISA positive (E) cases or egg and ELISA negative but IDT

Table 2. Experimental groups of subjected residents by 3 examinations

Egg detection	Intradermal test	ELISA	Groups	No.(%) of cases
+	+	+	P	44 (60.3)
+	+	−		1 (1.4)
+	−	+		2 (2.7)
+	−	−		2 (2.7)
−	+	+	IE	7 (9.6)
−	+	−	I	2 (2.7)
−	−	+	E	6 (8.2)
−	−	−	N	11 (15.1)
Total				73 (100)

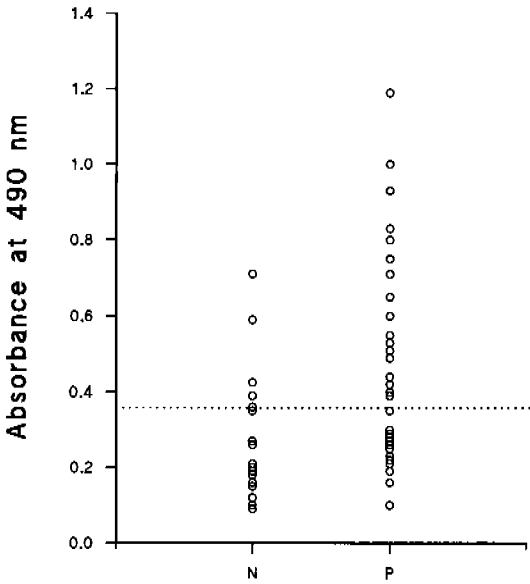

Fig. 2. Absorbance levels of IgG antibody by ELISA to crude antigen of *C. sinensis*. Egg positive cases (P) and negative cases (N). The positive margin was 0.38.

positive (I) cases also showed the bands of 94, 80, 72, 68, 52, and 40 kDa. The blotting of egg, IDT, and ELISA negative (N) cases showed the bands of 72, 68, 52, and 47 kDa. The bands of 94, 80, 72, 68, 52, 47, and 40 kDa were regarded as non-specific. The blotting with Ig(GMA) antibodies showed the strong non-specific reaction.

5. Immunoblot patterns with IgG antibody (Fig. 4A)

Immunoblotting with serum IgG antibody showed many bands between 94 and 28 kDa in P cases. Especially 94, 66, 62, 55, 43, 40,

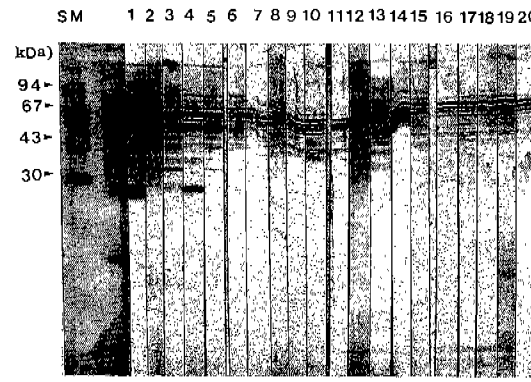

Fig. 3. Immunoblot of human Ig(GMA) antibodies to *C. sinensis* crude antigen in 4 groups. Lanes 1-5, P cases; lanes 6-10, IE cases; lanes 11-13, E cases; lanes 14-15, I cases; lanes 16-20, N cases.

34, and 28 kDa bands reacted strongly. The bands of 94, 66, 62, and 55 reacted in IE cases but very faint. In cases of E, I, and N cases, no bands were visualized.

6. Immunoblot patterns with IgE antibody (Fig. 4B)

In P cases, 64, 62, 52, 47, 44, 34, 28, and 26 kDa bands appeared, and 64, 62, 52, and 47 kDa bands also appeared in IE, I, E, and N cases. For 44, 34, 28 and 26 kDa antigenic bands of *C. sinensis*, the infected serum produced IgE antibody reactions.

7. Immunoblot patterns with IgM antibody (Fig. 4C)

The blotting showed 78, 72, 68, 60, and 44 kDa bands but no differences were recognized by the P, IE, I, E, and N cases. Therefore, the

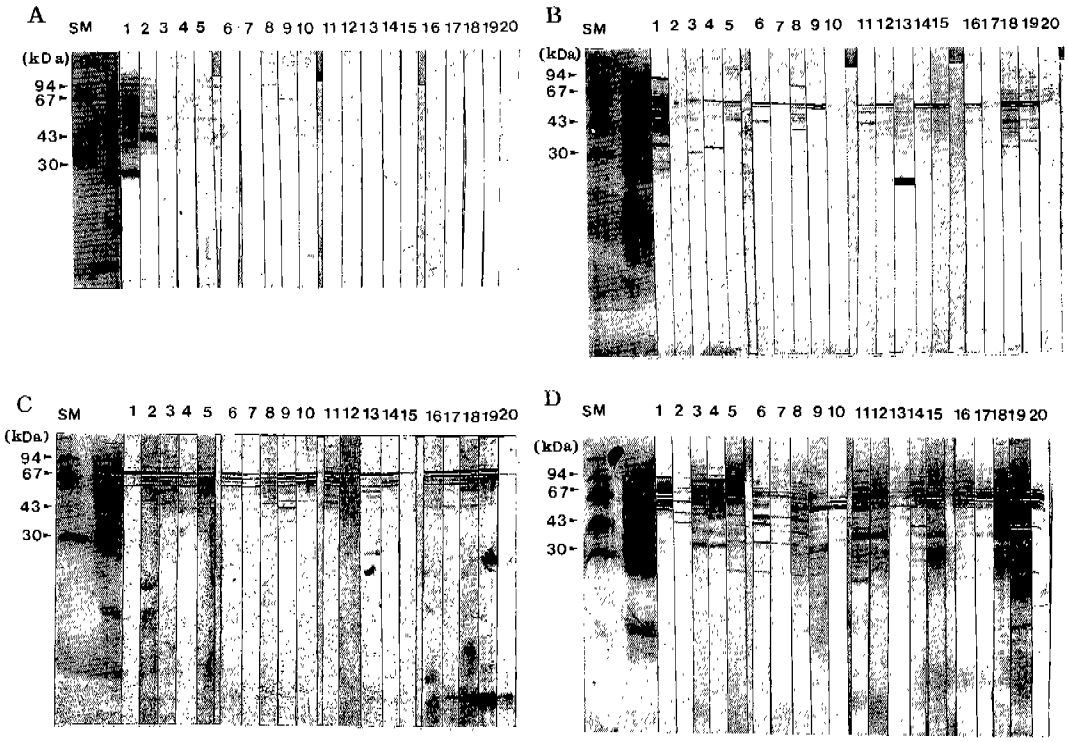

Fig. 4. Immunoblot of human IgG **(A)**, IgE **(B)**, IgM **(C)**, and IgA **(D)** antibodies to *C. sinensis* crude antigen in 4 groups. Lanes 1-5, P cases; lanes 6-10, IE cases; lanes 11-13, E cases; lanes 14-15, I cases; lanes 16-20, N cases.

bands visualized in the IgM antibody reaction were regarded as of no significance.

8. Immunoblot patterns with IgA antibody (Fig. 4D)

Fifteen to 20 bands appeared between 94-26 kDa in all of the examined cases. The basic pattern showed no differences in the P, IE, I, E, and N cases.

DISCUSSION

The protein bands in crude antigen of *C. sinensis* were known to be more than 20 in SDS-PAGE (Lee *et al.*, 1988; Kim, 1994). The present finding with two proteinase inhibitors revealed that immunoblot pattern of infected human serum to *C. sinensis* crude antigen was greatly varied by activity of endogenous proteinases. The E-64 supplemented antigen better preserved high molecular bands than the PMSF supplemented one did. This suggests that cysteine proteinase of *C. sinensis* more digests high molecular protein bands than serine proteinase does. The activity of cysteine proteinase was alreadey proved by Song and Rege (1991). This activity of endogenous cysteine proteinase must be considered during preparation of the antigen to keep its consistency.

The present immunoblotting scheme gave us the confidence that infection of *C. sinensis* induces serum IgG and IgE antibodies. Specific antibodies of other classes, IgM and IgA antibodies, were not enough in their amount to be detected in the serum.

Kim (1994) recorded that 25 protein bands of *C. sinensis* were antigenic to experimentally infected rabbit sera. However, all of the bands are hardly specific. Immunoblotting of the present study could evaluate the antigenic bands by reaction with human sera of different groups of egg detection, intradermal test, and ELISA. Serum antibodies reacted to 11 bands between 94 and 14 kDa bands in all of the 3-test positive (P) cases. Serum IgG antibody of

most of the 3-test negative (N) cases reacted to the bands of 72 and 68 kDa, and that of egg negative but intradermal test or ELISA positive (IE, I, E) cases also reacted to the 94, 80, 72, 68, 52, and 47 kDa bands. The reaction with these bands should be regarded as non-specific. According to the present results, the primary target antigenic bands of *C. sinensis* were 43, 34, and 28-25 kDa for specific IgG antibody and 43, 34, 28, and 26 kDa for specific IgE antibody. The K2 antigen, estimated as 12.5 kDa, was a proposedly promising band, which represented active infection (Kim, 1994). The present study resolved antigen bands mainly over 14 kDa, but the antigen reaction was also recognized at the bottom of the gel around 10 kDa. The molecule at the bottom might include the K2 antigen. This small molecule at the bottom should be separated more and evaluated of the characteristics.

The bands of false positive reaction may be interpreted in 3 ways. One is an outcome of false egg negative cases by stool examination. The false egg negatives can be the cases of low burden of infection which are missed by limited sensitivity of one cellophane thick smear screening examination. The second is that some residents may be parasitologically cured by chemotherapy but still serologically positive. Negative conversion of serology is known to occur at least 7 months after treatment by ELISA (Hong, 1988). The third is the cross reaction with the serum antibodies induced by other parasite antigen. For example, *C. sinensis* and *Paragonimus westermani* are well known as sharing cross reacting antigens.

The primary target antigen bands of *C. sinensis* in the present study should be evaluated by immunoblotting with sequential sera after treatment. The bands which are verified as specific to active infection should be used for diagnosis in the future.

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=초록=

면역이적법에 의한 간흡충 항원분획과 감염자의 항체반응 양상

홍성태[1], 고원규[2], 이미정[1], 이종수[3], 이순형[1]

서울대학교 의과대학 기생충학교실 및 감염병연구소[1],
인제대학교 의과대학 기생충학교실[2], 국립보건원 의동물과[3]

우리 나라에서 가장 감염자가 많은 기생충인 간흡충의 현 감염에 특이한 항원 분획을 찾고자 면역이적법을 이용하여 감염자의 혈청을 검색하였다. 실험적으로 토끼에서 얻은 간흡충 성충의 조항원에 시스테인계 단백질분해효소 억제제인 E-64를 첨가하였을 때 큰 분자량의 분획을 가장 잘 보존하여 200-14 kDa의 범위에 20개 이상의 분획을 관찰하였다. 이 조항원을 이용하여 경북 상주군 주민의 혈청을 면역이적법으로 검사하였다. 대변검사, 피내반응검사, 효소면역법검사(ELISA)를 이용하여 검사한 73명 중 충란양성자 49명의 혈청 내에 특이 IgG와 IgE 항체가 생성되었고, IgM과 IgA 항체는 특이하게 반응하지 않았다. 여러 항원 분획 중에서 43, 34, 28-25 kDa 항원이 현 감염에 특이한 분획이고, 항체와 반응한 94, 80, 72, 68, 64, 62, 52, 47, and 40 kDa 분획은 특이하지 않은 분획임을 확인하였다. 추후에 각 항원과 이에 대한 혈청반응에 대하여 특성을 더 연구할 필요가 있으며, 특히 치료후 추적검사를 통하여 혈청내 항체의 소실을 구명하여야 할 것이다.

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