CONCISE COMMUNICATION

Recombinant *Paragonimus westermani* Yolk Ferritin Is a Useful Serodiagnostic Antigen

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A recombinant protein of *Paragonimus westermani* yolk ferritin was bacterially produced from a previously cloned complementary DNA and was used as an antigen for an enzyme-linked immunosorbent assay (ELISA) against paragonimiasis- and other helminth-infected sera to evaluate its serodiagnostic potential. The ELISA revealed that paragonimiasis westermani had 88.2% sensitivity and 100% specificity. The positive and negative predictive values of the ELISA were calculated to be 100% and 97.1%, respectively. Sera from cats experimentally infected with *P. westermani* began to produce immunoglobulin G antibodies against the yolk ferritin at 13 weeks after infection, which suggests that the corresponding antigen was derived from the vitellaria in accordance with maturation of *P. westermani*. These results indicate that the recombinant *P. westermani* yolk ferritin is a potent serodiagnostic reagent for paragonimiasis westermani from an early stage of the infection.

Paragonimiasis is a disease caused by lung flukes of genus *Paragonimus* helminths. Pulmonary paragonimiasis, caused by *P. westermani* infection, is endemic in the Far East, southeastern Asia, and South America and remains a serious public health problem [1]. The diagnosis of human paragonimiasis westermani is dependent on detection of characteristic eggs in a sputum and/or stool examination. Although there have been some reports about serodiagnosis of the disease by using crude worm extracts as antigens, cross-reactions with other trematode infections, such as schistosomiasis, fascioliasis, or clonorchiasis, decreased the reliability of this diagnostic method [2, 3]. Purified antigens, such as cysteine proteinases of *P. westermani,* increased sensitivity to paragonimiasis sera and reduced cross-reactivity to fascioliasis sera [4]. Because purification of an antigenic protein from crude preparations to achieve homogeneity is a labor-intensive and time-consuming process, production of specific antigenic molecules into recombinant forms by using appropriate expression systems may be a good alternative way of alleviating the hindrances.

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We cloned a *P. westermani* yolk ferritin cDNA (GenBank accession no. AF188720), produced its recombinant protein in a bacterial system, and characterized its biochemical and molecular biological properties (data not shown). Ferritin is an iron-storage protein identified in a wide range of organisms, from bacteria to mammals [5]. Serodiagnostic potentials of ferritin have been reported to exist in *Echinococcus granulosus* [6] and *Taenia saginata* [7]. We investigate here whether recombinant *P. westermani* yolk ferritin may be useful for serodiagnosis of paragonimiasis westermani.

Materials and Methods

Production of recombinant protein. The coding region of *P. westermani* yolk ferritin cDNA was cut out by appropriate restriction enzymes and was subcloned into bacterial expression plasmid vector pRSET (Invitrogen). *Escherichia coli* strain BL21(DE3)-pLysS (Novagen) was transformed with the recombined pRSET. Overexpression of the recombinant protein was induced by adding isopropylthio-β-galactoside to the culture medium. Since the recombinant protein was located in insoluble inclusion bodies, it was purified by following the method of van Wuytswinkel et al. [8], with a partial modification. In brief, the bacterial cells were pelleted, resuspended in 20 m*M* Tris/maleate buffer (pH 8.0), and sonicated on ice. The bacterial lysate was centrifuged at 12,000 *g* for 10 min at 4°C. The pellet was washed in 20 m*M* Tris/maleate buffer (pH 8.0) containing 1 mg/mL deoxycholate, 1 m*M* EDTA, and 0.2 mg/mL lysozyme and was centrifuged, as aforementioned. The pellet was resuspended in 20 m*M* Tris/maleate (pH 8.0) containing 8 *M* urea and 0.2% (vol/vol) Triton X-100 and was again

centrifuged. The supernatant was saved and was dialyzed against 20 m*M* Tris/maleate (pH 8.0) overnight at room temperature. It was loaded onto Ni-NTA resin (Qiagen) pre-equilibrated with 20 m*M* Tris/maleate (pH 8.0). The resin was washed 3 times with a buffer (50 m*M* NaH_2PO_4, 300 m*M* NaCl, and 20 m*M* imidazole [pH 8.0]). The recombinant protein was eluted with the same buffer, but containing 400 m*M* imidazole, and was dialyzed against 20 m*M* Tris/maleate buffer (pH 8.0) overnight at room temperature. The recombinant protein thus produced was 28-kDa fusion protein with 4-kDa tag protein. Protein concentration was determined by use of Protein Assay Kit II (BioRad).

ELISA. The wells of a 96-well microplate were sensitized with 1 μg/100 μL of the recombinant *P. westermani* yolk ferritin at 4°C overnight. The wells were incubated with sera from humans infected with paragonimiasis westermani ($n = 34$), clonorchiasis sinensis ($n = 32$), opisthorchiasis viverrinii ($n = 9$), schistosomiasis japonicum ($n = 30$), fascioliasis hepatica ($n = 10$), cysticercosis cellulosae ($n = 20$), and sparganosis ($n = 25$) and with sera from uninfected humans ($n = 10$) at a 1:200 dilution at 37°C for 2 h. They were then incubated with peroxidase-conjugated anti–human IgG (Cappel Laboratories) at a 1:2000 dilution for 1 h. Color was developed by using a substrate, *o*-phenylene diamine, and absorbance was measured at 490 nm (A_{490}) with a microplate reader (model 3100; Bio-Rad Laboratories). The data obtained were analyzed for diagnostic sensitivity, specificity, and positive and negative predictive values [9], which were defined and calculated as follows: true negative = no. of control sera (other helminthiasis–infected and healthy control sera) that were negative by the assay; true positive = no. of proven paragonimiasis-infected sera that were positive by the assay; false negative = no. of proven paragonimiasis-infected sera that were negative by the assay; false positive = no. of control sera that were positive by the assay; sensitivity = [no. of true positives/(no. of true positives + no. of false negatives)] × 100; specificity = [no. of true negatives/(no. of false positives + no. of true negatives)] × 100; positive predictive value = [no. of true positives/(no. of true positives + no. of false positives)] × 100; negative predictive value = [no. of true negatives/(no. of true negatives + no. of false negatives)] × 100.

Immunoblot. The recombinant *P. westermani* yolk ferritin was separated by 12.5% SDS-PAGE and was electrotransferred onto a nitrocellulose membrane. Each stripped membrane was incubated, at a 1:200 dilution, with the pooled cat sera, which were collected at 0, 2, 4, 6, 8, 10, 13, and 15 weeks after the experimental infections with *P. westermani* metacercariae. The sera were incubated with peroxidase-conjugated anti–cat IgG (Cappel Laboratories) at a 1:2000 dilution, and color was developed by using 4-chloro-1-naphthol as a substrate.

Results

Absorbances of ELISA, obtained by using the recombinant *P. westermani* yolk ferritin as an antigen, are plotted in figure 1. The cutoff absorbance for a positive reaction was set at (A_{490}) = 0.214, which was a mean + 10 SD of the absorbances obtained from uninfected control sera. Of 34 paragonimiasis-infected sera, 30 were determined to be positive, representing a sensitivity of 88.2% (absorbance range, 0.068–0.660; mean ± SD, 0.469 ± 0.139). Absorbances of sera of the other helminthiases were below the cutoff absorbance. The mean of the absorbance to paragonimiasis serum was >5 times higher than those of the other helminthiasis sera. Since there were no cross-reactions to other helminthiasis sera, 100% was the specificity of the ELISA using the recombinant *P. westermani* yolk ferritin. The cutoff value employed, 0.214, which was even lower than the conventional cutoff value of 0.25 [10], produced good positive and negative predictive values of 100% and 97.1%, respectively.

IgG antibodies reacting to the recombinant *P. westermani* yolk ferritin were detected by an immunoblot in experimental cat sera after 13 weeks of the metacercarial infection (figure 2).

Discussion

As for molecular biological properties, *P. westermani* yolk ferritin shared a low sequence homology with those of vertebrate and invertebrate animals (data not shown). However, a ferroxidase center was predicted to be formed with a coordination of the highly conserved iron-binding amino acid residues [5], conferring an enzymatic function to the molecule. From a serodiagnostic point of view, the low homology of *P. westermani* yolk ferritin to those of invertebrates, including helminthic parasites, seems to favor its antigenic specificity.

E. granulosus ferritin was reported earlier to show a specific antigenicity with cystic and alveolar echinococcosis sera but not with cysticercosis sera [6], whereas *T. saginata* ferritin cross-reacted to both cysticercosis and hydatidosis sera [7]. Although

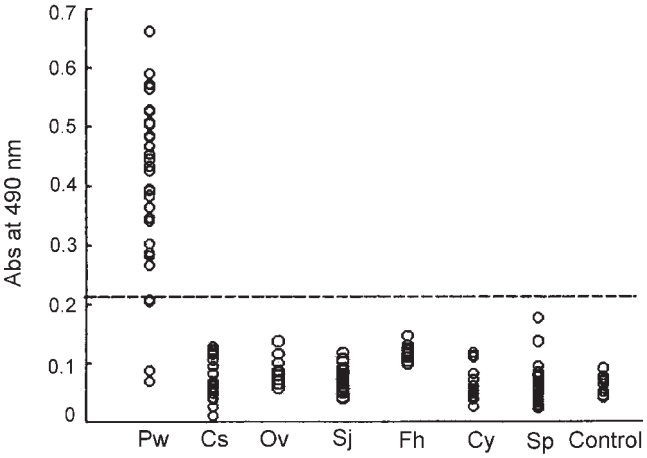

Figure 1. ELISA using recombinant *Paragonimus westermani* yolk ferritin in sera from patients with paragonimiasis westermani or various helminthiases. Each circle (○) represents the mean absorbance (Abs) of 3 wells. Pw, paragonimiasis westermani; Cs, clonorchiasis sinensis; Ov, opisthorchiasis viverrinii; Sj, schistosomiasis japonicum; Fh, fascioliasis hepatica; Cy, cysticercosis cellulosae; Sp, sparganosis; control, uninfected human. Cutoff value at absorbance 0.214 is shown *(dashed line)*.

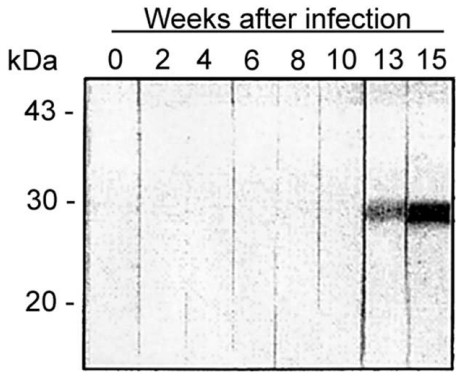

Figure 2. Immunoblot of recombinant *Paragonimus westermani* yolk ferritin probed with pooled sera of cats infected with *P. westermani*. Antibody reactivity was detected after 13 weeks of infection.

cestode ferritins share antigenic epitopes among the systematically related taxa, the recombinant *P. westermani* yolk ferritin do not cross-react to them (figure 1).

The ferritin was localized in vitellaria and eggs of adult *P. westermani* (data not shown). Yolk materials produced in vitellaria are provided to ova and packed in the eggs. In cats, *P. westermani* grows up to be an egg-producing adult between 7 and 8 weeks after the infection [1]. An increase in IgG antibody levels in the cat sera to the egg extract of *P. westermani* was attributed to a 28-kDa molecule after 13 weeks of the infection [11]. The antibody response to the recombinant *P. westermani* yolk ferritin, shown in figure 2 in regard to the 28-kDa molecule, is in accord with the egg production of the worm, with a transition of 4–5 weeks.

Eggs of *P. westermani* remain alive in the pulmonary (their natural habitat) and in extrapulmonary tissues for prolonged periods after the fluke dies. A seropositive result obtained by using egg antigens of various types, including yolk ferritin, may imply that the patient is in a prolonged chronic stage of the infection. We propose here that the recombinant *P. westermani* yolk ferritin is a potent antigen for serodiagnosis of human paragonimiasis westermani in early and prolonged stages.

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