The major tegumental antigen of *Fasciola hepatica* contains repeated elements

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SUMMARY

In order to provide a better understanding of the interaction between the liver fluke (*Fasciola hepatica*) and the immune system of its mammalian host immunoreactive λ bacteriophage clones containing *F. hepatica* cDNA have been isolated. Plasmids from these clones were sequenced and found to encode a family of proteins containing certain common elements†. All the clones contained a coding repeating sequence (RRRXCA) which is conserved at the nucleic acid level followed by a non-repeating element coding for the C terminal used by the proteins which shows conservation of amino acids at certain positions. Antisera raised against a β-galactosidase fusion protein with one of these sequences as a terminal extension was used to localize the immunoreactive antigens. Binding was predominantly in the tegument of the juvenile fluke but was reduced in the adult tegument. The wall of the uterus showed strong reactivity in the adult. Rats immunized with the β-galactosidase fusion protein showed enhanced resistance to challenge infections. The role of these antigens in the host response to infection by *F. hepatica* is discussed.

Key words: *Fasciola hepatica*, tegument, repeats, immunoevasion.

INTRODUCTION

The tegument of *Fasciola hepatica* provides the interface at which the host immune system interacts with the parasite. Its structure has been determined by microscopy to be a syncytium formed by the fusion of specialized tegumental cells located beneath the longitudinal and lateral muscle layers (Threadgold, 1967; Bennett & Threadgold, 1975). These cells produce secretory bodies which are transported towards the trilaminate apical surface of the syncytium where they discharge their contents (Hanna, 1980*a*). Studies based on electron microscopical, immunological and biochemical observations have shown that the type of vesicle and its contents change with the differentiation of the fluke in the mammalian host. Initially T0 bodies are produced but during the migratory stage there is a switch to T1 bodies and on reaching the bile duct T2 bodies become predominant (Bennett & Threadgold, 1975). It has been shown that although the T0 and T1 bodies can be distinguished morphologically their contents are antigenically indistinguishable (Hanna & Trudgett, 1983). There is a continuous sloughing of the tegumental proteins and this has been proposed as an immunoevasion mechanism (Hanna, 1980*b*). The tegument constitutes the main antigenic stimulus during the initial stages of infection in rodent based experimental models; all of 50 monoclonal cell lines generated during responses to infection were specific for tegumental components (Hanna & Trudgett, 1983) although other proteins of the fluke can be rendered immunogenic by administration with adjuvants (Hanna, Trudgett & Anderson, 1988). Antigen prepared by immunosorption using these monoclonal antibodies is capable, when incorporated in an ELISA, of detecting antibody responses in infected cattle and sheep as early as 2 weeks post-infection, thus indicating that the immune response mounted by natural hosts during the migratory stage of the flukes' development also involves these antigens (Trudgett, Anderson & Hanna, 1988). Further indication of the importance of tegumental material in the biology of *F. hepatica* comes from our finding that the tegument of the juvenile migratory fluke shares epitopes with structures in the reproductive system of the adult. We have proposed that this may underlie the phenomenon of 'concomitant immunity' observed in rats infected with *F. hepatica* (Keegan & Trudgett, 1992).

Despite the central position of tegumental proteins in the interaction between the fluke and its mammalian host there have been few attempts to

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† The nucleotide sequence data reported in this paper are available in the GenBank database with the accession number AF153056.

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Clone	No. of Repeats	Sequence
D6	4	CCA GAG CCA AAG GGA GAC GGA GAT CCA AAG
		P E P K G D G D P K
T21	>7	CCA GAG CCA GAA CCA AAG CCA GGG
		P E P E P K P G
U130	>8	CCA GAG CCA GGG CCA AAG CCA GAA GAT
		P E P G P K P E D
49mp and c200	>5 5	CCA GAG AAC AAG GAA GGA GAA
		P E N K E G E
T24	>6	CCA GAA CCA AAG CCA GGG
		P E P K P G
T24	>4	CCA GAG CCA GGG CCA GAG
		P E P G P E

Fig. 1. Variety of repeating elements found in immunoreactive clones. Underlining indicates a substance P-like motif.

investigate these entities using the techniques of molecular biology. A recent review (Spithill & Dalton, 1998) of research into vaccine strategies to combat fascioliasis described results with 4 candidate vaccines, fatty acid binding proteins, glutathione-S-transferase, cathepsin L and liver fluke haemoglobin. None of these molecules are located in the tegument. In this study we report the characterization of a number of clones derived from fluke cDNA and identified primarily by immunoscreening with sera from infected animals.

MATERIALS AND METHODS

Parasites

Flukes were obtained from 2 sources. Initially flukes from experimental infections in rats were used but later libraries used flukes from naturally acquired infections in cattle. Infected cattle livers were kindly supplied by a local abattoir.

RNA preparation

Following removal from animals infected 5 weeks previously, parasites were incubated for 2 h at 37 °C in 5 changes of PBS. RNA was isolated by cell lysis in a buffer containing guanidinium isothiocyanate, followed by caesium chloride gradient centrifugation using standard methods (Kaplan, Bernstein & Gioio, 1979). The polyadenylated RNA fraction (poly A-RNA) was purified by passing the RNA obtained over an oligo-dT cellulose column (Collaborative Research Inc., USA) as described (Sambrook, Fritsch & Maniatis, 1989).

cDNA library construction and screening

Poly A-RNA was used as the template for cDNA synthesis which was performed according to standard procedures (Gubler, 1987). λ gt 11 libraries were constructed from approximately 500 ng of blunt-ended double-stranded cDNA as described (Hunyh, Young, & Davis, 1985). Aliquots of the libraries were screened using a 1:250 dilution of serum taken 7 weeks post-infection from a sheep experimentally infected with 500 metacercariae. To reduce background activity to *E. coli* the serum was presorbed overnight at 4 °C with lysed *E. coli* linked to activated Sepharose 4B (Sigma, USA). This was followed by incubation in goat anti-sheep IgG alkaline phosphatase conjugate (Sigma, USA) at 1:500 dilution. The presence of bound enzyme was revealed by incubating the filters in nitroblue-tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate (both Sigma, USA) at pH 9·5. All immunoreactive plaques were rescreened 3 times with the antiserum.

Fusion protein production

Escherichia coli strain Y1089 was infected by phage at a multiplicity of infection of 500. Cultures consisting of recombinant lysogens were identified by their ability to grow at 32 °C but not at 42 °C. Recombinant fusion protein was prepared from a Y1089 lysogen (clone c200) using standard methods (Hunyh *et al.* 1985) and isolated by electroelution from a 12 % SDS polyacrylamide gel. Sera for use in immunolocalization studies (see below) was produced by inoculating Balb/c mice intraperitoneally with 2 injections of 15 μg of the recombinant fusion

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                                                         1         1
...........................CCA] [GAGCCA] [GGCCCA]
                               P     E  P     G  P

         2               1         1         1         1
[AAGCCTGAAGATCCA] [GAGCCA] [GGGCCA] [GACCCA] [GGGCCA]
  K  P  E  D  P     E  P     G  P     D  P     G  P

         2               1         1         1         1
[AAGCCTGAAGATCCA] [GAGCCA] [GGCCCA] [GAGCCA] [GGGCCA]
  K  P  E  D  P     E  P     G  P     E  P     G  P

         2               1         1         1         1
[AAGCCTGAAGATCCA] [GAGCCA] [GGGCCA] [GAGCCA] [GGGCCA]
  K  P  E  D  P     E  P     G  P     E  P     G  P

         2               1         1         1         1
[AAGCCTGAAGATCCA] [GAGCCA] [GGGCCA] [GAGCCA] [GGGCCA]
  K  P  E  D  P     E  P     G  P     E  P     G  P

         2               1         1         1         1
[AAGCCTGAAGATCCA] [GAGCCA] [GGGCCA] [GAGCCA] [GAACCA]
  K  P  E  D  P     E  P     G  P     E  P     E  P

   1        1        1        1
[AAGCCA] [GGGCCA] [GAGCCA] [GGGCCA]
  K  P     G  P     E  P     G  P

         2               1         1         1         1
[AAGCCTGAAGATCCA] [GAGCCA] [GGGCCA] [GAGCCA] [GAACCA]
  K  P  E  D  P     E  P     G  P     E  P     E  P

   1        1        1        1
[AAGCCA] [GGGCCA] [GAGCCA] [GGGCCA]  AAGC
  K  P     G  P     E  P     G  P     K

CTGAAGAAAACAGCACTACAGGTGGCACTTCGAGACTCTCCAATCACATGGCTTTC
P  E  E  N  S  T  T  G  G  T  S  R  L  S  N  H  M  A  F

CTCACGTTCCCATTAAAGTGTCTTGGATTTGTCCTGTAG
 L  T  F  P  L  K  C  L  G  F  V  L  U
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Fig. 2. Sequence from clone Ul30 showing periodicity of repeating elements. Order of repeating elements shows a periodic insertion of a larger element (2) within a series of smaller elements (1) i.e. (2) 4×(1) (2) 4×(1) (2) 4×(1) (2) 4×(1) (2) 8×(1) (2) 8×(1).

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CLONE

D6       NTTASSGGTSRLSNQMAFLTFPLKCLGLIQW

T24      EENSTTGGTSRLSNHMAFLTFPLKCLGLFVL

U117     TEATTTGGTSRLTNHMALLVFSLRSLRLIQ

48mp     RETTATGGTSRLTNHLAVLEFSLRCLGLIQW

c200..   RETTATGGTSRLTNHLAVLEF

43mp     RETTATGGTSRLTNHLAVLEFSLRCLGLIQ
              ********   * * * *   * *
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Fig. 3. Derived carboxy terminal sequences. Non-repeating C terminal showing conservation of certain residues (*).

protein in Freund's incomplete adjuvant (inoculum volume 0·1 ml) administered 2 weeks apart.

PCR amplification of the inserts

λ specific primers, which annealed approximately 30 bp from the *Eco*R1 site were synthesized on an Applied Biosystems (Warrington, England) synthesizer. The PCR reaction was performed on a Perkins–Elmer Cetus DNA thermal cycler using 0·01 μg of recombinant λ DNA as a template. Forty cycles of amplification were carried out. Each cycle consisted of 2 min at 55 °C, 2 min at 72 °C and 1 min at 92 °C. The resulting fragments were inserted by ligation into the TA cloning vector (Invitrogen,

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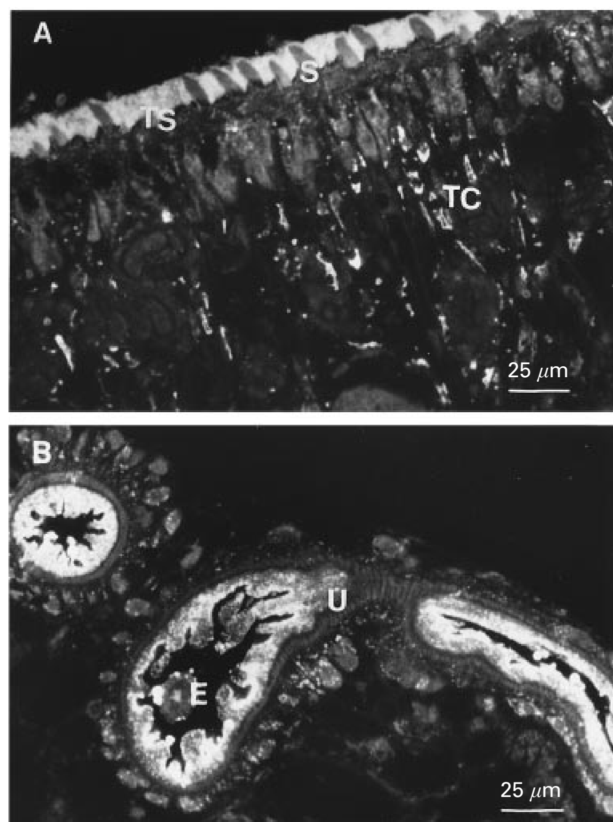

Fig. 4. Immunofluorescent localization of antigen. (A) Section from juvenile fluke. Ts, tegumental syncytium; Tc, tegumental cells; S, spine. (B) Section from adult fluke. U, Uterus; E, egg in lumen of the uterus.

USA). Recombinant plasmids were sequenced using M13 forward and reverse primers used in conjunction with a PRISM™ (ABI) DyeDeoxy™ terminator cycle sequencing kit. Sequencing reaction products were analysed using a ABI automated sequencer and the manufacturer's recommended protocols.

Immunolocalization of antigens

Juvenile flukes and fragments of appropriate regions of adult flukes were fixed in paraformaldehyde, embedded in JB4 resin and sectioned as described previously (Hanna & Trudgett, 1983). Sera from immunized mice and appropriate controls were used at a dilution of 1:100 in PBS. FITC-labelled rabbit anti-mouse Ig (Sigma) was used to reveal specific antibody binding. Sections were examined and photographed using a Leitz Laborlux D microscope system.

Challenge infections of rats immunized with fusion protein

Each of 15 male Hooded Lister rats, aged 3–4 months, were inoculated intraperitoneally with 0·5 ml of Complete Freund's Adjuvant containing 50 μg of the recombinant fusion protein as prepared above. After 4 weeks they were boosted with a further 50 μg of the protein in Incomplete Freund's Adjuvant. A group of 15 control animals followed the same protocol but with β-galactosidase being substituted for the fusion protein. Four days after the booster inoculation all animals were challenged with 25 *F. hepatica* metacercariae delivered orally. After a further 3 weeks the rats were killed and their livers examined for the presence of flukes. The Mann–Whitney *U*-test was used to determine the significance of differences in the number of flukes recovered from the test and control groups.

Table 1. Resistance of immunized rats to challenge with *Fasciola hepatica*.

(Figures show the number of flukes recovered from individual rats at 3 weeks post-challenge. Two rats in the fusion protein immunized group died prior to challenge. Application of the Mann–Whitney *U* test shows the 2 groups to be significantly different ($P = 0{\cdot}034$).)

Flukes recovered	
β-galactosidase immunized rats (control)	Fusion protein immunized rats
14	5
5	8
10	4
12	1
3	10
8	8
10	13
8	8
8	3
13	3
5	5
11	2
10	8
7	
9	

RESULTS

Screening of libraries and sequencing of clones

More than 40 clones were identified as positive in screening, we have obtained partial sequences for inserts in all of these and complete sequences for a smaller number of clones. The inserts had the following features. A repeating element of 18, 21, 24, 27 or 30 nucleotides which was located 5′ to a relatively conserved nucleotide sequence of approximately 200 bp. The repetitive element was followed in some clones by a polyadenylation signal and poly A tract. The repeating element, which was generally repeated 4–7 times but could be present as 30 repeats in some clones, was preceded by 24 bp 3′ to a 30 bp sequence showing homology (67 % at the nucleotide level) with a consensus sequence of *F. hepatica* and

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Schistosoma mansoni 5′ trans-splice leaders. An example of the sequence of one of these clones (D6) is deposited with Genbank (Accession No. AF153056).

Examples of the repeating elements and the predicted encoded amino acid sequence found in a representative selection of the clones are shown in Fig. 1. The repeating elements appear to be built around a 6 nucleotide motif RRRXCA (where R represents purines) as demonstrated in Fig. 2. These may be interspersed with 5′ extended form of RRRXCA which appears to occur at regular intervals.

Fig. 3 depicts a representative selection of the derived carboxy terminals of the clones.

Localization of immunoreactive sequences

Sera from mice immunized with protein purified from clone c200 (containing 4 repeats and the majority of the non-repeating C-terminal region) which expresses a β-galactosidase fusion with a 63 amino acid residue as a C-terminal extension was applied to sections of juvenile and adult flukes. In juvenile flukes (4 weeks post-infection) binding was seen throughout the tegumental syncytium and in the cytoplasm of the tegumental cells (Fig. 4A). There was also binding present in the gut caecum (not shown). In adult tissue (15 weeks post-infection) there was little binding to the tegument but bright fluorescence in the uterine wall (Fig. 4B). Control antisera raised against β-galactosidase gave no reactivity.

Immunoprotective activity of the fusion protein

The number of flukes obtained from the rats immunized with either β-galactosidase or the fusion protein is shown in Table 1. There was considerable variation in fluke yield within the groups but the fusion protein immunized group were significantly less susceptible to challenge infection ($P = 0{\cdot}034$, Mann–Whitney *U* test).

DISCUSSION

The results presented in this study are in agreement with the hypothesis (Hanna & Trudgett, 1983) that infection by *F. hepatica* induces antibodies directed in the main against a tegumental component. All inserts identified by immunoscreening of cDNA libraries contained a family of related sequences with several notable characteristics. The outstanding feature of these cDNA clones was the presence of tandemly repeated thymidine-deficient sequences coding for proline residues embedded in polar amino acids. There was conservation of the sequence at the nucleic acid level and deviation from the usual pattern of codon usage–proline in the repeating element was always encoded by CCA although this codon is only used for 40% of proline residues in other known *Fasciola* sequences (Panaccio & Good, 1998). The mechanism used to generate and maintain these sequences is unclear but almost all repeated sequences analysed so far are built up from repeats of the motif RRRXCA or an extension of this. Expressed sequences with tandemly arranged repeats have been reported in *F. hepatica* by Marin and colleagues (Marin *et al*. 1992). In this case the repeating element, which does not appear to be related to the repeats described in this report, was expressed in the gut.

There was diversity in the repeating element reported here and also in the carboxy-terminal non-repeating element. We have not determined whether this diversity was intra- or inter-fluke as the libraries were generated from several flukes. The most diverse sequences were obtained from flukes derived from cattle infected in the wild rather than rats infected with laboratory strains but this may reflect the development of our screening procedures rather than being a true representation of natural diversity.

The presence of what appears to be a trans-spliced leader at the 5′ end of some of the clones is of note. It has been suggested that the utilization of this mechanism allows the parasite to undergo rapid and co-ordinated switches in gene expression and thus differentiation (Panaccio & Trudgett, 1999). This would be in accordance with our understanding of the development of *F. hepatica* in its mammalian host. Antisera raised against the fusion protein derived from one of the clones identified by immunoscreening bound to the tegument and tegumental cells of juvenile flukes and the reproductive system of adults. This pattern of binding has been observed previously with sera from infected animals (Keegan & Trudgett, 1992) and we have postulated that the continued production of secretions in the bile duct by the adult fluke which share epitopes with tegumental components may serve to maintain immunity to infection in the gastrointestinal tract. As β-galactosidase fusion proteins are not glycosylated the epitopes in the fluke sections binding the antisera must be proteinaceous. A FASTA (Pearson & Lipman, 1988) search of the translated EMBL data bank (TREMBL) using several of the repeated elements revealed significant homologies with the IgG and IgE immunoreactive antigen recognized by sera from patients with strongyloidiasis (Ramachandran *et al*. 1998) and suggests that the repeating elements may function as epitopes. While the non-repeating carboxy ‘tail’ of the peptide may contain additional epitopes it also appears to have a conserved region around a potential protein kinase C phosphorylation site (SRL Fig. 3). Analysis of the derived sequence using an algorithm (Feller & de la Cruz, 1991) designed to predict T cell epitopes revealed that this region (GGTSRLTN) would form an amphipathic helix and, as such, may be

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capable of interacting with T cells and antigen-presenting cells. Rats immunized with a total of 100 μg of the β-galactosidase fusion protein containing 4 repeating elements from clone c200 and the non-repeating carboxy 'tail' were more resistant to challenge infection by *F. hepatica* than were β-galactosidase immunized controls. Whilst only partial immunity was induced this was comparable to that reported for other anti-*Fasciola* vaccines (Spithill & Dalton, 1998) and indicates that the epitopes associated with this protein are associated with the modulation of the host response to infection by the fluke.

There are several other features of the encoded sequence of the immunoreactive clones which may influence their interaction with the immune system of the host. If the repeating elements function as B cell epitopes as suggested above then each molecule would be able to bind several immunoglobulin molecules. This would be comparable to the well-characterized immunoevasive strategy employed by *Plasmodium* spp. (Godison *et al*. 1984) and would provide an example of a metazoan and an alveolate parasite evolving the similar immunoevasion molecules. The location of these antigens in the tegumental syncytium may mean that they are continuously shed by the fluke (Hanna, 1980*a*) during its migration through the host. This would act as an extremely efficient immunoevasive mechanism whilst the fluke was accessible to the full armamentarium of the immune system. On reaching the bile duct immunological pressure on the parasite is believed to be reduced and changes occur in the tegument to enable it to take on new functions (Hanna, 1980*b*). This may be reflected in the greatly reduced level of binding to the tegument seen when anti-fusion protein antisera was applied to sections of adult worms.

We observed a degree of protection against challenge following the inoculation of a fusion protein containing the repeating elements. It is probable that the interaction between the liver fluke and the immune system of its mammalian host is both complex and mutually interactive and the high degree of variation in challenge fluke numbers seen in individual rats may be a reflection of this complexity. This result, however, was obtained using a fusion protein which expressed the repeating element but not the motif Proline-Lysine-Proline (PKP) found in several of the other repeating elements. This motif, as part of the Substance P neuropeptide, has been reported to bind to mast cells and induce degranulation (Mazurek *et al*. 1981). The expression of repeating elements with the PKP motif introduces the possibility that the shed tegumental components act not only passively as repeating epitopes for B-cells but also actively with mast cells. There is some evidence that such mechanisms may be operative. Mast cells are known to increase in response to infection by *F. hepatica* (Charbon *et al*. 1991) and their degranulation promotes neutrophil infiltration and the release of a range of cytokines including IL-4, IL-5 (which promotes eosinophil differentiation) and tumour necrosis factor-α (Gordon & Galli, 1991; Bradding *et al*. 1993). Injection of athymic rodents with excretory–secretory (E–S) products from flukes has been reported to induce eosinophil production (Milbourne & Howell, 1997). Infections by *F. hepatica* are characterized by eosinophilia (Doy, Hughes & Harness, 1978; Burden *et al*. 1983; Milbourne & Howell, 1990; Keegan & Trudgett, 1992) and it has been recently shown that protection against infection by *F. hepatica* in rats is mediated by eosinophils in gut tissue (van Milgren *et al*. 1999) although their role in immunoprotection in the later stages of fascioliasis is less well defined. It is therefore possible that the cellular infiltration of the 'tracks' of the fluke seen in infected animals and other manifestations of cell-mediated immunity such as the predominance of T helper 2 stimulated responses are not directed exclusively by the immune system of the host but as part of an immunoevasive strategy that has evolved in the parasite.

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