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Research paper

Vaccination against *Fasciola hepatica* using cathepsin L3 and B3 proteases delivered alone or in combination

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ABSTRACT

No licensed vaccine is currently available for prevention of *Fasciola hepatica* infections. However, considering the alarming increase in drug resistance, there is an urgent need for a safe and fully effective vaccine against fasciolosis. Here, we tested if cathepsins L (FhCL3-1, FhCL3-2) and B (FhCB3) secreted by juvenile liver flukes are viable vaccine targets when delivered alone or in combination in a rat model. Since control over the early immune response is crucial for parasite's establishment in its host, it was hypothesised that targeting fluke juvenile stages may prove beneficial. Moreover, it was assumed that selected antigens will act in a cumulative manner to interfere with liver fluke migration and thereby will reduce *F. hepatica* infection. Recombinant FhCL3-1 and FhCL3-2 delivered alone reduced liver fluke burdens by 47 % and 63 %, respectively. A trivalent vaccine containing rFhCL3-1/CL3-2/CB3 did not increase the protective vaccine efficacy compared to the rFhCL3-2 vaccinated group (53 %), although, reductions in liver fluke wet weight (statistically significant) and liver damage score were most pronounced. Further, the highest IgG1 and IgG2a levels were seen in rFhCL3-2 vaccinated rats, the group for which the highest reduction in worm burden was demonstrated. Moreover, IgG1 and IgG2a levels in vaccinated rats were significantly elevated compared to those reported for control groups up to 4 week post-infection. While the mechanism of protection remains unknown, it appears that it depends on vaccine-induced antibodies directed against cathepsins. The obtained results imply that *F. hepatica* juvenile-specific cathepsins are promising vaccine candidates that induce responses that successfully target early migratory liver fluke stages. Now, the challenge is to evaluate these juvenile-specific cathepsins for use in livestock.

1. Introduction

Fasciola hepatica is a causative agent of fasciolosis, a disease that is not only an economic problem in livestock husbandry, but also poses a threat for human health (Cwiklinski et al., 2016). Since there is no approved vaccine against fasciolosis, liver fluke infection control relies almost exclusively on the use of anthelminthic drugs. However, high re-infection rates after drug treatment and the spread of triclabendazole resistance means this approach is no longer satisfactory (Kelley et al., 2016) and efforts to provide an effective vaccine against *F. hepatica* appear as a well-reasoned strategy. Among the many vaccine antigen candidates tested, *F. hepatica* cathepsins seem to be the most promising (Molina-Hernández et al., 2015). These proteins are involved in crucial parasite activities that facilitate parasitism, including tissue penetration, feeding, migration and immune evasion (Dalton et al., 2013). To date, vaccines containing cathepsins from adult liver fluke stages have shown partial efficacy (Toet et al., 2014). Numerous DNA and protein (native and recombinant) cathepsin vaccine preparations have been tested in both laboratory and target species (Buffoni et al., 2012; Dalton et al., 1996; Golden et al., 2010; Kęsik-Brodacka et al., 2017; Piacenza et al., 1999; Villa-Mancera et al., 2014; Wesołowska et al., 2013; Wędrychowicz et al., 2007, 2003; Zafra et al., 2013). However, a safe and fully effective vaccine has not yet been developed.

Since control over early immune responses is fundamental for the parasites establishment in its host, targeting of juvenile stages may prove beneficial. Indeed, rat vaccination with an antigen fraction derived from Newly Excysted Juveniles (NEJs) induced strong protective immunity against *F. hepatica* infection (van Milligen et al., 2000). Moreover, an immunogenic protein referred to as procathepsin L3 was identified and suggested to be an important target for early protection (Harmsen et al., 2004; Reszka et al., 2005). Further, juvenile-specific cathepsin B and cathepsin L1 g (ortholog of cathepsin L3) vaccine

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preparations tested in combination provided substantial protection in rats (Jayaraj et al., 2009). It seems reasonable to evaluate vaccination with more than one antigen to obtain optimal levels of protection. Hence, two newly characterised cathepsin L3 alleles (Zawistowska-Deniziak et al., 2013) delivered alone and in combination with cathepsin B3 were tested in a vaccine trial conducted on rats to verify if these juvenile-specific proteins are viable targets for immunotherapy.

2. Materials and methods

2.1. Protein expression and purification

The *FhCB3 (*GenBank™ EU090822.1) gene excluding the signal peptide sequence was cloned into pET28a(+) using primers containing HindIII and NheI restriction sites FhCB3-pETL 5′GCTAGCAAGCCAAA CTACAAACGGCA and FhCB3-pETR 5′AAGCTTGAGTGCAGGTAATCCG GCA. Construct integrity was confirmed by nucleotide sequence analysis. The recombinant vectors were introduced into *Escherichia coli* BL21. The constructs rFhCL3-1 and rFhCL3-2 (GenBank™ FJ617001.1, FJ617000.1) were cloned, expressed and purified as previously described (Zawistowska-Deniziak et al., 2013). Bacteria were cultured in LB medium. When the culture reached OD_{600} = 0.6 expression was induced for 4 h by adding 1 M IPTG. The recombinant proteins were purified by affinity chromatography using HIS-Select® HF Nickel Affinity Gel (Sigma) in the presence of 8 M urea and eluted in buffer containing 0.1 M Na_3PO_4, pH 4.5 according to the manufacturer's instructions. Purified rFhCL3-1, rFhCL3-2 and rFhCB3 were stored at −80 °C until further experiments. Proteins concentrations were determined with BCA Protein Assay Kit (Pierce).

2.2. Vaccine trial

The vaccine trial was carried out on Sprague Dawley rats that were 12-weeks-old at the beginning of the experiment. The trial involved six experimental groups, each containing six males. Rats were vaccinated with formulations indicated in Table 1 three times at two week intervals. Each dose was mixed with Imject Alum (IA) (Thermo Fisher Scientific) according to manufacturer's protocol. Further, the vaccine was administered subcutaneously in a final volume of 200 µl. Rats from the adjuvant control group were vaccinated with IA mixed with the protein elution buffer, whereas rats from infection and physiology groups remained unvaccinated. Two weeks after the third vaccination rats were challenged intragastrically with 35 two-month-old metacercariae (except for the physiology control group). Metacercariae (the Weybridge strain of *F. hepatica*) were obtained from a laboratory strain of *Galba truncatula* snails that have been maintained at the W. Stefański Institute of Parasitology (Januszkiewicz et al., 2015). From the day of challenge, rats were bled by tail vein and blood samples were collected at 0, 2, 4, 6 and 9 week post infection (WPI). At 9 WPI rats were euthanised. All procedures were approved by the 3rd Local Ethical Committee, Warsaw, Poland.

Table 1
The list of experimental groups and vaccine formulations.

Group	antigen dose
FhCL3-1/CL3-2/CB3/IA	6.6 µg of rFhCL3-1, 6.6 µg rFhCL3-2, 6.6 µg rFhCB3
FhCL3-1/IA	20 µg
FhCL3-2/IA	20 µg
physiology control	–
infection control	–
adjuvant control (IA)	–

2.3. Haematology analysis

Blood samples were subjected to analysis in an automated analyzer (Abacus Jun Vet). Total white blood cell count (WBC), lymphocyte, monocyte, neutrophil, eosinophil, and basophil populations as well as red blood cell parameters were investigated during the study.

2.4. Flow cytometry

Blood samples at a final cell concentration of 5×10^5/ml were incubated with the antibodies specific to CD4, CD8a, CD3 and CD45, labelled with APC, PE, FITC and PE-Cy5, respectively (BD PharMigen), at dilutions recommended by the manufacturer. Appropriate isotypic controls were also done. Following treatment with FACS lysing buffer (Becton Dickinson), cells were washed with phosphate buffered saline (PBS) and fixed with 0.5 % formaldehyde. Ten thousand gated events were counted in a FACS Calibur flow cytometer and analysed using CellQuest software. Lymphocytes were selected from the forward/side scatter plot followed by T cell subset identification: T helper cells (CD45+, CD3+, CD4+, CD8-) and T cytotoxic cells (CD45+, CD3+, CD4-, CD8+).

2.5. ELISA

ELISA plates were coated with recombinant FhCL3-1, FhCL3-2 or FhCB3 at 2.5 µg/ml in carbonate binding buffer and incubated overnight at 4° C. Following blocking with 5 % skim milk in PBS/Tween 20 (PBST) (1 h, 37 °C), plates were washed thrice and sera from individual rats were serially diluted starting from 1:100 dilution and incubated for 1 h at 37 °C. After washing, goat horseradish peroxidase (HRP)-conjugated anti-rat IgG1, IgG2a or IgG2b (1:100,000 Bethyl) antibodies were added and plates were incubated for 1 h at 37 °C. After six PBST washes, plates were developed using tetramethylbenzidine solution (Sigma), stopped with 2 M sulphuric acid and read at 450 nm on a spectrophotometer (HT Synergy, Biotek).

Absorbance (OD_{450}) values were taken from titres (1:2000-IgG1; 1:100-IgG2a, IgG2b) where the decreases in absorbances were observed and the reported values were higher than cut-off points (IgG1-0.20; IgG2a/2b-0.09).

2.6. Necropsy

At necropsy all rats were euthanised and their livers with the fragment of small intestine were removed to estimate liver damage scores. The degree of liver damage was evaluated by the same two team members according to the scale proposed by Raadsma et al. (2007) where 0 corresponds to no signs of damage, 1 represents minor damage (up to 5 % of the liver), 2 slight damage (up to 15 %), 3 moderate damage (up to 30 %), 4 heavy damage (up to 50 %) and 5 extensive damage (of more than 50 %).

After scoring, liver fluke burdens were evaluated. The bile ducts were open first and flukes were recovered. Then the whole liver and the fragment of small intestine were cut into smaller pieces, placed in warm water and gently fragmented with tweezers. Liver suspensions were examined for liver flukes in Petri dishes under a stereomicroscope. Flukes were counted and reduction rates were calculated according to the formula: P = (1-d/k) × 100 % (where d stands for the mean number of flukes found in vaccinated rats; k stands for the mean number of flukes in infection control rats). Moreover, flukes were measured and total parasite biomass from each processed liver was estimated.

2.7. Statistical analysis

All data was calculated as mean ± standard deviation (SD). Data was checked for normality and variance homogeneity, and non-