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Immune parameters to p67C antigen adjuvanted with ISA206VG correlate with protection against East Coast fever

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ABSTRACT

East Coast fever (ECF) is a lymphoproliferative disease caused by the tick-transmitted protozoan parasite *Theileria parva*. ECF is one of the most serious cattle tick-borne diseases in Sub-Saharan Africa. We have previously demonstrated that three doses of the C-terminal part of the sporozoite protein p67 (p67C) adjuvanted with ISA206VG confers partial protection against ECF at a herd level. We have tested the efficacy of two doses of this experimental vaccine, as reducing the vaccination regimen would facilitate its deployment in the field. We reconfirm that three antigen doses gave a significant level of protection to severe disease (46%, ECF score < 6) when compared with the control group, while two doses did not (23%). Animals receiving three doses of p67C developed higher antibody titers and $CD4^+$ T-cell proliferation indices, than those which received two doses. A new panel of immune parameters were tested in order to identify factors correlating with protection: $CD4^+$ proliferation index, total IgG, IgG1, IgG2 and IgM half maximal titers and neutralization capacity of the sera with and without complement. We show that some of the cellular and humoral immune responses provide preliminary correlates of protection.

1. Introduction

East Coast fever (ECF) is a lethal disease of cattle caused by the tick transmitted protozoan parasite *Theileria parva*. The disease is a constraint for the development of the livestock industry in eastern, central and southern Africa because of the high mortality and morbidity caused by the disease. Cattle that recover from infection develop life-long immunity to re-challenge. This enabled the development of a live vaccine for ECF called "Infection and Treatment Method" (ITM), which is based on the simultaneous inoculation of a lethal dose of sporozoites and a long-acting oxytetracycline. This method of vaccination was used to create the "Muguga-cocktail" vaccine, which consists of three different *T. parva* sporozoite isolates and results in broad-spectrum immunity to ECF. This vaccine is now commercially available. However, the process of ITM has several limitations such as the need of a liquid nitrogen cold chain, use of antibiotics and animals can become carriers of the vaccine strains. Further, the vaccine is difficult to produce and therefore relatively expensive. This has led to attempts of finding alternative ways of inducing immunity to ECF (reviewed in [1–4]).

It was previously demonstrated that MHC I-restricted $CD8^+$ cytotoxic T-lymphocytes played a critical role in protection primed by ITM immunization [5,6], but the humoral response also plays a role in mediating immunity. Sera from cattle immunized with *T. parva* Muguga (ITM) and repeatedly boosted by attaching adult infected ticks at intervals of two weeks, have high sporozoite-specific antibody titers capable of neutralizing infectivity of sporozoites *in vitro* [7,8] and *in vivo* [9], the latter determined by mixing sporozoites and sera before injection into cattle. This led to a search for sporozoite proteins involved in the infection process, which could be used for induction of neutralizing antibodies and, hence, used for vaccination. Monoclonal antibodies with sporozoite neutralizing activity recognized a major surface coat protein in sporozoites [7,10], called p67 due to its apparent molecular mass. The p67 protein is very conserved among cattle derived *T. parva* strains and consists of 709 amino acid residues, but it is polymorphic among buffalo derived stains [11,12]. Thus, it is likely that

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p67 can function as a cross-protective immunogen for an anti-sporozoite vaccine, at least among cattle derived *T. parva* strains, and possibly for buffalo derived strains, as all p67 alleles share a high degree of sequence identity [12].

Several *in vivo* vaccine trial experiments have been performed with various constructs of p67 using full-length and fragments of recombinant protein with a range of adjuvants and gene based antigen delivery systems (reviewed in [1]). A consistent problem encountered in expressing full length p67 in *E. coli* in a soluble format has been protein instability, which affected yield and quality [13]. An 80 amino-acid (AA) peptide from the C-terminal part of p67 (p67C), containing epitopes recognized by sporozoite neutralizing monoclonal antibodies [14], can be expressed in stable manner and in high yield [13]. When tested using syringe challenge, immunization with p67C protein resulted in similar levels of protection to a nearly full-length version of the protein, $p67_{635}$, 40 to 70% protection (ECF score < 6) against severe ECF relative to a control [13,15,16]. These results suggested that this fragment could replace near full-length protein. However, a general observation on the efficacy of immunization with p67 in field trials, where the challenge is achieved by infected ticks rather than a syringe challenge, was that protection levels relative to controls was lower, 20–30%, as approximately 50% of the animals in the control groups naturally recovered from tick challenge. The reason for this lower level of protection was not clear [15,17], but it indicates that the efficacy of p67 under laboratory conditions needs to be improved.

Previous experiments had primarily established the efficacy of three doses of 450 μg of p67C given at four week intervals. However, the number of p67C antigen doses was not assessed. One of the objectives of this study was to test the possibility of reducing the number of doses from three to two, which would make the vaccination regimen more applicable for use in the field and lower the cost. Moreover, previous studies have not identified strong immune correlates of protection, which is an obstacle for vaccine development. Parameters, previously tested for correlation were: antibody titers (either total immunoglobulin (Ig) or IgG), neutralizing antibody titers and peripheral blood mononuclear cell (PBMC) proliferation indices. Only in one report [16], a correlation was found between the capability of sera to neutralize sporozoite infectivity and the level of protection. Hence, another objective of this study was to expand the panel of immune parameters to determine correlates with protection. This included $CD4^+$ proliferation indices to p67C, total IgG, IgG1, IgG2 and IgM antibody half maximal titers, and a novel total antibody neutralizing assay, with and without complement, to help guide further improvements in optimizing the efficacy of p67C.

2. Material and methods

2.1. Experimental design of the in vivo experiment

Boran cattle (*Bos indicus*), 6 to 9 months old and negative for *T. parva* and *T. mutans* antibodies by ELISA [18] were used in the *in vivo* experiment. Thirty-two animals were randomly assigned into three experimental groups. In Group 1, ten animals were injected twice with 450 μg of purified p67C protein, and in Group 2, eleven animals were injected thrice with the same amount of protein. Antigen doses were administered subcutaneously 28 days apart. Group 1 animals were immunized for the first time at day 28 in the experiment, coinciding with the second dose of Group 2 animals. For Group 3, eleven animals were kept unvaccinated and used as a control group for challenge. The immunogen (p67C) was mixed with Montanide ISA206 VG adjuvant (Seppic) in a 1:1 ratio following the manufacturer's instructions. The final volume injected in each animal was 2 ml. His-Tag p67C protein was expressed and purified as previously described [13].

Twenty-one days after the last boost, all animals were given a syringe challenge of 1 ml of *T. parva* Muguga sporozoites (stabilate #3087), diluted 1/100 in stabilate diluent (Minimum Essential Medium (MEM) Eagle (Gibco), 3.5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich), 100 UI/ml penicillin (Sigma-Aldrich), 100 μg/ml streptomycin (Sigma-Aldrich) and 7.5% glycerol (v/v, Sigma-Aldrich). The stabilate, dilution and volume was identical to the ones used in earlier p67C experiments [11,19,20]. This dilution was reported to correspond to a lethal dose 70% (LD_{70}), which implies that 70% of the control animals will be susceptible to the disease (ECF score $\geq$ 6). The challenge dose was administered in all animals sub-cutaneously over the parotid lymph node (local drainage lymph node). We did not include an adjuvant-alone control group because it is known that the use of Montanide ISA206 VG does not result in any background protection [15]. It is important to note that the sporozoite stabilate was titrated in an *in vivo* experiment before the current experiment, since the stabilate had not been used for 9 years. It was found that the stabilate had retained its potency.

After the challenge, all experimental cattle were monitored daily for changes in rectal temperatures and other clinical manifestations of ECF and the ECF scores were calculated (Rowland's index) [21]. The index was used to define whether animals were susceptible (Index: 6–10, not protected) or immune (Index: 0–5.99, protected) to ECF, as previously described [15]. The humane end-point for the animals was determined by the institutional veterinarian based on clinical signs, such as: weakness, diarrhoea, staggering gait, dyspnoea and finally recumbency. The ECF scores included in the data analysis were from day 21 after the challenge. At this time point, the indices had stabilized and the experiment was terminated. Immune animals were monitored for an extra week in order to observe if there were further development in the indices. The animal experiment was approved by ILRI's Institute Animal Care and Use Committee (IACUC number 2014.09). One-way ANOVA was used for comparison of the ECF scores between groups (Rowland's Index), assumptions for normality were checked. The Fisher's exact test was used for testing significance between groups of protected versus non-protected animals (binary 0/1).

2.2. ELISAs for detection of p67C specific antibody isotypes/subtypes

p67C specific antibodies in bovine sera were detected by means of an ELISA assay. p67C protein was coated at 0.5 μg/ml (in phosphate buffered saline, PBS) overnight at 4 °C on Maxisorp ELISA plates (Nunc). After blocking the plates for an hour with 0.2% casein (Sigma-Aldrich) and 0.1% Tween-20 (Merck) in PBS (blocking buffer), sera were added to the plate in 3-fold dilution series in duplicates starting from 1/100. The presence of the bovine antibodies was detected using the following antibodies, depending on the isotype/subtype of interest: sheep anti-bovine IgM:HRP, sheep anti-bovine IgG:HRP, sheep anti-bovine IgG1:HRP or sheep anti-bovine IgG2:HRP (all from AbD Serotec), all used at a dilution of 1:1,000 in blocking buffer. The reaction was developed using the following substrate buffer: 2,2′-azino-di-[3-ethyl-benzothoazolines-6-sulfonic acid] diammonium salt (ABTS, Sigma-Aldrich) at 134 μg/ml, 0.075% H_2O_2 (Sigma-Aldrich), 175 mM Na_2HPO_4 (Sigma-Aldrich) and 200 mM citric acid (Sigma-Aldrich), at pH 4. The reaction was incubated for 30 min at 37 °C in the dark and read at 405 nm in the Synergy HT ELISA reader (BioTek). Four washes were performed between every step using PBS Tween-20 (0.1%).

2.3. Calculation of half maximal antibody titers

Bovine sera were diluted in 3-fold dilution series, starting from 1/100 up to 1/72,900 using blocking buffer. This generated a loga-