Isolation of *Fasciola hepatica* Tegument Antigens

GEORGE V. HILLYER

*Laboratory of Parasite Immunology, Department of Biology, University of Puerto Rico, Rio Piedras, Puerto Rico 00931*

*Fasciola hepatica* tegument antigens were isolated from intact worms in the cold by using Nonidet P-40. Proof of the tegumental nature of the antigens was shown by the peroxidase-antiperoxidase immunocytochemical technique at the light microscope level. The potential of *F. hepatica* tegument antigens for the immunodiagnosis of rabbit and human fascioliasis was shown by Ouchterlony immunodiffusion, although cross-reactivity was evident in one of six serum samples from patients infected with *Schistosoma mansoni*. A genus-specific *Fasciola* antigen was found in *F. hepatica* tegument. Finally, *F. hepatica* tegument contained antigens which protected mice from challenge infection with *S. mansoni*.

Most procedures used in the extraction of parasite antigens for serodiagnosis begin by the breaking up of parasite components. These complex mixtures are then applied to the serological test being used. Parasitic trematodes have a glycocalyx-tegument complex which continuously secretes macromolecules. For example, Threadgold (10) found that the glycocalyx of the *Fasciola hepatica* tegument consists of two parts, an inner continuous layer tightly bound to the apical plasma membrane and an outer fibrillar layer. Both layers are anionic and carbohydrate rich and thus contain glycoproteins and sialic acids. Wilson and Barnes (11) found that the plant lectin concanavalin A bound to the glycocalyx (membranocalyx) of *Schistosoma mansoni*: this surface coat had a half-life of 2 to 3 h. Thus glycocalyx antigens are probably continuously secreted by the parasite, stimulating host response. It would be expected, then, that isolation of tegument antigens would be a simple approach to the collection of antigens which may be useful starting material for immunochemical purification of antigens which can then be applied to the immunodiagnosis of parasitic infections. In addition, since they are being shed by the parasite surface they may also be functional in protective immunity.

This study concerns the isolation of *F. hepatica* tegument antigens (Fht) obtained from worms and their use in the serodiagnosis of experimental infections.

**MATERIALS AND METHODS**

**Antigens.** Bovine livers condemned by the U.S. Department of Agriculture because of fascioliasis were obtained at the slaughterhouse in Barrio Cubay, Canóvanas, P.R. The *F. hepatica* adult worms were removed from the bile ducts and washed repeatedly in ice-cold 0.01 M phosphate-buffered saline (PBS), pH 7.0. Groups of 10 worms were transferred to small beakers, covered with 25 ml of the nonionic detergent Nonidet P-40 (1% NP-40 in PBS), and incubated at either 4 or 37°C for 1 h. The NP-40 was obtained from BDH Chemicals Ltd., Poole, England. The fluid was then collected and centrifuged for 4 h at 50,000 × g (8°C). The supernatants were designated Fh4 (4°C) or Fh37 (37°C). Protein determinations were done by a micro-Lowry technique with bovine serum albumin as a standard and as described in Keleti and Lederer (7). The protein concentrations determined were as follows: Fh4 (4°C), 925 μg/ml; Fh37 (37°C), 1,375 μg/ml. Samples of each antigen preparation were then concentrated to 8 to 10 mg/ml by vacuum dialysis against PBS at 4°C.

*F. hepatica* worms, obtained from the liver of a New Zealand white rabbit infected for 34 weeks, were washed as above. One worm was incubated either in 5 ml of PBS or in 5 ml of 1% NP-40 in PBS for 1 h at 37°C. The fluid from each was then centrifuged as above, and the supernatant was collected. The protein concentrations determined were as follows: PBS supernatant, 260 μg/ml; NP-40-PBS supernatant, 803 μg/ml. A sample of the NP-40-PBS supernatant was also concentrated to 8 to 10 mg/ml by vacuum dialysis.

*F. hepatica* worm extracts were prepared as described previously by Hillyer (2) and used in Ouchterlony immunodiffusion at a concentration of 16 mg (dry weight)/ml.

**Sera.** Rabbits were infected with *F. hepatica* metacercariae obtained from laboratory-reared *Luminae cubensis* snails (kindly donated by Anne Frame and Pedro Bendezú, Inter-American University, San Juan Campus). They were bled repeatedly for the collection of serum for up to 20 weeks postinfection. Rabbits were hyperimmunized with *F. hepatica* crude worm extracts (bovine) for the collection of serum (anti-*F. hepatica* serum). A typical immunization protocol involved a first inoculation in five subcutaneous sites of a total of 10 mg (dry weight) of antigen emulsified in Freund complete adjuvant fol-
loved by four or more inoculations, each with 5 mg of antigen in Freund incomplete adjuvant.

Rabbits were immunized three times each with 1 mg of protein of Fh (bovine) per ml obtained as described above at 4°C (anti-F. hepatica tegument serum). The first inoculation (five sites) was done with an equal volume (1 ml) of Freund complete adjuvant, and the subsequent inoculations were done with Freund incomplete adjuvant. Bleedings were done 1 week after the final inoculation for the collection of serum.

Rabbit antiserum to F. hepatica arc 2, which is genus specific (9), was kindly donated by André Capron.

Rabbit antisera were also prepared to several F. hepatica antigen preparations which have been shown to protect mice from challenge infections with S. mansoni cercariae (summarized in references 4 and 6). These include F. hepatica antigens which bind to concanavalin-A-Sepharose 4B, denoted FhConA (5), and a purified F. hepatica antigen which cross-reacts with S. mansoni, denoted FhVon (5).

Human sera were obtained from six individuals from France with confirmed fascioliasis (sera kindly donated by André Capron), from six individuals from Egypt with medium (300 eggs per g) to high (1,680 eggs per g) excretion levels of S. mansoni (sera kindly donated by M. A. El Alamy), and six individuals from Puerto Rico with no detectable intestinal parasites.

Serological tests. Ouchterlony immunodiffusion was done with 1% agarose as described previously (2) either on glass plates or on Gel Bond (FMC Corp., Marine Colloids Division, Rockland, Maine).

Peroxidase-antiperoxidase immunocytochemical technique. At 21 days postinfection, F. hepatica worms were removed from mouse livers, fixed 15 min in 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3), and then placed in phosphate buffer. Worms were dehydrated in ethanol, embedded in paraffin, and sectioned at 6 to 10 μm. Sections were deparaffinized in xylene, air dried, and then exposed to the peroxidase-antiperoxidase technique of Sternberger et al. (8). Primary rabbit serum against Fh was used at a 1:1,000 dilution. Sections were not additionally stained.

RESULTS AND DISCUSSION

F. hepatica worms (bovine) incubated in NP-40 at 37°C shed approximately 1.5 times more Lowry-reactive material than those incubated at 4°C. When the protein concentrations were adjusted and both antigen preparations were compared by Ouchterlony immunodiffusion against the serum from a rabbit immunized with F. hepatica worm extracts or a rabbit infected with F. hepatica for 12 weeks, comparable lines of precipitation were seen. Since incubation at 37°C may result in proteolysis due to activation of enzymes, isolation of Fh was subsequently done only at 4°C.

F. hepatica worms (rabbit) incubated in PBS at 37°C shed Lowry-reactive material, although this was only 32% of that obtained under similar conditions when 1% NP-40 was added. When these antigens were reacted by Ouchterlony immunodiffusion against a rabbit anti-F. hepatica whole worm serum the complexity of the system became apparent. Many of these tegument antigens are logically also found in the F. hepatica whole worm extracts (Fig. 1). This also suggests that the important immunodiagnostic antigens

![](http://jcm.asm.org/)

**Fig. 1.** Ouchterlony immunodiffusion comparing a rabbit serum to F. hepatica crude worm extract (aFh) with various antigen preparations of F. hepatica (see text). FhWWE is a crude worm extract. Fh were obtained by incubation with 1% NP-40 and were of bovine origin; the preparation was at a protein concentration of 8 mg/ml. FhPBS were obtained in the cold after incubation with PBS; the antigen concentration of FhPBS was 260 μg/ml. Note that most FhNP-40 linked with FhWWE, indicating identity.

![](http://jcm.asm.org/)

**Fig. 2.** Same conditions as in Fig. 1, except that the serum was obtained from a rabbit infected with F. hepatica (Rf) for 12 weeks. Note that two major antigens are seen where Fh were used at a concentration of 8 mg/ml; a third antigen's best seen where Fh were diluted 10-fold.
FIG. 3. Ouchterlony immunodiffusion of the serum from a rabbit infected with *F. hepatica* for 10 to 20 weeks (outer wells) reacted with *Fh*<sub>t</sub> at a concentration of 8 mg/ml. This rabbit was first positive to *Fh*<sub>t</sub> at 4, but not 2, weeks postinfection.

FIG. 4. Ouchterlony immunodiffusion of the sera from six patients (a to f) with confirmed fascioliasis reacted against *Fh*<sub>t</sub> (center well) at a concentration of 8 mg/ml. Note the presence of at least three different antigen-antibody systems in serum c.

of crude *F. hepatica* are also found on the tegument. The Lowry-reactive material obtained by incubating the worms in PBS alone appeared poorly reactive although this may be a factor of concentration. When these antigens were reacted with the serum from the rabbit infected with *F. hepatica* for 12 weeks, a much simpler pattern was obtained (Fig. 2). Thus, 2 major *Fh*<sub>t</sub> were apparent at 8 mg/ml, and a third was clearly evident when the antigen was diluted 10-fold.

It was then of interest to see whether *Fh*<sub>t</sub> were useful as a preparation for the immunodiagnosis of rabbit fascioliasis. Sera from rabbits infected with *F. hepatica* were positive by 4 (but not 2) weeks of infection when reacted with 8 mg of *Fh*<sub>t</sub> (bovine) per ml. A typical rabbit antibody response is seen in Fig. 3, which shows the precipitin patterns from 10 to 20 weeks of infection. As many of three lines of precipitation were clearly seen at 14 weeks.

That *Fh*<sub>t</sub> may be useful for the immunodiagnosis of human fascioliasis is also evident. All six cases of human fascioliasis gave at least one line of precipitation against *Fh*<sub>t</sub>. At least three antigen-antibody systems were evident (Fig. 4, a).
F. hepatica worms have been shown to comprise a complex mosaic of antigens, and many of these have been enumerated by immunoelectrophoresis (1). The one denoted arc 2 has been shown to be genus specific (9). The anti-Fh serum was compared with an anti-Fasciola arc 2 serum by Ouchterlony immunodiffusion by reacting both with a crude F. hepatica worm extract. The Fasciola-specific antibody linked with one of the Fh antibodies, indicating identity (Fig. 5). Thus, Fasciola arc 2 is clearly a tegument antigen.

F. hepatica antigens have been shown to protect mice from challenge infection with S. mansoni cercariae (reviewed in references 4 and 6). Three antisera against different F. hepatica antigen preparations which induce protection against challenge with S. mansoni all reacted with Fh (Fig. 6). Significantly, one of the antibodies was common to the three sera (5). This suggests that the F. hepatica antigens which protect against S. mansoni are tegument antigens. I have shown that infection with F. hepatica also protects mice from challenge with S. mansoni (3). Thus, it is possible that the mechanism of protection may in part be due to the shedding of the Fh.

Finally, the method of isolation of Fh using detergents at cold temperatures suggested that only surface (tegument) antigens were being isolated. With the peroxidase-antiperoxidase immunocytochemical technique (8), an anti-Fh serum was reacted with sections of F. hepatica worms isolated from infected mice. The localization was exclusively on the worm surface (Fig. 7). Thus, Fh are truly tegument antigens.

Additional analysis of these antigens is warranted both for the improvement of serodiagnostic tests for fascioliasis and for studies on immunity to schistosomes. These studies are currently under way.

ACKNOWLEDGMENTS

These studies were supported by Public Health Service grant no. RR-8102-08 from the National Institutes of Health and administered by the Division of Research Resources, the Edna McConnell Clark Foundation, and the University of
Puerto Rico Office of Graduate Studies and Research.

Special thanks to Elliot Kieff for suggesting the initiation of this study. The technical assistance of Zulma Sánchez de Gavilanes and Richard DeMaree is gratefully acknowledged.

LITERATURE CITED


