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Research paper

Intranasal delivery of a formulation containing stage-specific recombinant proteins of *Fasciola hepatica* cathepsin L5 and cathepsin B2 triggers an anti-fecundity effect and an adjuvant-mediated reduction in fluke burden in sheep

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ABSTRACT

Fasciola hepatica infection continues to be a major problem in the agriculture sector, particularly in sheep and cattle. Cathepsin L and B proteases are major components of the excretory/secretory material of the parasite, and their roles in several important aspects of parasite invasion and survival has led to their use as targets in rational vaccine design. Previous studies in rats demonstrated that the use of stage-specific antigens, cathepsin B2 and cathepsin L5, as part of a multivalent vaccine, was able to confer significant protection against challenge. In the present study, recombinant versions of cathepsin L5 and cathepsin B2 produced in yeast were used in combination to vaccinate sheep. Intramuscular and intranasal forms of administration were applied, and sheep were subsequently challenged with 150 *F. hepatica* metacercariae. Intramuscular vaccination was able to induce a strong systemic antibody response against both antigens, but failed to confer significant protection. Conversely, no elevated antibody response was detected against the vaccine antigens following nasal vaccination; however, a reduction in parasite egg viability (> 92%) and a statistically significant ($p = 0.006$), predominantly adjuvant-mediated reduction in worm burdens was observed.

1. Introduction

The liver flukes *Fasciola hepatica* and *F. gigantica* are major veterinary parasites, with a worldwide distribution and the ability to infect a range of hosts (Norbury et al., 2012b). The effects of infection can range from sudden death to chronic underperformance producing marked economic effects; infected sheep show impaired live weight gain and wool production (Torgerson and Claxton, 1999). Parasitism of humans is also an emerging disease with estimates of infection ranging from 2.4 to 17 million (Hopkins, 1992; Rim et al., 1994).

Current methods of parasite control are predominantly based on the use of anthelmintic drugs, with triclabendazole the drug of choice. However, issues with food safety, reinfection, and the development of drug resistant strains have led to an increasing search for a suitable vaccine (Kelley et al., 2016). Despite research spanning several decades there is still no commercially available vaccine to combat *F. hepatica* infection (Molina-Hernández et al., 2015).

Fasciola spp. excrete and secrete a broad range of molecules while residing in the host. Cysteine proteases are some of the major proteins produced by flukes and often operate at the host-parasite interface. These proteases have varying substrate specificities (Norbury et al., 2011, 2012a; Stack et al., 2011) and are involved in important parasite functions including excystment, migration and tissue invasion, feeding, and immune evasion and modulation (Norbury et al., 2012b; Dalton et al., 2013).

Cathepsin L proteases present in the excretory-secretory (ES) material of adult *F. hepatica* have been some of the most extensively studied parasite proteins. Cathepsin L proteases are secreted by all developmental stages of fluke in the mammalian host, although different repertoires of cathepsin L proteases are secreted by the different fluke developmental stages (Cancela et al., 2008; Robinson et al., 2009). One such protease, *F. hepatica* cathepsin L5 (FhCL5) constitutes approximately 7% and 5–10% of the secreted protease content of immature and adult flukes, respectively (Robinson et al., 2009; Morphew et al., 2011). In recent years more research has been directed towards understanding cathepsin B proteases (Beckham et al., 2009; Smooker et al., 2010), and

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while some degree of temporal expression is also evident for cathepsin B proteases, they are predominantly expressed by earlier life stages of the fluke (Robinson et al., 2008, 2009; Smooker et al., 2010). *Fasciola* spp. cathepsin proteases are considered key targets for vaccine or drug development, and represent some of the most promising vaccine candidates for control of liver fluke (Toet et al., 2014; Yap and Smooker, 2016).

During the immature developmental stages, the parasite migrates through the host tissue and is considered to be more exposed and vulnerable to the host immune response. The juvenile stages cause the most significant damage and disease pathology, and generating a protective response prior to development of this damage is desirable. As the protein repertoire of the parasite changes greatly as the fluke matures, this complicates attempts at vaccine development. The use of an antigen expressed throughout the life cycle, or alternatively, the use of a multivalent vaccine containing several antigens, may therefore be preferable. Multivalent vaccines may be the way forward in combating this parasite, with certain multivalent vaccines (but not all) shown to be able to increase efficacy compared to a single antigen vaccine (Dalton et al., 1996; Piacenza et al., 1999; Jayaraj et al., 2009; Toet et al., 2014).

Different adjuvants can have a profound effect on vaccine efficacy among *F. hepatica* vaccines (Maggioli et al., 2011). Quil-A®, derived from saponin, promotes a T helper (Th) 1 immune response which is considered beneficial to generating protection against *F. hepatica.* Quil-A® alone has also been shown to produce a protective effect when administered in the absence of specific antigen (Haçariz et al., 2009b). Immunostimulating complexes (ISCOMs) are nanoparticles comprised of cholesterol, phospholipids, a saponin-derived adjuvant (such as Quil-A®) and an antigen. ISCOMs formulated lacking an antigen, here referred to as ISC-adjuvant, can also be mixed with antigens prior to vaccination (Sun et al., 2009).

Mucosal vaccination can offer several advantages over typical intramuscular or subcutaneous vaccination routes, including ease of administration and reduced adverse effects. Vaccine studies utilising oral or intranasal administration of recombinant protein or cDNA vaccines, including those utilising *F. hepatica* cathepsin proteases can confer high levels of protection in rats, cattle and sheep (Wedrychowicz et al., 2003, 2007; Kęsik et al., 2007). The results from these and other studies indicate that mucosal immunity plays an important role in protection against many parasites, and mucosal vaccination appears to be a promising avenue of research for the development of many anti-parasite vaccines, including those against *F. hepatica*.

Previously, a vaccine incorporating the stage-specific recombinant proteins *F. hepatica* cathepsin L5 (FhCL5) and cathepsin B2 (FhCB2), that were produced using a yeast expression system, was able to induce a strong protective response in rats against *F. hepatica*, with over 83% protection (Jayaraj et al., 2009). In this study, we used these antigens incorporated into vaccines with either Quil-A® or ISC-adjuvant adjuvants, and administered to sheep either by intramuscular injection or nasal delivery.

2. Material and methods

2.1. Antigen production

The cloning of the two antigens, FhCL5 and FhCB2, into the pFLAG expression system has been previously described (Smooker et al., 2000; Law et al., 2003). The antigens were expressed using the yeast *Saccharomyces cerevisiae* BJ3505 and purified on nickel-charged resin by virtue of the 6 × his-tag present on the recombinant proteins using established protocols (Jayaraj et al., 2009). Protein concentration was determined by Bradford assay, and purity was assessed by SDS-PAGE.

2.2. Vaccine formulation

Two vaccine formulations containing antigens were used. The intramuscular vaccine (IMV) formulation contained 150 µg of each of FhCL5 and FhCB2, mixed with 1 mg/ml of Quil-A® (Brenntag Biosector, Denmark) in PBS. Total dose volume per sheep was 1 ml per vaccination. The nasal vaccine (NV) formulation contained 75 µg of each of FhCL5 and FhCB2, 2 mg of CpG-oligodeoxynucleotide (ODN) 2135 (5′-TCGTCGTTTGTCGTTTTGTCGTT) (Sigma-Aldrich, UK), mixed with 750 µg of ISC-adjuvant (kindly supplied by Zoetis) in PBS. Each sheep received 600 µl (300 µl in each nostril) per vaccination. CpG-ODN are short single-stranded synthetic DNA molecules that contain motifs of cytosine and guanine dinucleotides linked by a phosphodiester or phosphorothioate backbone, that can act as immunostimulants when unmethylated (Hanagata, 2017). The CpG-ODN2135 was constructed with a full phosphorothioate backbone. The particular CpG-ODN sequence was selected for this experiment as it is reported to induce the strongest proliferation in the peripheral blood mononuclear cells of sheep (Rankin et al., 2001). Nasal delivery was administered using a syringe-mounted 10 cm catheter constructed as outlined by Vujanic et al. (2012) to ensure delivery deep into the sheep nasal cavity.

2.3. Experimental animals and parasites

Forty male, castrated, Corriedale lambs were used in the vaccination experiment. The sheep were 5 months old at commencement of vaccination. Sheep were housed in the Institute of Parasitology research station at Łomna Las. All procedures were approved by the Third Local Ethical Committee, Warsaw, and performed under the guidance of a local veterinarian. Sheep were confirmed fluke-free through faecal egg count and screening of sera by ELISA. Sheep were examined for signs of infection on two occasions, 7 weeks apart, prior to the beginning of the trial. Faecal sedimentation was undertaken with a Flukefinder® (Richard Dixon, US) as described previously by Nzalawahe et al. (2014). ELISA using sheep sera (1:100 dilution) and affinity purified rabbit polyclonal anti-sheep IgG heavy and light chain horseradish peroxidase (HRP) conjugate (1:100,000 dilution) (Bethyl Laboratories, USA) was undertaken as described in detail below, except plates were coated with 5 µg/ml of a mixture of the antigens FhCL5 and FhCB2. Sera from sheep known to be infected or uninfected by *F. hepatica* were used as controls. Additionally, sheep were treated with albendazole (Vermitan 10%) twice at 0.5 ml/10 kg two months prior to the beginning of the trial.

A strain of *F. hepatica*, termed 'CVL Weybridge' and originally obtained from the UK, has been maintained in our laboratory since 2003 through experimental infections of sheep and *Galba truncatula* snails (Januszkiewicz et al., 2015). Two-month-old metacercariae were used for infections.

2.4. Vaccinations, sampling and challenge

Sheep were divided randomly into 5 groups of 8. Each group were administered either; intramuscular vaccine (IMV), an intramuscular control (IM) which was the same as IMV except lacking antigens, nasal vaccine (NV), or nasal control (N) which was the same as NV except lacking antigens. The final group was a control group (C) and was left unvaccinated. Each group of sheep (except controls) received 3 doses of vaccine, at 4-week intervals. Five weeks after the final vaccination, all sheep were orally challenged with 150 metacercariae.

Sheep were weighed prior to each vaccination, then at 2-week intervals post-challenge. Blood samples were collected by jugular venipuncture every 2 weeks starting prior to the first vaccination, continuing until the end of the trial. Blood was collected into vacutainers (with and without EDTA, or with heparin). To obtain sera, blood samples were allowed to clot at 4 °C, then spun at 1500 × *g*, 4 °C, 20 min and stored at -80 °C. Nasal mucosa washings were also collected