A Fasciola hepatica-derived fatty acid binding protein induces protection against schistosomiasis caused by Schistosoma bovis using the adjuvant adaptation (ADAD) vaccination system

Belén Vicente a, Julio López-Abána a,⇑, José Rojas-Caraballo a, Luis Pérez del Villar a, George V. Hillyer b, Antonio R. Martínez-Fernández c, Antonio Muro a

Laboratorio de Inmunología y Parasitología Molecular, Centro de Investigación de Enfermedades Tropicales de la, Universidad de Salamanca (IBSAL-CIETUS), Avda Licenciado Méndez Nieto s/n, 37007 Salamanca, Spain
Department of Pathology and Laboratory Medicine (Suite 617-A), University of Puerto Rico School of Medicine, P.O. Box 365067, San Juan, PR 00936-5067, Puerto Rico
Departamento de Parasitología, Facultad de Farmacia, Universidad Complutense, Pza. Ramón y Cajal s/n, 28040 Madrid, Spain

Highlights

- rFh15 protein induces a reduction in the parasite burden in S. bovis murine model.
- Vaccine effectiveness against S. bovis depends on adjuvant formulation.
- ADAD system using PAL as immunomodulator offer a new vaccination strategy.
- rFh15 formulated on ADAD system induced high levels of IL-1 and IL-6 cytokines.

Abstract

Several efforts have been made to identify anti-schistosomiasis vaccine candidates and new vaccination systems. The fatty acid binding protein (FAPB) has been shown to induce a high level of protection in trematode infection. The adjuvant adaptation (ADAD) vaccination system was used in this study, including recombinant FAPB, a natural immunomodulator and saponins. Mice immunised with the ADAD system were able to up-regulate proinflammatory cytokines (IL-1 and IL-6) and induce high IgG2a levels. Moreover, there was a significant reduction in worm burden, egg liver and hepatic lesion in vaccinated mice in two independent experiments involving Schistosoma bovis infected mice. The foregoing data shows that ADAD system using FAPB provide a good alternative for triggering an effective immune response against animal schistosomiasis.

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1. Introduction

Blood flukes from the genus Schistosoma are a significant cause of disease in tropical and subtropical regions, (WHO, 2011; Gryseels, 2012). Schistosoma infection in animals, especially Schistosoma bovis, continues to be a veterinary problem in many endemic areas in Africa and Asia although prevalence data has not been well documented (Vercruysse and Gabriel, 2005). S. bovis belongs to the Schistosoma haematobium group and both species share biological features and infect the same Bulinus intermediate host (Lawton et al., 2011; Webster et al., 2013). S. bovis is also considered an analogue of S. haematobium from an immunological point of view and has been successfully used in diagnosing human

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schistosomiasis (Pardo et al., 2004). Due to such similarities, S. bovis appears to be an interesting model in schistosomiasis vaccine development (Agnew et al., 1989a,b).

Praziquantel is currently the most effective drug for treating schistosomiasis; however, it does not prevent reinfection and has low efficacy against early stages (Rollinson et al., 2013). A recent meta-analysis has suggested that incorporating artemisinin derivatives into schistosomiasis treatment combined with praziquantel may improve cure rates in endemic areas (Pérez del Villar et al., 2012); linking vaccination with chemotherapy would reduce overall morbidity and limit the impact of re-infection (Bergquist et al., 2008). The rationale behind anti-schistosomiasis vaccine development has been based on the natural protection of animals and humans living in endemic areas; the efficacy of irradiated cercariae vaccines in experimental trials has thus been ascertained (Hewitson et al., 2005). Several proteins have been proposed as vaccine candidates, such as S. bovis glutathione-S transferase (GST) and 14–3–3, and Fasciola hepatica cross-reacting fatty acid binding proteins (FABP). Using S. bovis GST in classical Freund’s adjuvant has led to a reduction in faecal and tissue eggs in cattle (Bushara et al., 1993); whilst, a significant reduction of worm burdens and faecal eggs has been reported in goat and sheep (Boulanger et al., 1999) and reduced egg hatchability in mice (Viana da Costa et al., 1999). A large reduction in worm burden has been observed using F. hepatica native or recombinant FABP with Freund’s adjuvant in mice (Abán et al., 1999; Abáné et al., 2000).

Our group has proposed the “Adjuvant Adaptation” (ADAD) vaccination system as an alternative to using Freund’s adjuvant in vaccination against F. hepatica (Martínez-Fernández et al., 2004). The ADAD adjuvant system combines an antigen with non-haemolytic Quillaja saponaria (Qs) saponins as adjuvant and an immunomodulator together with non-mineral oil (Montanide ISA 763AVG) to form an emulsion for providing a long-term delivery system. A hydroalcoholic extract from the rhizome of the fern Phlebodium pseudoaureum (PAL) was used as immunomodulator as down-regulation has been shown in the Th-response in mice immunised with Anisakis simplex, Trichinella spiralis and F. hepatica antigens (López-Abán et al., 2012). Using of FABP formulated in the ADAD system with PAL as immunomodulator has led to a significant reduction in worm recovery and eggs in the faeces of sheep challenged with F. hepatica (Martínez-Fernández et al., 2004; López-Abán et al., 2007). Furthermore, vaccination with the S. bovis 14–3–3; protein using ADAD with PAL has resulted in a significant reduction in adult worm recovery and less liver damage in mice following homologous challenge (Uribe et al., 2007).

The present study has explored the immunological effect of the recombinant 15 kDa F. hepatica protein (rFh15) using the ADAD vaccination system with PAL as immunomodulator in BALB/c mice. rFh15 efficacy as a vaccine candidate was then investigated in two independent protection studies against S. bovis infection in BALB/c mice. Our results offer insights into an immunological response against S. bovis infection using rFh15 formulated in the ADAD vaccination system as a new vaccination alternative.

2. Materials and methods

2.1. Mice and parasites

Ninety seven-week old BALB/c female mice (Charles River Laboratories, Barcelona, Spain) weighing 18–20 g were used. They were kept in a temperature and humidity controlled environment throughout the experimental period; a 12 h light/dark cycle was used and the mice were given water and food ad libitum in the University of Salamanca’s Animal Experimentation facilities. All animals were treated according to the provisions of current European law regarding animal handling and experimentation. The procedures were approved by the Universidad de Salamanca’s Ethics Committee (Protocol n° 8402). All efforts were made to minimise animal suffering. The S. bovis strain from Salamanca (Spain) was maintained in the Animal Pathology Department (IRNASA-CSIC, Salamanca) in Planorbis metidjensis as intermediate host and sheep as definitive host (Oleaga and Ramajo, 2004); 4–7 mm of diameter snails were collected in the wild, individually infected with 5 miracidia and reared in batches of 200 individuals.

2.2. Antigen preparation

Soluble S. bovis (S0b) adult worm antigens (AWA) were produced, (Abán et al., 1999); worms were suspended in sterile phosphate-buffered saline (PBS), 20 worms/ml, with 1 mM phenylmethylsulphonyl fluoride (PMSF), homogenised, frozen and thawed thrice and then sonicated with three 1-min cycles at 70 kHz. The suspension was spun at 50,000g for 30 min at 4 °C. Protein concentration was determined by using a Micro BCA Protein Assay Kit (Pierce, Rockford, Illinois).

2.3. rFh15 recombinant protein expression

Total F. hepatica RNA was isolated from adult flukes using an RNeasy Protect Mini Kit (Qiagen GmbH, Hilden, Germany) and used for cDNA synthesis with a First Strand cDNA Synthesis Kit (Roche Diagnostic, Indianapolis, Indiana). The rFh15 gene was amplified using the following primers: forward 5’-CGATCTCATGGCTGACCTTTGTCGG-3’ and reverse 5’-CCGACGGCTTTGACGAGTG-3’ in 30 PCR cycles as follows: 40 s at 94 °C, 40 s at 52 °C, and 1 min at 72 °C (López-Abán et al., 2012) Restriction sites were added from BamHI on the forward primer and Xhol on the reverse one. PCR products were then purified with a StrataPrep DNA Gel Extraction Kit (Stratagene, Madrid, Spain) and cloned into pGEX-4T2 vector (Amersham Pharmacia Biotech, Uppsala, Sweden) with a Schistosoma japonicum GST sequence for further detection and purification by affinity chromatography. Recombinant plasmid DNA was purified using a Nucleo Spin Plasmid Kit (Macherey–Nagel, Düren, Germany) and then was sequenced at the Universidade Salamanca’s central Facilities to verify cloned insert integrity. pGEX-4T2 recombinant construct-containing Escherichia coli BL21 cells were grown for 12 h at 37 °C in 50 ml of Luria Bertani medium with 0.1 mg/ml ampicillin (final concentration); this culture was then used to inoculate 1 L of LB medium at 37 °C until reaching 0.600 absorbance. Recombinant protein expression was induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 5 h at 37 °C. The cells were obtained by spinning at 10,000g for 30 min at 4 °C. The protein was solubilised by adding PBS with 1 mM PMSF and 1% Triton X-100, followed by sonication. Supernatant plus recombinant protein was centrifuged at 10,000g for 3 min at 4 °C. The protein was purified by affinity chromatography with glutathione Sepharose 4B resin. Non-retained proteins were eluted with PBS and the rFh15 protein was eluted by adding PBS with 50 Unit/ml thrombin (Amersham Biosciences), fractions were analysed by SDS–PAGE and proteins quantified by using a Micro BCA Protein Assay Kit.

2.4. The ADAD vaccination system

The purified rFh15 protein was formulated in a micelle consisting of Quillaja saponaria non-haemolytic saponins (Qs, Sigma, St Louis) and Phlebodium pseudoaureum hydroalcoholic extract (PAL) (ASAC Pharmaceutical International Alicante, Spain). This micelle was then emulsified in non-mineral oil (Montanide ISA763A VG, SEPPIC, Paris, France) as an oil-in-water (70/30) formulation and injected subcutaneously. The ADAD vaccination system involved
a set of two subcutaneous injections (Martínez-Fernández et al., 2004); the first, (adaptation), contained Qs and PAL emulsified in the aforementioned non-mineral oil without the recombinant protein, whilst the second (administered 5 days after adaptation) contained the rFh15 protein with Qs and/or PAL in the emulsion oil. Individual doses per injection included 600 μg PAL, 20 μg Qs and 20 μg rFh15 in a final 200 μl emulsion injection volume.

2.5. Immunological assessment of rFh15 using the ADAD vaccination system in BALB/c mice: immunisation schedule and sample collection

Three groups of 6 female BALB/c mice were randomly divided into groups for exploring the immunological role of rFh15 in the ADAD vaccination system: untreated, treated with PAL+Qs and immunised with PAL+Qs+rFh15. Two boosters were administered on days 14 and 28 after the first immunisation. The mice were anesthetized with isoflurane and euthanized by cervical dislocation two weeks after the third immunisation. Their spleens were removed during necropsy and then perfused with sterile PBS for obtaining splenocytes for culture and cytokine profile analysis and to ascertain the splenocyte population by flow cytometry. Blood samples were collected for antibody detection before each immunisation and during necropsy.

2.6. Vaccination experiments

The vaccination trials involved cercariae from different snail batches in two independent experiments (A and B) at two different points in time. BALB/c mice were randomly divided into four groups of 9 animals as follows: G1 uninfected, G2 S. bovis infected, G3 injected with ADAD plus PAL+Qs and infected and G4 vaccinated with ADAD plus PAL+Qs+rFh15 and infected. A booster was given two weeks after the first immunisation; each mouse was challenged by tail immersion in 150 S. bovis cercariae for 45 min 2 weeks after the second immunisation. All mice were killed with 60 mg/kg intraperitoneal pentobarbital and perfused 8 weeks post-infection. Parasite burden was evaluated in each group by culturing mice obtained splenocytes for culture and cytokine profile analysis and to ascertain the splenocyte population by flow cytometry. Blood samples were collected for antibody detection before each immunisation and during necropsy.

2.7. Cytokine measurement

Mouse splenocytes obtained from immunisation experiments were cultured in a 96-well plate at 1 × 10^6 cells per well concentration in complete medium (RPMI 1640 medium containing 10% heat-inactivated foetal calf serum, 5 mM l-glutamine and antibiotics: 100 units/ml penicillin and 100 μg/ml streptomycin) (López-Abán et al., 2007). Splenocytes from immunisation and control mice were individually re-stimulated with rFh15 at 10 μg/ml final concentration for 72 h at 37 °C in a humidified atmosphere with 5% CO₂. Controls containing splenocytes from untreated mice were also prepared. Culture supernatants were recovered for cytokine determination (after the incubation period) by quantitative detection flow cytometry assay using a Mouse Th1/Th2 10 Plex FlowCytomix Kit (Bender Med Systems, Vienna, Austria). Different sized fluorescent beads coated with antibodies raised against interferon-γ (IFNγ), interleukin (IL) 1α, IL-2, IL-4, IL-6, IL-10, IL-17 and tumour necrosis factor α (TNFα) were incubated with the samples for 2 h at room temperature. After washing the beads to remove unbound antibodies, a secondary biotin-conjugated anti-cytokine antibody mixture was added and incubated for 1 h at room temperature. Streptavidin–phycoerythrin conjugate was then used to bind the biotin conjugate. Sample fluorescence was analysed on a BD FACScalibur flow cytometer (Becton Dickinson) at the Universidad de Salamanca’s Flow Cytometry Central Service. A total of 8000 events were recorded and data were processed using FlowCytomix Pro 3.0 software (Bender, Med systems). Cytokine concentration was calculated from standard curves using known mouse recombinant cytokines concentrations.

2.8. Flow cytometry analysis of T-cell splenocytes populations

Mouse splenocytes were incubated with the blocking anti-CD16/CD32 monoclonal antibody for 5 min at room temperature and stained with commercially-available fluorochrome-conjugated antibodies at 1/50 dilution in PBS plus 2% foetal calf serum for 30 min at 4 °C. Rat anti-mouse CD45-theridin chlorophyll protein (PerCP)-cyanine dye (Cy5.5), CD4-fluorescein isothiocyanate (FITC), CD8-phycocerythrin (PE), CD45R/B220-allophycocyanin (APC), CD197-PE (CCR7), CD62L-APC and hamster anti-mouse CD27 APC (BD Pharmingen, USA) were used. The cells were washed in PBS with 2% foetal calf serum and then spun at 1000g for 5 min; the supernatant was discarded. Cells were fixed with 100 μl of a 2% paraformaldehyde solution for 1 h at 4 °C. A FACScalibur flow cytometer was used for phenotype analysis. Data was collected regarding 30,000 events (gated by forward and side scatter) and analysed using Cytomix Flow Cytometry Analysis software (INVA technologies Pty Ltd).

2.9. rFh15 and SoSb specific antibody production

Indirect ELISA (Abán et al., 1999) was used for measuring specific anti-rFh15 and anti-SoSbWA antibody production for immunological assessment and protection experiments. Briefly, 96-well polystyrene plates (Costar) were coated with 2.5 μg of SoSbWA or 2.0 μg rFh15 antigen and then blocked with 2% bovine serum albumin. Sera were then added at 1:100 dilution, followed by adding peroxidase-labelled anti-mouse IgG, IgG1, IgG2a, IgM and IgE antibodies at 1:1000 dilution (Sigma). The reaction was developed with H₂O₂ and orthophenylenediamine (Sigma) and the absorbance was then measured at 492 nm on an Ear400FT ELISA reader (Lab Instruments).

2.10. Statistical analysis

The results were expressed as the mean and standard error of the mean (SEM). Normal distribution was checked using the Kolmogorov–Smirnov test. Differences between groups were found using a one-way ANOVA test and Tukey’s honest significance test (HSD) or Kruskal Wallis test. Statistical analysis was considered significant at p < 0.05 level. Software SPSS 21 (IBM) was used for data analysis.

3. Results

3.1. Using rFh15 in the ADAD vaccination system induced high IL-1α and IL-6 levels but did not induce a Th2 or regulatory response

Innate inflammatory (TNFα, IL-6), Th1 (IFNγ, IL-1α, IL-2), Th2 (IL-4), regulatory (IL-10) and Th17 (IL-17) profile production was...
measured in cultured spleen cell supernatant. It was seen that the PAL+Qs+rFh15 combination elicited a significant increase in both IL-1α (1042 ± 39 pg/ml cf 527 ± 65 pg/ml p = 0.001) and IL-6 (1803 ± 130 pg/ml cf 964 ± 118 pg/ml p = 0.012) compared to untreated controls. However no differences were detected concerning Th2, Th17 and/or regulatory cytokine levels. Mice treated only with PAL+Qs had significantly increased IL-1α compared with untreated controls (Fig. 1). Regarding splenocyte percentage populations in PAL+Qs+rFh15 immunised mice, a slight reduction was observed in CD4, CD27 and B220, even though the differences between experimental groups were not statistically significant amongst the experimental groups (data not shown).

3.2. Immunisation with rFh15-ADAD induced a specific antibody response

Specific antibodies (IgG, IgG1, IgG2a, IgM and IgE) against recombinant Fh15 antigen were evaluated by ELISA (Fig. 2). Significant IgG, IgG1, IgE and IgM enhancement was observed after the second immunisation in PAL+Qs+rFh15 immunised mice compared to untreated mice. Interestingly, significant IgG2a production was only detected at the endpoint of the experiment (p < 0.05).

3.3. Protection against S. bovis infection in BALB/c mice vaccinated with rFh15 using the ADAD vaccination system

Two experiments were carried out independently for evaluating protection involving rFh15 and the ADAD vaccination system (experiments A and B). The adult worm population became reduced by 67–72% in mice immunised with the complete vaccine (PAL+Qs+rFh15); specifically, worm recovery rates for immunised groups compared to the infected group were 12.5 cf 3.5 (p = 0.001) in experiment A and 12.4 cf 4.1 (p = 0.001) in experiment B. Female recovery was thus reduced between 60% and 72% (p < 0.05) and male recovery between 68% and 75% (p < 0.05) (Table 1). Reductions in eggs per gram in liver and intestine varied from 60% to 93% in experiment A and 61–65% in experiment B, (p < 0.05) (Table 2). Moreover, the relationship between eggs and the number of females was evaluated as a measurement of the anti-fecundity effect, though no statistically significant reductions were observed. The degree of hepatic surface affected by granuloma reaction was quantified on 100 mm² liver surface, a significant reduction in PAL+Qs+rFh15 vaccinated mice (47–80%) being detected compared to infected mice (p = 0.001) (Table 1). Moreover, it was found that 38% (experiment A) and 50% (experiment B) of vaccinated mice had no appreciable lesions in their livers. Such data suggested the protection-inducing ability of rFh15 formulated in the ADAD vaccination system against S. bovis infection.

3.4. Antibody profile in protection against S. bovis in BALB/c mice vaccinated with rFh15 using the ADAD vaccination system

Specific IgG1 and IgG2a anti-rFh15 levels, measured as optical density (OD), were analysed by ELISA in the sera of mice from experiment B before infection to confirm immunisation effectiveness. A strong IgG1 humoral response against rFh15 was detected in vaccinated mice compared to either control or PAL+Qs treated experimental groups (p < 0.05). No significant differences were
detected regarding anti-rFh15 IgG2a levels (Fig. 3). Specific IgG1 and IgG2a isotypes against SoSbAWA were measured during experiment B. Significant differences were only found between groups 8-weeks p.i. Interestingly, infected mice treated with PAL+Qs had significantly higher IgG1 levels than infected controls. A significant increase in IgG2a was observed in PAL+Qs+rFh15-vaccinated mice compared to the infected control group (Fig. 3).

4. Discussion

Fatty acid binding proteins (FABP) Fh12, rFh15 and Sm14 have been recognised as important vaccine candidates as they have shown immunological cross-protection against schistosomes and F. hepatica infection. Such FABPs' amino acid sequences have a high degree of identity specifically, between F. hepatica rFh15 and

Fig. 2. Serum anti-rFh15 specific IgG, subtypes IgG1 and IgG2a, IgE and IgM antibody levels (mean ± SEM) during the immunisation experiment by ELISA. BALB/c mice were immunised with PAL+Qs+rFh15 using the adjuvant adaptation (ADAD) vaccination system. Untreated and PAL+Qs treated controls were used. OD optical density. *p < 0.05 compared to untreated control. #immmunisation.

Table 1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total worms (mean ± SEM)</th>
<th>Reduction (%)</th>
<th>Females (mean ± SEM)</th>
<th>Reduction (%)</th>
<th>Males (mean ± SEM)</th>
<th>Reduction (%)</th>
<th>Hepatic damage (mean ± SEM)</th>
<th>Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Infected</td>
<td>12.5 ± 1.9</td>
<td>-</td>
<td>2.5 ± 1.3</td>
<td>-</td>
<td>10.0 ± 1.6</td>
<td>-</td>
<td>78.9 ± 14.8</td>
<td>-</td>
</tr>
<tr>
<td>PAL+Qs</td>
<td>7.0 ± 1.5</td>
<td>44</td>
<td>1.4 ± 0.9</td>
<td>44</td>
<td>5.6 ± 1.0</td>
<td>44</td>
<td>58.2 ± 16.0</td>
<td>26</td>
</tr>
<tr>
<td>PAL+Qs+Fh15</td>
<td>3.5 ± 1.8</td>
<td>72</td>
<td>1.0 ± 0.7</td>
<td>60</td>
<td>2.5 ± 1.5</td>
<td>75</td>
<td>16.0 ± 8.6</td>
<td>80</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Infected</td>
<td>12.4 ± 1.4</td>
<td>-</td>
<td>5.3 ± 0.6</td>
<td>-</td>
<td>7.1 ± 0.6</td>
<td>-</td>
<td>27.5 ± 4.9</td>
<td>-</td>
</tr>
<tr>
<td>PAL+Qs</td>
<td>12.3 ± 1.8</td>
<td>n.r.</td>
<td>5.7 ± 0.8</td>
<td>n.r.</td>
<td>6.6 ± 1.1</td>
<td>6</td>
<td>21.5 ± 1.5</td>
<td>22</td>
</tr>
<tr>
<td>PAL+Qs+Fh15</td>
<td>4.1 ± 1.5</td>
<td>67</td>
<td>2.3 ± 1.0</td>
<td>72</td>
<td>1.5 ± 0.6</td>
<td>68</td>
<td>14.6 ± 7.3</td>
<td>46</td>
</tr>
</tbody>
</table>

SEM: standard error of the mean. n.r. No-reduction. *p < 0.05 compared to respective infection controls.
IL-1 levels were detected in both groups treated with PAL+Qs with SEM: standard error of the mean. n.r. No-reduction.

Reduction in number of eggs per gram (EPG) in tissues and fecundity considering liver and intestine in rFh15-vaccinated BALB/c mice using the adjuvant adaptation (ADAD).

Schistosoma mansoni which has been widely used for evaluating immunoprotection with immunological response in this adjuvant system in a mouse model for was initially evaluated to improve characterising the antigen’s immunomodulator (Martínez-Fernández et al., 2004) and against schistosomiasis (Uribe et al., 2007). Other new adjuvant system approaches i.e. CpG-ODN (Teixeira de Melo et al., 2013), a combination of CpG and squalene-based oil-in-water emulsions, such as MF59, stimulate IL-1 (Mosca et al., 2008). Moreover, IL-1 seems to coordinate the immune system’s early response to exogenous and endogenous pathogens, serving as a prototypic proinflammatory cytokine (Goldbach-Mansky and Kastner, 2009). However, maintaining a proinflammatory immune response may lead to progressive pathogenic fibrosis in other trematode infections, such as that caused by Opisthorchis viverrini (Sripa et al., 2012). The presence of IL-6 is considered a key factor in initiating an inflammatory response against S. mansoni because of its ability to induce an innate immune response (Rutitzky and Stadecker, 2011). Up-regulation of proinflammatory cytokines (i.e. IL-6) in Sm-P80-immunised mice has been associated with 60% reduction in worm burden in vaccination experiments (Torben et al., 2012; Zhang et al., 2011). Moreover, it is known that IL-6 enhances antibody response, since it can regulate the B-cell growth, differentiation and survival, and sustain an antibody response (Morel et al., 2011). Such data has suggested that rFh15, together with PAL and formulated in the ADAD vaccination system, has induced IL-6 and IL-1 expression. Immunisation with PAL+Qs+rFh15 elicited a strong serological specific response against rFh15 (IgG, IgG1, IgG2a, IgM and IgE).

Once an rFh15-induced immune response was evaluated in healthy mice, the ability of rFh15 to induce protection was tested in S. bovis-challenged BALB/c mice using the ADAD vaccination system with Qs and PAL in two separate experiments. High rFh15-induced protection levels were observed in the present work in terms of adult worm recovery rates (i.e. becoming reduced from 67% to 72%). Hepatic damage was macroscopically evaluated, revealing reduced granuloma formation in liver (47–80%). Our group has previously described comparable reduction rates using native and recombinant FABP (Fh12 and rhFh15) formulated in Freund’s adjuvant (Abâné et al., 2000) and confirm cross-reacting specific response against rFh15 (IgG, IgG1, IgG2a, IgM and IgE).

These protection levels were better than those obtained using GST in cattle, goats, sheep and mice (Bushara et al., 1993; Boulanger et al., 1999; Viana da Costa et al., 1999) and higher than protection reached with the 14–3–3 protein against S. bovis infection (Uribe et al., 2007). A significant increase in IgG2a in protected mice was observed regarding a humoral immune response against SoSbAWA in vaccination experiments. The ADAD system with PAL seems to activate an early pro-inflammatory immune response which could be involved in immunoprotection against S. bovis challenge. ADAD with PAL was able to induce a high level of protection in Th2 biased BALB/c mice in this work, whereas the classical Freund’s adjuvant was only able to induce protection with increased IFNγ and IgG2a against S. bovis in C57BL mice but not in BALB/c using FABPs (Abâné et al., 1999; Abâné et al., 2000; Uribe et al., 2007). Other new adjuvant system approaches i.e. CpG-ODN (Teixeira de Melo et al., 2013)), a combination of CpG

### Table 2

Reduction in number of eggs per gram (EPG) in tissues and fecundity considering liver and intestine in rFh15-vaccinated BALB/c mice using the adjuvant adaptation (ADAD).

<table>
<thead>
<tr>
<th>Groups</th>
<th>EPG in liver (mean ± SEM)</th>
<th>Reduction (%)</th>
<th>EPG intestine (mean ± SEM)</th>
<th>Reduction (%)</th>
<th>Liver EPG/female (mean ± SEM)</th>
<th>Reduction (%)</th>
<th>Intestine EPG/female (mean ± SEM)</th>
<th>Reduction (%)</th>
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<tbody>
<tr>
<td><strong>Experiment A</strong></td>
<td></td>
<td></td>
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<tr>
<td>Infected</td>
<td>1004 ± 377</td>
<td>–</td>
<td>977 ± 318</td>
<td>–</td>
<td>401 ± 66</td>
<td>–</td>
<td>390 ± 64</td>
<td>–</td>
</tr>
<tr>
<td>PAL+Qs</td>
<td>1077 ± 523</td>
<td>n.r.</td>
<td>1074 ± 476</td>
<td>n.r.</td>
<td>769 ± 109</td>
<td>n.r.</td>
<td>767 ± 180</td>
<td>n.r.</td>
</tr>
<tr>
<td>PAL+Qs+Fh15</td>
<td>73 ± 63</td>
<td>93</td>
<td>379 ± 340</td>
<td>61</td>
<td>73 ± 85 ns</td>
<td>82</td>
<td>379 ± 16</td>
<td>18</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Infected</td>
<td>1107 ± 275</td>
<td>–</td>
<td>709 ± 237</td>
<td>–</td>
<td>209 ± 56</td>
<td>–</td>
<td>134 ± 32</td>
<td>–</td>
</tr>
<tr>
<td>PAL+Qs</td>
<td>1298 ± 184</td>
<td>n.r.</td>
<td>1280 ± 387</td>
<td>n.r.</td>
<td>228 ± 25</td>
<td>n.r.</td>
<td>225 ± 104</td>
<td>n.r.</td>
</tr>
<tr>
<td>PAL+Qs+Fh15</td>
<td>443 ± 331</td>
<td>60</td>
<td>248 ± 116</td>
<td>65</td>
<td>193 ± 108</td>
<td>8</td>
<td>108 ± 46</td>
<td>19</td>
</tr>
</tbody>
</table>

SEM: standard error of the mean. n.r. No-reduction.

*p < 0.05 compared to respective infection controls.

**Fig. 3.** Serum specific IgG1 and IgG2a antibody levels by ELISA against rFh15 protein before challenge and against S. bovis soluble adult worm antigen (SoSbAWA) 8 weeks post-challenge. BALB/c mice were vaccinated with rhFh15 using the adjuvant adaptation system (ADAD), challenged with 150 S. bovis cercariae and perfused 8 week post-challenge. Data from experiment B is shown. OD optical density. *p < 0.05 compared to infected controls.
and R848 (Wang et al., 2013), cationic polymer carriers such as polyamidoamine (PAMAM) dendrimers (Wang et al., 2014) or the intramuscular injection of an adenoaviral vector (Dai et al., 2014) have all induced up-regulation of Th1 response and improved the immunoprotection against experimental schistosomiasis. This study has shown that the rFh15 protein formulated in the ADAD vaccination system with QS and PAL was able to up-regulate proinflammatory cytokines (IL-1 and IL-6), thereby inducing an immune response having increased IgG2a levels. It has also shown that rFh15 produced a significant reduction in worm burden in two independent protection experiments against S. bovis. This suggested that rFh15 could be used in vaccination experiments aimed at parasite clearance and reducing worm burden in an appropriate formulation.

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References


Dai, Y., Wang, X., Zhao, S., Tang, L., Zhang, L., Dai, Y., Li, H., et al., 2014. PAMAM-Lys, a novel vaccine carrier, for carefully revising the text. We would like to thank Jason Garry for carefully revising the text. We would like to thank Jason Garry for carefully revising the text.