Experimental Parasitology 160 (2016) 31–38

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Full length article

Adjuvant-enhanced antibody and cellular responses to inclusion bodies expressing FhSAP2 correlates with protection of mice to *Fasciola hepatica*

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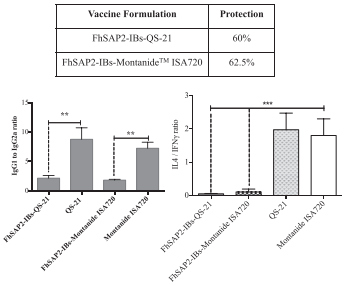
HIGHLIGHTS

- Inclusion bodies (IBs) containing FhSAP2 were tested in a mouse model of fascioliasis.
- FhSAP2-IBs emulsified in Montanide™ ISA720 or QS-21 induces partial immunity in mice.
- FhSAP2-IBs in Montanide™ ISA720 or QS-21 induces high levels of IgG1 and IgG2a antibodies.
- FhSAP2-IBs in Montanide™ ISA720 or QS-21 induces higher levels of IFNγ than IL-4.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 July 2015
Received in revised form
5 November 2015
Accepted 18 November 2015
Available online 26 November 2015

Keywords:
Fasciola hepatica
Vaccination
Inclusion bodies
Saposin-like proteins

ABSTRACT

Fasciola hepatica saposin-like protein-2 (FhSAP2) is a protein differentially expressed in various developmental stages of *F. hepatica*. Recombinant FhSAP2 has demonstrated the induction of partial protection in mice and rabbits when it is administered subcutaneously (SC) in Freund's adjuvant. Because FhSAP2 is overexpressed in bacteria in the form of inclusion bodies (IBs), we isolated IBs expressing FhSAP2 and tested their immunogenicity when administered SC in mice emulsified in two different adjuvants: QS-21 and Montanide TM ISA720. Animals received three injections containing 20 μg of protein two weeks apart and 4 weeks after the third injection, mice were infected with 10 *F. hepatica* metacercariae by oral route. The percentages of protection induced by FhSAP2-IBs were estimated to be between 60.0 and 62.5% when compared with adjuvant-vaccinated, infected controls. By determining the levels of IgG1 and IgG2a antibodies and IL-4 and IFNγ cytokines in the serum of experimental animals, it was found that both Th1 and Th2 immune responses were significantly increased in the FhSAP2-IBs vaccinated groups compared with the adjuvant-vaccinated, infected control groups. The adjuvant-vaccinated groups had significantly lower IgG1 to IgG2a ratios and lower IL-4 to IFNγ ratios than the FhSAP2-IBs vaccinated animals, which is indicative of higher levels of Th2 immune responses. Irrespective to the adjuvant used, animals vaccinated with FhSAP2-IBs exhibited significantly higher survival percentage and less liver damage than the adjuvant-control groups. This study suggests that FhSAP2 has potential as vaccine against *F. hepatica* and that the protection elicited by this molecule could be linked to a mechanism driven by the CD4-Th1 cells.

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http://dx.doi.org/10.1016/j.exppara.2015.11.002
0014-4894/Published by Elsevier Ltd.

1. Introduction

There is a need to develop a vaccine against *Fasciola hepatica*, a parasitic trematode distributed worldwide responsible for enormous economical losses estimated in more than 3 billion dollars yearly (Mas-Coma, 2005). Globally between 2.4 and 17 million individuals are infected with *Fasciola* species and more than 180 million people are at risk of infection (Mas-Coma et al., 1999a, 1999b; Mas-Coma, 2005). Over the last decade a large number of native and recombinant parasite proteins have been identified and tested as vaccine in a number of animals models. Some of these molecules includes leucinaminopeptidase (Maggioli et al., 2011a), cathepsin-L (Chantree et al., 2013; Golden et al., 2010; Piacenza et al., 1999), thioredoxin-glutathion reductase (Maggioli et al., 2011b), glutathione S-transferase (Sexton et al., 1990, 1994), fatty acid binding protein (Casanueva et al., 2001; Martinez-Fernandez et al., 2004) and saposin-like protein-2 (Espino et al., 2010; Kueakhai et al., 2013). All these vaccine candidates have induced partial levels of protection that range between 46.9 and 83% in different animal models, which indicates that a vaccine against *F. hepatica* is not a chimera but an attainable goal. However, despite of significant advances, no vaccine candidate has yet advanced to a clinical trial phase. Much of this could be attributed to differences in the structural characteristics of the antigens (e.g. hydrophobicity, specific localization in the parasite, biological functions, etc.), the feasibility to produce it at large amount in homogeneous form, differences in the vaccination protocols (doses and route of administration); the correct selection of the adjuvant and the lack of knowledge about the correlates of protection. In the current study, we explored the effectiveness of inclusion bodies (IBs) expressing saposin-like protein-2 (FhSAP2) as a cheaper and reliable vehicle to deliver antigenic molecules in homogeneous form.

Saposin-like proteins are a family of lipid interacting proteins that binds onto the cell membrane to induce cell lysis (Bruhn, 2005). *Fasciola* species use these lytic proteins to cause lysis of the hosts' erythrocytes and leuckocytes so that their contents can be digested further for the parasite's nourishment (Espino and Hillyer, 2003). In *F. hepatica*, there are two isoforms (SAP1 and SAP2) (Espino and Hillyer, 2003; Reed et al., 2000). SAP1 is expressed in immature and adult stages (Reed et al., 2000), whereas SAP2 is highly expressed in newly excysted juvenile, 3-week juvenile up to adult stages and also in non-embryonated eggs (Caban-Hernandez and Espino, 2013; Espino and Hillyer, 2003). Some characteristics of the protein moiety of FhSAP2 facilitate the formation of protein aggregates as inclusion bodies (IBs) during the expression process in *Escherichia coli.* The primary structure of FhSAP2 consists of 101 amino acids that are rich in hydrophobic amino acids (~39.6%) some of which are exposed and available to interact with similar exposed residues on other cellular proteins. The FhSAP2 also exhibits six cysteine residues at highly conserved positions and therefore, FhSAP2 is a protein that has disulfide bonds (Espino and Hillyer, 2003). During the expression of FhSAP2 in bacteria, the exposure to the reducing environment of the bacterial cytosol may inhibit the formation of these disulfide bonds, which also strongly contributes to the IBs formation (Hartley and Kane, 1988). Because >90% of fusion protein is expressed as inclusion bodies, only relatively low yields of fusion protein (0.3–0.5 mg/l of culture) could be obtained from the supernatants of bacterial lysates. This amount is inadequate for -large-scale immunization experiments and is not cost-effective for commercial manufacture. Preparation of IBs is cheaper and easier than affinity purification of FhSAP2-fusion protein, which is time-consuming and tedious.

In a previous experiment, we found that purified recombinant FhSAP2 can protect mice against a liver fluke infection when administered subcutaneously in Freund's adjuvant (Espino et al., 2010). In this study, we designed experiments to examine whether inclusion bodies (IBs) containing recombinant FhSAP2 could be as immunogenic as the purified protein when delivered subcutaneously and whether they are capable of inducing protection in a mouse model of fascioliasis. Because Freund's complete adjuvant (FCA), an oil-base adjuvant that contain mycobacteria (Freund, 1951), produces excessive inflammation (Broderson, 1989), it is not permitted in commercial vaccine formulations for human or veterinary use. In the current study, we will use FhSAP2-IBs as a model antigen to evaluate immune responses when formulated in non-toxic adjuvants like QS-21 and Montanide TM ISA720.

2. Materials and methods

2.1. Recombinant protein expression and inclusion bodies isolation

The cDNA of FhSAP2 gene (Accession No. AF28693) was subcloned from the adult *F. hepatica* cDNA library (Espino and Hillyer, 2003). The FhSAP2 cDNA was cloned into pBAD His-B expression vector and transformed into *E. coli* TOP10 (Invitrogen). The recombinant FhSAP2 expression was induced with 0.02% L-arabinose for 4 h at 37 °C. The bacterial cell pellets were suspended in a lysis buffer (500 mM NaCl, 50 mM Tris–HCl, 10 mM EDTA, 5 mM β-mercaptoethanol, 0.35 mg/ml lysozyme, pH 8.0) and incubated for 30 min at 20 °C. Triton X-100 was added to the concentration of 1% and the suspension was sonicated. After sonication, the suspension was centrifuged at 12,000-x *g* for 30 min. The IB pellets were suspended in PBS containing 1% Triton X-100, sonicated and centrifuged at 12,000-x *g* for 30 min. This procedure was repeated twice and subsequently the IBs were washed two times with PBS and characterized by electrophoresis in 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentration of recombinant FhSAP2 in relation to all proteins expressed by *E. coli* were measured by densitometry scanning of a Comassie Brillian Blue G stained SDS-PAGE using GelDoc Scan Software. Proteins were electrotransferred to a nitrocellulose (NC) sheet (0.2 μm; Bio-Rad) at 4 °C for 2 h. After blocking for 1 h in PBS containing 0.05% Tween-20 (PBST) and 5% skim milk, the NC membrane was incubated overnight in a mouse anti-Xpress™ epitope-peroxidase labeled antibody (Invitrogen) diluted 1:5,000, which recognizes a non-conformational epitope formed (Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys) at the amino end of the fusion protein. A positive brown signal for FhSAP2-fusion protein was visualized using diaminobenzidine as a substrate.

2.2. Obtaining of adult F. hepatica excretory-secretory proteins (ES)

F. hepatica ES products were prepared by culturing live adult flukes in RPMI medium for 24 h at 4 °C. The medium was centrifuged at 6000-x g at 4 °C and concentrated 10-fold using ultrafiltration membrane (YM-10) in an AMICON system. The protein concentration was estimated using the bicinchoninic acid method (BCA kit, Pearce, Inc).

2.3. Adjuvants

In the current study we analyzed two different adjuvants. QS-21 (kindly donated by Agenus Inc. Lexington, MA, USA) is an immunological adjuvant derived from a natural source: the bark of the South American tree *Quijalla saponaria* (QS) Molina, which has been identified as a saponin with potent adjuvant activity and low toxicity (Kensil et al., 1991). QS-21 has shown to stimulate a strong antibody response to T-dependent protein antigens in mice (Cribbs et al., 2003). Montanide ™ ISA720 (Seppic, Inc. Fairfield, NJ) is a