

Comparison of Purified 12 kDa and Recombinant 15 kDa *Fasciola hepatica* Antigens Related to a *Schistosoma mansoni* Fatty Acid Binding Protein

George V Hillyer

Laboratory of Parasite Immunology and Pathology, Department of Pathology and Laboratory Medicine, UPR School of Medicine, GPO Box 365067, San Juan, Puerto Rico 00936-5067

Vaccines in schistosomiasis using homologous antigens have been studied extensively in experimentally infected mammalian hosts. Vaccines using heterologous antigens have received comparatively less attention. This review summarizes recent work on a heterologous 12 kDa Fasciola hepatica antigenic polypeptide which cross reacts with Schistosoma mansoni. A cDNA has been cloned and sequenced, and the predicted amino acid sequence of the recombinant protein has been shown to have significant (44 %) identity with a 14 kDa S. mansoni fatty acid binding protein. Thus in the parasitic trematodes fatty acid binding proteins may be potential vaccine candidates. The F. hepatica recombinant protein has been overexpressed and purified and denoted rFh15. Preliminary studies show that rFh15 migrates more slowly (i.e. may be slightly larger) than nFh12 on SDS-PAGE and has a predicted pI of 6.01 vs. observed pI of 5.45. Mice infected with F. hepatica develop antibodies to nFh12 by 2 weeks of infection vs. 6 weeks of infection to rFh15; on the other hand, mice with schistosomiasis mansoni develop antibodies to both nFh12 and rFh15 by 6 weeks of infection. Both the F. hepatica and S. mansoni cross-reactive antigens may be cross-protective antigens with the protection inducing capability against both species.

Key words: *Fasciola hepatica* - *Schistosoma mansoni* - heterologous resistance - *Fasciola/Schistosoma* cross-reactivity - fatty acid binding protein

VACCINES IN SCHISTOSOMIASIS

Several vaccine candidates have been identified in *Schistosoma mansoni* directed against the schistosomulum as well as against other life cycle stages. Some of the more promising antigens have now reached a more advanced stage of development including in the case of glutathione S-transferase, the stage of industrial manufacture and safety testing (Capron 1992, Bergquist et al. 1994). Another approach has been to study closely related cross-reacting antigens from another trematode, *Fasciola hepatica*. One 12 kDa antigen has been shown to induce in outbred albino mice significant levels of resistance to challenge infection with *S. mansoni* (Hillyer et al. 1988a, 1990). This *Fasciola* antigen is most probably related to a recently described immunoprotective fatty acid binding protein from *S. mansoni*. This review covers recent studies with this heterologous antigen from *F. hepatica*.

HETEROLOGOUS RESISTANCE IN SCHISTOSOMIASIS AND FASCIOLIASIS

Many studies have demonstrated that infection with one species of parasite can induce significant reduction in infection with another parasite (heterologous immunity or resistance). This reduction is manifested by reduced parasite burdens relative to the challenge infection, reduced egg laying (anti-fecundity), reduced pathology, or a combination of these. Studies on heterologous resistance in relation to schistosomes and *Fasciola* were summarized at the 2nd International Symposium on Schistosomiasis (Hillyer 1987). Using the murine model investigators have shown that infection with *F. hepatica* induces high levels of resistance to challenge infection with *S. mansoni* and vice versa (Hillyer 1987, Christensen et al. 1987, Hillyer et al. 1988b). This heterologous resistance has an immunologic basis since the *Fasciola* antigen(s) conferring resistance cross-reacts with *S. mansoni* (Hillyer et al. 1988a,b).

RESISTANCE TO SCHISTOSOMES USING DEFINED, HETEROLOGOUS *FASCIOLA* ANTIGENS

A *F. hepatica* 12 kDa (nFh12) polypeptide purified from adult worm extracts induces in mice

significant levels of resistance to challenge infection with this parasite. In four separate experiments, outbred albino NIH (GP) mice immunized with nFh12 and challenged percutaneously with *S. mansoni* cercariae developed 47, 52, 60, and 77 % fewer schistosome worms than controls (Hillyer et al. 1988a, 1990). The term nFh12 is used to define the purified native polypeptide. On SDS-PAGE this polypeptide migrates faster than the 14 kDa MW marker and its migration is calculated to be ca. 12 kDa. It is a potent immunogen which must be expressed early after the transformation of the metacercaria cyst stage to the juvenile stage because different animal species (mice, calves, rabbits) infected with *F. hepatica* develop antibodies to Fh12 by 2 weeks of infection (Hillyer et al. 1988b). It is a cross-reactive antigen because mice infected with *S. mansoni* develop antibodies to Fh12 by 5 weeks of infection.

MOLECULAR CLONING OF A cDNA EXPRESSING RECOMBINANT nFh12 (rFh15)

A lambda gt11 *F. hepatica* cDNA library was constructed from poly(A)⁺ RNA extracted from adult worms. The unamplified library had a titer of 2.2×10^4 plaque-forming units/ml and 99 % recombinants. Primary screening of 300,000 phage plaques from the amplified library with an anti-Fh12 antiserum resulted in 33 positive clones. After plaque purification, 17 recombinant phages were selected for further analysis. One cDNA was sequenced and the predicted amino acid sequence revealed an open reading frame encoding a 132 amino acid protein with a predicted molecular weight of 14,700 Da (Rodríguez-Pérez et al. 1992). This recombinant antigen was denoted rFh15. It has a predicted molecular weight of 14.7 kDa, 132 amino acids, and an isoelectric point of 6.01. These parameters and others including the predicted amino acid composition are summarized in Fig. 1.

Importantly, the predicted amino acid sequence of rFh15 has significant identity (44 %) to a 14.8 kDa *S. mansoni* fatty acid binding protein (Moser et al. 1991). Because of the similarity of the *Fasciola/Schistosoma* protection inducing, cross reactive antigen (rFh15) with Sm14, our prediction has been that the schistosome homologue must also be a protection inducing molecule against *S. mansoni* and, possibly, *F. hepatica* (Rodríguez-Pérez et al. 1992).

CLONING, OVEREXPRESSION AND PURIFICATION OF rFh15

The Fh15 coding sequences were amplified by polymerase chain reaction, and the purified, amplified Fh15 DNA and the vector pGEX2TK were digested with BAM HI. The gel purified, digested PCR product was ligated into the BAM HI digested

Predicted Structural Class of the Whole Protein: Alpha Deléage & Roux Modification of Nishikawa & Ooi 1987

Analysis	Whole Protein
Molecular Weight	14712.40 m.w.
Length	132
1 microgram =	67.970 pMoles
Molar Extinction coefficient	14060±5%
1 A(280) =	1.05 mg/ml
Isoelectric Point	6.01
Charge at pH 7	-0.94

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	43	37.48	32.58
Acidic (DE)	20	16.88	15.15
Basic (KR)	19	16.75	14.39
Polar (NCGSTY)	31	21.85	23.48
Hydrophobic (AILFWV)	41	30.87	31.06
A Ala	6	2.90	4.55
C Cys	1	0.70	0.76
D Asp	7	5.48	5.30
E Glu	13	11.41	9.85
F Phe	6	6.00	4.55
G Gly	9	3.49	6.82
H His	1	0.93	0.76
I Ile	7	5.39	5.30
K Lys	18	15.68	13.64
L Leu	6	4.62	4.55
M Met	8	7.13	6.06
N Asn	3	2.33	2.27
P Pro	3	1.98	2.27
Q Gln	1	0.87	0.76
R Arg	1	1.06	0.76
S Ser	8	4.74	6.06
T Thr	16	10.99	12.12
V Val	14	9.43	10.61
W Trp	2	2.53	1.52
Y Tyr	2	2.22	1.52
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	0	0.00	0.00

Fig. 1: predicted molecular weight, number of amino acids, isoelectric point, and amino acid composition of rFh15.

and dephosphorilated pGEX2TK. The overexpressed *S. japonicum* glutathione S-transferase (SjGST)-rFh15 fusion protein was affinity purified using glutathione agarose by competitive elution with excess reduced glutathione (Smith & Johnson 1988). Pure rFh15 is obtained after thrombin cleavage of the fusion protein and subsequent purification using a second glutathione agarose column (Hillyer, Laxer, Soler de Galanes, García-Blanco in prep.). Major details of this purification procedure are found in Fig. 2.

PRELIMINARY COMPARISONS OF nFh12 AND rFh15

When pure nFh12 and rFh15 are compared on a 15 % SDS-PAGE gel, both migrate faster than the 14 kDa molecular weight standard, but nFh12 always migrates slightly faster than rFh15 (Fig. 3).

OVEREXPRESSION AND ISOLATION OF rFh 15

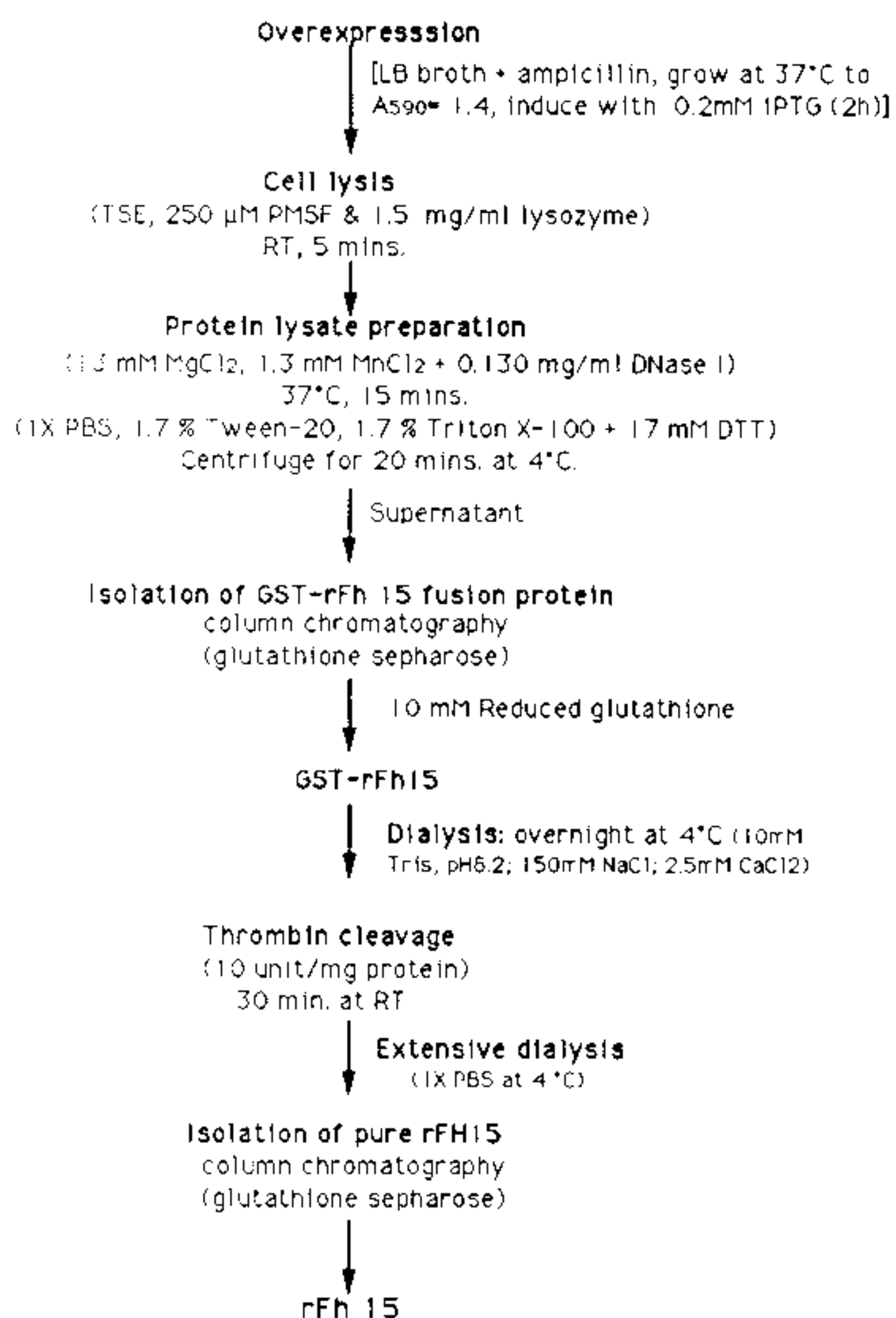


Fig. 2: flow chart detailing steps on the overexpression and purification of rFh15 (see text for details).

This suggests that rFh15 may have a slightly higher MW than nFh12. The predicted pI of rFh15 is 6.0; the observed pI using a BioRad Rotofor with 4-6 pH ampholytes is 5.45 (Hillyer, Laxer, Soler de Galanes, García-Blanco in prep.). Direct comparisons of the sequences of both proteins will resolve these differences and are in progress.

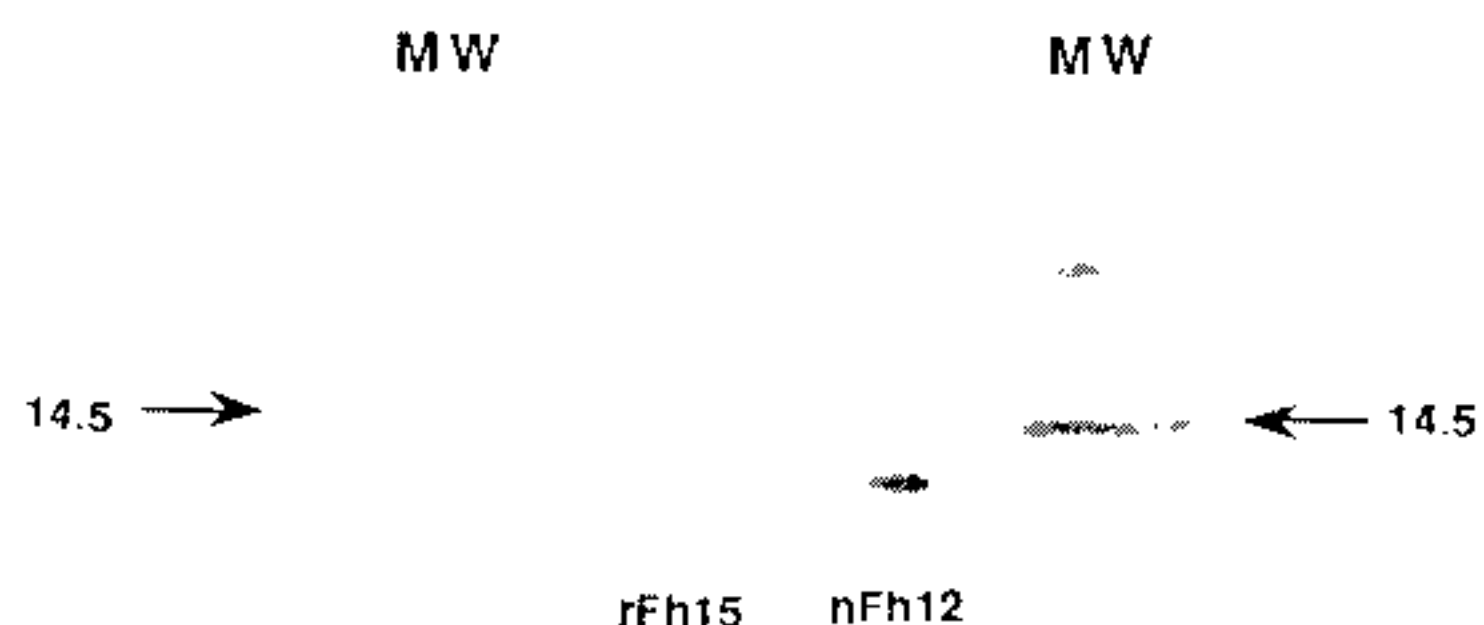


Fig. 3. comparison of the migration of nFh12 and rFh15 in a 15 % SDS-PAGE gel. The 14.5 kDa molecular weight markers (BioRad) in ascending order are 14.5 (arrows), 21.5 and 31 kDa. Note that nFh12 migrates slightly faster than rFh15, with both migrating faster than the 14.5 kDa marker.

Other results on the time of appearance of antibodies during infections support differences between nFh12 and rFh15. For example, mice, calves, and rabbits develop antibodies to *F. hepatica* excretion-secretion (FhES) antigens and to nFh12 by two weeks of infections (Hillyer et al. 1988b, Hillyer & Soler de Galanes 1991). In contrast, in rabbits with fascioliasis antibodies to rFh15 appear much later, i.e. by the 6th week of infection (Fig. 4). Mice infected with *S. mansoni* develop antibodies which are cross reactive with FhES and nFh12 (Hillyer et al. 1988b, Hillyer & Soler de Galanes 1991), as well as to rFh15 (Fig. 5) by the 6th week of infection. Thus the differences in timing of appearance of antibodies between nFh12 and rFh15 are in the fascioliasis, but not schistosomiasis, infections.

It was also of interest to determine the reactivity of different rabbit antisera with purified rFh15. These are seen in Fig. 6. Antisera to *F. hepatica* excretion-secretion, whole worm antigens, and *F. gigantica* whole worm antigens, clearly react with rFh15, as do antisera to nFh12 and rFh15. Antisera to *F. hepatica* glutathione S-transferase did not react with rFh15. Antisera to *S. bovis* and to *Paragonimus westermani* had lesser reactivity, but cross reactivity was clearly evident. These last two are of special interest with regard to heterologous resistance because Hillyer and Serrano (1983) demonstrated that mice immunized with *P. westermani* adult worm extracts developed resistance to challenge infection with *S. mansoni*. More recently, Rodríguez-Osorio et al. (1993) showed that sheep infected with *F. hepatica* for 10 weeks developed

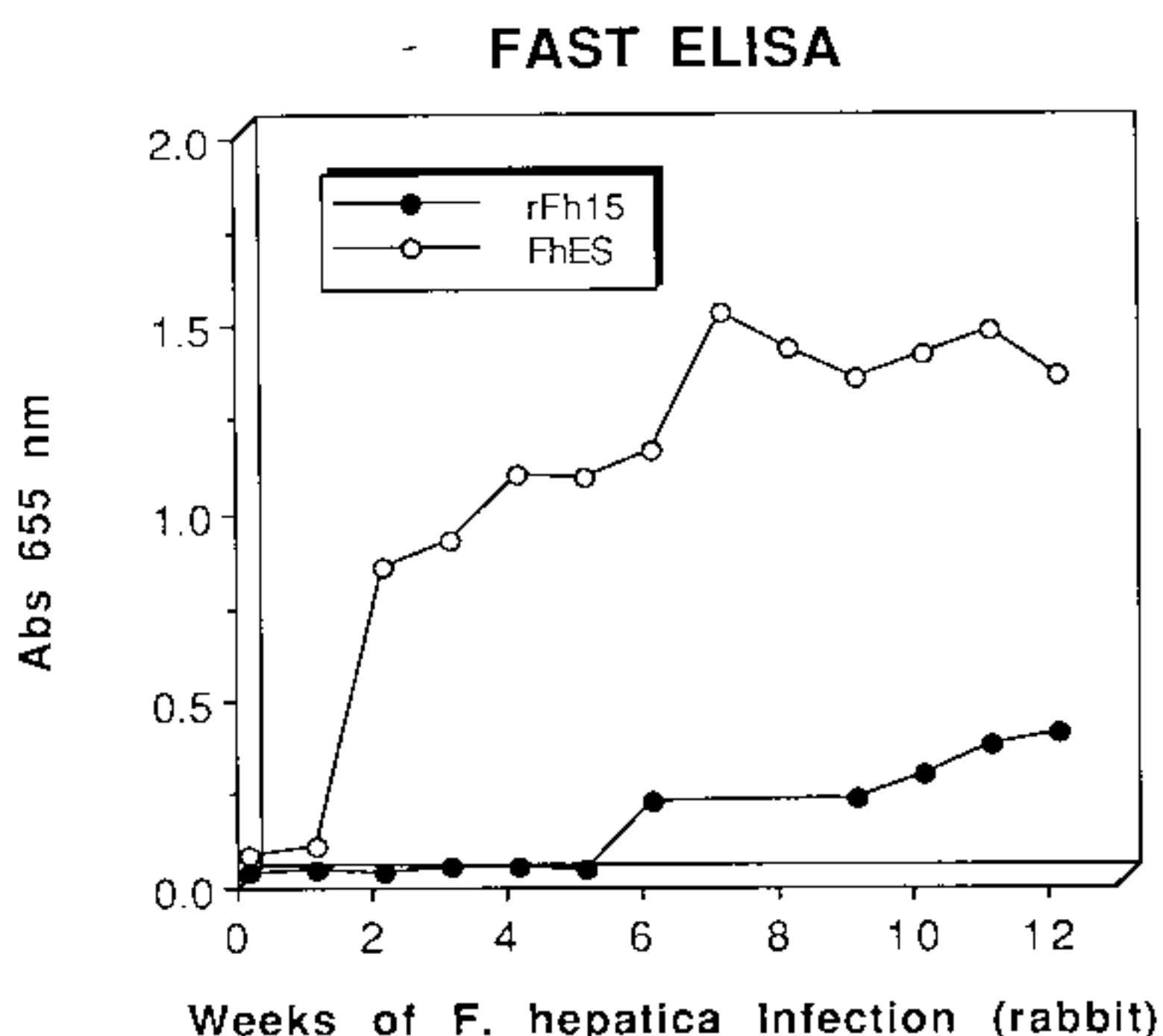


Fig. 4. FAST-ELISA comparing the time of appearance of antibodies to rFh15 and to *Fasciola hepatica* excretion-secretion antigens in the serum from a rabbit infected with *F. hepatica*. Antibodies to FhES appear by 2 weeks of infection whereas antibodies to rFh15 appear at 6 weeks of infection.

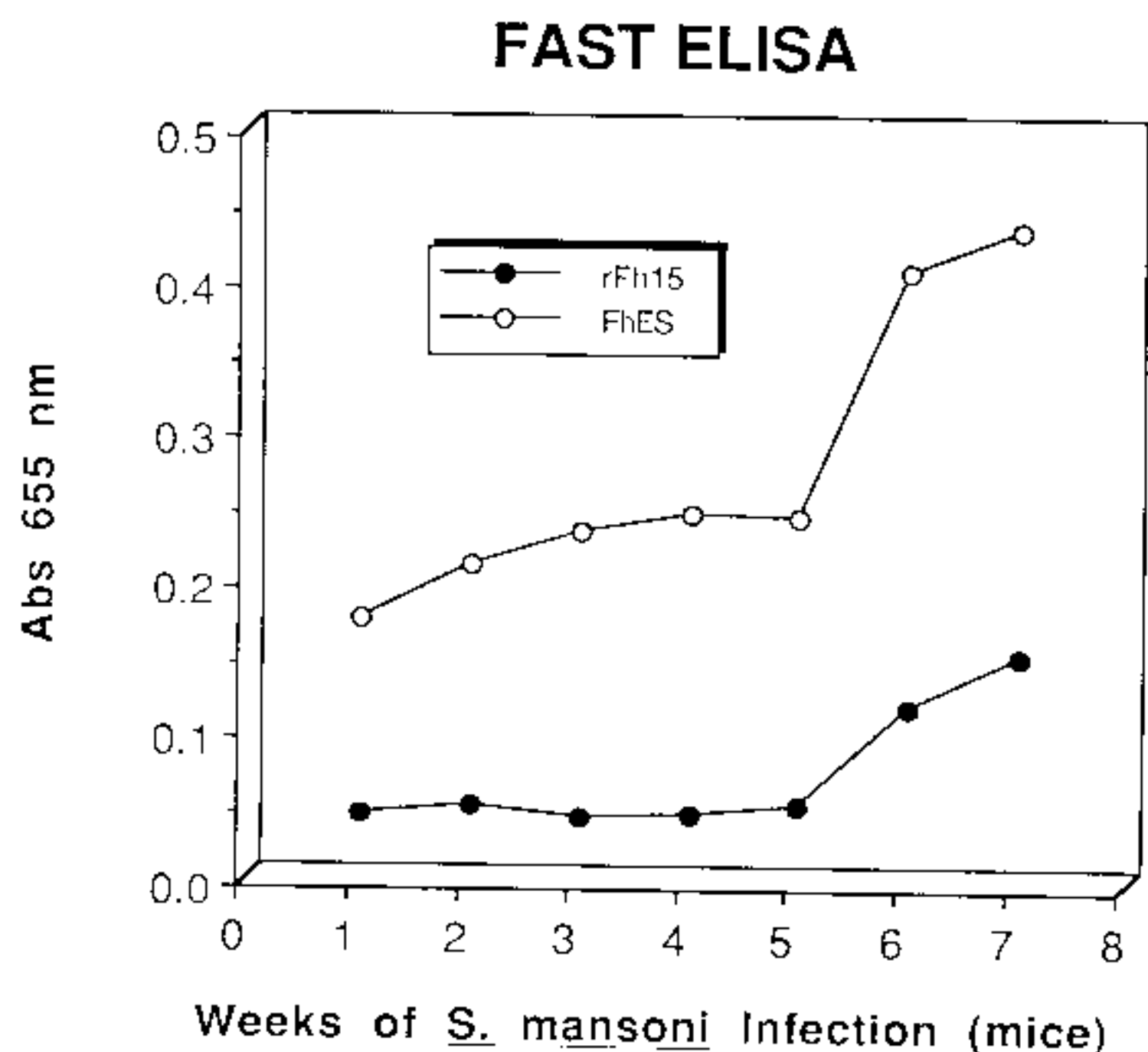


Fig. 5. FAST-ELISA comparing the time of appearance of antibodies to rFh15 and to *Fasciola hepatica* excretion-secretion antigens in pooled serum from mice infected with *Schistosoma mansoni*. Cross reacting antibodies to both FhES and rFh15 appear at 6 weeks of *S. mansoni* infection.

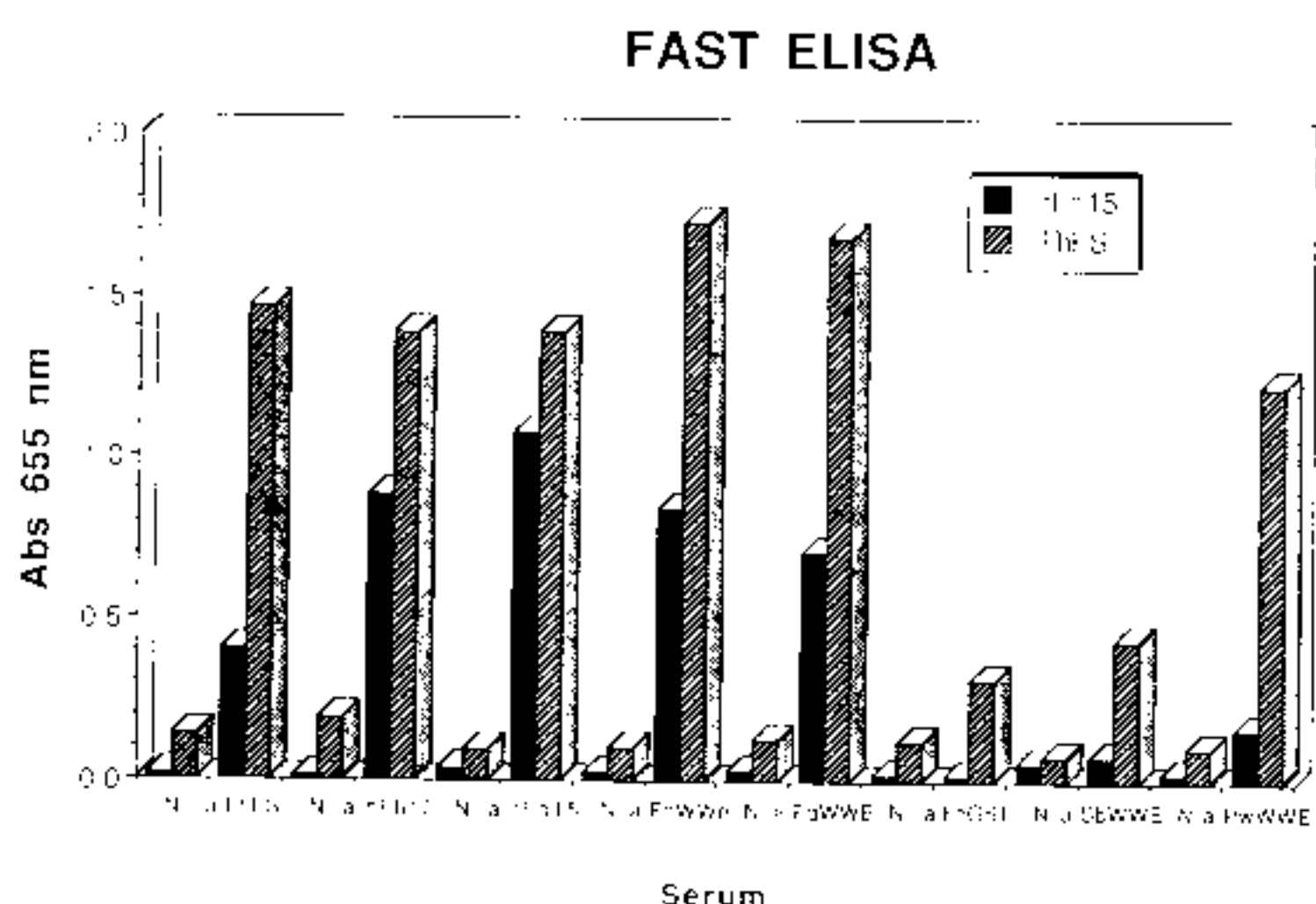


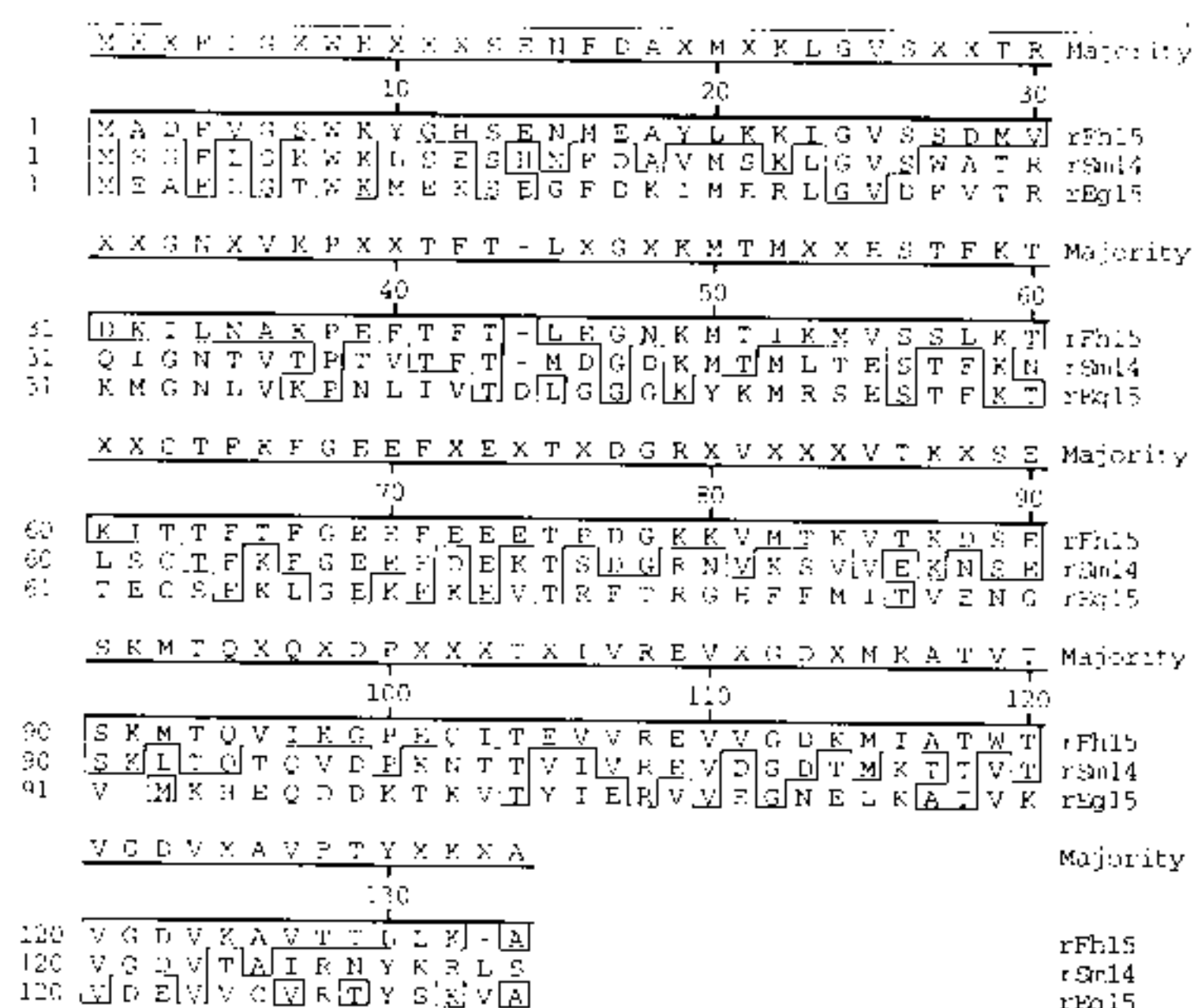
Fig. 6. FAST-ELISA comparing the reactivities of rabbit antisera to different trematode antigens. N = the normal rabbit serum to the left of its immune serum. a = antiserum against: *Fasciola hepatica* excretion secretion (FhES) whole worm extracts (FhWWE), glutathione S-transferase (FhGST), the purified 12 kDa antigen (nFh12), the 15 kDa recombinant protein (rFh15), *F. gigantica* whole worm extracts (FgWWE), *Schistosoma bovis* adult worm extract (SbWWE), and *Paragonimus westermani* whole worm extract (PwWWE).

87 % less *S. bovis* worms than controls. This is similar to the finding that sheep infected with *S. mansoni* for 10 weeks acquired resistance to challenge infection with *F. hepatica* (Haroun & Hillyer 1988). Heterologous resistance has also been demonstrated by Yagi et al. (1986) who found that *F. gigantica* infections protected cattle against challenge infection with *S. bovis*. Because the homologues of rFh15 (or nFh12) may be involved in protective heterologous immunity, further studies are required to demonstrate that they are also present in the heterologous antigen preparations used to prepare the antisera described above.

It should be noted that rabbit antisera against Fh12 differ in their reactivities depending on the method of isolation of antigen. For example, an anti-Fh12 antiserum developed by immunization with Fh12 excised from SDS-PAGE gels when reacted with crude *F. hepatica* adult worm extracts (FhWWE) will yield a single band on SDS-PAGE at the 12 kDa position by Western Blot. This same antiserum will fail to react with FhWWE in FAST-ELISA. In contrast, anti-Fh12 developed by immunization with Fh12 purified by a combination of gel filtration and isoelectric focusing will react with SDS-PAGE fractionated FhWWE in Western Blot and also by FAST-ELISA (data not shown). These results suggest that conformational epitopes must be taken into account when preparing antisera to antigens isolated in different manners.

FATTY ACID BINDING PROTEINS AS VACCINES IN FASCIOLIASIS AND SCHISTOSOMIASIS

Two fatty acid binding proteins have been described from adult worm parasitic platyhelminthes: a protein with 133 amino acids from *S. mansoni* denoted Sm14 (Moser et al. 1991), and a 15.5 kDa protein from *Echinococcus granulosus* denoted Eg15 (Esteves et al. 1993). Amino acid sequence alignment and comparison of rFh15 with Sm14 shows 44 % identity among them and thus suggests that the *F. hepatica* antigen may also be a fatty acid binding protein. Moreover, extensive studies by M Tendler and collaborators in Brazil have shown that mice and rabbits immunized with recombinant Sm14 develop significantly less *S. mansoni* worms after challenge than controls. Similarly, mice immunized with recombinant Sm14 develop high levels of resistance to challenge with *F. hepatica* (M Tendler, Intl. Symp. on Schistosomiasis, Rio de Janeiro, Dec. 1993). Moreover, our own studies have shown that nFh12 protects mice against challenge with *S. mansoni* (Hillyer et al. 1988a, 1990). Are these three FABPs common protective antigens? A comparison of the deduced amino acid sequences of all three are shown in Fig. 7. It is clear that rFh15 is more closely related to Sm14 than to Eg15 with 43.9 % identity between the trematode sequences; but there is only 23.5 % identity between rFh15 and rEg15. Moreover, direct comparison of Sm14 with rEg15 only shows a 33.8 % identity. These differences may be sufficient to preclude rEg15 as a vaccine candidate for cestodes as are the trematode proteins if immunity is based on primary sequence alone. But if protection is a function of blocking the biological activity of the fatty acid binding protein, then the sequence divergence may be less important than the function of the protein. For example, Sacchetti and Gordon (1993) have



Designation: "Designation #1": Box residues that match rFh15 exactly.

Fig. 7. amino acid sequence comparison of three related recombinant platyhelminth fatty acid binding proteins: *Fasciola hepatica* (rFh15), *Schistosoma mansoni* (rSm14), *Echinococcus granulosus* (rEg15). The three amino acid sequences were aligned by the Clustal method using DNASTAR, Inc. (Madison, Wisconsin) comprehensive sequence analysis software for the Apple Macintosh. Identical amino acid sequences have a frame surrounding them. The results show that rFh15 is closer to rSm14 than to rEg15 (see text).

shown that comparisons of numerous mammalian and avian fatty acid binding proteins reveal similar conformations even though multiple sequence alignments reveal that they have as little as 20 % amino acid sequence identity. Thus the protection inducing activity of Eg15 needs to be tested directly.

Homologous recombinant antigens which are potential vaccines against schistosomes have been tested extensively (Capron 1992, Bergquist et al. 1994). The trematode fatty acid binding proteins may soon join this list of vaccine candidates.

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