Evaluation of Immunoglobulin G Subclass Antibodies against Recombinant Fasciola gigantica Cathepsin L1 in an Enzyme-Linked Immunosorbent Assay for Serodiagnosis of Human Fasciolosis

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Received 12 June 2005/Returned for modification 13 July 2005/Accepted 28 July 2005

A cystatin capture enzyme-linked immunosorbent assay (ELISA) using recombinant Fasciola gigantica cathepsin L1 antigen was developed to detect specific immunoglobulin G (IgG) subclass antibodies (IgG1, IgG2, IgG3, and IgG4) and was evaluated for its diagnostic potential for human fasciolosis. In an analysis of the sera of 13 patients infected with F. gigantica, 209 patients with other parasitic infections, 32 cholangiocarcinoma patients, and 42 healthy controls, the IgG4-ELISA gave the highest diagnostic values. The sensitivity, specificity, accuracy, and positive and negative predictive values of this method based on the detection of IgG4 antibody were 100%, 99.3%, 99.3%, 86.7%, and 100%, respectively. The results revealed that restricting the ELISA to the detection of specific IgG4 antibody enhanced the specificity and accuracy for the serodiagnosis of human fasciolosis.

Fasciolosis is caused by liver flukes of the genus Fasciola, of which F. hepatica and F. gigantica are the most common representatives (38). Both have a worldwide distribution, but F. hepatica predominates in temperate zones while F. gigantica is found primarily in tropical regions (3). The disease is recognized as a serious public health problem by the World Health Organization (38), and an estimated 17 million people are infected worldwide (28).

The diagnosis of F. hepatica infections is usually based on fecal egg counts or serological assays of which some are based on the detection of cathepsin L proteases of these flukes. Serological tests for the diagnosis of fasciolosis have been developed as standard assays using native F. hepatica cathepsin L1 (31, 32, 34, 36) or recombinant cathepsin L1 as marker antigens (5, 7, 31) as well as selected B-cell epitopes from F. hepatica cathepsin L1 (7, 8). The development of an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of human fasciolosis based on the detection of serum immunoglobulin G4 (IgG4) antibody reactive to native or recombinant F. hepatica cathepsin L1 showed excellent potential (31, 32, 36).

Despite recent studies involving the diagnosis of human fasciolosis using native F. gigantica excretory-secretory antigen (20, 26, 27), native F. gigantica cysteine proteinase (35) or recombinant F. gigantica cathepsin L1 (rCTL1) (37), specific IgG subclass antibodies to F. gigantica rCTL1 antigens as targets for an ELISA have not been studied sufficiently and need further investigation. In the present study, we evaluated four IgG subclass antibodies (IgG1, IgG2, IgG3, and IgG4) against F. gigantica rCTL1 in a cystatin capture ELISA for the serodiagnosis of human fasciolosis. The aim was to determine whether the detection of any subclasses of IgG antibodies could be used to improve the specificity and accuracy of this immunodiagnostic technique.

MATERIALS AND METHODS

Preparation of recombinant protein antigen for the cystatin capture ELISA. The F. gigantica rCTL1 antigen was prepared as previously described (37). Briefly, the expression of calmodulin binding peptide fused with cathepsin L1 in transformed Escherichia coli BL21 gold (DE3; Stratagene, La Jolla, CA) was induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30°C. The cells were then harvested and the cell pellet was resuspended in cooled 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Triton X and 1% sarcosine. Then it was sonicated and the resulting suspension was centrifuged at 15,000 × g for 10 min at 4°C. Recombinant protein produced as inclusion bodies was obtained from the pellet. The pellet was then washed several times with 0.01 M PBS, pH 7.4, and resuspended in solubilizing solution (50 mM Tris-HCl, pH 8.0, containing 50 mM NaCl, 5 mM EDTA, 10 mM dithiothreitol, and 8 M urea) for 30 min. After centrifugation at 15,000 × g for 30 min, the first supernatant fraction was discarded. The remaining pellet was repeatedly solubilized and centrifuged two times. The second (S2) and third (S3) supernatant fractions containing rCTL1 were selected for use as antigen for the cystatin capture ELISA. The S2 and S3 fractions were pooled, dialeyzed, lyophilized, and then resuspended in 1% sodium dodecyl sulfate with a final concentration of 2 mg/ml. The resulting suspension was stored at 4°C until being used. A bacterial lysate from E. coli BL21 gold (DE3) transformed with the pCAL-n-FLAG expression vector (Stratagene, La Jolla, CA) was prepared and used as control extraction for demonstrating no contamination of the cysteine proteinases from the bacterial cells. The protein concentration of the samples was determined by the method of Bradford (4) using bovine serum albumin (BSA) as the standard.

Cystatin capture ELISA. The method was performed as previously described (37) with some modifications. Each well of the ELISA plate was sensitized with 0.25 µg of chicken egg cystatin (Sigma Chemical Co., St. Louis, MO) in 0.1 ml of 0.1 M carbonate buffer, pH 9.6, at 4°C overnight. The wells were washed five times with 10 mM PBS, pH 7.4, containing 0.05% Tween 20 (PBS-T) and blocked with 2% BSA in PBS-T for 1 h at room temperature. After washing with PBS-T, 3 µg of rCTL1 antigen diluted in 100 µl of 1% BSA in PBS-T was added to the wells.
well and the plate was incubated at 4°C overnight. Following another washing step with PBS-T, the wells were incubated at 37°C for 1 h with 0.1 ml of human serum diluted 1:200 with 1% BSA in PBS-T. After washing with PBS-T, the peroxidase-conjugated anti-human IgG (IgG1 = 1:2, 1:4) subclasses [Zymed, South San Francisco, CA] diluted 1:1,000 (for IgG1, IgG2, and IgG3) and diluted 1:20,000 (for IgG4) with 1% BSA in PBS-T were used as secondary antibody.

The wells were then washed with PBS-T and incubated with the 0.1 ml of O-phenylenediamine dihydrochloride substrate for 30 min. The reaction was stopped with 0.05 ml of 8 N H4SO4, and the absorbance was measured at 492 nm using a microplate ELISA reader (Tecan, Salzburg, Austria).

**Human sera.** Sera were obtained from serum banks of different departments of the Faculty of Medicine, Khon Kaen University (1992 to 2003). Each serum was aliquoted and stored at -70°C until used. The study protocol was approved by the Human Research Ethics Committee of Khon Kaen University. Informed consent was obtained from all adult patients, parents, or legal guardians. Thirteen serum samples were obtained from parasitologically confirmed cases of *F. gigantica* as well as control sera (other parasitoses, cholangiocarcinoma, and healthy controls) that were negative by the assay; false positive was the number of control samples that were positive by the assay; true positive was the number of proven fasciolosis samples that were positive by the assay; false negative was the number of proven fasciolosis samples that were negative by the assay.

**RESULTS**

For the analysis of specific IgG subclasses against *F. gigantica* rCTL1 antigen, the absorbance values and mean optical density (OD) ± SD of all serum groups are shown in Table 1 and Fig. 1. The mean OD values of the specific IgG subclass were significantly higher than for parasitic diseases other than fasciolosis, cholangiocarcinoma cases and the healthy controls groups (P < 0.001). Using the mean absorbance value plus 4 SD of the negative control sera from healthy controls as the cutoff limit between positivity and negativity for fasciolosis, the diagnostic sensitivity, specificity, accuracy and predictive values were calculated and expressed using the method of Galen (12). These values were calculated and expressed as follows: sensitivity was equal to the number of true positives/(number of true positives + number of false negatives) × 100; specificity was equal to the number of true negatives/(number of false positives + number of true negatives) × 100; accuracy was equal to the number of true positives + number of true negatives/(number of true positives + number of false positives + number of true negatives) × 100; positive predictive value was equal to the number of true positives/(number of false positives + number of true positives) × 100; negative predictive value was equal to the number of true negatives/(number of false positives + number of true negatives) × 100; 100; true negative was the number of control samples (other parasitoses, cholangiocarcinoma, and healthy controls) that were negative by the assay; true positive was the number of proven fasciolosis samples that were positive by the assay; false positive was the number of control samples that were positive by the assay and false negative was the number of proven fasciolosis samples that were negative by the assay.

**TABLE 1. Absorbance values of IgG subclass antibodies to F. gigantica rCTL1 by cystatin capture ELISA and positivity percentage of each serum sample**

| Type of serum       | IgG1 (Mean ± SD) | IgG2 (Mean ± SD) | IgG3 (Mean ± SD) | IgG4 (Mean ± SD) |
|---------------------|-----------------|-----------------|-----------------|-----------------|
| Fasciolosis         | 0.358 (46.2)    | 0.583 (40.0)    | 0.383 (23.1)    | 1.154 (100)     |
| Paragonimiasis      | 0.098 (4.0)     | 0.455 (3.0)     | 0.225 (8.0)     | 0.096 (4.0)     |
| Opisthorchiasis      | 0.060 (3.0)     | 0.291 (3.0)     | 0.207 (4.0)     | 0.20 (4.0)      |
| Ascariasis           | 0.087 (6.6)     | 0.159 (7.1)     | 0.097 (7.1)     | 0.049 (4.0)     |
| Hookworm infection  | 0.072 (0.7)     | 0.148 (0.7)     | 0.137 (0.7)     | 0.105 (0.7)     |
| Strongyloidaisis     | 0.051 (3.3)     | 0.352 (3.3)     | 0.121 (3.3)     | 0.031 (3.3)     |
| Capillariaisis       | 0.055 (0.4)     | 0.072 (0.0)     | 0.098 (0.0)     | 0.069 (0.0)     |
| Gnathostomiasis      | 0.052 (0.0)     | 0.176 (0.0)     | 0.138 (0.0)     | 0.035 (0.0)     |
| Angiostrongyliaisis  | 0.033 (0.0)     | 0.269 (0.0)     | 0.133 (0.0)     | 0.027 (0.0)     |
| Cysticeriosis        | 0.082 (0.0)     | 0.585 (20.0)    | 0.277 (20.0)    | 0.069 (20.0)    |
| Malaria*             | 0.226 (35.7)    | 0.504 (7.1)     | 0.485 (28.6)    | 0.044 (28.6)    |
| Trichinosis          | 0.169 (29.4)    | 0.501 (11.8)    | 0.503 (17.6)    | 0.024 (17.6)    |
| Echinostomiasis      | 0.078 (0.0)     | 0.213 (0.0)     | 0.185 (0.0)     | 0.033 (0.0)     |
| Taeniasis            | 0.030 (0.0)     | 0.079 (0.0)     | 0.105 (0.0)     | 0.033 (0.0)     |
| Trichurus            | 0.118 (0.0)     | 0.044 (0.0)     | 0.059 (0.0)     | 0.022 (0.0)     |
| Amoebiasis           | 0.087 (0.0)     | 0.070 (0.0)     | 0.070 (0.0)     | 0.018 (0.0)     |
| Giardiasis           | 0.224 (50.0)    | 0.038 (0.0)     | 0.038 (0.0)     | 0.034 (0.0)     |
| Other parasitoses*   | 0.126 (13.3)    | 0.072 (0.0)     | 0.071 (0.0)     | 0.040 (0.0)     |
| Cholangiocarcinoma   | 0.174 (12.5)    | 0.649 (6.2)     | 0.586 (37.5)    | 0.043 (37.5)    |
| Healthy controls     | 0.086 (0.0)     | 0.344 (0.0)     | 0.173 (0.0)     | 0.052 (0.0)     |

*See Materials and Methods.*

**Data analysis.** The statistical analysis was performed by Student’s t test using the statistical software of Sigma Stat (San Rafael, CA). The mean absorbance value plus 4 standard deviations (SD) of the negative control sera from healthy controls was used as the cutoff limit between positivity and negativity for fasciolosis. The diagnostic sensitivity, specificity, accuracy and predictive values were calculated and expressed using the method of Galen (12). These values were calculated and expressed as follows: sensitivity was equal to the number of true positives/(number of true positives + number of false negatives) × 100; specificity was equal to the number of true negatives/(number of false positives + number of true negatives) × 100; accuracy was equal to the number of true positives + number of true negatives/(number of true positives + number of false positives + number of true negatives) × 100; positive predictive value was equal to the number of true positives/(number of false positives + number of true positives) × 100; negative predictive value was equal to the number of true negatives/(number of false positives + number of true negatives) × 100; true negative was the number of control samples (other parasitoses, cholangiocarcinoma, and healthy controls) that were negative by the assay; true positive was the number of proven fasciolosis samples that were positive by the assay; false positive was the number of control samples that were positive by the assay and false negative was the number of proven fasciolosis samples that were negative by the assay.
(DE3) transformed with the pCAL-n-FLAG vector as control extraction, the mean OD values of proven fasciolosis cases in each IgG antibody subclasses were not statistically significant different from the healthy controls groups (P < 0.001) (data not shown).

DISCUSSION

The specificity and sensitivity of the serodiagnosis of filariasis (6, 10, 33), strongyloidiasis (14), chronic schistosomiasis (21, 22), trichuriasis (23) and hookworm infection (24) have been increased by the detection of IgG subclass antibodies to antigens of these parasites. Recently, Hassan et al. (16, 17) showed that out of the anti-Fasciola subclasses, IgG4 antibody is the most specific for an accurate diagnosis of human fasciolosis. In addition, an IgG4-ELISA based on the detection of serum antibody reactive to native or recombinant F. hepatica cathepsin L1 demonstrated an excellent potential for the diagnosis of F. hepatica infection (31, 32, 36). Recently, we also

| Type of IgG subclass | Sensitivity (%) | Specificity (%) | Accuracy (%) | Predictive value (%) |
|----------------------|----------------|----------------|--------------|---------------------|
|                      |                |                |              | Positive           | Negative          |
| IgG1                 | 46.2           | 93.3           | 91.2         | 24.0               | 97.4              |
| IgG2                 | 0              | 97.9           | 93.6         | 0                  | 95.5              |
| IgG3                 | 23.1           | 92.2           | 89.2         | 12.0               | 96.3              |
| IgG4                 | 100            | 99.3           | 99.3         | 86.7               | 100               |
demonstrated the detection of total specific IgG antibodies by F. gigantica rCTL1-cystatin capture ELISA with diagnostic values of 100% sensitivity, 98.92% specificity, 98.97% accuracy, 81.25% PPV and 100% NPV, respectively (37).

That study was designed to evaluate total specific IgG antibodies against F. gigantica rCTL1 for the serodiagnosis of human fasciolosis whereas the present study focused on IgG subclasses and found that the detection of IgG4 antibody against F. gigantica rCTL1 antigen in human sera could improve the degree of specificity (99.3%) and accuracy (99.3%). We also demonstrated that there was no contamination with cysteine proteases from bacterial lysate by obtaining low optical density values against control extraction bacteria. Two cases of paragonimiasis gave positive results, which could either be due to a subclinical infection with Fasciola spp. or to the rCTL1 sharing some epitopes with the Paragonimus trematodes. Nevertheless, the IgG4-ELISA presents a significant serological advantage, as it can differentiate between human fasciolosis and gnathostomiasis, a disease with high prevalence in Asia (29, 30).

The human immune response to parasitic infections exhibits different distributions of IgG subclasses antibodies in different infected groups (9, 25). The genetic background of the infected individual and the intrinsic properties of the antigen itself and/or defined cytokines play a role in determining the main subclass of the antibody response (13). This study demonstrated the prominent role of specific IgG4 antibody in human fasciolosis caused by F. gigantica. It has been suggested that IgG4 antibodies are especially prominent in the total IgG response when antigenic exposure is chronic (1) or associated with a T-helper-cell type 2 response (13). This theory was supported by similar findings of several studies showing raised specific IgG4 antibody levels in individuals with chronic schistosomiasis (21), filariasis (33), angiostrongyliasis (19), strongyloidiasis (14), clonorchiasis (18) and hookworm infection (24).

In generally, clinical manifestations of human fasciolosis are only present in chronic cases, which is why almost all of the fasciolosis serum samples used in the present study were collected from chronic fasciolosis patients. This is possibly supported by the theory that specific IgG4 antibody becomes prominent through chronic antigenic stimulation. Interestingly, the concomitant high levels of IgG4 and IgE antibodies were also shown in helminthic infections (2). IgG4 possible serves to modulate IgE-mediated allergic activity as a blocking antibody (15) either by blocking mast cell degranulation through competing with cell-bound IgE for specific allergens or by inhibiting the binding of IgE molecules to specific receptors on the surface of mast cells or basophiles. Additionally, the IgG4 antibody may interfere with the complement activation by IgG1 (21).

In summary, a cystatin capture ELISA based on the detection of the IgG4 response to F. gigantica rCTL1 could be developed as a serodiagnostic test for human fasciolosis and has the potential to be employed in future large-scale surveys to determine the extent of fasciolosis throughout Southeast Asia where it is prevalent.

ACKNOWLEDGMENTS

This research was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (grant no. PHD/0155/2542) to Chairat Tantrawatpan and Wanchai Maleewong and by a grant from Khon Kaen University. We thank Mark Rosleib for improving the English presentation of the manuscript.

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