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Fasciola hepatica:

Identification of CD4⁺ T-Helper Epitopes from the 11.5kDa Saposin-like Protein SAP-2 Using Synthetic Peptides

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Abstract

Fasciola hepatica saposin-like protein (FhSAP-2) is a novel antigen expressed at an early stage of infection and has been shown to induce in rabbits a significant protection to infection with *F. hepatica*. There are no studies to identify the immunologically relevant regions of FhSAP-2. In this work the amino acid sequence of FhSAP-2 was analyzed to identify potential T-cell epitopes. A predictive algorithm identified 4 possible sites. Experimental determination of the T-cell epitopes was achieved using a panel of overlapping peptides spanning the entire sequence of FhSAP-2, which was evaluated for their ability to induce lymphoproliferative responses of spleen cells from 8 immunized BALB/c (H-2^d) mice. Five different epitopes were identified. There was minimal agreement between theoretical and experimental approaches. It was found that peptides containing amino acid residues AVTFA and IDIDLCDICT as part of their structure induce high levels of IL-2 and IFN γ in vitro and was classified as Th1 epitopes. Peptides that contain the residues ADQTV, CIEFVQQEVD and YIIDHVDQHN induced significant amount of IL-4 and IL-2 were considered as containers of Th0 epitopes. Identification of prominent T-cell epitopes from FhSAP-2 offers the possibility of understanding how the CD4⁺ T cell response is involved in protection against fasciolosis and how it is implicated in susceptibility to infection.

Keywords

Fasciola hepatica saposin-like protein family; Mapping of T-cell epitopes

Introduction

Fasciolosis caused by *Fasciola hepatica*, represents a recognized unsolved agricultural problem responsible for economic losses estimated around \$3 billion US per year to rural agricultural communities and commercial producers worldwide, including the United States (Chen and Mott, 1990; Espino and Duménigo, 2003). Recent reports indicate that *F. hepatica* is also a major human pathogen and is increasingly recognized as causing significant human diseases, with 2-4 million people infected with this parasite (Chen and Mott, 1990;

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Bjorland *et al.* 1995; Espino and Duménigo, 2003). Although the flukicide triclabendazole is an effective drug for the treatment of fasciolosis, the prohibitive cost of treatment and the drug resistance of parasites have become a problem (Suhardono *et al.* 1991; Overend and Bowen, 1995; Fairweather 2005). Several potentially protective antigens have been isolated and one of these is a secreted protein named FhSAP-2 (Hillyer, 2005). It is an 11.5kDa polypeptide belonging to the *F. hepatica* saposin-like protein family (Espino and Hillyer, 2003). Other saposins have been identified in *F. hepatica* and *F. gigantica* (Reed *et al.* 2000; Grams *et al.* 2006).

Recombinant FhSAP-2 prepared from *Escherichia coli* was used to immunize rabbits and induced a worm reduction of 83% (Espino and Hillyer, 2004). Mice vaccinated with a DNA vaccine expressing FhSAP-2 induced a mixed Th1/Th2 response (Espino *et al.* 2005). Therefore it can be suggested that FhSAP-2 contains T-cell epitopes, some of which may elicit protective responses. However, the effects of protection from the Th1 or Th2 polarization induced by FhSAP-2, which will lead to induction of cellular and humoral immunity, are still not clear nor there is a report on the identification of T cell epitopes from FhSAP-2 antigen. In order to localize murine T-cell epitopes on the FhSAP-2 protein overlapping synthetic peptides spanning the entire sequence of FhSAP-2 were used to detect *in vitro* proliferative responses from BALB/c (H-2d) immunized with peptides or recombinant protein. Determination of the cytokines IL-2, IL-4 and IFN γ secreted by sensitized lymphocytes in culture as well as the type of antibody response elicited as consequence of immunization, served as criteria for classification of epitopes into the Th1 or Th2 phenotype.

Material and Methods

Recombinant FhSAP-2 preparation

FhSAP-2 was cloned into pBAD HisB, expressed in *E. coli* as fusion protein with a His-tag and then purified by Ni²⁺ column affinity chromatography as previously described (Espino and Hillyer, 2003). Protein concentrations were determined using the Pierce BCA reagent according to the manufacturer's instructions.

Epitope prediction and structural analysis

The amino acid sequence of the protein moiety of FhSAP-2, as deduced from the nucleotide sequence of its encoding cDNA, was entered into the AMPHI software for prediction of T-cell Class II epitopes (Margalit *et al.* 1987). The FhSAP-2 sequence was also analyzed for solvent accessibility using the PHDacc program (Rost and Sender, 1994) and for secondary structure using the SOPM program (Geourjon and Deleage, 1994).

Peptide synthesis and carrier coupling

A series of 17 peptides spanning the full length 101-amino acid sequence of FhSAP-2 was synthesized. Peptides were synthesized as 15-mers, with adjacent peptides overlapping by 10 amino acids and termed sequentially as P2 to P18. The N-terminal peptide (P2) was synthesized as a 10-mer without overlapping with other peptide. Peptide synthesis was carried out by Fmoc chemistry under continuous flow conditions using PEG-Polystyrene resins. At the completion of synthesis, peptides were cleaved from the resin and de-protected using TFA/DTT/H₂O/Triisopropyl silane (88/5/5/2) cleavage cocktail. Peptides were then precipitated from the cleavage cocktail using cold diethyl ether. The precipitate was washed three times with the cold ether and then dissolved in a crude buffer containing H₂O/ACN/HOAc (75/20/5) prior to lyophilization. The quality of peptides was analysed by reverse phase chromatography on a Supelco Bio Wide Pore column and by MALDI-TOF mass spectrometry. To increase the immunogenicity of peptides five mg of each peptide was coupled to keyhole limpet hemocyanin (KLH; Pierce, Rockford, III). Synthetic KLH-peptide conjugates were suspended

in acidic or basic 0.1M phosphate buffer (pH 6.0 or pH 8.0) and pooled such that each peptide was at a final concentration of 70µg/ml.

Immunization of mice

Female BALB/c (H-2d) mice aged 6-8 weeks were purchased from Harland Inc, Indianapolis, Indiana, and kept under conventional germ-free conditions in the animal care facility of the University of Puerto Rico, School of Medicine and treated according to international regulations for the care of laboratory animals. A group of eight mice received three injections of 20µg of FhSAP-2 emulsified in classical Titer Max adjuvant (Sigma, St. Louis, USA). Another group of mice received three injections with 70µg of a pool of peptides emulsified in the adjuvant. All the injections were given 15 days apart and mice were necropsied 15 days after the last immunization. A negative control group received three injections of KLH in PBS emulsified in adjuvant. Mice were periodically bled from the retro-orbital venous plexus to determine, respectively, anti-FhSAP-2 or anti-peptide antibody levels. Serum samples were stored at -20°C until used.

Medium and reagents

The medium used for all cell cultures was RPMI-1640 (Gibco, USA) supplemented with fetal calf serum (final concentration 10%), L-glutamine (final concentration 2mM), 2-mercaptoethanol (5×10^{-5} M), HEPES (25mM), ampicillin (100 U/ml) and streptomycin (100µg/ml). Concanavalin A (ConA) was obtained from Sigma (St. Louis, USA).

Preparation and cultivation of spleen cells

At necropsy the spleen from mice was removed aseptically. The suspension of single spleen cells was prepared after removing erythrocytes by hypotonic lysis and resuspended in RPMI-1640 medium by vigorous pipetting. The single cell suspension was spread and added in triplicate at 200µl/well (4×10^5 cells/ml) into the 96-well flat-bottomed tissue culture plates at, then cultured at 37°C in a humidified atmosphere with 5% CO₂.

Splenocyte proliferation assay

After a preliminary experiment to determine the optimal peptide concentration for *in vitro* stimulation, wells containing the cell suspension (4×10^5 cells/ml) in RPMI-1640 medium were stimulated with 5µg/ml of FhSAP-2, 10µg/ml of each single peptide, or 10µg/ml of ConA and incubated at 37°C in 5% CO₂. After incubation for 72hrs, [³H] Thymidine ([³H]TdR) was added at 0.5µCi/well. The cells were cultured continuously for an additional 16hr and harvested. The incorporated [³H]TdR was measured in a liquid scintillation counter (Epics XL, Beckman Coulter) after harvesting the cell cultures onto glass filters. The incorporation of thymidine calculated as mean counts per minute (CPM) was an indication of cell proliferation. The stimulation index was determined by dividing the CPM incorporated by antigen stimulated cells by the CPM incorporated by the same cells without antigen stimulation. A stimulation index of greater than 2 was considered a positive response and peptides that stimulated the proliferation were considered as 'responder peptides'. The stimulation index induced by each peptide was compared with the negative control group using Student's *t*-test and responder peptides with *P*<0.05 were classified as broadly reactive Th epitopes.

Flow cytometry analysis

To investigate the cell population that proliferates in response to immunization with a single responder peptide, groups of 5 mice each were individually immunized with those peptides that in the proliferation assay induced a stimulation index above cut off. Each injection was performed with 70µg of single KLH-peptide emulsified in adjuvant and 15 days after the last immunization animals were necropsied for spleen collection and lymphocyte isolation as

described above. For immunofluorescence staining with a single labeling, 10^6 cells were incubated with fluorescein isothiocyanate (FITC)-conjugated rat monoclonal antibodies against CD3, CD4 or with phycoerythrin (PE)-conjugated rat monoclonal antibody against CD8 (Pharmingen, San Diego, CA, USA) for 30min at 4°C. Flow cytometry analysis was performed with a FACSort (BD Biosciences, San Jose, CA) equipped with a 488 nm argon laser and detectors for forward scatter (FSC) and 90° light scatter (side scatter, SSC) and for FL1 (band pass filter wavelength 530 nm) and FL2 (585 nm) fluorescence emission in the green and red/orange respectively. Splenocytes stained with single colors, with FITC- or PE-labelled antibodies were detected by FL1 or FL2, respectively. Splenocytes were identified by their characteristic appearance on a dot plot of FSC versus SSC and electronically gated to exclude platelets and dead-cell debris. T-cells were gated on the basis of CD3 staining. Significant differences between the mean % of CD4⁺ compared to the % of CD8⁺ cells are indicated with an asterisk ($P<0.05$).

***In vitro* cytokine detection**

For *in vitro* cytokine detection, the splenocyte cell suspension (4×10^5 cells/ml) was dispensed in triplicate into 96-well flat bottomed tissue culture plates at 200 µl/well, and incubated at 37°C with 5% CO₂ in the presence of 10 µg/ml ConA, 10 µg/ml synthetic peptide or 5 µg/ml FhSAP-2. After 72hr, the supernatants were harvested for detection of IL-2, IL-4 and IFN γ . Cytokine levels were determined using an ELISA Kit (R & D Diagnostic, MN) according to the manufacturer's instructions. Each cytokine determination was performed in triplicate and the results expressed as mean of the cytokine concentration for each determination. Results were analyzed with Student's *t*-test. P values of 0.05 or less were considered to be significant.

ELISA for antibody detection

Sera from immunized mice were tested by ELISA for measuring the levels of peptide-specific total IgE, or IgG as well as levels of the peptide-specific IgG₁ and IgG_{2a} antibody isotype. Assays were performed essentially as previously described (Espino et al. 2005). Briefly, microtiter plates (Costar, Corning Costar Corp, Cambridge, Mass.) were coated overnight at 4°C with single peptide (20 µg/ml) in carbonate buffer. Non-specific reactions were blocked with 3% albumin in PBS containing 0.05%-Tween 20 (PBST). To determine total IgG and IgE antibody as well as IgG₁ and IgG_{2a} antibody titres the serum samples collected were serially diluted in PBST. The detecting antibodies were rat anti-IgE mouse or goat anti-IgG mouse peroxidase conjugate. Color was developed using *o*-phenylenediamine as substrate and the OD of each well was measured at 492nm. Each antibody assay was repeated three times and the tests were conducted on two different days. The results were expressed as inverse of antibody titres. Titre of antibody was defined as the maximum dilution that renders absorbance value over the cut off value established for the assay (mean OD $\pm$ 2SD = 0.2) calculated after testing sera from non-vaccinated mice. Statistical analysis of the data was also done by using Student's *t*-test.

Results

Structural analysis and theoretical prediction of epitopes in FhSAP-2

The AMPHI program identifies regions of amphipathic helix that have the potential to act as T-cell epitopes according to the charge and polarity pattern (Margalit et al. 1987). Four such regions were identified in the amino acid residues 21-30, 41-50, 56-70 and 81-90 of FhSAP-2 (Figure 1). The SOPM Program identified five α -helix regions in the protein moiety of FhSAP-2 which extended from amino acid residues 3-18; 30-46; 49-63; 69-88 and 91-98. Analysis of the sequence for solvent accessibility using the PHDacc algorithm predicted that, with the exception of some regions, most of the amino acid residues within FhSAP-2 protein are accessible to solvent (data not shown).

Experimental identification of T-cell epitopes

The T cell epitopes recognized by cells from mice immunized with peptides were determined using proliferation assays with the set of 15-mer overlapping peptides. A total of 11 from 17 peptides induced proliferation (Figure 2). Peptides P3 (residues 11-25), P5 (residues 21-35), P6 (residues 26-40), P10 (residues 46-60), P14 (residues 67-81), P15 (residues 71-85), and P17 (residues 81-95) showed significantly greater stimulation ($P<0.05$) than the baseline values obtained with the other peptides. Peptides P4 (residues 16-30), P7 (residues 32-45), P9 (residues 41-55) and P16 (residues 76-90) showed a stimulation index above the cut off but their mean value was not statistically different to the control value. Peptides P2 (residues 1-10), P11 (residues 51-65), and P18 (residues 87-101) did not induce proliferation. Overall, responder peptides from the N-terminal region (P3, P5 and P6) induced a significantly higher proliferation index than responder peptides from the C-terminal region (P14, P15 and P17) ($P<0.05$). Peptide P10 showed a stimulation index of magnitude similar to those observed with peptides from the N-terminal region.

Individual animals showed similar T-cell proliferative responses towards FhSAP-2 but showed a wide diversity in both the responses to the peptides which induced proliferation of their T-cells as well as the magnitude of the proliferation induced. Mice immunized with FhSAP-2 also recognized at least one peptide. Cells from FhSAP-2 immunised mice proliferated in response to FhSAP-2 but some failed to proliferate in response to any of the synthetic peptides. Several peptide epitopes (P3, P5, P6, P10, P14, P15 and P17) were recognized by more than 3 animals. In most cases the proliferation with peptides was lower than with FhSAP-2. All the epitopes which were identified experimentally are shown in Figure 1. An epitope not predicted by AMPHI algorithm was identified spanning residues 11-15 (peptide P3). Three peptides capable of causing proliferation contained the region 21-30 identified by the algorithm (P4, P5 and P6). Another two peptides (P9 and P10) contained the region 41-50, and peptides P16 and P17 contained the region 81-90 identified by the AMPHI algorithm. Epitopes on peptides P5, P6 and P10 were found six amino acids shifted from their predicted position. The theoretical epitope predicted by the algorithm within region 56-70 was not experimentally found within responder peptides.

Flow cytometry analysis of T splenocytes

Overall, the percentage of CD4⁺ cells was higher than percentage of CD8⁺ in animals vaccinated with FhSAP-2 or vaccinated with most peptides as compared to controls. Peptide P3 elicited a higher percentage of CD8⁺ cells relative to CD4⁺ cells (Figure 3).

Cytokine production by proliferating T cells

To evaluate the cytokine profile in response to synthetic peptides, spleen lymphocytes from immunized mice were obtained and cultured in the presence of single peptide the culture supernatants were collected and stored to determine the levels of cytokines produced. IL-4 was used as a Th2 cytokine marker and IL-2 and IFN γ as a Th1 cytokine signature. As expected, peptides that did not induce proliferation failed to induce detectable amounts of cytokine (data not shown). T-cells stimulated with peptides P3, P5, P6, P14 and P15 secrete significantly higher levels of IL-2 and IFN γ than IL-4 into the culture supernatants. By contrast, T-cells stimulated with P4 and P9 secreted higher levels of IL-4 than IL-2 or IFN γ . T-cells stimulated with P10 and P16 secreted significantly higher levels of IL-4 than IFN γ but similar levels of IL-2 and IL-4. T-cells stimulated with P7 and P17 secreted similar amounts of all cytokines (Figure 4).

Antibody reactivity to single peptides

The specific IgG and IgE antibody was evaluated by ELISA in the serum of peptide-immunized animals. Total antibody titres against peptides P3, P4, P5, P6, P9, P10, P14, P15, P16 and P17 were detectable after the first immunization and increased steadily after each injection to reach titers that ranged from 1/5,000 to 1/25,000 (data not shown). Antibody titres of 1/500 to 1/3,500 were detected against peptides P7, P8 and P18 but they were not significantly different from controls (data not shown). Peptides P2, P11 and P18 were not reactive against sera from immunized mice (data not shown). High titers of IgE antibody ($>11,000$) were detected against P3, P4, P10 and P16. Low titers of IgE (from 3000 to 7000) were detected against peptides P9 and P17. No specific IgE was observed with peptides P5, P6, P7, P14 and P15 (Fig. 5A). When total IgG titres were dissected between IgG_{2a} and IgG₁ subclasses similar antibody titres of both antibodies against most peptides were observed without an apparent predominance of an isotype over another. The pattern of IgG isotypes observed against responder peptides is shown in Figure 5B.

Discussion

It is believed that the epitope-induced Th1-type response may play an important role in anti-*Fasciola* immunity in ruminants by producing cytokines such as IFN γ and IL-2 (Oldham and Williams, 1985; Mulcahy *et al.* 1998; Zhang *et al.* 2005). Identification of T-cell epitopes from *Fasciola* antigens offers the possibility of understanding how the CD4⁺ T cell response is involved in protection against *F. hepatica* and how it is implicated in susceptibility to infection. To our knowledge, there are no studies directed to the characterization of T-cell epitopes from *Fasciola* antigens. Recently, we reported the characterization of a novel *F. hepatica* antigen termed FhSAP-2, which possesses several interesting features that make it an attractive molecule for vaccination (Espino and Hillyer, 2003). FhSAP-2 appears to be highly immunogenic in rabbits and mice. By its structural homology with the amoebapores of *Entamoeba histolytica* (Leippe *et al.* 1994; Zhai and Saier, 2000), *F. hepatica* (Reed *et al.* 2000), porcine NK-lysin (Andersson *et al.* 1995) and several other related proteins (O'Brien and Kishimoto, 1991; Sano *et al.* 1992; Bruno *et al.* 1995; Stenger *et al.* 1998) FhSAP-2 fall in the saposin-like / NK-lysin protein family of *F. hepatica*. In addition, this molecule exhibits a potent lytic activity on human erythrocytes and peripheral blood mononuclear cell (Espino and Hillyer, 2003), and induces partial protection against a challenge *F. hepatica* infection in rabbits (Espino and Hillyer, 2004). Correct identification of epitopes in FhSAP-2 will help in the characterization of targets peptides for immunization-vaccination experiments. The aim of this study was to identify T-cell epitopes on the protein moiety of FhSAP-2 recognized by spleen lymphocytes of BALB/c (H-2d) mice and determine which epitopes induce a Th1 or Th2 phenotype.

The outcome of an immunological response to infection or immunization can be determined by the degree of activation in at least two distinct CD4⁺ T helper cell subsets, T helper cells type 1 (Th1) and T helper cells type 2 (Th2) following antigen stimulation (Murphy *et al.* 2002). Both Th1 and Th2 responses can be induced during the course of an acquired immune response and the series of events which take place are controlled by a network of immune hormones termed cytokines. Generally Th1 cells secrete type 1-like cytokines (i.e. IFN γ , IL-2 and IL-12) that induce the production of IgG_{2a} from B-cells (Romagnani *et al.* 1999). In contrast, Th2 cells secrete type 2-like cytokines (i.e. IL-4, IL-5, IL-13) which stimulate the production of IgE and IgG₁ antibodies from B-cells (Romagnani *et al.* 1999, Jankovic *et al.* 2001; Mena *et al.* 2002, Murphy *et al.* 2002; Maizels *et al.* 2004). Cross-regulation can occur between the two T helper cells subsets where the cytokines produced by one subset can inhibit the production and / or activity of cytokines produced by the other subset and, consequently, polarizes the overall predominating immune response (Mosmann *et al.* 1989). In the present

study we used the antibody responses elicited against each single peptide, together with the determination of IFN γ , IL-2 and IL-4 cytokine levels for defining the Th1 or Th2 phenotype of the epitopes on FhSAP-2.

The study proceeded in two steps. First, the entire amino acid sequence of FhSAP-2 was analysed using software to predict the localization of potential T-cell epitopes and then, we attempted to confirm this prediction with an experimental study using synthetic overlapping peptides that span the entire amino acid sequence of FhSAP-2. Our experimental study was capable of identifying one epitope that was not predicted by the AMPHI algorithm as well as determining that two of the observed epitopes are localized six-amino acids downstream from their predicted position. Algorithms use only primary sequence data to predict epitopes, making no allowance for secondary or tertiary structure. For this reason many authors use the peptide synthesis method for decreasing the possibility of missed epitopes (Williams et al. 2001; Sarkar et al. 2002; Schulze et al. 2003; Gaspole et al. 2005).

One of the most reactive peptides from the N-terminal region is P3 (residues ¹¹AVTFASFDVPSKQPT²⁵), which induced a high stimulation index within T-cells from all 8 animals studied. This peptide shares the residues ¹⁶SFDVPSKQPT²⁵ with the poorly reactive peptide P4 (residues ¹⁶SFDVPSKQPTIDIDL³⁰). Thus, the residues AVTFA only contained in P3 appear to determine the high stimulation index of this peptide. Since P3 induced a Th1-like cytokine pattern characterized by significant amount of IL-2 and IFN γ we considered that P3 contains a dominant Th1 epitope present in residues ¹¹AVTFA¹⁵ or which overlap the boundaries of the P3/P4 peptides, that was not predicted by the algorithm. The cytokine profile secreted by cells stimulated by P4 (higher IL-4 than IL-2 or IFN γ) as well as the high levels of IgE detected are typically a Th2 response. However, since this peptide showed a very low proliferation index; we considered that the ¹⁶SFDVPSKQPT²⁵ epitope contained in P4 is a non-dominant Th2 epitope. Peptide P3 overlaps with the Th2 epitope identified on P4, and this could explain the high levels of IgE observed against P3.

On the other hand, peptide P5 (residues ²¹SKQPTIDIDLCDICT³⁵) shares the residues ²⁶IDIDLCDICT³⁵ with peptide P6 (residues ²⁶IDIDLCDICTNTMDV⁴⁰). Both peptides were shown to be highly reactive and to induce higher amount of IL-2 and IFN γ , than IL-4 as well as an antibody response characterized by very low levels of IgE and higher levels of IgG_{2a} and IgG₁. Therefore we considered that peptides P5 and P6 contain a dominant Th1 epitope within residues ²⁶IDIDLCDICT³⁵. According to the AMPHI algorithm this epitope was predicted on residues 21-30, however, we found it six amino acid residues downstream from its theoretical position.

Other important region of FhSAP-2 moiety was identified within residues ⁴⁶ADQTVEEHIGYLVKY⁶⁰ of peptide P10, which induced proliferation in T-cell from most animals. Interesting, peptide P10 share residues ⁴⁶ADQTVEEHIG⁵⁵ with the poorly reactive peptide P9 and share the residues ⁵¹EEHIGYLVKY⁶⁰ with the non-reactive peptide P11. In addition, large amount of IgE antibody were detected specifically against this peptide. Therefore, we considered that the third T-cell epitope of FhSAP-2 is localized within the residues ⁴⁶ADQTV⁵⁰ or overlaps the boundaries of the P9/P10 peptides. This experimental epitope also appears to be six amino acids downstream from its predicted position (residues 41-50). Since P10 induced high levels of IL-4 and IL-2 it appears to contain a dominant Th0 epitope.

Another theoretical epitope was predicted within amino acid residues 56-70 but our data did not confirm this region as a T-cell epitope. These residues are entirely contained within peptide P12 (residues ⁵⁶YLVKYLCKIARSQDA⁷⁰) and partially contained within the peptides P11 and P13 which were classified as non-responders. The highly reactive peptide P14

(residues ⁶⁶RSQDACIEFVQQEVD⁸⁰) shares the residues ⁷¹CIEFVQQEVD⁸⁰ with the highly reactive peptide P15 (residues ⁷¹CIEFVQQEVDYIIDH⁸⁵). We consider that the epitope contained within residues ⁷¹CIEFVQQEVD⁸⁰ is dominant. The cytokine profile induced by P14 and P15 appear to be consistent with a Th0-type epitope to judge by the similar amounts of IL-2, IFN γ and IL-4 observed. In addition, these peptides elicited a very low IgE antibody response with a balanced IgG_{2a}/IgG₁ antibody response. On the other hand, peptide P16 (residues ⁷⁶QQEVDYIIDHVDQHN⁹⁰) shares the residues ⁸¹YIIDHVDQHN⁹⁰ with peptide P17 (residues ⁸¹YIIDHVDQHNATEIC⁹⁵) and P17 shares the residues ⁸⁶VDQHNATEIC⁹⁵ with the non responder peptide P18. Since P17 is a highly responder peptide that induced similar amount of all cytokines we considered that residues ⁸¹YIIDHVDQHN⁹⁰ are a Th0 epitope. Importantly, the last theoretical epitope predicted by AMPHI algorithm was identified exactly within this region.

In a previous study in which the lineal B-cells epitopes on the protein moiety of FhSAP-2 were mapped, we demonstrated the presence of two dominant lineal B-cell epitopes within amino acid residues 21-30 (P4) and 76-85 (P16) that are highly reactive with sera from rabbits and mice infected with *F. hepatica* and *Schistosoma mansoni*, respectively (Torres and Espino, 2006). In the present study we demonstrated that these B-cell epitopes are flanked and overlapped with Th epitopes. Therefore, it is obvious that T-cell epitopes described herein may interact with such B-cell epitopes, influencing the repertoire and specificity of antibody producing cells.

It is interesting to highlight that four from the five dominant epitopes were restricted to the amino terminal (peptides P3, P5-P6) and carboxy terminal (peptides P14-P15 and P16-P17). This pattern of epitope distribution appears to be a function of the tertiary structure of the antigen and is in accord with the finding of Meister et al. (1995) who reported that T cell epitopes have a striking tendency to cluster within short segments of an antigen. Sercarz et al. (1993) proposed that T-cell epitopes are predominantly located on the most accessible regions of an antigen and that these accessible regions are rapidly bound by MHC molecules while the remainder of the antigen is discharged. Epitopes recognized within FhSAP-2 appear to follow this hypothesis. The FhSAP-2 molecule is a polypeptide that contains six predicted α -helical domains (Espino and Hillyer, 2003). The α -helical domain localized towards the N-terminal region of the protein contains a large number of hydrophobic amino acids (P2: residues ¹MNPLFVLM¹⁰). As it was expected, this region is predicted to contain few surface accessible residues.

Previous studies relating to the epitope mapping of *Schistosoma japonicum* 28kDa Glutathione S-transferase have shown T-cell epitopes to be around 21 amino acid residues long (Li et al. 2005). However, other studies have shown reactive T-cell epitopes relating to *S. mansoni*, *Plasmodium*, or other parasite antigens to be as short as 15 or even 10 residues (Hiroko et al. 2000; Caro-Aguilar et al. 2002; López-Moreno et al. 2003; Fonseca et al. 2005) similar to the size of the 10-mers IDIDLCDICT, CIEFVQQEVD, or YIIDHVDQHN described here. In addition, the epitope identified in peptide P3 appears to be shorter (AVTFA) than the others. It is necessary however; to perform mapping studies using peptides of shorter length, which overlap the peptide boundaries or which conform to known MHC binding motifs to determine the minimal epitope.

It is also important to highlight that our study was performed with a single genetic background mouse strain. Perhaps, some peptides considered herein as Th epitopes may or may not represent T cells epitopes in other mouse strains or animals such as sheep and cattle. Identification of promiscuous epitopes will be more beneficial for further vaccination studies. Therefore, it would be interesting to define the residues critical for CD4⁺ T cell activation and proliferation that could determine a similar activation and proliferation pattern with cells from

other mice of genetically different background, or sheep, as has been suggested by several authors (Hiroko *et al.* 2000; Williams *et al.* 2000; Schulze *et al.* 2003).

There are no previous reports about CD4⁺ T cell epitopes derived from *F. hepatica* antigens although Muro *et al.* predicted epitope on *F. hepatica* GST (Muro *et al.* 1993). Our knowledge about the cellular responses in human or animal infections, as well as of experimental models of *F. hepatica* is scarce, in spite of the demonstration that CD4⁺ T cell response plays a critical role in fasciolosis (Oldham and Williams, 1985). In this context, the reactivity of peptides P3, P5, P6 defined as Th1 epitopes offers the possibility of manipulating the outcome of CD4⁺ T cell responses in fasciolosis, for the development of a vaccine or to study the peptide specific CD4⁺ T cell receptor repertoire. It would also be necessary to determine how the immune response induced by Th1 peptides correlates with the level of protection induced by FhSAP-2 vaccine. Studies directed to address these important issues will be performed shortly.

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Abbreviations

FhSAP-2, *Fasciola hepatica* saposin-like protein; IL-4, interleukin 4; IFN γ , γ interferon; Th1, T-helper cell response type-1; Th2, T-helper cell response type-2; Con A, Concanavalin A.

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Figure-1.

Prediction of T-cell epitopes, and α -helical structure applied to the primary structure of FhSAP-2 (GenBank AF286903). The α -helix domains predicted by the SOPM algorithm are highlighted. The predicted T-cell epitopes appear underlined with a dashed line. Regions containing dominant T cell epitopes experimentally identified on the FhSAP-2 protein moiety appear boxed. Non-dominant epitopes appear underlined with a hard line. Dominant epitopes are regions capable of causing proliferation index above cut off (>2) with statistical differences ($P < 0.05$) from controls which were classified as broadly reactive Th epitopes peptides. Non-dominant epitopes are regions capable of causing a proliferation index above cut off with no statistical differences from controls.

Figure-2.

Typical proliferative response of T cells derived from BALB/c mice immunized with FhSAP-2 and a pool of seventeen overlapped peptides (P2 to P18) spanning the amino acid sequence of FhSAP-2. The stimulation index induced by FhSAP-2 or each peptide was compared with the negative control group (n=8 mice) across all the eight animal groups using Student's *t*-test. The horizontal dashed line represents a stimulation index of 2, the cut off for a positive response. Numbers above columns indicate the number of animals whose T cells were reactive with a given peptide. Asterisk over the bar indicates statistical differences ($P < 0.05$) between the mean proliferation index from experimental animals and controls.

Figure-3.

Percent of CD4⁺ and CD8⁺ in spleen cells from mice immunized with FhSAP-2 and single peptides that induced proliferation index above cut off (>2.0). Negative control (NC) represents immunized BALB/c mice with PBS in adjuvant. The values represent the mean $\pm$ SD obtained from each of three individual experiments. Significant differences between mean of CD4⁺ compared to CD8⁺ are indicated with an asterisk ($P < 0.05$).

Figure-4.

Cytokine secretion profiles of peptide-sensitized splenic cells stimulated by FhSAP-2 or single peptides. Animals were immunized three times s.c. with 20 μ g of FhSAP-2 or 70 μ g of pool of peptides. Fifteen days after the last injection, the splenocytes were prepared and assayed for *in vitro* secretion of cytokines after stimulation with FhSAP-2 (5 μ g) or single peptides (10 μ g). The cytokine levels are expressed as net mean $\pm$ SD of triplicate cultures after subtracting the mean of medium only. The results are representative of 3 separate experiments with similar results.

Figure-5.

(A) Titres of IgE antibody, and (B) IgG₁ and IgG_{2a} antibody isotypes induced to a given peptide in mice immunized with a pool of peptides. Results represent mean $\pm$ SD three independent experiments. The cut off value established for the assay (mean OD₄₉₂ $\pm$ 2SD = 0.2) was calculated after testing sera from non-vaccinated mice.