Vaccine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

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

Antibody recognition of cathepsin L1-derived peptides in *Fasciola hepatica*-infected and/or vaccinated cattle and identification of protective linear B-cell epitopes

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ARTICLE INFO

Article history:
Received 27 October 2017
Received in revised form 20 December 2017
Accepted 10 January 2018
Available online xxxx

Keywords:
Fasciola hepatica
Vaccine
Epitope mapping
Cathepsin L1
Peptides
Cattle

ABSTRACT

Fasciola hepatica infection causes important economic losses in livestock and food industries around the world. In the Republic of Ireland *F. hepatica* infection has an 76% prevalence in cattle. Due to the increase of anti-helminthic resistance, a vaccine-based approach to control of Fasciolosis is urgently needed. A recombinant version of the cysteine protease cathepsin L1 (rmFhCL1) from *F. hepatica* has been a vaccine candidate for many years. We have found that vaccination of cattle with this immunodominant antigen has provided protection against infection in some experimental trials, but not in others. Differential epitope recognition between animals could be a source of variable levels of vaccine protection. Therefore, we have characterised for first time linear B-cell epitopes recognised within the FhCL1 protein using sera from *F. hepatica*-infected and/or vaccinated cattle from two independent trials. Results showed that all *F. hepatica* infected animals recognised the region 19–31 of FhCL1, which is situated in the N-terminal part of the pro-peptide. Vaccinated animals that showed fluke burden reduction elicited antibodies that bound to the regions 120–137, 145–155, 161–171 of FhCL1, which were not recognised by non-protected animals. This data, together with the high production of specific IgG2 in animals showing vaccine efficacy, suggest important targets for vaccine development.

1. Introduction

The trematode parasite *Fasciola hepatica* causes fasciolosis in livestock on every continent of the world. The disease results in important economic losses to the agricultural community globally, as well as being an highly prevalent food-borne zoonosis, with 180 million of people at risk [1–4]. *F. hepatica* infection has a prevalence of 76% in cattle in the Republic of Ireland, and an estimated prevalence of 78% in the UK [5,6]. Due to the increase in anthelmintic resistance in parasite populations, and the inherent difficulties in developing anthelmintics [7–9], a vaccine-based approach to aid in the control of fasciolosis is urgently needed.

There has been many protein candidates identified as potential vaccines against *F. hepatica*, such as fatty acid-binding proteins (FhFABP) [10–12] and glutathione S-transferases (FhGST) [13,14]. Thioredoxin peroxidase (FhPrx) was shown to induce variable levels of protection in goats [15]. Other antigens, such as leucine aminopeptidase (FhLAP), have also been demonstrated to induce high levels of protection after vaccination in sheep [16,17]. Another group of proteases, the cathepsins, have been a major vaccine target due to their proteolytic actions and potential for immunoregulation [18]. Members of this family are secreted by the juvenile parasite stage (FhCL3) and adult parasite (FhCL1, FhCL2, FhCL5) [19]. FhCL1 and FhCL2, in their native state were shown to induce 50–55% protection in cattle when used alone, and 72.4% reduction in fluke burden when administered with an haem-containing (Hb) fraction in cattle [20–22]. FhCL1 is the major component found within the excretory and secretory products from adult *F. hepatica* and it is involved in blood feeding [23,24], as well as acting to

Abbreviations: rmFhCL1, recombinant *Fasciola hepatica* Cathepsin L1; FhCL1, *Fasciola hepatica* Cathepsin L1.

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https://doi.org/10.1016/j.vaccine.2018.01.020
0264-410X/

suppress pro-inflammatory cytokines [25,26]. FhCL1 is found as an inactive procathepsin L1 in secretory vesicles in the parasite gut and only after secretion in the lumen is activated by autocatalytic cleavage of its propeptide [23,24].

A recombinant mutant version of FhCL1 (rmFhCL1), expressed in *Saccharomyces cerevisiae* or *Pichia pastoris* [23,27], which does not autocatalytically activate, is useful as a reliable immunodiagnostic tool in *Fasciola hepatica* infections in cattle [5,28]. rmFhCL1 has also been used as a vaccine antigen that was capable of reducing fluke burdens in cattle by 48.2% [29]; However, other trials have not shown a similar reduction in fluke burden, although in a study with goats, a significant decrease in liver pathology was found [30–32].

Inconsistency in vaccine efficacy between trials hinders development of a vaccine. These differences may result from multiple factors, including adjuvant effects, *F. hepatica* strain or immunological state of the animal. Differential epitope recognition by individual animals could also be a potential source of variable levels of protection both within and between trials of *F. hepatica* vaccines. Hence, epitope mapping studies are potentially useful tool in the quest for a commercialisable vaccine to protect livestock against fasciolosis.

To date, B-cell epitope mapping studies on *F. hepatica* antigens have been carried out on the *F. hepatica* Glutathione S-Transferase (FhGST) in sheep [33], *F. hepatica* saposin-like protein (Fh-SAP2) in rabbits [34], and, more recently, a range of *F. hepatica* antigens in mice [35]. In the case of *F. hepatica* cathepsins, Harmsen et al. (2004) described specific regions of FhCL1 and FhCL3 used to immunize rats which induced 40–64% fluke burden reduction [36]. Villa-Mancera (2008, 2011 and 2014) developed synthetic peptide mimotopes based on the FhCL1 protein sequence that could induce fluke burden reduction in sheep [37,38], mice [39] and goats [40]. In cattle, Cornelissen (1999) described peptides of FhCL1 that could be used as immunodiagnostics for *F. hepatica* infection [41]. Here, we characterise for the first time linear B-cell epitopes recognised within the FhCL1 protein by antibodies in the sera from, both *Fasciola hepatica*-infected and infected plus vaccinated cattle, in two independent trials. We identify specific peptides that are the sites of immunodominant epitopes with potential for future subunit vaccines.

2. Materials and methods

2.1. Experimental design, vaccination and F. hepatica infection

Ten male castrated Holstein-Friesian cattle for each trial, between 6 and 8 months of age in Trial 1 (Kalamazoo) and between 5 and 11 months old for Trial 2 (Dublin), were purchased from areas where *F. hepatica* infection was not reported. Animals were housed under uniform conditions at the experimental research facility (Trial 1) at Kalamazoo (USA) and at University College Dublin (UCD) Lyons Research Farm (Newcastle, County Kildare, Ireland) (Trial 2). In both experiments, to ensure that animals were free from *F. hepatica* infection before starting the study, animals were serologically screened by ELISA using recombinant mutant *F. hepatica* cathepsin L1 (rmFhCL1) and by faecal egg examination, as previously described [29]. Animals from each trial were then randomly divided into two groups, 5 animals in a control group, and 5 in a vaccinated group.

For the vaccine preparation, recombinant *F. hepatica* cathepsin L1 (rmFhCL1) mutant was expressed in *Pichia pastoris* as previously described [23]. Recombinant *F. hepatica* Cathepsin L3 (rmFhCL3) was a purified recombinant protein expressed by Chinese Hamster Ovary (CHO) cells. In both trials, a combination of rmFhCL1 and rmFhCL3 antigens was used to formulate a vaccine containing 200 μg of each antigen per dose plus 2 ml of adjuvant (ZA1) (Zoetis Adjuvant propriety). For the control group, 2 ml of a sterile saline solution was administered as a sham vaccine. The vaccines were kept at 4 °C during the storage and transport. In both trials, animals were vaccinated subcutaneously with a 19G needle, two times with a three (Trial 1) and two (Trial 2) weeks-interval.

In Trial 1, animals were infected with a total of 720 *F. hepatica* metacercariae orally administering 40 metacercariae in a gelatine bolus every second day over a period of 6 weeks starting 3 weeks post-2nd vaccination. Blood samples were collected by jugular venepuncture at Day 0, 3 weeks post-2nd vaccination (pre-infection phase), at 7 weeks post-infection (7 wpi) and at 13 weeks post-infection (13 wpi). This project was approved by the Kalamazoo Institutional Animal Care and Use Committee.

In Trial 2, animals were infected with a total of 200 *F. hepatica* metacercariae (Baldwin Aquatics, (Oregon) at 2 weeks post-2nd vaccination over two consecutive days (100 metacercariae per day). The metacercariae were dispersed in 10 ml of dH_2O and were administered by oral route *via* a 20 ml syringe. Blood samples were collected at Day 0, 2 weeks post-2nd vaccination (pre-infection), 2 weeks post-infection (2 wpi), 6 weeks post-infection (6 wpi), 10 weeks post-infection (10 wpi) and 14 weeks post-infection (14 wpi). This trial was approved/licenced by the UCD Animal Research Ethics Committee/Health Products Regulatory Agency (AE18982/P048), University College Dublin, Ireland.

Animals were euthanized and the livers collected at 13 (Trial 1) or 14 weeks post-infection (Trial 2). Flukes in each liver were counted as previously described [42]. In these studies carried out previously, the vaccinated group in Trial 1 showed a fluke burden reduction of 37.6% in comparison with the non-vaccinated group, whereas in Trial 2 vaccination did not elicit reduction in fluke burden compared to controls.

2.2. Measurement of IgG1 and IgG2 anti- rmFhCL1 by ELISA

IgG1 and IgG2 levels were measured in serum from all time points in both trials by using an in-house ELISA. 96 well-plates (Corning) were coated with 5 μg/ml of rmFhCL1 in carbonate-coating buffer at pH 9.6, and incubated overnight at 37 °C. Next, plates were washed three times with Phosphate Buffered Saline plus 0.05% Tween 20 (PBS-T) and then blocked by adding 100 μl of 5% milk in PBS-T for 30 min at 37 °C. After washing three times, serum samples diluted 1:20 in PBS-T were added into the wells (in 100 μl volume) in duplicate and then serial dilutions (1:3) were carried out. Plates were incubated for 30 min at 37 °C and washed as before, HRP-conjugated monoclonal anti-IgG1 (Prionics) or anti-IgG2 (Bio-rad) were added at a concentration of 1:100 (anti-IgG1) in PBS-T or 1:1000 (anti-IgG2) in PBS without Tween. After incubation at the same conditions as previously described, and washing, 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma) were added for 10 min in the dark. Finally, 50 μl of Stop solution (H_2SO_4) were added onto each well and absorbance was measured at 450 nm. Negative and positive serum controls were included in each plate. Endpoint titres were calculated for each sample and then transformed into log10.

2.3. Linear B-Cell epitope mapping of FhCL1

For epitope mapping studies, with the purpose of comparing both trials, the following time points were selected: 3wks post-2nd vaccination (pre-infection), 7 wpi and 13 wpi (Trial 1), and 2 wks post-2nd vaccination (pre-infection), 6 wpi and 14 wpi (Trial 2).

A total of 160 overlapping peptides of FhCL1, 9 amino-acids in length, and with an overlap of 7 amino-acids between successive peptides were synthesised (Mimotopes Pty. Ltd. Australia), each

Please cite this article in press as: Garza-Cuartero L et al. Antibody recognition of cathepsin L1-derived peptides in *Fasciola hepatica*-infected and/or vaccinated cattle and identification of protective linear B-cell epitopes. Vaccine (2018), https://doi.org/10.1016/j.vaccine.2018.01.020