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Fasciola antigens as vaccines against fascioliasis and schistosomiasis

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Abstract

Fascioliasis is an important trematode infection of herbivores worldwide with increasing evidence of prevalence as a disease of humans. Vaccination studies with purified native and recombinant *Fasciola* antigens suggest that this approach to diminished morbidity and mortality and reduced transmission is a realistic goal. Among the major potential vaccine candidates are fatty acid binding protein (FABP), cysteine (cathepsin) proteases, haemoglobulin, leucine aminopeptidase, and a saposin-like protein. In the case of *Fasciola hepatica* FABP, cross-reaction and cross-protection against *Schistosoma mansoni* is an important feature. In addition to protective effects with significant worm burden reductions, some vaccine candidates also have anti-fecundity (smaller flukes), anti-pathology (less liver lesions), and anti-embryonation effects. Optimism is tempered by the fact that fascioliasis in humans is an orphan disease and in need of governmental and foundation support.

Introduction

Fascioliasis is an infection of herbivores caused primarily by the parasitic trematodes *Fasciola hepatica* and *F. gigantica*. The former has a worldwide distribution mainly in the temperate climates; the latter is primarily of tropical climates in Africa and Asia. Fascioliasis is a true zoonosis with increasing evidence of human infections worldwide, but especially in the rural poor. Human disease due to *F. hepatica* is endemic in the Altiplano region of Bolivia with high prevalence rates in humans and their sheep and cattle (Hillyer *et al.*, 1992; Bjorland *et al.*, 1995; Hillyer & Apt, 1997; Mas-Coma *et al.*, 1999). High human infection rates have also been reported in Peru, Egypt (Curtale *et al.*, 2000), Iran (Moghaddam *et al.*, 2004), and Vietnam where, although infections in herbivorous animals had been known since *c.* 1895, in 1978 only two human cases had been identified. Yet since 1991 the number of reported cases increased yearly. Thus, from 1997 through 2001 some 500 new cases were identified using eosinophilia and ELISA titres as markers of infection, with abdominal upper quadrant pain as a marker a close third. In this same study only 14 of 285 (4%) fascioliasis cases had *F. hepatica* in stools (Hien *et al.*, 2001). Outbreaks have been reported in developed countries including France, Portugal, Spain and Australia. Human and animal infections with the tropical liver fluke *F. gigantica* have been reported in the former Soviet Union, Africa, Asia and South East Asia including Thailand and Vietnam. A comprehensive summary of human fascioliasis worldwide is found in Mas-Coma & Bargues (1997) and Esteban *et al.* (1998). A single drug, triclabendazole, is available for chemotherapy of fascioliasis but is costly; in the case of herbivores it needs to be used repeatedly, and some drug resistance is appearing. This makes vaccination an important alternative in the control and morbidity reduction of fascioliasis in livestock and other herbivores.

Studies in natural hosts such as sheep and cattle provide strong evidence that ruminants acquire resistance to both *F. hepatica* and *F. gigantica* infection following vaccination using irradiated metacercariae or parasite extracts (Haroun & Hillyer, 1986; Spithill *et al.*, 1999b), or with defined antigens (Hillyer *et al.*, 1987; Spithill *et al.*, 1999b; Mulcahy & Dalton, 2001).

Infected animals show lower weight gain, anaemia, reduced fertility, reduced milk production, lowered feed conversion efficiency, and a diminished work capacity.

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This last trait significantly impacts on production of crops in South East Asia and Africa where ruminants provide 80% of the draught power (Spithill *et al.*, 1999a). Thus vaccination resulting in reduced fluke burdens will increase the efficiency of draught, milk and meat animals significantly.

Vaccines – *Fasciola hepatica*

About half a dozen purified, native and recombinant *F. hepatica* antigens have been shown to have immunoprophylactic potential against fascioliasis. These include fatty acid binding proteins (FABP), glutathione S-transferases (GST), cathepsin L proteases (catL), haemoglobin, leucine aminopeptidase (LAP) and a saposin-like protein denoted SAP-2 (Spithill & Dalton, 1998, and below). Most of these cross-react with, and are also vaccine candidates against schistosomes of humans, especially *Schistosoma mansoni*.

Fatty acid binding proteins

Fatty acid binding proteins are a large family of proteins involved in the binding and transportation of a variety of hydrophobic ligands. The most striking feature of the cytoplasmic FABPs is the conservation of size: all known members range between 14 and 16 kDa in mass and 127–133 amino acids in length. FABPs were the first defined and purified antigen fractions to be tested as a vaccine against fascioliasis. The recognition of this antigen as protective came from a series of experiments begun 30 years ago by Hillyer and collaborators in which a subset of *Fasciola* antigens could be purified by virtue of their cross-reactivity of antibodies against *S. mansoni* and were shown to cross-protect against *S. mansoni*; those antigens that did not bind to the anti-*S. mansoni* antibodies did not cross-protect (reviewed in Hillyer, 1976, 1984). A more purified subset of these cross-reactive *Fasciola* antigens, denoted Fh/SmIII(M), with the FABP antigen being a major component, was able to protect both mice with up to 78% reduction and calves with up to 55% reduction against challenge infection with *F. hepatica* (Hillyer, 1985; Hillyer *et al.*, 1987). The above-referenced cross-protection also applied to mice challenged with *S. mansoni*, with up to 81% fluke reductions in the vaccinated mice, thereby confirming the conservation of immunoprotective epitopes between *Fasciola* and *Schistosoma* antigens (Hillyer, 1979).

The antigen in the Fh/SmIII(M) fraction that was protective against murine schistosomiasis mansoni (up to 77% reduction in mice) was identified as a 12 kDa protein and was denoted Fh12 (Hillyer, 1987; Hillyer *et al.*, 1988a,b). Using a mono-specific, polyclonal antiserum to Fh12, a cDNA from an adult *F. hepatica* cDNA expression library was isolated that predicted a 14.7 kDa recombinant protein (denoted rFh15, see below) with high similarity to a family of FABPs (Rodriguez-Perez *et al.*, 1992; Hillyer, 1995), including significant homology to a 14.8 *S. mansoni* FABP (rSm14, in Moser *et al.*, 1991).

Subsequently, Tendler *et al.* (1996) reported that vaccination with rSm14 significantly protected rabbits and mice against infection with *S. mansoni* and completely protected against murine fascioliasis. Additional studies (Almeida *et al.*, 2003) demonstrated that sheep and mice vaccinated with rSm14 were significantly protected against challenge infection with *F. hepatica* metacercariae, and were completely free of the histopathological hepatic damage related to liver fluke infection. Finally, they were able to demonstrate that vaccination trials in Swiss mice challenged with *S. mansoni* cercariae or *F. hepatica* metacercariae showed that peptides that included the sequences VTVGDVTA or EKNSESKLTQ were capable of inducing levels of protection equivalent to the recombinant form of Sm14 (Vilar *et al.*, 2003). A BLASTQ2 pairing of the deduced amino acid sequence in the Swiss-Prot database of Tendler's rSm14 (P29498) and Hillyer's rFh15 (Q7M4G0) shows that the corresponding sequence TKDSESKMTQ located between amino acid residues 84 and 93 of rFh15 has homology to nine of the ten amino acids contained in the reported Sm peptide EKNSESKLTQ with seven identical, two conserved, and one non-homologous amino acid residues. Moreover, the corresponding *F. hepatica* sequence WTVGDVKA located between amino acid residues 118 and 125 has homology to six of the eight amino acids contained in the *S. mansoni* peptide VTVGDVTA with six identical and two non-homologous amino acid residues.

Interestingly, monoclonal antibodies developed against *F. gigantica* FABP all cross-reacted with *S. mansoni* whole worms (Sirisriro *et al.*, 2002). Whether this can be translated into cross-reactive immunoprophylaxis against both *S. mansoni* and *F. gigantica* remains to be determined.

The cloning, sequencing, characterization and purification of rFh15 have been described (Rodriguez-Perez *et al.*, 1992; Hillyer, 1995). The purification of rFh15 and the native molecule (nFh12) prompted a series of experiments to compare their immunoprophylactic potential with the native, purified antigen with collaborators in Salamanca, Spain. Muro *et al.* (1997) vaccinated rabbits with rFh12 or rFh15 in Freund's adjuvant to see their effects to challenge with *F. hepatica* metacercariae. Here a longer (4 week vs. 2 week) lag time after the second immunization resulted in higher levels of protection, with nFh12 only achieving statistically significant fluke reductions over controls (40%). However, rabbits immunized with either nFh12 or rFh15 developed smaller flukes, and had milder liver lesions than controls. Casanueva *et al.* (2001) repeated the above using only rFh15 but extending the lag times to 12 and 20 weeks after the second immunization. Significant fluke reductions were obtained directly related to the lag time (43% fluke reduction with 12 week lag; 76% reduction with 20 week lag). Moreover, the percentage of immature flukes was highest in the rFh15 vaccinated rabbits (66% reduction with 12 week lag; 84% reduction with 20 week lag). Finally, liver lesions were least in the rFh15 vaccinated rabbits. The authors concluded that a recombinant *F. hepatica* FABP induced protective (worm burden reductions), anti-fecundity (immature flukes), and anti-pathology (less liver lesions) effects in rabbits and may serve as a model for the immunoprophylaxis of fascioliasis.

Lopez-Aban *et al.* (1999) showed cross-protection of nFh12 versus *S. bovis* in mice. In this study, C57/BL mice immunized with nFh12 in Freund's adjuvant developed

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87–96% less *S. bovis* flukes than controls. Moreover, vaccination of mice with the native protein in adjuvant increased the production of IFNgamma by splenocytes when compared with uninfected mice suggesting a shift to Th1 protective responses. They also found (Lopez-Aban *et al.*, 2000) that C57/BL mice vaccinated with rFh15 in Freund's developed 72% less *S. bovis* worms than controls; moreover, vaccinated mice had slight liver lesions as compared with the generally severe lesions of control/infected mice. No protection was afforded when NMRI or BALB/c mice were used. In the first published experiment on the immunoprophylactic effect of native and recombinant *Fasciola* FABP in Castillian breed sheep, antibody levels rose rapidly in those immunized with either antigen in Freund's adjuvant. Although no differences were found in fluke burden reductions, the worm size and faecal egg counts were significantly diminished in those immunized with either antigen, suggesting an anti-fecundity effect (Ramajo *et al.*, 2001). In a subsequent study, a novel adjuvant/immunomodulator system was used with nFh12 as a vaccine against Castillian breed sheep challenged with *F. hepatica* metacercariae (Martinez-Fernandez *et al.*, 2004). This vaccination protocol consisted of a set of two injections. The first contains a micelle in which two components are included, saponin from *Quillaja saponaria* and or Anapsos, a *Polypodium leucotomos* hydroalcoholic extract, both emulsified in a non-mineral oil (Montanide) in a 30/70 water/oil emulsion, subcutaneously injected. The second injection has the same components plus the nFh12 FABP. Six weeks later the sheep were challenged with 100 *F. hepatica* metacercariae. The vaccinated sheep at necropsy presented lower fluke recovery (24.5%), lower number of eggs in bile fluid (58.1%), and faeces (40.3%) over control groups. Moreover, the recovered flukes were shorter (32.7%), immature (34%) and with lower body mass (31.6%) over the non-complete vaccinated sheep. BALB/c and CD-1 mice given lethal (5) doses of metacercariae all died by 6 weeks, while 40% of those given the complete vaccination protocol with nFh12 survived.

All of the above suggests that native and recombinant *F. hepatica* FABPs induce significant levels of protection in different animal models against infection with *F. hepatica* and cross-protection against *S. mansoni* and *S. bovis* with anti-fluke, anti-fecundity and anti-pathology effects. But are the native and recombinant molecules the same? Antibodies to nFh12 appear earlier in both *F. hepatica* and *S. mansoni* infections (Hillyer *et al.*, 1988b; Hillyer, 1995). The native, purified Fh12 has been shown to be a protein complex of at least eight isoforms with identical molecular mass. However, what appears to be the equivalent of rFh15 is one of the least immunogenic forms and may, in fact, be one of the least immunoprotective forms (Espino & Hillyer, 2001).

Glutathione S-transferases

The GSTs comprise a family of isoenzymes involved in the cellular detoxification of a broad range of chemical substrates. The GSTs of *F. hepatica* were chosen as candidate vaccine antigens on the basis that homologous GST proteins from *S. mansoni* and *S. japonicum* were shown to protect laboratory animals (see Spithill & Dalton, 1998). Purification of *F. hepatica* GSTs by Spithill *et al.* (1999b) have shown a high degree of heterogeneity finding eight native and four recombinant isoenzymes. Initial studies by this group in 1990 showed that sheep, which received multiple vaccinations with native *F. hepatica* GST in FCA, showed a 57% reduction in worm burden, marking the first demonstration in sheep against *F. hepatica* using a defined antigen. In extensive additional studies of this group in the 1990s, it was not possible to consistently induce a protective response despite using comparable vaccination protocols (Spithill *et al.*, 1999b). Recently, De Bont *et al.* (2003) at the Institut Pasteur de Lille tested recombinant *S. bovis* GST with aluminum hydroxide, Quil A, or FCA as adjuvant and obtained no protection in cattle against *F. hepatica*.

Cysteine (cathepsin) proteases

The cysteine proteases comprise a large family that include cathepsin L and B that have been studied in relation to parasite invasion, feeding, immune evasion and vaccine potential. The *Fasciola* cathepsin L proteases have been proposed to play a number of functional roles including promoting tissue penetration, nutrient acquisition, egg production, and immune evasion by cleavage of the Fc region of the antibody immunoglobulin. The efficacy of cathepsin L as a vaccine in sheep was first demonstrated by Wijffels and collaborators (see Spithill *et al.*, 1999b). Here, although no reduction in fluke recoveries was found, faecal egg counts were significantly reduced in the vaccinated sheep.

The efficacy of two isolated secreted cathepsin L homologues, cathepsin L1 and cathepsin L2, were tested alone or in concert with *Fasciola* haemoglobin in cattle (Mulcahy *et al.*, 1988). Both cathepsin L preparations elicited protection against infection, with the highest being 72% fluke reduction when cathepsin L2 was used with haemoglobin. Moreover, cathepsin L1 was found to induce reduction in egg fecundity. The results suggested that protective immune responses were of the Th1 type involving the IgG2 isotype, interferon gamma-activated macrophages and cytotoxic T cells. An interesting observation was that, although IgG2 antibodies appeared important in protective immune responses to *F. hepatica*, they may be protective only if a certain magnitude of response is achieved, because this study showed that calves with lower fluke burdens tended to have higher affinity IgG2 antibodies. Therefore, a prolonged immunization protocol that allows a greater time for the maturation of the immune response and the development of higher-affinity antibodies may be an important factor in generating protective immune responses. Perhaps this is the reason the animals immunized with *F. hepatica* FABPs, described above, require longer lag times after vaccination to obtain higher worm burden reductions.

Subsequently, Dalton *et al.* (2003b), in a large series of vaccine trials on both sheep and cattle with purified, native *F. hepatica* cathepsin L1 and L2 showed that these enzymes could induce protection, ranging from 33 to 79%, to challenge infection with *F. hepatica* metacercariae, and a very potent anti-embryonation/hatch rate effect that would block parasite transmission. They reported that there were 13 *Fasciola* cathepsin L cDNAs deposited

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in the public databases representing a gene family of at least seven distinct members. How many of these have immunoprophylactic effects in fascioliasis remains to be determined. However, it should be noted that lowered fluke recoveries are not the only marker of a successful vaccine. Anti-fecundity, anti-embryonation and lower faecal egg counts are another feature of cathepsin L vaccines in cattle and sheep (Mulcahy & Dalton, 2001).

Van Milligen *et al.* (2000) obtained an antigen fraction derived from newly existed *F. hepatica* juveniles containing an immunoreactive 32-dDa protein which when inoculated into rats intraperitonealy or intramuscularly without adjuvant induced almost complete protection to challenge with reductions of over 90% in comparison to naïve control rats. This antigen was subsequently found to have 70% sequence homology with cathepsin L1 and L2, but still containing the propeptide (Harmsen *et al.*, 2004).

Leucine aminopeptidase

Piacenza *et al.* (1999) in Uruguay found that vaccination of sheep with LAP, a metalloprotease from *F. hepatica*, induced the production of neutralizing antibodies and elicited 89% protection against fascioliasis. Although highest protection was found with LAP alone, cocktails with cathepsin L1 and cathepsin L2 and LAP were more protective than cathepsin L1 or L2 alone. Reduced liver damage, as assessed by the level of the liver enzyme gamma-glutamyl transferase was observed in all vaccinated groups.

Saposin-like proteins

Recently, Espino & Hillyer (2003) isolated a 436 bp-cDNA from an adult *F. hepatica* cDNA expression library by screening with the serum from a rabbit infected with *F. hepatica* for 4 weeks. The ORF encodes a 101 amino acid polypeptide with calculated molecular weight of 11.5 kDa and a putative isoelectric point of 4.63. The deduced amino acid sequence revealed significant homology with a *F. hepatica* NK-lysin and saposin-like protein denoted by Reed *et al.* (2000) as FhSAP-1. For this reason, this newly described protein was denoted FhSAP-2. Rabbits vaccinated with FhSAP-2 developed 81.2% less flukes than controls. Moreover, *F. hepatica* egg counts in faeces as well as in bile collected from the gall bladders from vaccinated animals were lower, 83.8% and 73% respectively, over controls. The vaccinated rabbits also had significantly lower amounts of parasite antigen in stool and bile samples than controls. Lastly, evaluation of macroscopic liver lesions revealed that the rabbits vaccinated with rFhSAP-2 had milder lesions than the infected-control rabbits. These findings support the hypothesis that this novel rFhSAP-2 protein has immunoprophylactic potential against fascioliasis in rabbits including anti-fecundity and anti-pathology effects (Espino & Hillyer, 2004).

DNA vaccines

Smooker *et al.* (1999) evaluated in BALB/c mice the humoral responses following vaccination with DNA constructs encoding *F. hepatica* GST47 cDNA subcloned into two DNA vaccine vectors, VR1012 and VR1020 which direct expression to the cytoplasmic and extra-cellular compartments, respectively. Expression was confirmed by transfection into COS 7 cells. Vaccination with the construct designed for secretion resulted in an increased humoral response compared to vaccination with the non-secretory construct. The total humoral response after vaccination with the secretory construct was not dependent on the route of vaccination but the isotype profile did differ among the groups: intramuscular vaccination with the construct directing cytoplasmic expression yielded an IgG2a dominant Th1-type response, whereas intradermal vaccination with the construct directing cytoplasmic expression resulted in a IgG/IgE dominant Th2-type response. Finally, intramuscular vaccination with the secretory construct resulted in a mixed Th1/Th2 isotype response. Thus the immunogenicity of a DNA vaccine based on *Fasciola* GST, as well as the isotype of the response against GST, is determined by the mode of vaccine administration.

Kofta *et al.* (2000) designed a DNA vaccine using a pcDNA 3.1 vector carrying DNA encoding for a cysteine proteinase of *F. hepatica*. Male rats vaccinated intramuscularly with cysteine proteinase cDNA revealed 100% protection against *F. hepatica*; female rats had a reduction of 74%. They subsequently found that intranasal vaccination resulted in 61–75 flukes over controls and that this route seemed to favour a Th2 regulated antibody response; intramuscular and intraperitoneal immunizations favoured mixed Th1 and Th2 antibody responses (Wedrychowicz *et al.*, 2003).

Smooker *et al.* (2001) found that both recombinant *F. gigantica* FABP and recombinant *F. hepatica* CatL5 (both obtained from cDNA clones) were shown to be expressed in COS 7 cells and induced a humoral response when delivered as secretory constructs in BALB/c mice. rFgFABP induced an IgG1 dominant response, with significant IgE, IgG2a, and IgG2b responses also present, indicating a mixed Th1/Th2 response. In contrast, the delivery of the FABP as a non-secreted construct did not result in the induction of a measurable humoral response.

Espino *et al.* (2005) evaluated the humoral and cellular responses to DNA vaccination using pFLAG-CMV series linked to rFhSAP-2. Vaccination with cytoplasmic constructs induced high humoral and cellular immune responses whereas vaccination with secretory DNA construct resulted in lower antibody levels as compared to the cytoplasmic construct or rFhSAP-2 in adjuvant alone.

Vaccines – *Fasciola gigantica*

Comparatively less is known about vaccines against *F. gigantica*. Estuningsih *et al.* (1997) found low but significant reductions in fluke burdens (31%) and fluke wet weight (36%) in Brahman cross cattle vaccinated with purified native FgFABP in Freunds' adjuvant, with no correlation of total antibody titres to FABP and protection. In this study, *F. gigantica* native glutathione S-transferase, cathepsin L, paramyosin, and a recombinant FABP all failed as vaccines when formulated in one or more of nine adjuvants. More recently, Paykari *et al.* (2002) failed to

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obtain significant protection in sheep vaccinated with native, purified FgGST in aluminum hydroxide or saponin as adjuvants.

Fascioliasis vaccines – a reality?

Given the high levels of efficacy of some vaccines in sheep and cattle, control by immunoprophylaxis should be an achievable goal, although defining a proper adjuvant is still an issue. As described above, candidate vaccines result not only in reduced fluke burdens, but smaller flukes, fewer eggs, less liver pathology, all leading to better well-being of the livestock. Mean levels in cattle have been shown to be in the 40–70% fluke reduction. Because economic loss is correlated with fluke burden, a burden of 30–80 flukes causes losses in weight gain in cattle, suggesting that a vaccine inducing a mean worm burden reduction as low as 43% will reverse economic losses only in herds exposed to burdens of around 53–140 flukes. Fluke burdens of 40–140 were shown to be common in cattle in the USA, whereas in the UK only 3% of cattle livers had more than 50 flukes. In Iran and Nigeria, mean fluke burdens of 68–99 were reported in cattle (in Spithill & Dalton, 1998). Thus vaccines are economically viable.

The fact that important potential vaccine candidates against fascioliasis have been identified suggests that now the time is ripe to look at combinations of these successful candidates as chimeric proteins or as DNA prime and peptide boost vaccines. Recombinant homologues can be identified and obtained via numerous expression systems that are widely available (Dalton *et al*., 2003a). The future of fascioliasis vaccines in farm animals looks bright and research in this field should be encouraged. But the major difficulty for studies on vaccines for this orphan disease still is lack of funding.

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