In 40 cases of human paragonimiasis caused by *Paragonimus westermani* (20 cases), *P. miyazakii* (10 cases), and *P. skrjabini* (10 cases), responses of serum immunoglobulin G (IgG), IgG subclasses, and IgE were analyzed by immunoblotting with crude antigens prepared from egg, 4-week-old juvenile, and adult forms of *P. westermani*. The 32- and 35-kDa proteins in the adult extracts showed specific reactions regardless of the causative species (39 of 40 cases; 98%). Sera of patients infected with *P. westermani* and *P. miyazakii* reacted strongly with the 28-, 46-, and 94-kDa proteins of egg extracts, while those from patients infected with *P. skrjabini* reacted faintly. No sera from patients with other trematodiasis (0 of 15 cases), cestodiasis (0 of 20 cases), or lung cancer (0 of 5 cases) or from healthy controls (0 of 10 individuals) showed positive reactions. Analysis by IgG subclass revealed that IgG4 (33 of 40 cases; 83%) and IgG1 (29 of 40 cases; 73%) antibodies in the patient sera recognized the 32- and 35-kDa proteins predominantly. IgG3 reaction was found in 50% (10 of 20 cases) and 30% (3 of 10 cases) of the sera of patients infected with *P. westermani* and *P. miyazakii*, respectively. In an IgE immunoblot, 83% (33 of 40 cases) of the sera from paragonimiasis patients reacted with the 32- and 35-kDa proteins while no sera from patients with heterologous diseases and healthy controls showed a positive reaction. Both 32- and 35-kDa proteins in adult extracts of *P. westermani* were highly reliable for serodiagnosis of human paragonimiasis.

*Paragonimus westermani*, type species of the genus *Paragonimus*, is an important cause of chronic inflammatory diseases of the lung, central nervous system, and abdominal cavity (24). In addition, *P. miyazakii* and *P. skrjabini* elicit pleural and subcutaneous lesions in infected individuals in Japan and China and *P. heterotremus* causes lung infections in Southeast Asia. Lung infections caused by the parasites should be differentiated from tuberculosis, especially in East and Southeast Asia, where both diseases are endemic (24, 29, 31).

Raising a suspicion of paragonimiasis is the first step leading to the diagnosis, which can be confirmed by egg detection. Egg examination is not, however, highly sensitive due to intermittent discharge in many patients. Furthermore, for extrapulmonary lesions, parasitological diagnosis is impossible unless a biopsy is carried out. The antibody test, developed to overcome these difficulties, has been proven to be effective in diagnosis of paragonimiasis. Enzyme-linked immunosorbent assays or immunoblots showed a high degree of reliability and were shown to be applicable to sera, pleural effusions, or cerebrospinal fluid (2, 3, 7, 9, 12, 15, 22, 26).

Crude extracts of adult worms were shown to be most effective in serodiagnosis because these extracts contain many compartmental proteins which could detect the polyclonal antibody produced by the patients (2, 4, 9, 11, 12, 26). Meanwhile, excretory-secretory products, partially purified cysteine proteases, and egg extracts have been used to detect specific antibody and appear to be highly useful diagnostic antigens for paragonimiasis (7, 10, 14, 18, 22, 23, 27). However, their antigenic properties are yet to be properly evaluated, especially in sera of patients infected with different species of *Paragonimus* (16). Moreover, little information on the analysis of immunoglobulin G (IgG) subclasses and IgE immune responses in paragonimiasis is available. In this study, we evaluated the antigenicity of crude extracts from egg, juvenile, and adult stages of *P. westermani* and compared antibody responses to these extracts in sera of patients infected with *P. westermani*, *P. miyazakii*, and *P. skrjabini*.

**MATERIALS AND METHODS**

**Preparation of crude extracts of *P. westermani***. Cats and dogs were infected with metacecarias, which were collected from freshwater crayfish, *Cambaroides similis*. Four weeks after infection, the juvenile worms were harvested from the peritoneal and thoracic cavities of cats. At 16 weeks after infection, adult worms were collected from dog lungs (5). Eggs of *P. westermani*, obtained either by incubating the adult worms in physiological saline overnight at 37°C or by flushing the infected dog lung, were purified as described previously (13). The eggs, juveniles, and adults were ground with a Teflon pestle-homogenizer in physiological saline and centrifuged at 500 × *g* for 5 min followed by 20,000 × *g* for 1 h at 4°C. The resulting supernatants were used as the crude extracts and stored at −70°C until use. Protein content was measured by using bovine serum albumin as a standard (20).

**Serum samples**. A total of 40 serum samples from patients with paragonimiasis, consisting of 10 patients each infected with *Paragonimus westermani* from Korea and Japan (10 patients from each country), *P. miyazakii* (Japan), and *P. skrjabini* (China), was used. Three patients in Korea were diagnosed by egg detection, and seven patients were diagnosed by their histories of eating crabs, radiological findings, and positive antibody tests by enzyme-linked immunosorbent assay when hemoptysis, chronic cough, or chest pain had been manifested for 10 months to 2 years (4, 8). Japanese patients with *P. westermani* and *P. westermani*; P. miyazakii; P. skrjabini; Y. Yang; Y. Baechung; S. Kasuya; Y.-H. Liu; and S.-Y. Cho

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miyazakii infections were diagnosed by positive differential double immunodiffusion and complement fixation tests and by their histories of eating freshwater crabs (Eriocheir japonicus and/or Geothelphusa dehaani). All patients manifested pulmonary symptoms such as pleuritic pain, hemoptysis, and/or dyspnea for a maximum 3 years. Peripheral eosinophilia and elevation of serum IgE levels were observed in all cases. *P. skrjabini* infection was diagnosed by finding of migratory subcutaneous nodules, a history of ingestion of raw freshwater crabs, and positive antibody tests. The interval between the detection of subcutaneous nodules and diagnosis was not available on an individual basis. As negative controls, five serum samples each from patients with *Schistosoma japonicum* schistosomiasis (patients from China), clonorchiasis (Korea), fascioliasis (Japan), cysticercosis (Korea), alveolar echinococcosis (China), cystic echinococcosis (Jordan), sparganosis (Korea), and cytology-proven lung cancer were used. In addition, 10 healthy controls (students of Gifu University, Gifu, Japan) who denied exposure to any possible infection sources were included in the study. All serum samples were stored at $-70^\circ$C until use.

**Immunoblot for IgG and IgG subclasses.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a commercially available precast 4- to 20% gradient gel (no. 01-102, -106, -020, and -026; SDS-PAGE Mini; TEFCO, Tokyo, Japan) was carried out as described elsewhere (17). The resolved proteins were electroblotted onto polyvinylidene difluoride (PVDF) microporous membrane (Millipore, Bedford, Mass.). Patient sera, diluted 1:200, were probed overnight, peroxidase-conjugated anti-human IgG (heavy- and light-chain specific; Cappel, West Chester, Pa.) and monoclonal antibodies against human IgG subclasses (IgG1, -G2, -G3, and -G4; Zymed, San Francisco, Calif.) were diluted 1:1,000 and incubated for 2 h. The blots were developed with 0.03% (wt/vol) 4-chloro-1-naphthol (4C1N) containing 0.03% H$_2$O$_2$ in phosphate buffer (0.1 M, pH 7.4).

**IgE immunoblot.** SDS-PAGE and transfer-blot to PVDF membrane were performed as described above. The patient sera were diluted 1:25 and probed overnight. Peroxidase-conjugated anti-human IgE (e-chain specific; Cappel), diluted 1:500, was further incubated for 4 h. The blots were developed as described above by using 4C1N.

**RESULTS**

Figure 1 shows immunoblot findings obtained with crude extracts of egg (lanes Egg), 4-week-old juvenile (lanes 4-wk), and 16-week-old adult (lanes 16-wk) stages of *P. westermani*. The pooled sera from groups of 10 patients, each group infected with *P. westermani* (Fig. 1, panels PwJ and PwK) or *P. miyazakii* (panel Pm), revealed strong immunoreactive bands at 28, 46, and 94 kDa with the egg extracts, whereas sera from patients infected with *P. skrjabini* (panel Ps) reacted weakly. The juvenile extracts reacted strongly with sera of patients with paragonimiasis, but they showed cross-reactions with other patient sera, especially those of patients with fascioliasis and schistosomiasis, with 30- and 31-kDa bands (Fig. 1, lanes 4-wk of panels Fh and Sj). In adult extracts, bands at 6, 17, 26, 27, 28, 32, 35, 46, and 94 kDa exhibited strong reactions to sera of patients infected with the three different *Paragonimus* species. Of these, the 32- and 35-kDa bands showed the strongest and most frequent reactions. The diagnostic sensitivity and specificity of these paired bands were 98% (39 of 40 patients) and 100% (0 of 50 patients), respectively.

The cross-reactivity of the adult extracts was further examined by employing individual infection sera (Fig. 2). Sera of...
patients infected with *S. japonicum*, *Clonorchis sinensis*, and *Fasciola hepatica* exhibited nonspecific positive reactions to several bands, including those of 6, 17, 30, 31, and over 100 kDa, while no sera showed positive reactions to the 32- and 35-kDa bands. The sera of patients with cystic echinococcosis, alveolar echinococcosis, cystic echinococcosis, and sparganosis showed a few reactions to the antigenic bands below 10 kDa. The sera from patients with lung cancer and healthy controls did not show positive reactions (immunoblots of lung cancer patients are not shown).

Figure 3 demonstrates recognition by IgG subclasses of adult extracts of *P. westermani*. The sera from patients infected with *P. westermani* and *P. miyazakii* reacted mainly to both 32- and 35-kDa bands with IgG4 (18 of 20 [90%] for patients with *P. westermani* paragonimiasis; 8 of 10 [80%] for patients with *P. miyazakii* paragonimiasis) and IgG1 (16 of 20 [80%] for patients with *P. westermani* paragonimiasis; 9 of 10 [90%] for patients with *P. miyazakii* paragonimiasis). Sera of patients with *P. skrjabini* infections reacted weakly to 32- and 35-kDa bands with IgG4 (7 of 10 [70%]) and IgG1 (4 of 10 [40%]) (panel Ps in Fig. 3). In addition, sera of patients infected with *P. westermani* (10 of 20, 50%) or *P. miyazakii* (3 of 10, 30%) showed IgG3 antibody reaction, while those of patients infected with *P. skrjabini* showed minimal reactions (1 of 10, 10%). IgG2 subclass reaction was observed in only 8% (3 of 40) of the sera examined.

When specific IgE reactions against the adult extracts were analyzed by immunoblot, sera of patients with *P. westermani* and *P. miyazakii* infections were found to react to 17-, 26-, 28-, 32-, and 35-kDa bands. Those of patients infected with *P. skrjabini* reacted mainly to the 32- and 35-kDa bands and faintly to those of 17, 26, and 28 kDa (Fig. 4). The 32- and 35-kDa bands exhibited the most specific and strongest reactions to paragonimiasis sera, irrespective of the species (18 of 20 [90%] for patients with *P. westermani* paragonimiasis, 8 of 10 [80%] for patients with *P. miyazakii* paragonimiasis, and 7 of 10 [70%] for patients with *P. skrjabini* paragonimiasis; overall positivity rate 83%). A few sera of patients with other parasitic diseases and the normal control showed weak reactions to either the 6-, 14-, or 17-kDa band, as shown in Fig. 4.

**DISCUSSION**

In this study, crude extracts from egg, juvenile, and adult stages of *P. westermani* were examined for their ability to be captured by specific IgG, IgG subclasses, and IgE antibodies in sera of patients infected with *P. westermani*, *P. miyazakii*, and *P. skrjabini*. Irrespective of the causative species, all the patient sera tested exhibited specific reactions to 32- and 35-kDa proteins in extracts of adult *P. westermani*, with IgG4 being the predominant reactive antibody (Fig. 3). These two bands were shown to elicit strong reactions when extracts were obtained from adults after 8 weeks of experimental paragonimiasis (18). An IgE immunoblot demonstrated also that most sera examined (83%) revealed specific reactions to these two bands (Fig. 4). Taken together, both the 32- and 35-kDa proteins in the adult extracts were highly specific and sensitive for diagnosis of paragonimiasis from the early stage to chronic infection and showed common antigenic epitopes for IgG and IgE. The present result is partly in agreement with results of analyses of antigenic epitopes in *P. heterotremus*, in which the 31.5-kDa protein was found to be specific for homologous sera (21–23).

While the sera of patients infected with *P. westermani* and *P. miyazakii* reacted strongly to 28-, 46-, and 94-kDa proteins in the egg extracts, those of patients infected with *P. skrjabini* reacted weakly (Fig. 1 and 3). This result suggested that *P. miyazakii* matures in the human host, though the eggs were hardly detectable due to the organism’s location in the given host. Because *P. skrjabini* infections were recognized as subcutaneous nodules before the worms had grown to adult form, antibody responses against the egg antigen might be either absent or weak. It was also shown that sera of patients with *P. westermani* and *P. miyazakii* paragonimiasis revealed similar levels of serum antibody to the partially purified antigen of adult *P. westermani* (7).

The paragonimiasis sera recognized the 32- and 35-kDa proteins strongly with IgG4. In addition, 50 and 30% of the sera infected with *P. westermani* and *P. miyazakii* showed IgG3 responses, while those from patients with *P. skrjabini* infection showed negligible IgG3 reactions. Because IgG4 isotype switching was correlated with the duration of infection and
clinical manifestation (19, 28), the present result matched well with the common clinical characteristics of chronic and persistent infections, which continued stimulation of the host immune system. In patients with P. westermani and P. miyazakii paragonimiasis, pulmonary symptoms were long-standing, being manifested for 8 months to 3 years. A chronic course of infection in paragonimiasis patients was also associated with elevation of IgG4 subclass levels. High IgG4 responses have been described to occur in patients with chronic schistosomiasis and other cestode infections (1, 6, 25). It is yet to be determined whether a relatively weak IgG4 response in P. skrjabini paragonimiasis reflects an early infection or a difference in antibody recognition in the patient sera.

In this study, the juvenile extracts showed cross-reactions with sera from patients with fascioliasis and S. japonicum infections, whereas the adult extract exhibited negligible cross-reactions. On dilution to 1:100, sera from patients with fascioliasis, schistosomiasis, and clonorchiasis revealed cross-reactions, whereas none of the sera of patients with other parasitic infections exhibited positive reactions. M₄, molecular mass (in kilodaltons). The letters a to e each represent a different protein.

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