Isolation of *Fasciola hepatica* Tegument Antigens

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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 (Fh_i) 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 Cubuy, 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 Fh_i (4°C) or Fh_i (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: Fh_i (4°C), 925 µg/ml; Fh_i (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 *Lymnaea 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-

lowed 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_t* (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 *Fh_{ConA}* (E1-1), and a purified *F. hepatica* antigen which cross-reacts with *S. mansoni*, denoted *Fh_{Sm}* III(M) (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 μ m. 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_t* 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_t* 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

FIG. 1. Ouchterlony immunodiffusion comparing a rabbit serum to *F. hepatica* crude worm extract (α Fh) with various antigen preparations of *F. hepatica* (see text). *FhWWE* is a crude worm extract. *Fh_t* were obtained by incubation with 1% NP-40 and were of bovine origin; the preparation was at a protein concentration of 8 or 0.8 mg/ml. *Fh_{PBS}* were obtained in the cold after incubation with PBS; the antigen concentration of *Fh_{PBS}* was 260 μ g/ml. Note that most *Fh_{NP-40}* linked with *FhWWE*, indicating identity.

FIG. 2. Same conditions as in Fig. 1, except that the serum was obtained from a rabbit infected with *F. hepatica* (RFh) for 12 weeks. Note that two major antigens are seen where *Fh_t* were used at a concentration of 8 mg/ml; a third antigen is best seen where *Fh_t* 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_t at a concentration of 8 mg/ml. This rabbit was first positive to Fh_t 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_t (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_t 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_t were useful as a preparation for the immunodiagnosis of rabbit fascioliasis. Sera from rabbits infected

FIG. 5. Ouchterlony immunodiffusion of *F. hepatica* adult worm extract ($FhWWE$) reacted against a rabbit anti- Fh_t serum (aFh_t) and a rabbit serum containing specific antibody to *Fasciola arc 2* ($aFh_{arc 2}$) (see text). Note that the arc 2 antibody links with one Fh_t antibody, indicating identity.

FIG. 6. Reactivity of three different rabbit sera against Fh_t by Ouchterlony immunodiffusion. Serum 1 contained a pure antibody prepared from *F. hepatica* which cross-reacted with *S. mansoni*; serum 2 contained antibodies to *F. hepatica* antigens which bound to concanavalin A-Sepharose 4B and were eluted with α -methylglucopyranoside; serum 3 contained antibodies to *F. hepatica* adult worm extracts. Note linkage of a common antibody in all three sera. The antigens used to prepare each of the sera have been shown to protect mice from challenge infection with *S. mansoni*.

with *F. hepatica* were positive by 4 (but not 2) weeks of infection when reacted with 8 mg of Fh_t (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_t 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_t . At least three antigen-antibody systems were evident (Fig. 4,

FIG. 7. Peroxidase-antiperoxidase immunocytochemical method reacting a rabbit anti-Fh_t serum with a section of an *F. hepatica* worm obtained from a mouse infected for 21 days. Note that the dark grains are uneven and only appear on the worm surface. The bar represents 20 μ m.

serum c.) None of the normal controls were positive against Fh_t. However, one of the six *S. mansoni* cases did react with Fh_t. Thus, the source of this cross-reactivity must be identified and absorbed out to increase the specificity of the antigen preparation.

F. hepatica worm extracts 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_t 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_t 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_t (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_t.

Finally, the method of isolation of Fh_t using detergents at cold temperatures suggested that only surface (tegument) antigens were being isolated. With the peroxidase-antiperoxidase immunocytochemical technique (8), an anti-Fh_t 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_t 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.

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