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Original article

Bacterial membranes enhance the immunogenicity and protective capacity of the surface exposed tick Subolesin-*Anaplasma marginale* MSP1a chimeric antigen

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ABSTRACT

Ticks are vectors of diseases that affect humans and animals worldwide. Tick vaccines have been proposed as a cost-effective and environmentally sound alternative for tick control. Recently, the *Rhipicephalus microplus* Subolesin (SUB)-*Anaplasma marginale* MSP1a chimeric antigen was produced in *Escherichia coli* as membrane-bound and exposed protein and used to protect vaccinated cattle against tick infestations. In this research, lipidomics and proteomics characterization of the *E. coli* membrane-bound SUB-MSP1a antigen showed the presence of components with potential adjuvant effect. Furthermore, vaccination with membrane-free SUB-MSP1a and bacterial membranes containing SUB-MSP1a showed that bacterial membranes enhance the immunogenicity of the SUB-MSP1a antigen in animal models. *R. microplus* female ticks were capillary-fed with sera from pigs orally immunized with membrane-free SUB, membrane bound SUB-MSP1a and saline control. Ticks ingested antibodies added to the blood meal and the effect of these antibodies on reduction of tick weight was shown for membrane bound SUB-MSP1a but not SUB when compared to control. Using the simple and cost-effective process developed for the purification of membrane-bound SUB-MSP1a, endotoxin levels were within limits accepted for recombinant vaccines. These results provide further support for the development of tick vaccines using *E. coli* membranes exposing chimeric antigens such as SUB-MSP1a.

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1. Introduction

Diseases caused by arthropod-borne pathogens greatly impact human and animal health. Ticks are considered to be second to mosquitoes as vectors of pathogens to humans and the most important vectors of pathogens that cause disease in cattle (de la Fuente et al., 2007). Control of tick infestations has been based primarily on the use of chemical acaricides that has resulted in selection of acaricide-resistant ticks and environmental pollution (de la Fuente et al., 2007). Vaccination with the *Rhipicephalus microplus* BM85/BM95 gut antigen emerged as an alternative for tick control that has shown the advantage of being cost-effective while reducing acaricide applications and the drawbacks associated with their use (de la Fuente et al., 2007; Willadsen, 2006).

Subolesin (SUB), the ortholog of insect and vertebrate Akirins (AKR) is an evolutionary conserved protein that was recently discovered in *Ixodes scapularis* as a tick protective antigen (Almazán et al., 2003; de la Fuente et al., 2011). SUB is involved in tick innate immune response and in other molecular pathways including those required for feeding, reproduction and pathogen infection and multiplication (de la Fuente et al., 2011). Vaccination with recombinant SUB/AKR showed a reduction in tick, mosquito, sand fly and poultry red mite infestations by reducing ectoparasite numbers, weight and/or oviposition and tick infection with different pathogens such as *Anaplasma phagocytophilum*, *Anaplasma marginale*, *Babesia bigemina* and *Borrelia burgdorferi* (de la Fuente et al., 2013). Recently, vaccination with recombinant *Aedes albopictus* AKR reduced *Plasmodium berghei* infection in mosquitoes fed on immunized mice when compared to controls (da Costa et al., 2014). These results suggested that SUB/AKR is a good candidate antigen for the development of vaccines for the control of multiple arthropod vectors and vector-borne diseases (de la Fuente et al., 2011, 2013; da Costa et al., 2014; Moreno-Cid et al., 2013).

Proteins exposed on the cell membrane such as major surface proteins are good targets for vaccine development. Recently, recombinant chimeras comprising tick proteins such as BM95 immunogenic peptides and SUB fused to the N-terminal region of the *A. marginale* Major Surface Protein 1a (MSP1a) (BM95-MSP1a and SUB-MSP1a chimeras, respectively) were produced in *Escherichia coli* as membrane-bound and exposed proteins (Canales et al., 2008; Almazán et al., 2012). Furthermore, the use of bacterial membranes containing the membrane-bound BM95-MSP1a or SUB-MSP1a proteins as vaccines resulted in the control of *R. microplus* and *R annulatus* infestations in cattle (Almazán et al., 2012; Canales et al., 2009). This system provides a novel, simple and cost-effective approach for the production of tick protective antigens by surface displaying antigenic protein chimera on the *E. coli* membrane and demonstrated the possibility of using recombinant bacterial membrane fractions in vaccine preparations to protect cattle against tick infestations (Almazán et al., 2012; Canales et al., 2009, 2010).

However, important issues still need to be addressed before *E. coli* membranes with surface-exposed antigens such as the SUB-MSP1a chimera could be used in commercial vaccine formulations. These issues include the characterization of the role of *E. coli* membranes in enhancing the immunogenicity of membrane-bound SUB-MSP1a chimeric antigen and the potential problems associated with the content of bacterial endotoxins in the SUB-MSP1a antigen preparation. In this study, we addressed these issues by characterizing the composition and immunogenicity of the membrane-bound SUB-MSP1a chimeric antigen. The results showed that the purified membrane-bound SUB-MSP1a chimeric antigen is more immunogenic than the membrane-free SUB-MSP1a and SUB antigens with endotoxin levels acceptable for vaccine formulations, therefore providing further support for the development of tick vaccines using *E. coli* membranes exposing chimeric antigens such as SUB-MSP1a.

2. Materials and methods

2.1. Production of recombinant SUB-MSP1a chimeric antigen and SUB in E. coli

Unless otherwise indicated, all reagents used in this work were purchased either from Sigma–Aldrich (St. Louis, MO, USA) or VWR International Eurolab S.L. (Mollet del Vallés, Barcelona, Spain). For the production of the membrane-bound *R. microplus* SUB-MSP1a chimera, recombinant *E. coli* JM109 cells transformed with the pMBXAF3 expression vector were used (Almazán et al., 2012). In this construct, as for the BM95-MSP1a chimera (Canales et al., 2008), the inserted SUB coding region is fused to MSP1a and is under the control of the inducible tac promoter (Almazán et al., 2012). Recombinant *E. coli* were propagated in 1 l flasks containing 250 ml Luria–Bertani (LB) broth supplemented with 10 g tryptone l^{-1}, 5 g yeast extract l^{-1}, 10 g NaCl l^{-1}, 50 μg/ml ampicillin and 0.4% glucose (Laboratorios CONDA S.A., Madrid, Spain) for 2 h at 37 °C and 200 rpm and then for 5.5 h after addition of 0.5 mM final concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) for induction of recombinant protein production as previously described (Canales et al., 2008). Cell growth was monitored by measuring OD at 600 nm. Protein concentration was determined using bicinchoninic acid (BCA). The cells were harvested by centrifugation at 10,000 × *g* for 15 min at 4 °C and then 1 g of cell pellet was resuspended in 5 ml of disruption buffer (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM PMSF, 5 mM $MgCl_2 \cdot 6H_2O$ and 0.1% (v/v) Triton X-100) and disrupted using a cell sonicator (Model MS73; Bandelin Sonopuls, Berlin, Germany). After disruption, the insoluble and soluble protein fractions containing the membrane-bound and membrane-free SUB-MSP1a, respectively were collected by centrifugation at 21,500 × *g* for 15 min at 4 °C and stored at −20 °C until used for characterization and vaccine formulations. Recombinant SUB was produced in *E. coli* and purified by Ni affinity chromatography to >95% purity as previously described (Almazán et al., 2010).

2.2. Polyacrylamide gel electrophoresis and western blot for the characterization of recombinant SUB-MSP1a

The recombinant SUB-MSP1a was analyzed by SDS-PAGE and western blot. Ten micrograms of total proteins were loaded onto a 12% SDS-polyacrylamide gel (Criterion XT precast gels, Bio-Rad, Hercules, CA, USA) and either stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane for western blot analysis. The percent of total cell proteins corresponding to recombinant SUB-MSP1a was determined in the stained SDS-polyacrilamide gel by densitometric analysis using ImageJ 1.44p (National institute of Health, USA).

For western blot analysis, proteins in the gel were transferred to a nitrocellulose membrane during 1 h at 12 V in a Mini-Genie Electroblotter semi-dry transfer unit (Idea Scientific, Corvallis, OR, USA). The membrane was blocked with 5% skim milk for 1 h at room temperature, washed three times in TBS and probed with rabbit antibodies. Serum from rabbits immunized with recombinant *R. microplus* SUB (Almazán et al., 2010) was diluted 1:500 in 3% BSA in TBS and the membrane was incubated with the diluted sera for 1 h at room temperature, and washed three times with TBS. The membrane was then incubated with an anti-rabbit horseradish peroxidase (HRP) conjugate (Sigma, St. Louis, MO, USA) diluted 1:1000 in TBS. The membrane was washed three times with TBS and finally developed with TMB stabilized substrate for HRP (Promega) for 20 min. Recombinant *A. phagocytophilum* superoxide dismutase

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