PARASITE-SPECIFIC ANTIBODY PROFILE IN HUMAN FASCIOLIASIS: APPLICATION FOR IMMUNODIAGNOSIS OF INFECTION

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Abstract. The antibody isotype response to an adult Fasciola worm antigen preparation (FWAP) was examined in sera from 60 Egyptians with parasitologically confirmed fascioliasis by an ELISA. The FWAP-specific IgG1 and IgG4 antibodies were found in 97–100% of the patients. The ratio of the mean absorbance values between infected patients and healthy controls was 9.7 and 29.7 for IgG1 and IgG4 antibodies, respectively. The IgM, IgA, IgG2, and IgG3 antibodies were less dominant. In contrast to IgG1 antibodies, which were often detected in sera from patients infected with Schistosoma, Echinococcus granulosus, Ascaris lumbricoides, Ancylostoma duodenale, or Hymenolepis nana, FWAP-specific IgG4 antibodies were detected exclusively in the sera of patients with fascioliasis. The data thus support the conclusion that an IgG4/ELISA with crude FWAP as antigen may be used for sensitive and accurate immunodiagnosis of human fascioliasis.

Fascioliasis is essentially a disease of ruminants but is increasingly observed in humans in Egypt, the Middle East, Africa, South Europe, South America, Puerto Rico, Australia, and China. The disease is due to infection with the metacercariae of Fasciola hepatica and/or F. gigantica. The fluke devastates the liver while dwelling in the bile ducts and gall bladder. The infection results in biliary cirrhosis, sclerosing cholangitis associated with destructive jaundice, liver abscesses, and other serious hepatic and ectopic clinical manifestations. In the biliary tree, the hermaphroditic fluke reaches sexual maturity and begins to lay eggs that reach the small intestine and are excreted with the stools to continue the life cycle in the snail Lymnaea. Rapid and accurate diagnosis of the infection is of primary importance for starting immediate chemotherapy before the host endures irreversible damage of the liver tissue, and contributes to the spread of the infection. However, diagnosis of human fascioliasis is very difficult since the clinical picture is highly variable with different degrees of eosinophilia, abdominal pain, pyrexia of undetermined origin, and hepatic nodules, which are difficult to distinguish from liver metastases or liver abscesses. Patients may present with extra-hepatic abnormalities such as pulmonary infiltrate, pleuropedicarditis, meningitis, or lymphoedema. These manifestations can be caused by other parasitic infections such as schistosomiasis, Ascaris lumbricoides abscess of the liver, liver infection with Clonorchis sinensis or Opisthorchis viverrini, complications from Echinococcus infection of the liver, capillaria hepatica, or toxocariasis. Definitive diagnosis relies essentially on a fecal examination for the presence of specific eggs. Parasitologic stool examination is a notably difficult, insensitive, and unreliable method for diagnosis of human fascioliasis since it is known that Fasciola worms excrete eggs in an intermittent manner. In addition, this technique does not allow the detection of early-stage prepatent infection, which lasts approximately 3 months.

Detection of antibodies to specific Fasciola worm antigens can monitor prepatent and patent infection. Specific ELISA methods have been developed using adult fluke crude extracts or excretory-secretory products (ES) as antigen for the detection of serum antibodies. However, these assays show limited specificity since Fasciola share cross-reactive antigens with many parasites, namely Schistosoma and Echinococcus. Specificity could be markedly improved by using proper anti-isotype secondary antibodies. Unfortunately, information on the serum antibody isotype response in human fascioliasis is scarce. The antibody isotype profile varies significantly depending on the characteristics of the immunogen and other factors such as cytokines. In natural infection with parasites, the host is dealing with a multiplicity of different antigens, resulting in polyspecific responses. However, each immunoglobulin isotype recognizes distinct antigenic epitopes. In the present work we have analyzed the Fasciola-specific antibody isotype response in sera from 60 patients with parasitologically confirmed fascioliasis by ELISA. The possibility of subsequent specific immunodiagnostic application for human fascioliasis was evaluated in patients with fascioliasis in parallel with patients infected with other parasites.

SUBJECTS, MATERIALS AND METHODS

Study population. Subjects presenting at the Tropical Medicine Research Institute, General Organization for Teaching Hospitals, and the Theodore Bilharz Research Institute complaining of abdominal pain, body weight loss, dyspepsia, fever, and diarrhea were given repeated parasitologic stool examinations using the Kato-Katz technique. Sixty patients showing the characteristic large operculated Fasciola eggs in stool, and having no other parasites, were included in this study. There were 49 women and 11 men, with an age range of 17–54 years. All Fasciola-infected patients were treated with a single dose (10 mg/kg of body weight) of triclabendazole (Fasini®; Ciba Geigy, Basel, Switzerland). Monitoring of cure was performed by parasitologic examination at 1, 2, and 3 months after treatment, and patients showing eggs in stool in at least 1 of 5 daily analyses at every interval received another dose of Fasini® (4 of 60 patients). Venous blood for serum preparation was obtained from each of the 60 patients before treatment. Blood samples could be obtained from 30 of the 60 patients at 1, 2, and 3 months after treatment.
Blood samples were also collected from 20 parasite-free, healthy donors and from patients with parasitologically confirmed schistosomiasis mansoni or haematobium (50), hydatidosis (25), ascariasis lumbricoides (7), ancylostomiasis duodenale (7), or Hymenolepis nana (7) infections. Healthy donors and parasite-infected patients were matched for age, sex, and social conditions with the fascioliasis patients. Serum aliquots of each donor were stored at −70°C and thawed only once. Informed consent was obtained from all healthy adult participants and from parents of minors. The project was approved by the Tropical Medicine Research Institute, and the Theodore Bilharz Research Institute.

**Adult Fasciola worm antigen preparation (FWAP).** Approximately 20 adult flukes obtained from infected bovine livers at a local slaughterhouse were washed repeatedly and then homogenized in serum-free, ice-cold phosphate-buffered saline (PBS), pH 7.2. The homogenate was centrifuged at 5,000 × g for 1 h at 4°C and the supernate was used as crude FWAP. Protein content was estimated by the Bradford assay.

**Assessment of anti-FWAP isotype response by ELISA.** Wells of polystyrene plates (Costar, Cambridge, MA) were coated with 500 ng of crude FWAP (100 μl/well of a suspension of 5 μg of FWAP/ml of binding buffer [0.05 M carbonate buffer, pH 4.6]), blocked with 1% bovine serum albumin in PBS, washed with PBS, 0.05% Tween 20, and incubated with serum undiluted for IgE, diluted 1:500 for IgM and total IgG, 1:250 for total IgA, 1:100 for IgG1 and IgG4, and 1:25 for IgG2 and IgG3. Peroxidase-labeled anti-human immunoglobulin conjugates: anti-IgM, μ chain-specific; anti-IgG, gamma chain-specific, anti-IgG1, anti-IgG2, anti-IgG3, anti-IgG4 subclass-specific, respectively; anti-IgA, α chain-specific, and anti-IgE, ε chain-specific, all obtained from the Binding Site (Birmingham, United Kingdom), were used at 1:1,000 dilutions. In some experiments, FWAP was subjected to mild periodate oxidation at acidic pH to degrade carbohydrate determinants without altering protein or lipid epitopes as described, and sera were tested in parallel against intact and periodate-treated molecules. Reactivity was read spectrophotometrically at 492 nm after adding o-phenylenediamine (Sigma Chemical Co., St. Louis, MO) substrate. Sera giving absorbance values higher than the cut-off value (mean absorbance of wells with serum from healthy, parasite-free donors + 2 SD) were considered positive. Sensitivity, specificity, and efficacy of immunodiagnosis were evaluated as described.

### RESULTS

**The FWAP-specific antibody profile in patients with fascioliasis.** Approximately 20 adult liver flukes yielded a total of 600 ± 38 mg of protein (mean ± SD of 2 different preparations). Parasite-specific immunoglobulin classes and subclasses in sera from patients infected with *Fasciola sp.* were assayed by ELISA using a single batch of crude FWAP. The data obtained in 2 separate experiments were essentially identical and showed that patients responded to FWAP by production of IgM-, IgG-, IgA-, and IgE-specific antibodies. Ninety-seven percent of the patients had parasite-specific IgG antibodies, while IgM, IgA and IgE antibodies were detected in 64%, 79%, and 92% of the patients, respectively. The IgG1 and IgG4 antibodies dominated the IgG antibody response, being expressed in 100% of the patients tested. The ratio of the mean absorbance values between infected patients and healthy controls was 9.7 and 29.7 for IgG1 and IgG4 antibodies, respectively. The IgG2 and IgG3 antibody response was less dominant since approximately 47% of the patients failed to produce FWAP-specific IgG2 or IgG3 antibodies (Table 1). Isotype variability could not be correlated with sex, age, or clinical symptoms. No significant changes were observed in the isotype profile at 1, 2, or 3 months after treatment.

Parasite-specific IgM, IgG1, IgG4, and IgE antibodies appeared to be directed against both periodate-sensitive and -insensitive FWAP epitopes. The IgA and IgG3 antibodies were directed essentially towards periodate-insensitive determinants, while IgG2 recognized essentially carbohydrate epitopes (Table 2).

**The FWAP-specific IgG1 and IgG4 antibody profile in patients with parasitic diseases.** The FWAP-specific IgG1 and IgG4 antibodies were examined, in parallel, in sera of healthy, parasite-free control donors (20) and of patients with fascioliasis (60), schistosomiasis mansoni or haematobium (50), hydatidosis (25), ascariasis (7), and *A. duodenale* (7) or *H. nana* (7) infections by ELISA. The data of 3 repeat experiments demonstrated that IgG1 antibodies to FWAP were detected in 36–100% of patients infected with parasites other than *Fasciola sp.* Conversely, FWAP-specific IgG4 an-

### Table 1

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Controls</th>
<th>Patients</th>
<th>Fold increase</th>
<th>% positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>0.326 ± 0.161</td>
<td>0.800 ± 0.415</td>
<td>2.45</td>
<td>64</td>
</tr>
<tr>
<td>IgG</td>
<td>0.198 ± 0.081</td>
<td>0.916 ± 0.383</td>
<td>4.62</td>
<td>97</td>
</tr>
<tr>
<td>IgA</td>
<td>0.152 ± 0.098</td>
<td>0.491 ± 0.172</td>
<td>3.20</td>
<td>79</td>
</tr>
<tr>
<td>IgE</td>
<td>0.059 ± 0.079</td>
<td>0.195 ± 0.132</td>
<td>3.30</td>
<td>92</td>
</tr>
<tr>
<td>IgG1</td>
<td>0.075 ± 0.028</td>
<td>0.728 ± 0.440</td>
<td>9.70</td>
<td>97</td>
</tr>
<tr>
<td>IgG2</td>
<td>0.022 ± 0.016</td>
<td>0.085 ± 0.045</td>
<td>3.86</td>
<td>53</td>
</tr>
<tr>
<td>IgG3</td>
<td>0.081 ± 0.075</td>
<td>0.193 ± 0.151</td>
<td>2.38</td>
<td>53</td>
</tr>
<tr>
<td>IgG4</td>
<td>0.028 ± 0.044</td>
<td>0.832 ± 0.296</td>
<td>29.71</td>
<td>100</td>
</tr>
</tbody>
</table>

* Mean ± SD absorbance (492 nm) of all control donors (n = 20) and of all patients with fascioliasis (n = 80).
† Increase in mean absorbance of patients relative to controls.
‡ Percent of patients with serum absorbance above the cut-off value.

### Table 2

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Mean ± SE absorbance against FWAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>IgG</td>
<td>1.16 ± 0.07</td>
</tr>
<tr>
<td>IgA</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>IgE</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>IgG1</td>
<td>1.08 ± 0.06</td>
</tr>
<tr>
<td>IgG2</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>IgG3</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>IgG4</td>
<td>1.21 ± 0.06</td>
</tr>
</tbody>
</table>

* Serum pooled from 10 patients with fascioliasis was tested by ELISA against FWAP before and after periodate oxidation.
† The results are presented as the mean ± SE absorbance (492 nm) of 3 separate experiments.
Figure 1. Specificity of the IgG1 (left) and IgG4 (right)/ELISA using crude Fasciola worm antigen preparation and sera from patients with parasitologically proven infection. Each point represents the absorbance (492 nm) of a single patient. The horizontal line depicts the cutoff value (mean absorbance of sera of 20 control, parasite-free donors ± 2 SD). The Student's t-test revealed highly significant differences between the sera of patients with fascioliasis and the other groups (P < 0.001). OD = optical density; H. = Hymenolepis.

Table 3
Sensitivity, specificity, and efficacy of IgG1 and IgG4/ELISA for immunodiagnosis of human fascioliasis*

<table>
<thead>
<tr>
<th>Isotype</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG1</td>
<td>100%</td>
<td>56.2%</td>
<td>78.1</td>
</tr>
<tr>
<td>IgG4</td>
<td>100%</td>
<td>97.0%</td>
<td>98.5</td>
</tr>
</tbody>
</table>

* Calculated as described by Fleiss.

In contrast, IgG antibodies were abundant and predominated by IgG1 and IgG4, while IgG2- and IgG3-specific antibodies were detected in only 53% of the patients. In ELISA studies using adult liver fluke ES products and cathepsin L1 cysteine proteinase (CL1) for antigens, IgG1 and IgG4 antibodies were also found to predominate in the human response while IgG2 and IgG3 reactions had low absorbance values. Comparable results were observed in human filariasis and schistosomiasis since IgG1 and IgG4 tended to dominate the IgG response to worm antigen preparations followed by IgG2 and IgG3.

The data indicate that FWAP-specific IgG1 and IgG4 antibodies best identified the patients with fascioliasis. Assays conducted to investigate the specificity of the response indicated that IgG1 antibodies to crude FWAP could be detected in 36–100% of donors infected with parasites other than Fasciola sp. Thus, it was evident that the IgG1 isotype response could not be used for accurate immunodiagnosis of fascioliasis. In contrast, FWAP-specific IgG4 antibodies ap-
peared to be found exclusively in patients with fascioliasis. This finding was surprising since polyclonal and antigen-specific IgG4 are preponderant in the human antibody response during chronic parasite infections.\textsuperscript{26,33} However, antigens exclusively specific to Fasciola elicit a copious IgG4 antibody response. An IgG4/ELISA using a single adult liver fluke antigen, CL1, was recently proposed as a standardized diagnostic test for human fascioliasis,\textsuperscript{18} however CL1 failed to identify all individuals reacting with whole ES products. Additionally, production of sufficient quantities of pure CL1 is a complex, time-consuming, and expensive process.\textsuperscript{18} To overcome this problem, a capture ELISA was developed using chicken cytoatin to bind cysteine proteinases from adult fluke ES. However, the assay that used peroxidase-conjugated anti-human total IgG showed low specificity,\textsuperscript{34} thus pointing to the fundamental importance of the antibody isoform for improving the specificity of the diagnostic test. It is important to note that highly restricted cross-reactions between Onchocerca volvulus and other nematodes have been reported for IgE and IgG4 antibodies.\textsuperscript{35,36}

Based on the above information, we propose that a crude FWAP/IgG4 ELISA may be used as an economical, rapid, sensitive, and specific test for serologic diagnosis of human fascioliasis. However, as for human schistosomiasis,\textsuperscript{31} FWAP-specific IgG4 levels did not decrease significantly following chemotherapy. This finding indicates that even the most accurate methods for detection of specific antibodies cannot be used to monitor the outcome of drug treatment, or differentiate between past exposure to the parasite and the presence of an active infection. Immunoologic methods based on detection of circulating or coproantigens are needed for detection of patent infections with Fasciola sp. and follow-up of patients after chemotherapy.\textsuperscript{37,38}

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