Identification of IgE-Reacting *Clonorchis Sinensis* Antigens

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**Abstract**

*Clonorchis sinensis* is a liver fluke and it is the most prevalent human parasite in Korea at present. The parasite infection induces immune responses, characteristically an increased production of parasite-specific IgE in the host. Major IgE-reacting *C. sinensis* antigens in infected humans have been protein bands with MWs of 15, 28, 37, 45, 51, 56, 62, 66, 74, 97 and 160 KD identified by immunoblot analysis. Individual variations of the IgE binding pattern to *C. sinensis* antigens have also been documented. Using immune BALB/c mouse sera, IgE-reacting protein bands have been visualized with MWs of 28, 74, 86, 160 and several >200 KD. One of the most strongly reacted *C. sinensis* antigenic proteins with a molecular weight of 28 KD was purified by gel filtration and preparative electrophoresis. Using a monoclonal antibody produced against the antigenic protein, the protein was localized in the parasite's intestine, and also found to be contained in excretory-secretory antigens.

**Key Words:** *Clonorchis sinensis*, IgE, hypersensitivity, excretory-secretory antigens

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**INTRODUCTION**

*Clonorchis sinensis* is a liver fluke of humans or animals in Eastern Asia, including China, northern Vietnam and Korea. According to a nationwide survey done in 1997, the prevalence rate of *C. sinensis* infection in humans was 1.4% in Korea, being the most frequently found helminthic parasite in humans at present in Korea. Interestingly, a recent survey on intestinal parasite infections in China showed that people of Korean ethnicity had a higher infection rate of *C. sinensis* than any other minority people living in China. The facts are believed to be closely related with Koreans' eating habits, especially the consuming of raw fresh-water fish.

Infection with *C. sinensis* causes pathological changes of the bile duct and surrounding liver tissue, such as desquamation, proliferation, glandular change, metaplasia of the cholangial epithelium, gall-stone formation and cholangial carcinoma. Once infected, antigenic materials are challenged to the immune system of the host, so pathogenic mechanisms of clonorchiasis can be elucidated through studies on the antigenic materials of *C. sinensis*. The humoral immune response, i.e. an increased production of parasite-specific immunoglobulins, has been well proven in laboratories by employing several immunological assays, such as immunofluorescent assay (IFA) or enzyme-linked immunosorbent assay (ELISA). Immunoblot analysis also showed that more than 10 protein bands reacted with parasite-specific antibodies in infected animals or humans.

Generally, helminthic parasite infections induce characteristic immune responses, such increased production of interleukin (IL)-4 or IL-5 resulting in a significant increase of IgE production or eosinophilia resembling the responses in allergy. IgE-reacting helminth antigens play similar roles with the allergens causing clinical symptoms in allergy. The identification and characterization of IgE-reacting antigens should be an important research for elucidating the pathogenesis of immunopathology in helminth infections. About 8 major IgE-reacting *C. sinensis* antigens with a molecular weight (MW) from 66 to 15.5 KD have been previously reported by immunoblot analysis.

In this study we describe the identification of IgE-reacting antigens of *C. sinensis* in infected humans or immune mice by using enzyme-immunoblot analysis, as well as the localization of one of the most prominently reacted antigens by immunohistochemical technique using a monoclonal antibody produced against the protein.
MATERIALS AND METHODS

Parasites and antigens

*C. sinensis* metacercariae were collected from naturally-infected fish (*Pseudorasora parva*) caught in the Nakdong River, Korea, and administered orally to experimental rabbits. Eight weeks later, *C. sinensis* adult worms were obtained from the rabbits. The worms were homogenized in 0.01 M Tris-HCl buffer (pH 7.2), and centrifuged at 15,000 g for 1 hour. The supernatant was used as *C. sinensis* crude antigens for the experiment. To obtain excretory-secretory antigen (ESA), live worms were incubated in physiologic saline for 4 hours at 37°C immediately after collecting them from the bile ducts of a sacrificed rabbit. After centrifugation, the supernatant was taken, concentrated and stored at −20°C until used as the ESA.

Sera

Eight sera of parasitologically-confirmed cases of *C. sinensis* infection were employed from the sera stock of the Department of Parasitology, Yonsei University College of Medicine. The sera were also confirmed previously by ELISA, especially with high *C. sinensis*-specific IgG. In order to collect immune mouse sera, 6-week-old female BALB/c mice were immunized intraperitoneally with a mixture of 100 μg of *C. sinensis* crude extract and alum adjuvant 3 times as at intervals of 3 weeks. The sera were obtained at week 7.

Immunoblot analysis

The crude extract of *C. sinensis* adult worms was electrophoresed on 5–20% gradient SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were incubated in a 1 : 10 dilution of human sera or pooled immune mouse sera for IgE-immunoblot analysis. Washed 3 times, the membranes were then incubated with a 1 : 500 diluted alkaline phosphatase conjugated anti-human IgE (Sigma, St. Louis, MO, USA) or peroxidase conjugated anti-mouse IgE (Nordic Immunology, Tilburg, Netherlands). The color was developed with the substrate for alkaline phosphatase (66 μl of 50 mg/ml of nitrobluetetrazolium and 33 μl of 50 mg/ml of 3-bromo-4-chloro-5-indolyl-phosphate in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM MgCl2). For peroxidase, 2 mM diaminobenzidine solution containing 0.003% H2O2 was used as a substrate.

Purification of an IgE-reacting antigen

Gel filtration chromatography using Sephadex G-200 of *C. sinensis* crude extract yielded 5 fractions as described previously. Of these, the third fraction was selected in consideration of the immunoblot analysis finding. A protein band of 28 KD, being the most prominently reacted band with a pooled immune mouse sera, was cut out from the SDS-PAGE gel of the third fraction. The protein was electroeluted from the gel slice into Tris-HCl buffer (pH 8.3), and concentrated. The purity and IgE reactivity of the protein was reconfirmed by SDS-PAGE and immunoblot analysis.

Production of a monoclonal antibody (Mab)

Six-week-old BALB/c mice were immunized with the antigenic protein of 28 KD according to the same schedule for obtaining immune mouse sera against crude *C. sinensis* extract as described above, except that the third immunization was conducted with an antigen alone, without any adjuvant.

Cell fusion between myeloma cells and spleen cells from immune mice was performed, and selection of antibody secreting hybridoma clones was carried out essentially by the same method described previously. Briefly, 3 days after the third immunization, the spleen of a mouse was obtained. Hybridization was performed with myeloma cells (P3-X-63-Ag8. V653) using 50% polyethyleneglycol 4,000 (Merck, Munich, Germany). HAT media (RPMI 1640 containing hypoxanthine 13.6 μg/ml, aminopterin 0.174 μg/ml, thymidine 3.87 μg/ml and fetal calf serum 20%) was used to select fused cells. Positive clones were selected by ELISA. Isotyping was performed using an isotyping kit (Sigma, St. Louis, MO, USA).

Immunofluorescent microscopy

Live *C. sinensis* adult worms were washed thoroughly with PBS, and frozen quickly at −20°C. The frozen worm was cryocut into 7-μm thick sections, laid on the slides, and air-dried. After washing, the sections were incubated with an undiluted cell culture supernatant containing the Mab produced against the protein with a MW of 28 KD. Washed again, the sections were reacted with 1 : 40 diluted FITC (fluo-
rescent isothiocyanate)-conjugated anti-mouse IgG for 1 hour at 37°C. Then, the slides were observed under a fluorescent microscope.

RESULTS

Immunoblot analysis

IgE-immunoblot analysis showed that major C. sinensis antigens reacted with serum IgE in humans were protein bands with MWs of 15, 28, 37, 45, 51, 56, 62, 66, 74, 97 and 160 KD. Slight differences were found between this study and previous studies, presumably because different serum samples or reagents were used. The sera of 3 subjects (A, B, H) showed no distinct IgE-binding bands on immunoblot analysis, indicating an individual difference of the immune response against the same antigens of the infectious organism. No IgE-reacting bands were noticed in the sera of controls (Fig. 1). Using a pooled serum of immune BALB/c mice, IgE-reacting protein bands were visualized with MWs of 28, 74, 86, 160 and several >200 KD (Fig. 2). There were some differences noticed, together with similarities, in the immunoblot pattern of humans versus immune BALB/c mice.

Localization of an antigen with 28 KD in the parasite

Purification of a protein band with a MW of 28 KD on SDS-PAGE was performed. Based on the finding of the immunoblot analysis, the protein was considered to bind IgE very prominently, especially against immune mouse sera. A Mab (Cs28) was produced against the protein and a corresponding binding was shown on the immunoblot analysis (Fig. 3). The isotype of the Mab was IgG1. The Mab was used to localize the antigen inside a parasite by means of IFA. The Mab was bound to the parasite's intestine, including an intestinal epithelium with its contents in the lumen and possible supportive tissues.

![Fig. 1. IgE-immunoblot analysis of C. sinensis crude extract. Lane A to H: human sera of clonorchiasis, I: uninfected pooled sera. Note IgE-reacting bands of C. sinensis crude extract with MWs of 15, 28, 37, 45, 51, 56, 62, 66, 74, 97 and 160 KD.](image1)

![Fig. 2. IgE-immunoblot analysis of C. sinensis crude extract using a pooled serum of immune BALB/c mice. IgE-reacting protein bands were shown with MWs of 28, 74, 86, 160 and several >200 KD. A protein band with a MW of 28 KD on the immunoblot was relatively distinct.](image2)
or secretory glands around the intestine (Fig. 4). No binding was found on the regement, ovary, uterine eggs, vitelline follicles, seminal receptacles or testes recognized under the fluorescent microscope. The Mab also strongly reacted with C. sinensis ESA. The findings suggested that the antigen is produced from the intestine of the parasite, excreted outside of the parasite contained in the ESA, and challenged to the host immune system.

DISCUSSION

IgE is well known to be responsible for the pathogenesis of atopy or allergic diseases. On the other hand, the IgE response is a hallmark of helminthic infection. High serum total IgE or specific IgE levels were reported in various parasitic infections, such as schistosomiasis, ascariasis or onchocerciasis. An increased IgE level in clonorchiasis was also reported from experimental rats or humans. In a previous study, more than 90% of the clonorchiasis with an increased serum parasite-specific IgG showed increased serum IgE levels. A number of structurally diverse antigens preferentially stimulate the synthesis of IgE antibodies, but no unifying principle has been proposed that explains the nature of isotype selection. The production of IgE antibodies in parasitic infections is induced by parasites' products directly, or indirectly through Type 2 helper T cells or interleukin-4. The mechanism of IgE production in clonorchiasis has not been elu-

Fig. 3. IgG-immunoblot analysis of C. sinensis crude extract. Lane A: a monoclonal antibody produced against the IgE-reacting protein showed a band at 28 KD. B: immune mouse sera against C. sinensis crude extract also showed a strong IgG response around 28 KD.

Fig. 4. A monoclonal antibody produced against the protein with a MW of 28 KD bound to the intestine of C. sinensis elucidated by means of IFA. (A) A cross-sectioned parasite's intestine right posterior to the intestinal bifurcation with a fluorescent color (magnification, 1:100), (B) An adult worm of C. sinensis was cryo-cut (arrow) to produce the sectioned worm antigens of Fig. 4A (rectangle).
Humans vary considerably in their immune responses to parasites through repertoire control of the immune system genetically. Considerable variations in humans or a difference of the IgE binding pattern between humans and mice demonstrated in this study should be related to a genetic difference of the individuals. The major allergens recognized by the majority could reasonably be highlighted in the research. Regardless, the animal model like BALB/c mice in this study would be useful in the basic research on allergens because IgE production through immunization is consistently noticed and comparable to humans without an individual difference among humans. Although the IgE-reacting antigens of C. sinensis have not been characterized in detail until now, the intradermal test was standardized in the early 1960s. It had been commercialized before the characteristics of reaginic antibody was discovered, and is still popularly used in Korea. It is based on the IgE-mediated type I hypersensitivity reaction on the skin of infected individuals. The intradermal test is believed relatively sensitive, but not so specific. As mentioned above, the individual difference in immune reactions, especially the IgE production, presented another difficulty in interpreting the result.

The IgE response is generally considered a major host defense against parasites. For the past 20 years or so, immunology textbooks have routinely exhibited fanciful diagrams as to how IgE and eosinophils, macrophages or platelets cooperatively killed all helminth parasites. Although IgE has been considered to play an essential role in host defense against parasitic helminth infections such as Schistosoma mansoni, in vivo evidence of a protective function of IgE in infected hosts is not enough, and is still controversial. IL-4-deficient mice that produce negligible IgE levels did not differ significantly, and reduction of the IgE response in mice to a primary S. mansoni infection by anti-IgE treatment resulted in decreased worm burden and fecundity, suggesting that IgE plays a detrimental, rather than beneficial, role for the host in schistosomiasis. The IgE response has been demonstrated well in clonorchiasis, but no information has been available on the role of IgE against C. sinensis antigens until now. Although the experiment using C. sinensis as research material is not as feasible as research on S. mansoni because of the difficulty in maintaining the parasite in the laboratory, the role of IgE in clonorchiasis would be an important subject of future studies. Like other parasitic infections, it is speculated that IgE in C. sinensis-infected individuals would play an immunologically protective role, or at least be related with immunopathology in the local tissue.

Mabs were produced against C. sinensis antigens for improving the specificity of immunodiagnostic tests. The Mabs produced in the previous study were localized around the eggs, parenchyma, intestinal epithelium or a parasite tegument etc., depending on each Mab. The Mab produced in this study was only localized on the parasite intestine, indicating that the ESA would be important in serving as stimulators in IgE production in the bile duct directly or indirectly by triggering corresponding cytokine release. The Mab would then be useful in the characterization of the reacting antigen.

In summary, we identified IgE-reacting proteins in C. sinensis antigens with MWs of 15, 28, 37, 45, 51, 56, 62, 66, 74, 86, 97, 160 and several >200 KD in infected humans or immune mice, and found that one of the prominently shown IgE-reacting antigens with a MW of 28 KD was localized in the parasite intestine. The enzyme-immunoblot analysis implemented in this study was a feasible method to identify IgE-reacting parasitic antigens, since it is sensitive and needs no radioisotope like 125I used in the previous study on this parasite. The molecular and biochemical characteristics of these IgE-reacting helminths derived materials are under investigation, and the role and importance of IgE in clonorchiasis will require further adequately-designed research in the future.

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