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A *Clonorchis sinensis*-specific antigen that detects active human clonorchiasis

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Abstract: A *Clonorchis sinensis*-specific antigen in excretory-secretory product of *C. sinensis* (CsE) was assessed in human clonorchiasis by immunoblot. Thirty and 7 kDa antigens of CsE2, one of four different batches of CsEs reacted strongly with infection sera from clonorchiasis patients; however, the antigens reacted weakly with 6-month post-treatment sera from praziquantel-cured cases, but were still highly detected by the sera from praziquantel-failed patients, indicating that the 30 and 7 kDa antigens can detect antibodies during an active infection. The 30 kDa antigen showed some cross reactions with sera from patients with *Paragonimus westermani* and *Metagonimus yokogawai*, while the 7 kDa antigen did not, suggesting that the 7 kDa antigen has high specificity. The 30 kDa antigen reacted with some past clonorchiasis sera, whereas the 7 kDa antigen did not, supporting that antibodies to the 7 kDa antigen are not present in sera from past clonorchiasis patients. In an endemic area, 92% (23/25) of active clonorchiasis patients and 91% (10/11) of mixed infection patients with *C. sinensis* and *M. yokogawai* had IgG antibodies to the 7 kDa antigen, while 40% (6/15) of past clonorchiasis individuals and 43% (3/7) of metagonimiasis patients cross-reacted to the antigen. These data suggest that the 7 kDa antigen in an excretory-secretory antigen may serve as a marker of an active clonorchiasis with reliable specificities in past clonorchiasis, paragonimiasis and metagonimiasis.

Key words: *Clonorchis sinensis*, excretory-secretory antigen, 7 kDa antigen, IgG antibody response, immunoblot

INTRODUCTION

Human clonorchiasis remains highly endemic in Korea; high prevalence rates have been found in some rural areas although the mean egg positive rate of *Clonorchis sinensis* was 2.2% in a nationwide survey (MHW & KAH, 1992). According to a recent nationwide survey in China, *C. sinensis* appeared as one of the major helminths, as prevalent as 0.4% infection rate on stool examination (Hotez *et al.*, 1997).

The highly curative drug, praziquantel, was introduced in the early 1980s for the elimination of clonorchiasis in Korea (Rim, 1986). Nevertheless, the infection rates during the past 10 years assessed in 3 consecutive nationwide surveys have not decreased significantly; the egg positive rates of *C. sinensis* were 2.6% in 1981, 2.7% in 1986 and 2.2% in 1992. This may be due in part to reinfection of *C. sinensis* after praziquantel treatment, to abuse of the drug in individuals who are past clonorchiasis that were false-positive by skin test, and to the other

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trematode-infected cases whose stool eggs may easily be misinterpreted as *C. sinensis*.

The increased incidence and the need to control this disease in the epidemic fields have led to a search for more effective diagnosis than the stool egg examination. The serodiagnoses using skin test, IFA and ELISA have been developed (Kim *et al.*, 1969; Cho and Soh, 1974; Lee *et al.*, 1981).

Many studies on the *C. sinensis* antigen had focused their view to improve the skin test; nevertheless, veronal buffered saline (VBS) antigen (Kim *et al.*, 1969) has been used for the standard skin test antigen of human clonorchiasis in Korea since 1958.

The skin test using the crude VBS antigen has some problems to adopt it to the present status of changed epidemiology in which over one half of clonorchiasis patients had low worm-burden in EPG 100-900; on the other hand, the prevalence rate of human paragonimiasis dropped significantly, but metagonimiasis became the second most prevalent trematode disease in Korea (MHW & KAH, 1992). The false negative rate in clonorchiasis skin test was much higher in group of EPG 1-999 as 14.0% (Rim *et al.*, 1973), predicting that the VBS antigen could miss some clonorchiasis patients. Moreover, false positive reactors to clonorchiasis skin test have been found in the past clonorchiasis individuals who were already cured. In this context, more sensitive and specific antigen should be needed for the serodiagnosis of a present *C. sinensis* infection.

The characterization of the immune responses to *C. sinensis* antigens that are synthesized during the liver fluke life-cycle in the definitive host and down-regulated on the course of removal of this worm can yield information on the extent to which the present infection is immunologically different from the past illness.

To specifically identify the pertinent antigen for a present *C. sinensis* infection, Kim (1994) screened proteins in excretory-secretory antigen of *C. sinensis* in which a 12.5 kDa antigen, designated K2 antigen, reacted with infection sera, but it was not reactive with 6-month post-treatment sera from rabbits experimentally infected with *C. sinensis*. Recently, Hong *et al.* (1997) reported that *C. sinensis*-specific antigens of 43, 34 and 28 kDa, which were extracted from adult worms in the presence of protease inhibitors, were reacting with specific IgG antibodies in active clonorchiasis patients. Collectively, these studies showed that diverse *C. sinensis* proteins were expressed as *C. sinensis*-specific antigens in an active infection.

The author now determined that *C. sinensis*-specific antigens that are immunologically relevant to a present infection of human clonorchiasis were identified from the different batches of excretory-secretory antigens of *C. sinensis*, and characterized in immune responses related to present and past clonorchiasis, paragonimiasis and metagonimiasis.

MATERIALS AND METHODS

Crude antigens of *C. sinensis*

The whole worm extract of adult *C. sinensis* (CsW) was prepared by homogenizing 10-month-old worms in ice, centrifugation 10,000 *g* at 4°C and supplementation with phenylmethyl sulphonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO) to 0.5 mM in supernatant. The excretory-secretory antigens of *C. sinensis* (CsE1, CsE2, CsE3 and CsE4) were prepared according to the method of Sun and Gibson (1969) with some modification; the simple incubation of 50 living worms was done while they were alive in 10 ml 0.85% saline at 36°C and this metabolite was centrifuged 5,000 *g* at 4°C to eliminate the insoluble materials such as eggs. The CsE1 was from 10-month-old worms collected from rabbits, incubated for 18 hr and supplemented with PMSF; CsE2 was from 11-week-old worms from rabbits, incubated 18 hr and supplemented with PMSF; CsE3 was from 6-month-old worms from rats, incubated 17 hr and was not supplemented with PMSF; CsE4 was from 10-month-old worms from rats, incubated 40 hr and PMSF was not added.

Infection sera

The infection sera of human clonorchiasis were obtained from two different endemic localities in Koesan-gun, Chungbuk and

Koksong-gun, Chonnam in Korea. The clonorchiasis patients were diagnosed by formalin-ether concentration method of stool examination, skin test and ELISA. In Koesan-gun, the active clonorchiasis patients who were producing eggs were medicated with praziquantel by 40 mg/kg single-dose medication according to Rim (1986). The post-treatment sera collected on 6 months after chemotherapy were classified to each group of praziquantel-cured cases who showed egg-negative and praziquantel-failed cases who still passed the eggs in stool until 6 months after treatment. The past clonorchiasis sera were collected from the individuals in endemic areas who showed positive skin test but negative stool eggs and ELISA. The infection sera with *Metagonimus yokogawai* and the mixed infection sera with *C. sinensis* and *M. yokogawai* were obtained from the inhabitants in Koksong-gun along the Sumjin river, who were diagnosed by the formalin-ether concentration method. Individuals infected with *Paragonimus westermani* were screened by routine ELISA for the serodiagnosis of patients who were refered from pulmonary section of Internal Medicine of Chosun University Hospital.

Immunoblot and ELISA

To determine the presence of *C. sinensis*-specific IgG antibodies in the sera, Western blot was adopted according to the method of Kim (1994) with minor modification. Antigen preparations were electrophoresed on 10-15% gradient acrylamide mini-gels in the Laemmli buffers of sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE) with a mini-gel apparatus (Hoefer Scientific Instruments, San Francisco, CA). For immunoblotting, the proteins in the gels were electrotransfered onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) in a semi-dry electrotransfer apparatus (Hoefer). The blotted nitrocellulose membranes were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma) (PBS-T) and then incubated with sera diluted at 1:100 with PBS-T overnight. The membranes were incubated with peroxidase-conjugated goat anti-human IgG antibodies (Sigma) diluted at 1:1,000 with PBS-T for 3 hr. The blots were developed with 3,3'-diaminobenzidine (Bio-Rad) as a chromogen. For ELISA, the procedure was as described previously (Yang *et al.*, 1995).

RESULTS

Thirty and 7 kDa antigens of CsE2 probed with infection sera from clonorchiasis patients

To determine whether *C. sinensis*-specific antigens were reacting with antibodies in active infection sera but not in cases with cured clonorchiasis, Western blots of crude antigens of CsW and CsEs were incubated with paired infection and 6-month post-treatment sera from 3 praziquantel-cured cases and 3 praziquantel-failed cases. The infection sera reacted with numerous antigen bands ranged from 65 to 7 kDa in all the crude antigens (Fig. 1). However, the reactivity of the post-treatment sera was decreased in the cured cases (Fig. 1A), while it was not declined in the failed cases (Fig. 1B). Of the antigen bands, 30 kDa band of all the crude antigens was remarkably attenuated in the post-treatment sera from 3 cured cases (Fig. 1A, cases 1, 2 & 3), while was sustained until 6 months after medication in 2 failed cases (Fig. 1B, cases 1 & 2). The lowest and most prominent 7 kDa antigens of CsE2 and CsE4 disappeared in the post-treatment sera from 2 cured cases (Fig. 1A, cases 1 & 2), while those of CsE1 and CsE3 were still detectable in the post-treatment sera from all of them (Fig. 1A, cases 1, 2 & 3). On the other hand, immune responses to the 7 kDa antigens were not decreased in the post-treatment sera from all the failed cases (Fig. 1B, cases 1, 2 & 3). In addition, the 7 kDa antigen of CsE4 showed weaker antigenicity with 2 infection sera (Fig. 1A, cases 1 & 2).

Cross-reactivities of 30 and 7 kDa antigens of CsE2 with other paragonimiasis, metagonimiasis and normal control sera

The 30 kDa antigen of CsE2 reacted with sera from 3 patients with paragonimiasis, while the 7 kDa antigen did not react with 2

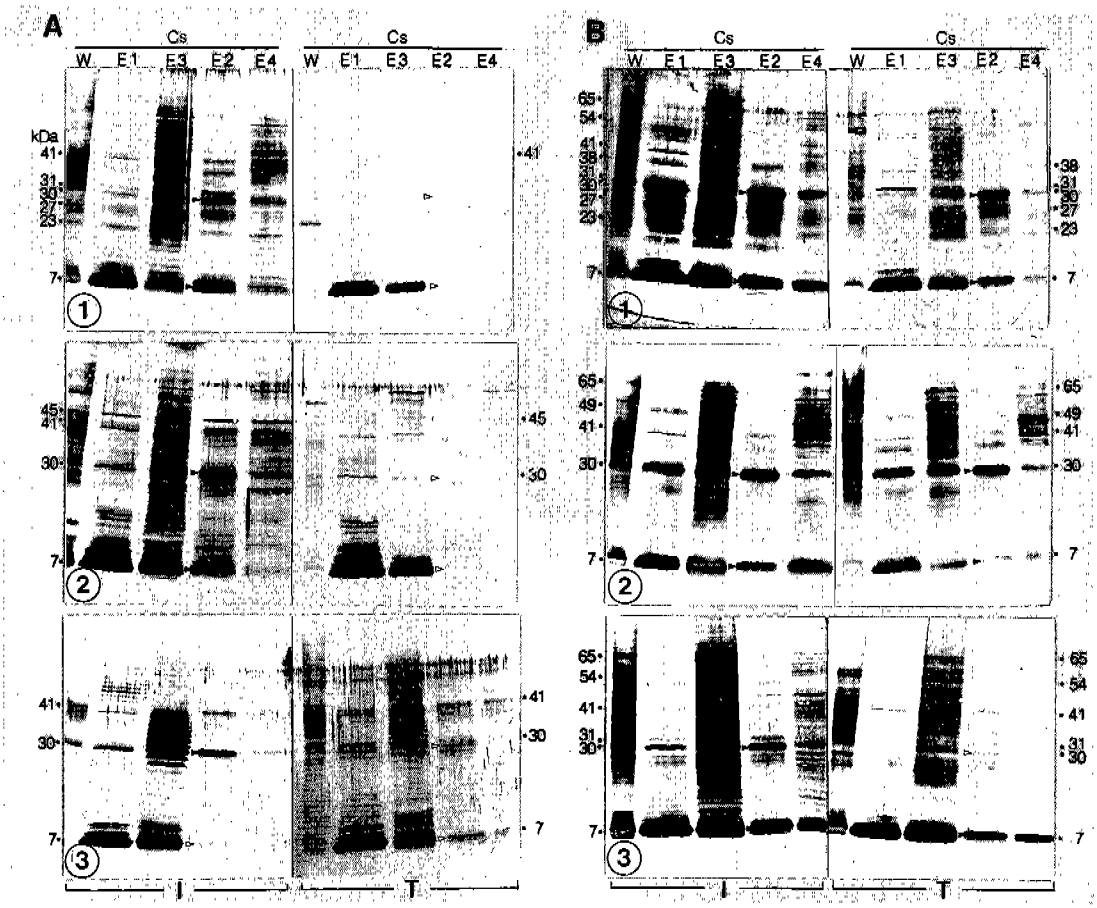

Fig. 1. Immunoblot analyses of CsW and CsE1-4 of *C. sinensis* probed with paired infection (I) and 6-month post-treatment (T) sera from praziquantel-cured (A) and praziquantel-failed (B) clonorchiasis patients. Amounts of 5 μg protein from each antigen were electrophoresed. Molecular masses in kDa were estimated with standard markers of Bio-Rad. Closed arrow-heads mean 30 and 7 kDa antigen bands that were observed remarkably; open ones mean these bands that were not discernible.

such sera (Fig. 2A, cases 1 & 2) and weakly reacted to one of them (Fig. 2A, case 3). The 7 kDa antigen of CsE2 did not react with 2 metagonimiasis sera (Fig. 2B, cases 1 & 3), but was faintly detected by one of them (Fig. 2B, case 2). The 30 kDa antigen of CsE2 reacted faintly with 3 normal sera, while the 7 kDa antigen was not detected by the same sera (Fig. 2C). The 7 kDa antigens of CsE1 and CsE3 reacted strongly with all sera from paragonimiasis, metagonimiasis and normal control (Fig. 2A, B & C).

Reactivity of 7 kDa antigen of CsE2 with the past clonorchiasis sera

To verify whether the IgG antibody response to the 7 kDa antigen was specific to a present infection of human clonorchiasis, the blots of CsW and CsEs were probed with the past clonorchiasis sera. The 7 kDa antigen of CsE2 did not react with the sera from 3 cases as positive as 80 mm^2 (Fig. 3, cases 1, 2 & 3) and also with the sera from 2 cases of 100 mm^2 in skin test (Fig. 3, cases 4 & 5). In contrast, 30 kDa antigen of CsE2 was detected by 3 past clonorchiasis sera (Fig. 3, cases 1, 2 & 5).

IgG antibody response to 7 kDa antigen of CsE2 can disappear by 6 months after treatment

The paired sera of infection and 6-month post-treatment from 10 praziquantel-cured

cases and 10 failed ones were tested for the specific IgG antibodies to the CsE2 antigen (Fig. 4). In the cured cases, 80% of the patients had antibodies to the 30 kDa antigen in their sera, and after medication 30% of them turned to be negative, while 90% of the patients had antibodies to the 7 kDa antigen, and 60% of them turned to be negative in response to medication (Table 1 & Fig. 4A). Contrary to that, in the failed cases, 100% of the patients had antibodies to the 30 kDa antigen, and after chemotherapy 100% of them still showed positive. Similarily, 100% of the patients had antibodies to the 7 kDa antigen, and 90% of them continuously showed positive after chemotherapy (Table 1 & Fig. 4B).

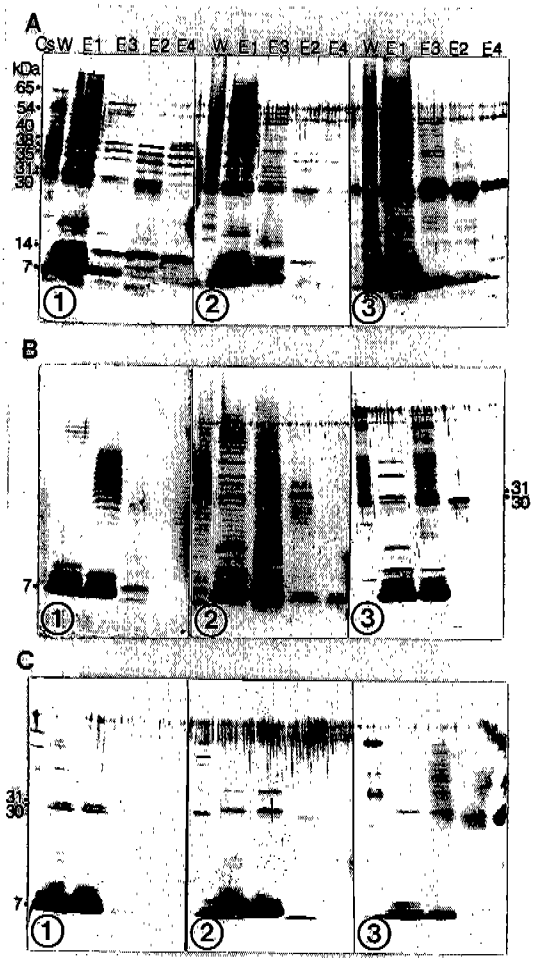

Fig. 2. Immunoblot analyses of CsW and CsE1-4 of *C. sinensis* reacted with paragonimiasis (A), metagonimiasis (B) and normal control sera (C).

The 7 kDa antigen of CsE2 detects antibodies during active infection of human clonorchiasis

The sera from patients in an endemic area with 15 past clonorchiasis, 25 active clonorchiasis, 7 metagonimiasis, and 11 mixed infection of *C. sinensis* and *M. yokogawai* were tested by immunoblot with the 7 kDa antigen of CsE (Table 2 & Fig. 5). A fraction of 40.0% (6/15) of past clonorchiasis, 92.0% (23/25) of active human clonorchiasis had IgG antibodies to the 7 kDa antigen (Fig. 5A & B). A fraction of 42.9% (3/7) of metagonimiasis showed cross-reactivity to the antigen, whereas 90.9% (10/11) of mixed infection appeared to have antibodies to the same antigen (Fig. 5C & D). Contrary to the result in another endemic area as seen in Fig. 4, immune response to the 30 kDa antigen of CsE2 was not detected in most of active clonorchiasis sera (Fig. 5B).

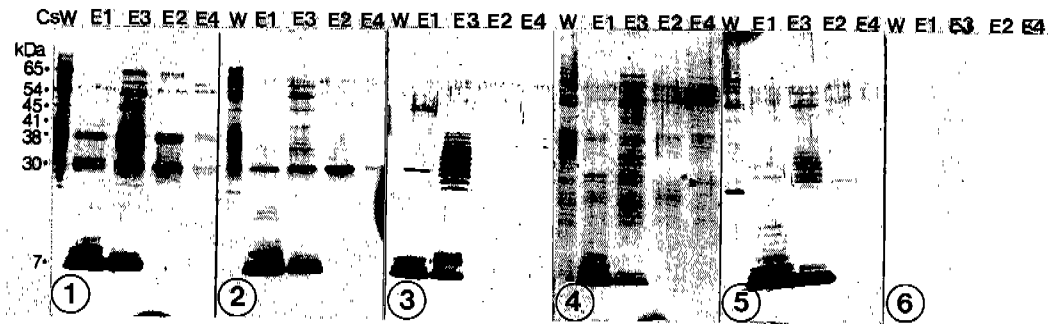

Fig. 3. Immunoblot analyses of CsW and CsE1-4 of *C. sinensis* reacted with the past clonorchiasis sera (1-5) and conjugate control (6).

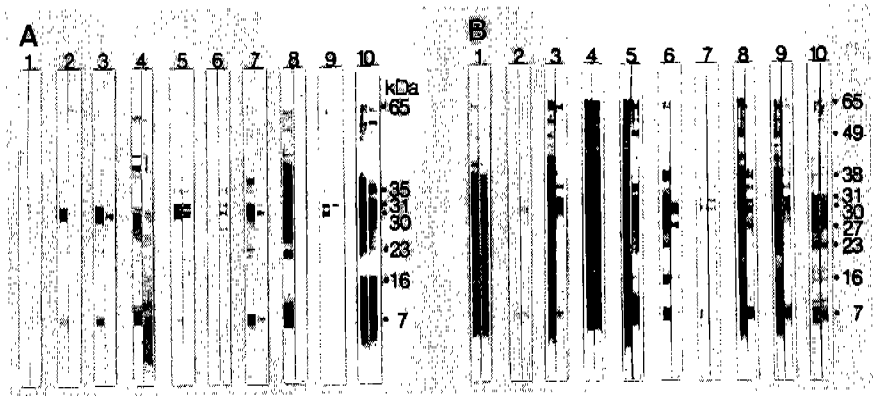

Fig. 4. Immunoblot analysis of CsE2 of *C. sinensis* reacted with paired infection (left strips) and 6-month post-treatment (right strips) sera from praziquantel-cured (A) and praziquantel-failed (B) clonorchiasis patients. Amount of 65 μg protein was electrophoresed in a preparative tract and 40 nitrocellulose strips were prepared.

Table 1. Immunoblot analyses of 30 and 7 kDa antigens of CsE2 probed with paired infection and 6-month post-treatment sera from praziquantel-cured and praziquantel-failed clonorchiasis patients

Subjected group	Status of infection	No. of cases	30 kDa antigen		7 kDa antigen	
			(+)ve	(-)ve	(+)ve	(-)ve
Praziquantel-cured	Infection	10	8	2	9	1
	Post-treat	10	7	3	4	6
Praziquantel-failed	Infection	10	10	0	10	0
	Post-treat	10	10	0	9	1

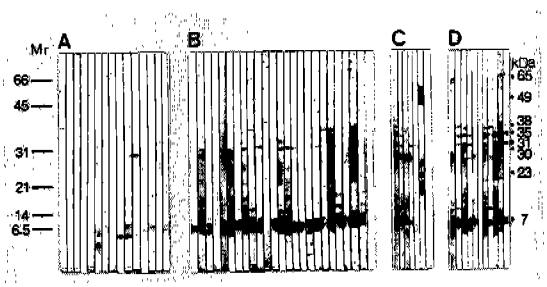

Fig. 5. Immunoblot analysis of CsE2 of *C. sinensis* reacted with sera from past clonorchiasis (A), active clonorchiasis (B), metagonimiasis (C), and mixed clonorchiasis and metagonimiasis patients (D).

Table 2. Immunoblot analysis of 7 kDa antigen of CsE2 probed with sera from various subjects in an endemic area of clonorchiasis and meta gonimiasis

Subject	No. of cases	No. of positive (%)
Past clonorchiasis	15	6 (40.0)
Active clonorchiasis	25	23 (92.0)
Active metagonimiasis	7	3 (42.9)
Mixed clonorchiasis and metagonimiasis	11	10 (90.9)

DISCUSSION

The purpose of this study was to evaluate the antigenicities of the component proteins in several batches of CsEs and to investigate a *C. sinensis*-specific antigen that detects antibodies during present infection of human clonorchiasis. The same molecular weight proteins that were located at the lowest portion on the 10-15% gradient gel of SDS-PAGE revealed different antigenicities; the 7 kDa antigens of CsE2 and CsE4 showed high specificities in that they were strongly reactive with the sera from active clonorchiasis patients, but did not react with the past

clonorchiasis, other trematode infection and normal control sera, whereas that of CsE1 and CsE3 gave a nonspecific reaction to all the sera examined. These data corroborated that the 7 kDa antigen in each CsE was composed of more than 2 component proteins which showed different antigenicities; one was *C. sinensis*-specific and the other was not specific. This result suggests strongly that one of the 7 kDa proteins in the excretory-secretory product include an immunologically intact molecule that is specific to the present clonorchiasis. The CsW antigen was shown to have different antigenic profile from that of CsEs, in which the 7 kDa antigen could be hardly recognized but near antigens at 8 and 5 kDa were observed.

Kim *et al.* (1991) demonstrated that the intestinal epithelium and the intestinal contents showed the strongest antigenicity among the body compartments of *C. sinensis* using immunohistochemical staining. This finding was corroborated by the early report on the importance of the excretory-secretory antigen in eliciting antibody response to *C. sinensis* infection (Sun and Gibson, 1969). Taken together, it seems likely that a excretory-secretory molecule liberated from the living worm play a major role in immune response to *C. sinensis* infection.

Yong *et al.* (1991) also reported that the 10 kDa antigen in the crude worm extract was probed by monoclonal antibody to *C. sinensis*. Since it was observed as the lowest band near the bottom of the gel, it could be similar to the 7 kDa antigen of this study. However, Hong *et al.* (1997) reported that they hardly observed 7 kDa or lower molecular weight antigen in their immunoblot, but found 43, 34 and 28-25 kDa antigens reacted to the present infection. In this report, the crude antigen of adult *C. sinensis* extracted by supplementation of a cysteine proteinase inhibitor, E-64, was made to preserve the larger molecules, so that they couldn't find the smaller components. Thus, the discrepancy seems more likely from the different methods of laboratories which may favor the loss of some antigens such as the 7 kDa antigen.

Min *et al.* (1980) have also observed that the lowest antigen in the crude worm extract of *C. sinensis* on 6-20% gradient SDS-PAGE was a 14 kDa or a lower molecular allergen which induced passive cutaneous anaphylaxis. In addition, they have confirmed these findings by the elevation of *C. sinensis*-specific IgE antibodies in clonorchiasis patients (Min and Soh, 1983). The 7 kDa antigens of CsW and CsE2, which were a little different in immunoblot profile, were considered to be very similar to the allergen because of their lowest position in SDS-PAGE and their strong antigenicities. There may, therefore, share the unique characteristics of the 7 kDa antigen or lower molecules near the bottom of gel that influence the IgE production and the immediate hypersensitivity in skin test of clonorchiasis, as well as inducing a specific IgG antibody response as shown in this study.

Both the 30 and 7 kDa antigens of CsE2 reacted strongly with the infection sera. Once the infection was cured, the IgG antibodies to these antigens disappeared by 6 months after chemotherapy (Figs. 1A & 4A), while they were still discernible until the same period in the failed cases (Figs. 1B & 4B). Thus, these antigens are regarded as antigenic molecules immunologically detectable an active clonorchiasis. The attenuated response to the 12.5 kDa molecular mass of K2 antigen was also observed in experimental rabbit clonorchiasis (Kim, 1994). The 12.5 kDa was precisely reestimated to 7 kDa as comparing with the 6.5 kDa marker protein (Bio-Rad) in this experiment. The 7 kDa antigen or K2 antigen was proven to be promising for serodiagnosis of a present infection through the studies on animal and human.

The 30 kDa antigen of CsE2 has been shown to be cross-reactive with antibodies in paragonimiasis and metagonimiasis patients (Fig. 2A & B). However, the 7 kDa antigen showed negligible reaction except for one case in each infection, so that it could be very specific to exclude other trematode infections. In addition, the 30 kDa antigen of CsE2 was observed to react to past clonorchiasis, while the 7 kDa antigen was not (Fig. 3). Thus, the 30 kDa antigen might sustain its antigenicity for a prolonged time and provoke the false positive reaction after the cure, whereas the 7 kDa antigen loses its reactivity in a short

period after the successful treatment. In fact, the 30 kDa antigen was still observed until 6 months after treatment in 70% of the cured cases; however, it was not the marker of past clonorchiasis in other endemic area since only 2 of 15 past clonorchiasis cases showed positive (Fig. 5A). The fundamental problems to be solved remain yet to understand the exact antibody responses to these antigens corresponding to the past clonorchiasis.

The 7 kDa, treatment-attenuated antigen of CsE2 is thought doubtlessly to react with *C. sinensis*-specific IgG antibodies in the infection stage but not with antibodies in post-treatment and other parasitic infections. As seen in Table 2 and Fig. 5, in terms of sensitivity and specificity, the 7 kDa antigen was prominently detected as positive as 92% of active clonorchiasis patients but faintly observed in 40% of past clonorchiasis individuals. In addition, it was weakly detected in 42.9% of metagonimiasis patients but strongly observed in 90.9% of mixed infection cases with both *C. sinensis* and *M. yokogawai*, indicating that the 7 kDa antigen was minimally related with metagonimiasis.

In conclusion, the 7 kDa antigen, which is an immunologically intact molecule in an excretory-secretory product, is responding to the present infection of *C. sinensis* and thus it can be a candidate for the serodiagnosis of an active infection of human clonorchiasis.

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=초록=

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조선대학교 의과대학 기생충학교실

인체 간흡충증의 현증감염 시기에 생산된 특이 IgG 항체와 반응하는 간흡충 특이항원을 규명하였다. 간흡충 성충의 조항원 및 서로 다른 조건에서 회수한 4가지 분비배설항원 (CsE)의 항원성을 면역이적법 소견으로 비교하였다. CsE2의 30, 7 kDa 항원이 간흡충 환자혈청과 강한 면역반응을 보였다. 이 항원들은 프라지콴텔 완치 후 6개월 혈청과는 반응하지 않았으나 투약 후 간흡충 충란이 검출된 환자의 치료 후 혈청과는 반응하여 현증감염의 면역반응과 관련된다고 판단하였다. 그러나, 30 kDa 항원은 폐흡충, 요코가와흡충 감염혈청과 교차반응하였고, 7 kDa 항원은 반응하지 않았다. 또, 30 kDa 항원은 일부 간흡충 과거 감염자 혈청과 반응하였으나 7 kDa 항원은 반응하지 않아 7 kDa 항원의 특이도가 높았다. 간흡충 유행지역에서 7 kDa 항원의 민감도를 파악한 바 간흡충 현증감염자 25명 중 23명 (92%), 간흡충 요코가와흡충 중복감염자 11명 중 10명 (91%)과 반응하였다. 반면, 간흡충 과거감염자 15명 중 6명 (40%), 요코가와흡충 감염자 7명 중 3명 (43%)과 반응하였다. 따라서, CsE2의 7 kDa 항원은 인체 간흡충 현증감염의 표식자가 되는 간흡충 특이항원으로 판단된다.

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