IMMUNOCHEMICAL CHARACTERIZATION AND DIAGNOSTIC POTENTIAL OF A 63-KILODALTON SCHISTOSOMA ANTlegen

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Abstract. Schistosoma circulating antigens were used for the detection of active infection. Anti-S. mansoni IgG2a monoclonal antibody (MAb) designated C5C4 was generated. The target epitope of this MAb was detected in adult worms, eggs, and cercariae antigenic extracts of S. mansoni and S. haematobium, had a molecular size of 63 kD, and was not detected in Fasciola hepatica and Ascaris. In addition, a 50-kD degradation product was identified only in the urine of infected individuals. Analysis by high-performance liquid chromatography of the purified antigen demonstrated only one peak. The 63-kD antigen was characterized as a protein containing 40.4% hydrophobic, 7.5% acidic, and 8.8% basic amino acids. The C5C4 MAb was used in a Fast Dot-ELISA for rapid and simple diagnosis of human schistosomiasis. The 63-kD circulating antigen was detected in 92% of urine samples from 330 S. mansoni-infected individuals, with 16% false-positive results among 130 noninfected individuals.

The identification and characterization of schistosome antigens in different developmental stages and the assessment of their role in host-parasite interactions are needed as a step toward the diagnosis of human schistosomiasis. Several investigators have isolated and characterized many of the schistosomiasis antigens in different developmental stages of the parasite that have a potential application in immunodiagnosis. In the present study, we have identified and characterized a schistosome antigen using a specific anti-Schistosoma mansoni mouse monoclonal antibody (MAb) and evaluated the suitability of this MAb in the immunodiagnosis of S. mansoni infection.

MATERIALS AND METHODS

Monoclonal antibody subtype and isotype determination. A hybridoma cell line designated C5C4 was produced from mice infected with S. mansoni as described by Attallah and others. An ELISA was used to characterize the anti-S. mansoni MAb (C5C4). A microtiter plate (Costar, Cambridge, MA) was coated with 50 µg/ml of this MAb in 0.05 M carbonate/bicarbonate buffer, pH 9.6. After blocking with 200 µl/well of 0.3% nonfat milk, a 1:500 dilution of mouse IgG1, IgG2a, IgG2b, and IgG3 MAbS produced in sheep (The Binding Site, Birmingham, United Kingdom) were added and incubated with MAb C5C4 diluted in the blocking buffer for 1 hr at 37°C. The blots were then washed three times (30 min/wash) in TBS, followed by a 2-hr incubation with goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma, St. Louis, MO) was added and incubated at 37°C for 1 hr. Sheep anti-mouse IgG–alkaline phosphatase conjugate (Sigma, St. Louis, MO) was added at a dilution of 1:1,000 for 1 hr at 37°C. Fifty microliters per well (1 mg/ml) of p-nitrophenyl phosphate in 0.1 M glycine buffer, pH 10.4, were added and the plate was incubated for 20 min at 37°C. The reaction was stopped with 3 N NaOH and the optical density was read at 405 nm using an EL 311 microplate autoreader (Bio-Tek Instruments, Winooski, VT).

Preparation of antigenic extracts. The S. mansoni and S. haematobium antigenic extracts of worms (SWAP), cercariae (CAP), and eggs (SEA) were prepared according to the method of Da Silva and Ferri. The same method was used for the preparation of Fasciola hepatica and Ascaris lumbricoides antigenic extracts. The protein content was determined using the Lowry method. Antigenic extracts were stored at −70°C until use.

Gel electrophoresis and electroelution. Fifty micrograms per lane of different antigenic extracts and urine samples were loaded on 10% vertical polyacrylamide slab gels (Bio-Rad Laboratories, Hercules, CA) according to the method of Laemmli. Gels were stained in a solution containing 0.2% Coomassie blue, 40% methanol, and 10% acetic acid, and destained in the same solution without the Coomassie blue stain until the desired background was obtained. The band of interest (63 kD) was cut and electroeluted from the polyacrylamide gels at 200 volts for 3 hr in a dialysis bag (Sigma). The protein content of the electroeluted antigen was then determined.

Western blotting. Resolved proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) were electrotransferred onto nitrocellulose filters (0.45-µm pore size; Sigma) in a protein transfer unit (Bio-Rad Laboratories) according to the method of Towbin and others. The nitrocellulose filter was blocked using a blocking buffer composed of 5% (w/v) nonfat dry milk dissolved in 0.05 M Tris-buffered saline (TBS), pH 7.4, rinsed in TBS, and incubated with MAb C5C4 diluted in the blocking buffer with constant shaking. The blots were washed three times (30 min/wash) in TBS, followed by a 2-hr incubation with goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma) diluted in TBS. The blots were then washed three times with TBS (15 min/wash) and soaked in alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl phosphate [BCIP], nitro blue tetrazolium [NBT], and 0.1 M Tris buffer, pH 9.6 [Kirkegaard and Perry Laboratories, Gaitherburg, MD]). The color reaction was observed within 20 min and the reaction was then stopped by dipping the blots in distilled water.

Biochemical treatments. The electroeluted 63-kD antigen was treated with protease and various chemical reagents, and a duplicate treated samples were then tested in a Fast Dot-ELISA with MAb C5C4 to establish whether these treatments effected the active epitope on the 63-kD antigen. Crude antigen (1 mg/ml) was incubated with 1 mg/ml of protease type VI A from Bacillus amylolyticus (Sigma) for 1 hr at 37°C, with 4% trichloroacetic acid (TCA) (v/v) for 1 hr at 4°C, and with 0.2 M NaOH and 0.2 M HCl (v/v) for 1 hr at room temperature. A periodate oxidation was carried out overnight with 20 mM sodium metaperiodate at room temperature, and the reaction was then stopped by adding an equal volume of 130 mM glycerol according to the
method of Hamburger and others. Bovine serum albumin and SWAP were tested in parallel.

**Analysis by reversed-phase high-performance liquid chromatography (HPLC).** The electroeluted antigen was tested with a Kontron Pc Integrator HPLC system equipped with a C4 (vydac, 46 × 15 cm, 300 Å) reversed-phase column (Kontron Instruments, Milan, Italy) and a UV detector. The mobile phases used included solvent A (50 mM phosphate buffer, pH 6) and solvent B (40% CH3 CN in 50 mM phosphate buffer, pH 6). The column was initially equilibrated at 0% at a flow rate of 1 ml/min. The separation was performed using a linear gradient of 0% to 100% in 40 min.

**Amino acid analysis.** A 20-μl volume of the electroeluted antigen was used for derivatization and dried under vacuum. Dried residues were hydrolyzed with 6 N HCl (BDH Chemicals, Ltd., Poole, United Kingdom) containing 1% phenol for 16 hr at 115°C. Samples were then dried under vacuum, and the procedure was completed according to the method of Buzzigoli and others.

**Fast dot-ELISA.** The assay was carried out according to the method of Attallah and others using a Hybri-Dot Manifold (Bethesda Research Laboratories, Gaithersburg, MD).

**Parasitologic examinations.** A total of 460 cases (100 females and 360 males) 10–62 years of age were parasitologically examined using both simple stool sedimentation analysis (it was done for two or three consecutive days for each individual) and rectal biopsy (specimens were taken from one or two sites). The study was approved by the Clinical Review Committee of the Gastro-Enterology Center, Mansoura University (Mansoura, Egypt). All cases were fully informed concerning the diagnostic procedures involved and the nature of the disease. Informed consent was obtained from all adults and from the parents of minors before participation in the study. Three hundred thirty cases were *S. mansoni*-infected individuals (120 cases had eggs in their stool samples and 210 cases had eggs in their rectal snips), and 130 cases had no eggs in their stool specimens and rectal biopsies. Urine samples of all 460 cases were tested for the 63-kD antigen using the Fast Dot-ELISA and MAb C5C4.

**RESULTS**

**Characterization of MAb C5C4.** An MAb designated C5C4 showed high reactivity against SWAP in the Fast Dot-ELISA. This MAb was tested by an ELISA using different alkaline phosphatase–conjugated antimouse immunoglobulin (IgM and IgG) classes. The MAb showed a positive result only with anti-IgG. The subclass of this MAb was determined by ELISA using anti-mouse IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3) and sheep anti-mouse IgG–alkaline phosphatase conjugate. The C5C4 MAb showed a positive reaction with the IgG2a subclass.

**Identification of the target antigen of MAb C5C4 among antigenic extracts from three developmental stages of the Schistosoma parasite and in urine samples.** Twenty micrograms per lane of *S. mansoni* and *S. haematobium* antigenic extracts (SWAP, CAP, and SEA) were subjected to SDS-PAGE, and Western blotting was carried out to determine the target epitope of MAb C5C4. It was found that the polypeptide band of 63 kD in SWAP and SEA reacted with MAb C5C4 (Figure 1). This MAb did not recognize any antigens in the adult worm antigenic extracts of *Fasciola hepatica* and *Ascaris* (Figure 1). Western blot analysis of urine samples from eight individuals infected with *S. mansoni* and two uninfected individuals showed that MAb C5C4 also recognized the 63-kD band in the urine of all infected patients, in addition to a degradation product of 50 kD. No reaction was observed in the uninfected individuals (Figure 2).

**Biochemical characterization.** To biochemically characterize the 63-kD antigen, it was treated with several chemical reagents and its reactivity was tested using the Fast Dot-ELISA. The antigen became unreactive after treatment with NaOH, HCl, and protease. Sodium periodate did not affect the antigen. The antigen was precipitated with TCA; the precipitate was still reactive and the TCA-soluble fraction was unreactive. For further characterization of this antigen, the electroeluted antigen was subjected to HPLC analysis using reversed-phase column. The electroeluted antigen showed only one peak (Figure 3). The amino acid composition analysis showed that the 63-kD antigen contains 40.4% hydrophobic amino acids, 25% acidic amino acids, and 8.8% basic amino acids (Table 1).

**Detection of the 63-kD antigen in urine samples using the Fast Dot-ELISA.** The Fast Dot-ELISA assay with MAb C5C4 detected the 63-kD antigen in urine of 304 of 330 *S. mansoni*-infected cases (sensitivity = 92%, Table 2). Among 130 uninfected controls, 21 cases showed false-positive results (specificity = 84%, Table 2). The positive predictive...
Characterization of a 63-kD S. Mansoni Antigen

Figure 2. Western blot analysis using monoclonal antibody (MAb) C5C4 of two urine samples from healthy individuals (lanes 1 and 2) and eight urine samples from Schistosoma mansoni-infected patients (lanes 3–10). The C5C4 MAb identified a 63-kD circulating antigen in urine samples of infected patients in addition to a degradation product of 50 kD in urine samples of some of the infected patients.

Table 1: Amino acid composition analysis for the 63-kD Schistosoma antigen

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Concentration (nmol/mg)</th>
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<tbody>
<tr>
<td>Aspartic acid</td>
<td>189.25</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>303.40</td>
</tr>
<tr>
<td>Serine</td>
<td>149.13</td>
</tr>
<tr>
<td>Glycine</td>
<td>1,962.79</td>
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<tr>
<td>Histidine</td>
<td>338.27</td>
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<tr>
<td>Arginine</td>
<td>184.50</td>
</tr>
<tr>
<td>Alanine</td>
<td>231.24</td>
</tr>
<tr>
<td>Proline</td>
<td>263.42</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>249.07</td>
</tr>
<tr>
<td>Valine</td>
<td>1,916.77</td>
</tr>
<tr>
<td>Methionine</td>
<td>203.87</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>182.46</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>174.05</td>
</tr>
<tr>
<td>Leucine</td>
<td>156.31</td>
</tr>
</tbody>
</table>

Table 2: Detection of 63-kD Schistosoma circulating antigen using the Fast Dot-ELISA in urine samples of parasitologically confirmed Schistosoma mansoni-infected and noninfected Egyptian individuals

<table>
<thead>
<tr>
<th>Parastology</th>
<th>Fast Dot-ELISA*</th>
<th>% positive†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected individuals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stool analysis</td>
<td>120 107</td>
<td>13 89</td>
</tr>
<tr>
<td>Rectal biopsy</td>
<td>210 197</td>
<td>13 94</td>
</tr>
<tr>
<td>Total</td>
<td>330 304</td>
<td>26 92</td>
</tr>
<tr>
<td>Noninfected individuals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stool analysis</td>
<td>39 2</td>
<td>37 5</td>
</tr>
<tr>
<td>Rectal biopsy</td>
<td>91 19‡</td>
<td>72 20</td>
</tr>
<tr>
<td>Total</td>
<td>130 21</td>
<td>109 16</td>
</tr>
</tbody>
</table>

* Fast Dot-ELISA based on monoclonal antibody C5C4 specific for a 63-kD schistosome antigen and 50-kD degradation product excreted in urine.
† Sensitivity = 304/(304 + 26) × 100 = 92%; specificity = 109/(109 + 21) × 100 = 84%; positive predictive value = 304/(304 + 21) × 100 = 94%; negative predictive value = 109/(109 + 26) × 100 = 81%; efficiency = (304 + 109)/460 × 100 = 90%.
‡ All had a history of schistosome infection.

Discussion

Characterization of the schistosome antigens shared in different stages of the parasite life cycle is of crucial importance for diagnosis and further development of a molecular-based vaccine. In the present study, MAb C5C4 recognized a single 63-kD antigen in three stages of the parasite life cycle (cercariae, adult worms, and eggs). Thus, this MAb can be used as a probe to diagnose schistosome infection. The 63-kD antigen was also detected in the urine samples of S. mansoni-infected patients, in addition to a degradation product of 50 kD. The 63-kD antigen was hydrolyzed by NaOH and HCl, precipitated by TCA, sensitive to protease, and insensitive to periodate. The electroeluted antigen showed only a single peak when analyzed by reversed-phase HPLC, indicating that this antigen contained a single polypeptide chain. The amino acid composition analysis showed that 40.4% of the amino acids of the 63-kD antigen are hydrophobic.

It is of interest that an antigen with a similar size has not been previously reported. Miller and others reported a 73-kD antigen as a component of the adult worm and of the antigenic extract of the 3-hr schistosomula. Attallah and others reported a 74-kD protein antigen as a component of the antigenic extracts of the cercariae, adult worms, and eggs. The anatomic localization of the 63-kD target antigen was determined using MAb C5C4 in the immunostaining of S. mansoni adult worms. The antigenic determinant was located in the gut, tegumental tubercles, and tegumental surface of adult S. mansoni worms. The target antigens can be expected to play a significant role in the detection of infection because of their location and excretion.

The intensity of schistosome infection is currently measured by quantitative egg counts, which are highly variable, and may depend on the immune status of the host. However, stool analysis needs to be confirmed by rectal biopsy, especially in cases of fibrosis resulting from trapping...
of *S. mansoni* eggs in the liver and intestinal tract. Detection of specific anti-schistosome antibodies provides some epidemiologic information, but in endemic areas it does not discriminate between previous and current infection due to the continuous exposure to infection sources. A high titer value may be found for extended periods of time even after successful treatment.\(^\text{17,18}\)

The excretion of the schistosome-circulating antigens in the urine of infected patients makes possible the use of noninvasive techniques for simple diagnosis of schistosomiasis.\(^\text{19–22}\) In addition, low fluctuations of urinary elimination of the circulating antigens have been observed.\(^\text{23,24}\) Attallah and others\(^\text{5}\) examined the presence of a 74-kD circulating antigen in the urine of infected and uninfected cases using Fast Dot-ELISA based on MAb BRL4.

In the present study, we describe the use of MAb C5C4 in detecting the 63-kD circulating antigen in urine samples of infected patients using the Fast Dot-ELISA as a diagnostic tool. In mass screening programs, the use of urine samples for a noninvasive diagnosis of the disease would be desirable. Our results indicate that the overall sensitivity of this assay for the detection of 63-kD circulating antigen was 92% among proven *S. mansoni*-infected individuals, the specificity was 84%, the positive predictive value was 94%, and the negative predictive value was 81%.

In the present study, the microscopic detection of schistosome eggs in stool and rectal biopsy specimens was considered the gold standard for diagnosis. It is difficult to detect ova in very mild infections and in chronic infections due to the intense fibrosis present around the eggs.\(^\text{25}\) Abdel-Hafez and Balbol\(^\text{26}\) recommended examining multiple rectal biopsies to increase the positivity rate. This may explain the false-positive results among some of the parasitologically uninfected individuals in our study in whom the rectal biopsy was taken only from one or two sites. In addition, many of these cases had a history of schistosomiasis and may still be infected. These results were consistent with those of Attallah and others,\(^\text{3}\) who reported a sensitivity of 93% and a specificity of 89% using another MAb specific for a 74-kD circulating antigen.

The results of the present study are relevant to the problems in clinical diagnosis, epidemiologic studies, and mass treatment of schistosome infections. Further studies on the diagnostic potential of MAb C5C4 among those infected with *S. haematobium* and its utility for immunoprophylaxis are planned.

Acknowledgments: We gratefully acknowledge Professor Farouk Ezzat, Professor Ahmed Soltan, and Dr. Hanan El-Mohamady for kind help.

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REFERENCES


Course title: Travel-Related Vaccine Preventable Illnesses
Location: Washington Hilton and Towers; Washington, DC
Dates: November 27–28, 1999
Sponsor: American Society of Tropical Medicine and Hygiene (ASTMH) in cooperation with the American Committee on Clinical Tropical Medicine and Travelers’ Health

Course description: This one and one-half day course will focus on current issues relating to the use of vaccines in travelers. The course will consider the use, indications, adverse events, efficacy, and cost effectiveness of vaccines such as those used to protect against hepatitis A & B, Japanese encephalitis, yellow fever, rabies, typhoid, meningococcal disease, anthrax and plague, among others. The course will also consider issues relating to the development and approval of vaccines, issues relating to the identification and education of travelers who may benefit from the administration of travel-related vaccines, and issues relating to the effectiveness of vaccine administration. Speakers are internationally recognized authorities on travel-related vaccines. The course will immediately precede, and occur in the same venue as, the ASTMH annual meeting. For additional information, please contact ASTMH at (847) 480-9592, e-mail astmh@astmh.org, or visit our web site at www.astmh.org.