Detection of Specific Serum IgE in Clonorchiasis Cases and Analysis of Clonorchis sinensis Allergens

Tai-Soon Yong, Dong-Soo Kim, Soo-Young Lee, Kyung-II Im and Ki Young Lee

Specific serum IgE levels of Clonorchis sinensis in infected humans were measured by avidin-biobind ELISA, and allergens from C. sinensis were identified by immunoblot and autoradiography. Then, allergens fractionated by Sephadex G-200 gel filtration were analyzed, and cross-reactive allergenic components of C. sinensis reacted with paragonimiasis sera were revealed. Fourteen out of 15 C. sinensis egg-positives were found to be serum IgE positive (absorbance > 0.27). Of 14 IgE-reacting allergen bands visualized, major allergens of 66, 61.5, 45, 37, 28.5, 23.5 and 15.5 KD were recognized by more than 50% of the sera of infected humans. The considerable individual variations of IgE immune responses to C. sinensis allergenic components were also noticed. C. sinensis extract was separated into 5 fractions by Sephadex G-200 gel filtration. Seventy-four KD allergen was recognized in the first fraction, 50, 45, 37, 29.5 and 28.5 KD in the third, and 15.5 KD in the fourth. Cross-reactive allergens with sera of paragonimiasis cases were identified as 66, 45, 28.5, 13 and 7.5 KD.

Key Words: IgE, Clonorchis sinensis, allergen, immunoblot, autoradiography, cross-reaction

Clonorchis sinensis, the Chinese liver fluke, is a common parasite, of which geographical distribution is largely confined to Eastern Asia. In Korea, it has been well known that the human infection of C. sinensis is widely distributed along the great rivers and streams (Soh et al. 1976). Allergens, that induce a IgE response of the host particularly, exist in the antigens of C. sinensis like other helminth parasites. Actually, most helminth parasites induce a significant IgE antibody in their mammalian hosts and a number of studies have suggested that IgE, possibly in association with eosinophils or other cells of the immune system, may be an essential element of the host protective immunity against helminth infections (Rigoni et al. 1986). Although no extensive studies on the allergen of C. sinensis have been carried out up to the present time, the skin test inducing IgE-mediated immediate hypersensitivity response (type I) of the host against intradermally injected antigens of this parasite has been frequently used for diagnosis or epidemiological survey on clonorchiasis over the past few decades in Korea (Ahn et al. 1975; Rim, 1986).

In this study, we attempted to measure clonorchis specific serum IgE levels in clonorchiasis cases and to analyze allergenic components of C. sinensis.

MATERIALS AND METHODS

Preparation of C. sinensis adult worm extract

C. sinensis metacercariae were collected from naturally infected Pseudorasbora parva,
and orally infected to experimental rabbits. Adult worms of *C. sinensis* were obtained from the bile duct of the rabbits 8 weeks after infection. Those were homogenized in 0.01 M tris-HCl buffer (pH 7.2) at 4°C for 30 minutes. The supernatant was obtained after centrifugation at 15,000g at 4°C for 1 hour. The supernatant was used as crude *C. sinensis* extract throughout this experiment. Protein concentration was determined by the method of Lowry et al. 1951.

**Serum specimens**

Fifteen sera of *C. sinensis* infected humans were obtained by the survey in an endemic area along the Nakdong-river. Infection of *C. sinensis* was confirmed by stool examination. Six sera of parasitologically confirmed paragonimiasis who were not infected with *C. sinensis* were selected from patients who visited the Severance Hospital, Yonsei University for the last 4 years. Ten control sera were obtained from humans who had no parasite eggs in their stool specimens and also who were serum IgG negative for paragonimiasis, clonorchiasis, cysticercosis or sparganosis in the ELISA test at our laboratory. And the sera were kept at -70°C until used.

**Detection of clonorchis specific serum IgE**

Avidin-biotin ELISA was used (Luczynska et al. 1989). Wells in polystyrene plate (Costar) were coated with one-hundred μl of *C. sinensis* extract (0.5 mg protein/ml in 0.05 M bicarbonate-carbonate buffer, pH 9.6 with 0.01% merthiolate) overnight at 37°C. After washing 3 times with 0.01 M phosphate-buffered saline (pH 7.4) containing 0.05% Tween 20 (PBS-T), blocking was performed with 1% bovine serum albumin in PBS-T for 1 hour at room temperature. Wells were washed with PBS-T, and 1:10 diluted human sera were incubated for 2 hours at 37°C. Wells were washed with PBS-T, and 1:1,000 diluted horseradish peroxidase streptavidin (Vector) was incubated for 30 minutes at 37°C. After washed with PBS-T, wells were developed for 30 minutes at room temperature in the dark using ABTS substrate kit (Vector) containing 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and H₂O₂. The optical density was read at 410 nm using ELISA Reader (Dynatech MR 300).

**Detection of clonorchis specific serum IgG**

Conventional ELISA was used to measure serum IgG levels directed against *C. sinensis* extract. One-hundred μl of *C. sinensis* extract (2 μg protein/ml) coated on wells in a polystyrene plate (Costar) were incubated overnight at 4°C. After being washed 3 times, 1:200 diluted human sera were incubated for 1 hour at 37°C. The wells were washed again, and incubated with 1:2,000 diluted peroxidase conjugated anti-human IgG (Sigma). After washing, the wells were developed using 100 μl/well of 0.05% orthophenylendiamine and 0.006% H₂O₂ 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 H₂SO₄. The optical density was read at 490 nm using the ELISA Reader.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer of proteins on nitrocellulose (NC) membrane**

SDS-PAGE was done according to the procedure of Laemmli (1970). A vertical electrophoresis system of 17 × 12 cm was used (Manhattan Co., Korea). Separating gel, 9 cm long and 1.5 mm thick, was prepared with 0.37 M tris-HCl buffer (pH 8.8) containing 0.1% SDS. And also linear gradient gel of 5–20% were made. Stacking gel, about 1 cm long, was prepared with 3% polyacrylamide in 0.125 M tris-HCl buffer (pH 6.8) containing 0.1% SDS. *C. sinensis* extract 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 extract (containing about 100 μg of protein) were applied to each well. Samples were electrophoresed at 20 mA for about 5 hours. Gels 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 for visualization of the protein bands. For immunoblotting with human sera, the 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). Electrophoresis was carried out at 50 volts, 4°C for 16 hours.

Identification of IgE reacting components of *C. sinensis* extract by immunoblot and autoradiography

After electrophoresis, the NC paper was washed on shaker 3 times with PBS (pH 7.4) containing 10% methanol for 1 hour. Blocking was followed with 3% bovine serum albumin in 10 mM Tris-HCl buffer, pH 7.4 for 4 hours at room temperature. After being washed with 0.9% NaCl 3 times, 1:10 diluted *C. sinensis* infected human sera were applied on a shaker at room temperature for 16 hours. After the wash with PBS-T 3 times each for 1 hour, 111-labelled anti-human IgE (30,000 cpm per strip) were reacted at room temperature for 16 hours. Washed again, the NC membrane strips were air-dried and exposed to X-ray film (Fuji) in an X-Omatic cassette at −70°C for 7–8 days. The film was developed and the bands were examined.

Identification of IgG reacting components of *C. sinensis* extract by enzyme-immunoblot

Enzyme-immunoblot was performed according to the procedures described by Tsang et al. 1983. After electrophoresis, the NC paper was washed on a shaker 3 times with PBS-T each for 10 minutes. Blocking was followed with 3% skim milk in PBS-T for 1 hour at 37°C. After being washed with PBS-T 3 times, 1:100 diluted *C. sinensis* infected human sera, the same sera that we used for IgE blotting, were applied at room temperature for 2 hours. After the wash as above, 1:2,000 diluted peroxidase conjugated anti-human IgG was reacted at room temperature for 2 hours. After being washed again, a substrate (50 mg of diaminobenzidine, 10 μl of 30% H2O2 and 100 ml of distilled water) was reacted until the band pattern could be visualized and was maintained for about 10 minutes. The reaction was stopped by washing the NC paper with distilled water.

Gel filtration and employment of separated extract for identifying IgE reacting components

*C. sinensis* extracts were ultracentrifuged at 15,000 g at 4°C for 1 hour using an Ultracentrifuge (Beckman). Sephadex G-200 gel filtration was performed using the supernatant as a sample. Freeze-dried Sephadex G-200 gel was put into distilled water, and boiled for 5 hours to make it swell up. The gel was poured into a glass column (16 mm x 16 mm) up to 85 cm high, and washed with tris-HCl buffer (pH 7.2) at a flow rate of 6 ml/hour for about 24 hours to reach an equilibrium. 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* extract 20 mg/4 ml) was applied, and monitored using an UV monitor at 206 nm. Each fraction (6 ml) was collected, and the buffer was flowed through the gel continuously until the absorbance was decreased to zero. Separated antigenic fractions were used for identifying IgE reacting components of *C. sinensis* extract by immunoblot and autoradiography as described above.

Identification of cross-reactive allergens using paragonimiasis sera

It was performed as described above in the procedure of IgE reacting components of *C. sinensis* extract by immunoblot and autoradiography except that confirmed 6 paragonimiasis sera instead of clonorchiasis sera were used.

RESULTS

Detection of anti-*C. sinensis* specific serum IgE and IgG

Specific serum IgE or IgG levels of *C. sinensis* infected humans were found to be significantly higher than those of normal controls. Fourteen out of 15 *C. sinensis* egg-positives were positives (absorbance > 0.27) in the specific IgE level, and all tested sera were found positive in the specific IgG level. The mean absorbance of IgE was 0.86 ± 0.32, and that of normal controls, 0.13 ± 0.07. The mean absorbance of IgG was 0.95 ± 0.36, and that of normal controls, 0.16 ± 0.08 (Fig. 1).
IgE reacting components of *C. sinensis* extract by immunoblot and autoradiography

Fig. 2 shows the protein bands of SDS-PAGE separated crude water-soluble *C. sinensis* adult worm extract. More than 30 protein bands were noted. IgE reacting components of *C. sinensis* extract identified by immunoblot and autoradiography were shown (Fig. 3). A total of 14 allergenic bands were visualized. Major allergenic components of 66, 61.5, 45, 37, 28.5, 23.5 and 15.5 KD were clearly seen, which were recognized by more than 50% of the infected (Table 1). No IgE reacting components were recognized by uninfected controls at all. The considerable individual variations of IgE immune responses to *C. sinensis* allergenic components were also noticed.

IgG reacting components of *C. sinensis* extract by enzyme-immunoblot

Fig. 4 shows IgG reacting components of *C. sinensis* extract identified by enzyme-immunoblot. More than 20 antigenic components were noted including relatively prominent bands of 74, 66, 61.5, 50, 45, 37, 31 and 28.5 KD. Individual variations of IgG immune responses to *C. sinensis* antigenic components were not so distinct in comparison with an IgE reacting pattern shown in Fig. 3.

Antigen fractions by Sephadex G-200 gel filtration

The first fraction of the extract appeared 10 hours after application of the sample, so the void volume was 60 ml. During 33 hours from the beginning of gel filtration, 5 largely separated fractions of the extract were collected (Fig. 5). Different protein bands of
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**Fig. 3.** IgE reacting components of _C. sinensis_ extract identified by immunoblot and autoradiography using infected human sera were shown. Major allergenic components of 66, 61.5, 45, 37, 28.5, 23.5 and 15.5 KD were clearly seen. (From lane A to F: clonorchiasis cases, lane G: pooled sera of 2 uninfected controls).

Table 1. Allergens of _C. sinensis_ recognized by human clonorchiasis sera identified by immunoblot and autoradiography (arranged from Fig. 3)

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* Major allergens (reacted with more than 50% of clonorchiasis cases)

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Fig. 4. IgG reacting components of C. sinensis extract identified by enzyme-immunoblot. There were noted more than 20 antigenic components including relatively prominent bands of 74, 66, 61.5, 50, 45, 37, 31 and 28.5 KD. (From lane A to E: clonorchiosis cases, lane N: protein bands blotted on NC membrane from acrylamide gels non-specifically stained with Amido-Black)

SDS-PAGE of fraction 1 to 5 were shown (Fig. 6). Fig. 7 shows IgE reacting components of C. sinensis extract fractions using Sephadex G-200 gel filtration followed by immunoblot and autoradiography. Seventy-four KD allergen was recognized in the first fraction, 50, 45, 37, 29.5 and 28.5 KD in the third, and 15.5 KD in the fourth.

Fig. 5. Elution profile of crude water-soluble C. sinensis adult worm extract separated by Sephadex G-200 gel filtration. There were 5 largely separated fractions were noted. 1: blue dextran(2,000KD), 2: γ-globulin(150 KD), 3: bovine serum albumin(66 KD) and 4: carbonic anhydrase(29 KD) were used as standard molecular weight markers.

Number 3
Identification of cross-reactive allergens using paragonimiasis sera

Cross-reactive allergens with sera of paragonimiasis cases were identified as 66, 45, 28.5, 13 and 7.5 KD. Some bands were identical with allergens recognized by *C. sinensis* infected human sera, but others were only recognized by *P. westermani* infected patients’ sera (Fig. 8).

**DISCUSSION**

It has already been recognized that one of the characteristic immune responses in parasite infection is to produce IgE. High serum total IgE or specific IgE levels were reported in various parasite infections such as schistosomiasis, cysticercosis, strongyloidiasis and filariasis (Johansson et al. 1968; Ito et al. 1972; McRury et al. 1986; Jassim et al. 1987; Nutman et al. 1988; Short et al. 1990).

Increasing levels of IgE caused by *C. sinensis* infection was previously reported from experimental rats (Min et al. 1980; Watanabe and Kobayashi, 1983, 1988) or humans (Min and Soh, 1983; Woolf et al. 1984; Yanagihara et al. 1989), and also confirmed by avidin-biotin ELISA in this study. The result obtained in this study was similar to previous reports showing increased serum IgE levels in clonorchiasis. There was one case in which the IgE value was below the limit that we had arbitrarily set. But it was
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still higher than the values of normal controls. Thus there would be no problem in including this case in the positive group. This may be due to time sequence and individual differences in immune response to parasites.

The avidin-biotin ELISA used in this study is an excellent tool to measure serum IgE, since it is sensitive, relatively easy to perform and it is unnecessary to use radioisotope (Lunzynska et al. 1989). The quantity of serum IgE, however, was impossible to compare with previous reports in which it was represented as IU/ml, but only as absorbance at 410nm in this study. Further studies are needed to resolve this problem.

IgE is known to be harmful in atopy or allergic diseases in humans (Maunsell et al. 1967; Park et al. 1989; Hong et al. 1991). In parasitic infections, IgE in association with cells of the immune system, such as mast cells, eosinophils, macrophages or platelets was reported to be protective (Murray et al. 1971; Dessaint et al. 1979; Joseph et al. 1986; Hagan et al. 1991), although the role of IgE is still controversial (Shet et al. 1990). Evidently, production of IgE in parasite infection is induced by parasites’ products. Briefly, if allergens of the parasite stimulate Type 2 helper T cells (Th2), Th2 produce interleukin-4 (IL-4), and IgE production follows by induction of IL-4 (Phillips et al. 1991). However, the detailed cellular mechanism of producing IgE has yet to be elucidated.

It must be a very interesting subject to investigate the characteristics of helminths’ products or allergens, that are different from IgG-inducing general extracts of bacterial or viral origins. Actually, the commercial skin test antigen of C. sinensis which was manufactured for diagnosis of clonorchiasis or paragonimiasis has been commonly used in the last few decades in Korea (Ahn et al. 1975).

The skin test by using IgE-mediated type I hypersensitivity in the diagnosis of clonorchiasis was standardized in the early 1960s, before the unique characteristic of reaginic antibody (IgE) was recognized (Ishizaka et al. 1966). In contrast with a practical application for diagnosis, research on the allergens of C. sinensis has been very limited until now. The immunoblot technique in association with autoradiography used in this study seemed to be a very powerful tool to show allergen bands of C. sinensis.

Humans vary considerably in the antigen specificity of their immune responses to parasites, and in the infection loads of individuals living even in the same environment. The possibility of the former has a genetic basis operating through repertoire control of the immune system (Tomlinson et al. 1989). Considerable individual variations of IgE immune responses to C. sinensis allergenic components as shown in this result should be related with genetic differences between individuals. It is noteworthy that the variations of IgE immune responses seemed to be more prominent than those of IgG. But, investigation of the antibody isotypes in details of each infected individual are needed in the future.

The autoradiography using each antigen obtained by sephadex G-200 gel filtration showed fractionated IgE-reacting allergens. Each allergen of different molecular weight was found in the early to late elutions, respectively. It means that allergens of C. sinensis are of different molecular weights in a large range, and those are possible to be separated by gel filtration. It also suggested the possibility of purifying allergens by similar techniques for further biochemical studies.

The cross-reactions in the immunodiagnosis of clonorchiasis and paragonimiasis were reported to be common (Cho et al. 1976; Yong et al. 1991). In this study, some allergens of C. sinensis cross-reacted with sera of paragonimiasis were identified evidently, although a limited number of cases were tested.

In conclusion, we found a high level of serum IgE in clonorchiasis cases using the avidin-biotin ELISA method and identified and analyzed IgE-reacting C. sinensis allergens in this study. In the future, more detailed analysis of the antigen should be pursued for the diagnosis and to understand biochemical characteristics of allergens.

REFERENCES

Ahn YK, Soh CT, Han JK: Comparison of dermal reaction with VBS and KST antigens of Clonorchis sinensis in reference to sensitivity
Kigoni EP, Elsas PP, Lenzi HL, Dessein AJ: IgE antibody and resistance to infection. II. Effect of IgE suppression on the early and late skin reaction and resistance of rats to Schistosoma mansoni infection. Eur J Immunol 16: 589-595, 1986
Analysis of Clonorchis Sinensis Allergens

Tomlinson LA, Christie JF, Fraser EM, McLaughlin D, McIntosh AE, Kennedy MW: MHC restriction of the antibody repertoire to secretory antigens, and a major allergen, of the nematode parasite Ascaris. J Immunol 143: 2349-2356, 1989

Tsang VCW, Peralta JM, Simons AR: Enzyme-linked immunoelectro-transfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. Methods Enzymol 92: 377-391, 1983


