The failure of a DNA prime/protein boost regime and CTLA-4 mediated targeting to improve the potency of a DNA vaccine encoding *Fasciola hepatica* phosphoglycerate kinase in sheep

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ABSTRACT

DNA vaccination in large animals has often been associated with poor immunogenicity, consequently several approaches have been evaluated to enhance its efficacy. Here, we tested a cDNA encoding a phosphoglycerate kinase from *Fasciola hepatica* (cDNA-FhPGK/pCMV) as a vaccine against ovine fasciolosis and investigated whether a DNA prime/protein boost regime or CTLA-4 (cytotoxic lymphocyte antigen 4) mediated targeting improved DNA vaccine efficacy. No statistically significant differences in the cellular responses were seen in either vaccine trial when compared with the respective control groups. However, specific antibody responses were considerably enhanced in DNA primed/protein boosted sheep, but not among CTLA-4 targeted cDNA-FhPGK/pCMV vaccinated animals. Nevertheless, increased titers of specific IgG1 did not contribute to protection against infection, with no differences in liver fluke recoveries reported. If DNA vaccines against fasciolosis in target species are to reach the market one day, more research in this area is needed.

1. Introduction

Fasciola hepatica infections pose a considerable threat to the health and economic value of livestock, mainly sheep and cattle (Cwiklinski et al., 2016). There is currently no approved vaccine against ovine or bovine fasciolosis and drugs are the only means of disease control. However, the emergence of drug resistance and the issue of drug residues entering the food chain are reasons to focus research on vaccine development. To date several vaccine studies in both laboratory and natural *F. hepatica* hosts have been conducted with some antigens showing potential (Toet et al., 2014; Molina-Hernández et al., 2015). Recently, genetic vaccination has provided excellent promise in the control of different diseases and a few DNA vaccines have already been approved for commercial use in companion and food animals (Redding and Weiner, 2009). Unique advantages of DNA vaccines, including their simplicity, safety, scalability, ease of transport and potentially cost-effective production, make this immunization approach a very attractive strategy. DNA-based vaccines encoding *F. hepatica* antigens have already shown their potential (Smooker et al., 1999, 2001; Wędrychowicz et al., 2003; Kennedy et al., 2006; Espino et al., 2010; Jaros et al., 2010; Jayaraj et al., 2012; Wesołowska et al., 2013). We

have previously demonstrated that rats vaccinated with cDNA encoding a phosphoglycerate kinase of *F. hepatica* (FhPGK) and subsequently exposed to liver fluke infection showed 48% and 54% reductions in worm burdens in two trials (Wesołowska et al., 2016; Wesołowska et al., 2018). However, cDNA-FhPGK/pCMV vaccinated sheep were not protected from experimental challenge (Wesołowska et al., 2016). The poor immunogenicity of genetic vaccines in large animals is still a major obstacle with this vaccination approach. Here, our aim was to investigate if a heterologous prime/boost strategy or use of a targeting molecule (cytotoxic lymphocyte antigen 4, CTLA-4) could enhance vaccine efficacy in sheep. The former approach applies to priming the immune response with the genetic form of the vaccine, and then boosting with the protein form commonly co-delivered with an adjuvant (McShane, 2002). The latter strategy is based on trafficking vaccine antigens directly to immune cells through the use of antigen-CTLA-4 fusion. CTLA-4 is known to bind with high affinity to B7 molecules present on the surface of antigen presenting cells (APCs), thus CTLA-4 fusion antigens are directly targeted to APCs. Both approaches for enhancing specific immune responses to genetic vaccines have already proven their beneficial potential in large animal models (Rothel et al., 1997; Kennedy et al., 2006).

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2. Materials and methods

2.1. Vaccine constructs

The cDNA-FhPGK/pCMV construct for vaccination was obtained as described previously (Jaros et al., 2010). In brief, a *F. hepatica* gene encoding FhPGK (GenBank, accession no. DQ112667) was subcloned into the pGEM®-T Easy Vector (Promega), and subsequently into pCMV/myc/ER Eukaryotic Vector (Invitrogen). The cDNA-FhPGK/pCMV construct was used to vaccinate sheep.

To obtain the cDNA-FhPGK-CTLA-4/pCMV construct, the cDNA encoding the ovine CTLA-4 (GenBank, accession no. NM_001009214.1) gene was cloned using specific primers CTL4ovL (5′-ACTGCAGATGGC TTGCTCTGGATTCCA-3′) and CTL4ovR (5′-TCAATTGATGGGAATAAA ATAAGGCT-3′). The fusion cDNA-FhPGK-CTLA-4/pCMV construct was obtained by using new fusion primers, FhPGK (5′-CTGGGTACCATGG GTCTGAAAAAGTTGAGT-3′) and CTLA-4 (5′-GCGGCCGCGGTACCATT GATGGGAATAAAATAAG-3′). Restriction digests of PCR products and vector were performed and followed by ligation. The accuracy of the fusion vector was confirmed by sequencing and restriction analyses.

2.2. Vaccination trial

Male Merino lambs were purchased from a fluke-free area and were shown to be free of infection by faecal analysis and ELISA using *F. hepatica* excretory-secretory material. Two vaccine trials were conducted, at the beginning of which animals were 5-months old. All experimental procedures were approved by the III Local Animal Experimentation Ethics Committee, Warsaw, Poland.

The first vaccine experiment was conducted to test heterologous prime/boost vaccination. Sheep were randomly allocated into two groups, each containing 8 male Merino sheep. Animals were immunized intramuscularly thrice at 4-week intervals. Sheep from the first group received one intramuscular injection with 100 μg of cDNA-FhPGK/ pCMV in 0.05% bupivacaine, and then two injections of 100 μg of FhPGK mixed with Montanide ISA 206 (SEPPIC-France), while animals from the second group received one pCMV and two adjuvant injections (control group). Four weeks after the third immunization sheep were orally challenged with 250 *F. hepatica* metacercariae (Weybridge, UK). The experiment ended at 12 weeks post infection (WPI) when animals were euthanized.

In the second vaccine trial sheep were randomly allocated into two groups, each containing 6 male Merino sheep. Animals from the first group were immunized thrice by intramuscular injection at 4-week intervals with 100 μg of cDNA-FhPGK-CTLA-4/pCMV construct administered in 0.05% bupivacaine. Sheep from the control group received three intramuscular injections of 100 μg of CTLA-4/pCMV construct in 0.05% bupivacaine at the same time points. Four weeks after the third immunization animals were challenged with 100 *F. hepatica* metacercariae and slaughtered at 12 WPI.

At necropsy, livers, bile ducts and gall bladders were removed and gross hepatic damage was scored 0–5 according to the scale proposed by Raadsma et al. (2007). Furthermore, flukes found in the bile ducts and gall bladders were removed, livers were cut into 1-cm-thick slices, carefully examined for parasites presence, and then worm burdens were determined.

2.3. Sampling

Blood samples were drawn from the subclavian vein from each sheep biweekly from the first immunization day to 12 WPI. Serum was separated and then stored at −70 °C until use.

Faecal samples were collected from each animal prior to vaccination, then every week starting from 6 WPI, then analyzed by the sedimentation method (Thienpont et al., 1986).

Haematology parameters were monitored during the study using an automated analyzer (Abacus JunVet). Moreover, the blood serum activities of the liver enzymes gamma glutamyl-transferase (GGT) and lactate dehydrogenase (LDH) were determined.

2.4. ELISA

Microtiter plates were coated with recombinant FhPGK that was obtained according to protocols described previously (Jaros et al., 2010). Microtiter plates were coated with 10 μg of the protein solution in carbohydrate buffer, incubated overnight at 4 °C, blocked with a 5% solution of soya milk in PBS (1 h, 37 °C), and washed thrice with PBS supplemented with 0.05% Tween20 (PBST). Sera from individual sheep were serially diluted along the plates starting from a 1:100 dilution and incubated (1 h, 37 °C). Following three consecutive PBST washes, a 1:100,000 dilution of horseradish peroxidase (HRP) conjugated anti-sheep IgG1 was added, and plates were incubated at 37 °C for 1 h. After six washes with PBST, plates were developed using tetramethylbenzidine solution (Sigma), then the reaction was stopped with 2 M sulphuric acid after 15 min and read at 450 nm on a spectrophotometer (HT Synergy, Biotek). The endpoint titers were defined as the reciprocal of the highest dilution yielding an OD450 of 0.28.

2.5. Statistical analysis

The Mann-Whitney U test was used to analyze data obtained from vaccinated versus control animals using STATISTICA 6.1 software. Comparisons were considered significant at p-values of < 0.05.

3. Results and discussion

DNA vaccine technology has been extensively studied in laboratory animals and obtained results have demonstrated that DNA vaccines are capable of inducing both cellular and humoral responses. Moreover, protection against experimental parasitic infection has been reported in a number of studies (Ahmad et al., 2009; Dai et al., 2009; Mazumder et al., 2011; Gu et al., 2014). Once it was verified that genetic vaccines had an immunoprotective potential in rodent models, trials including other species were considered viable. However, achievements in the area of anti-parasite DNA vaccines are hampered by their poor immunogenicity in large animals, such as sheep. In general, DNA vaccination does not induce strong cellular and humoral responses in target species. While the reasons underlying this hyporesponsiveness are not clear, it seems that a low transfection efficacy should be considered (Smooker et al., 2004). To improve the potency of DNA vaccines in large animals a number of approaches have been applied. These include the use of heterologous DNA primed/protein boost regime, cytokines as DNA vaccine adjuvants, CpG oligonucleotides, electroporation, and targeting molecules (Smooker et al., 2004).

Both DNA prime/protein boost regime and CTLA-4 mediated targeting have been previously tested in sheep. It has been demonstrated that intramuscular vaccination with a CTLA-4 construct encoding a procathepsin B from *F. hepatica* (FhCatB) enhanced the speed and magnitude of the antibody response before and after protein boosting when compared with non-targeted vaccination (Kennedy et al., 2006). Furthermore, studies in a murine model also confirmed that CTLA-4 fused FhCatB2 delivered as a DNA vaccine induced early and clear antibody responses (Jayaraj et al., 2012). However, protection against experimental liver fluke infection was not evaluated in those studies.

We recently tested the efficacy of a FhPGK delivered as a DNA vaccine in Merino sheep (Wesołowska et al., 2016) and observed neither a reduction in worm burden nor any differences in the course of immune responses between vaccinated and control animals following vaccination and liver fluke infection. Here, an attempt to overcome the poor immunogenicity of cDNA-FhPGK/pCMV by using a prime/boost regime and CTLA-4 targeted vaccination has been undertaken. Both DNA primed/protein boosted and cDNA-FhPGK-CTLA-4/pCMV

Table 1
Analysis of liver fluke recoveries in experimental sheep.

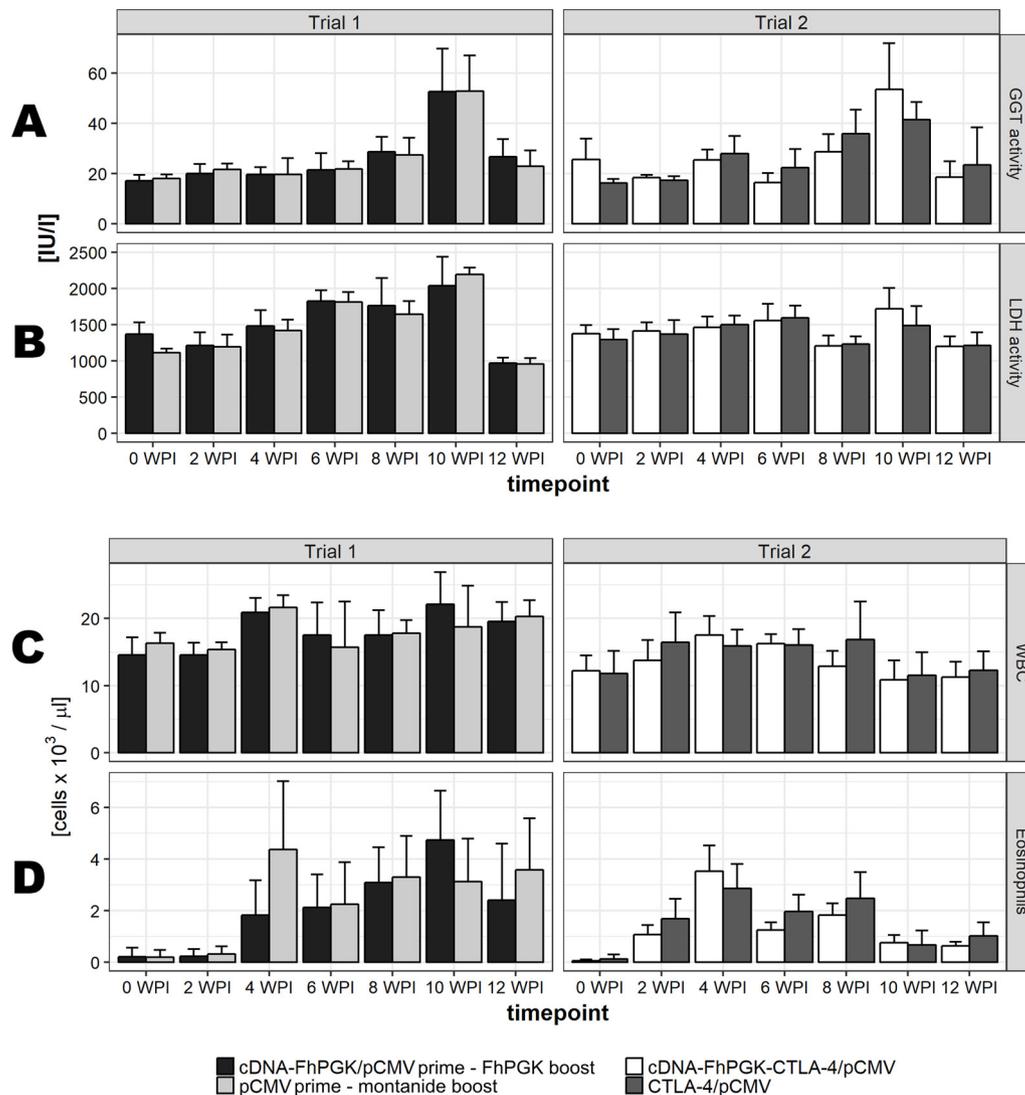| group	Liver fluke counts	Mean liver fluke count ± SD
cDNA-FhPGK/pCMV prime –FhPGK boost	136, 142, 150, 153, 154, 156, 157, 163	151.4 ± 8.7
pCMV prime – Montanie boost	87, 122, 130, 138, 166, 184, 188,189	150.5 ± 37.2
cDNA-FhPGK;CTLA-4/pCMV	37, 40, 51, 54, 64, 66	52.0 ± 11.9
CTLA-4/pCMV	19, 42, 48, 49, 64, 69	48.5 ± 17.7

Fig. 1. Liver enzyme, LDH (A) and GGT (B), activities in sera collected from vaccinated and control sheep throughout the study; WBC (C) and eosinophil (D) responses in blood collected from vaccinated and control sheep throughout the study.

vaccinated sheep were not protected from experimental challenge. Liver fluke recoveries were comparable in both vaccinated and control groups (Table 1). When previously testing cDNA-FhPGK/pCMV efficacy in a rat model, only female rats were protected from *F. hepatica* exposure (48% and 54% reduction in worm burden in two trials). No protection was observed for male rats (Wesołowska et al., 2016, 2018). Here, vaccine effectiveness and post-vaccination effects were investigated only in males. It remains unknown whether the protective effect observed in female rats could also be achieved in female sheep. Still, it is widely practiced that animal studies are conducted preferentially on males only, resulting in less evidence-based research in females. However, it must be emphasized that female exclusion may have serious implications for vaccine studies.

It is difficult to compare immune parameters directly between trials as different doses of metacercariae were used (250 and 100, respectively). After the first trial it was assumed that challenge with 250 metacercariae might have considerably altered the immune response as sheep are very sensitive to *F. hepatica*, and a lower infection dose in the second trial was used. Still, cellular responses induced by DNA primed/ protein boosted or cDNA-FhPGK-CTLA-4/pCMV vaccinated sheep were not significantly different from those generated by respective control groups. Puzzlingly, the level of liver damage (average score of 4 in each group, data not shown) was comparable between the two trials despite the different fluke burdens (averages of 150 versus 50 liver flukes). It remains unclear why the greater number of flukes observed in the first trial did not result in greater damage. Moreover, an analysis of LDH and GGT serum levels revealed no differences between vaccinated and control animals (Fig. 1 A, B).
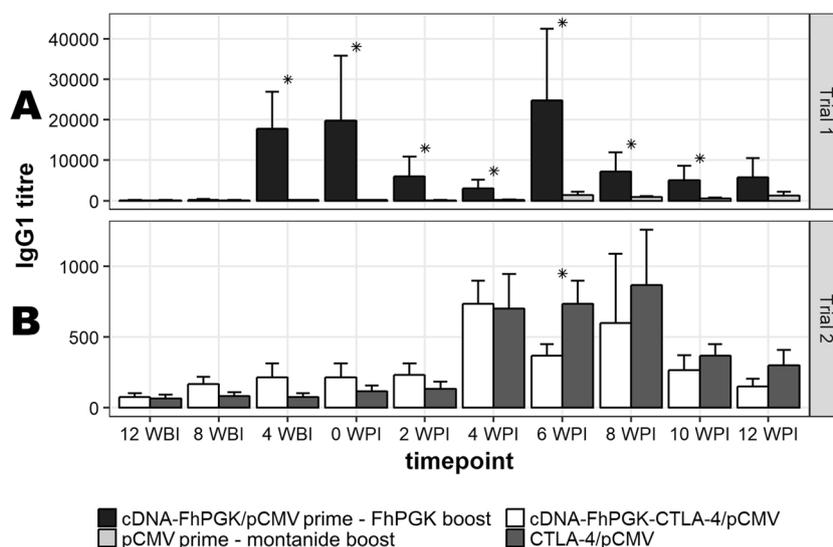
Fig. 2. IgG1 responses in vaccinated and control sheep throughout the study. Statistically significant results (p < 0.05) are marked with an asterisk.

Although the prime/boost regime and targeting via CTLA-4 failed to enhance cellular responses in vaccinated sheep (Fig. 1 C, D), an analysis of specific antibody responses in DNA primed/protein boosted animals revealed significantly increased IgG1 titers when compared with the control group (Fig. 2A). Moreover, humoral responses in sheep vaccinated according to the prime/boost regime were also different from those generated previously by cDNA-FhPGK/pCMV non-targeted vaccination (Wesołowska et al., 2016). It must be highlighted that antibody titers observed for the control group in the first trial (empty vector injection followed by two montanide injections) were similar to values observed for Merino sheep vaccinated thrice with pCMV (Wesołowska et al., 2016). During the pre-challenge period in DNA primed/protein boosted sheep a significant rise in IgG1 level was observed at 4 weeks before infection. A high level of FhPGK specific IgG1 was observed on the day of challenge, followed by a reduction in the humoral response before an apparent boosting effect occurred at 6 WPI in vaccinated sheep. The nature of the vaccine antigen was likely a factor in the decreased humoral response observed during the early infection stage. As FhPGK is a hidden antigen, significant boosting of vaccine induced immunity cannot be expected at the moment of pathogen entry, but rather at later infection stages when antigen is released by dead parasites. Increased titers of specific IgG1 did not correlate with protection against the challenge infection.

Furthermore, CTLA-4 targeting failed to enhance humoral responses (Fig. 2B). Antibody responses were modest when compared with the prime/boost regime. Moreover, the boosting effect at 6 WPI was not observed following CTLA-4 targeted vaccination. Potentially, this may be related to the lower fluke burdens as well as lower IgG1 titers observed for this group. The reasons for the poor immunogenicity of the FhPGK-CTLA-4 fusion protein remain unclear. It was confirmed that the fusion protein was expressed *in vitro* (data not published); however, it cannot be excluded that CTLA-4 fusion to FhPGK may have altered the conformation of the vaccine antigen and immunoprotective epitopes might not have been properly exposed to immune cells. Further, in the present study the CTLA-4 molecule was fused directly to the vaccine antigen, while in some early experiments the CTLA-4 molecule was additionally linked to a human IgG and therefore the fusion protein was expected to be produced as a much larger molecule (Chaplin et al., 1999; Kennedy et al., 2006). The larger molecule might potentially be more immunogenic and would have a higher avidity.

Another difference between the above-mentioned studies and the current study is that in previous experiments higher immunization doses were used (500 μg of DNA versus 100 μg). Here, the lower dose was used to enable direct comparison with a previous study where

100 μg of cDNA-FhPGK was administered (Wesołowska et al., 2016). It remains unknown whether using a higher dose would result in increased antibodies levels.

Another potential reason for the poor immunogenicity observed might be due to activation of inhibitory pathways downstream of the CTLA-4 and B7 target, resulting in failure to boost potent immune responses. In addition, other studies have demonstrated that the CTLA-4 targeting strategy is not always successful at improving DNA vaccine efficacy. It has been reported that vaccination of sheep with a DNA vaccine expressing CTLA-4 targeted 45 W antigen from *Taenia ovis* did not enhance immune responses (Drew et al., 2001).

The veterinary use of genetic vaccines has great potential. However, despite the promise shown in laboratory animals, poor immunogenicity of DNA vaccines in target animal species is still a challenge for practical DNA vaccine use. Reasons underlying the failure of genetic vaccination to promote adequate immune responses in large animals have to be elucidated. Nevertheless, DNA vaccines, owing to their unique advantages, represent an attractive approach for the control of parasitic diseases. Further research aimed at designing new vaccination regimens and identification of effective vaccine antigens is needed to provide enhancement for DNA vaccination in large animal models.

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