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Evaluation of the immune response to *Anaplasma marginale* MSP5 protein using a HSV-1 amplicon vector system or recombinant protein

Carlos Palacios [a], Susana Torioni de Echaide [b], Nora Mattion [a,*]

[a] Centro de Virología Animal, Instituto de Ciencia y Tecnología Dr. Cesar Milstein, CONICET, Saladillo 2468, C1440FFX, Ciudad de Buenos Aires, Argentina
[b] Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela, Ruta 34 Km 227, CP 2300, Rafaela, Santa Fe, Argentina

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ABSTRACT

Anaplasma marginale is an intraerythrocytic vector-borne infectious agent of cattle. Immunization with the current vaccine, based on parasitized erythrocytes with live *Anaplasma centrale*, shows some constraints and confers partial protection, suggesting the feasibility for the development of new generation of vaccines. The aim of the present study was to assess the effect of sequential immunization of BALB/c mice, with herpesvirus amplicon vector-based vaccines combined with protein-based vaccines, on the quality of the immune response against the major surface protein 5 of *A. marginale*. The highest antibody titers against MSP5 were elicited in mice that received two doses of adjuvanted recombinant protein ($p < 0.0001$). Mice treated with a heterologous prime-boost strategy generated sustained antibody titers at least up to 200 days, and a higher specific cellular response. The results presented here showed that sequential immunization with HSV-based vectors and purified antigen enhances the quality of the immune response against *A. marginale*.

1. Introduction

Anaplasmosis is a relevant vector-borne hemoparasitic rickettsial disease of ruminant livestock, caused by the intraerythrocytic rickettsia *Anaplasma marginale* (Dumler et al., 2001), in tropical and subtropical regions of the world, including South America (Brayton et al., 2009; Guglielmone, 1995; Suarez and Noh, 2011). It is transmitted either biologically by ixodid ticks or mechanically through needles and biting flies. The replication of the rickettsia in erythrocytes of infected cattle, results in anemia, weight loss, abortions, and eventually death. Infection is characterized by sequential cycles of rickettsemia, which occurs as a logarithmic increase in the parasite population, followed by a dramatic decline. Each cycle reflects the emergence of a new generation of bacteria expressing variants of the major surface protein 2 (MSP2) (French et al., 1999).

In cattle that survive clinical disease, the microorganisms persist indefinitely after infection, although at low levels, acting as reservoirs for further transmission (Aubry and Geale, 2011; Kuttler et al., 1984; Vidotto et al., 1994), and remaining protected against subsequent homologous challenge (Aubry and Geale, 2011).

The vaccine against bovine anaplasmosis, currently used in several countries, is based on live *A. centrale*, multiplied in splenectomized calves. Although one dose of this vaccine confers immunity for several years, it has the drawbacks of requiring exhaustive quality control to prevent the presence of other blood-borne organisms in the donor calves and the induction of only partial protection from challenge with virulent *A. marginale*.

New generation vaccines, such as those based on recombinant DNA methodologies, are not available yet for anaplasmosis control (OIE, 2012). Nevertheless, it has been reported that cattle immunized with an outer membrane fraction of *A. marginale* were protected from clinical disease following homologous challenge (Noh et al., 2008; Tebele et al., 1991), and that antibody titers against some members of outer membrane proteins, correlated with protection against anemia (Vidotto et al., 1994).

Several immunization strategies have been experimentally evaluated to protect cattle against anaplasmosis (Brown et al., 1998; McGuire et al., 1994; Tebele et al., 1991); however neither one prevents cattle from becoming persistently infected with *A. marginale*, as reviewed elsewhere (Aubry and Geale, 2011; Kocan et al., 2003).

Despite the advances in vaccine development, further studies are needed in order to evaluate the combination of novel antigen-delivery systems in the improvement of protection of cattle from *A. marginale* infection, and in blocking its biological transmission. Viral vectors represent an interesting delivery tool for DNA vaccines, which have not been extensively studied for anaplasmosis.

In this regard, HSV-1 amplicon vectors have a large transgene capacity (up to 150 kb), which allows the encapsidation of multiple genes or multiple copies of a transgene. Additional advantages of HSV-1 derived vectors include (i) low toxicity and low immunogenicity, in particular when helper virus-free amplicon vectors are used, (ii) high transduction efficiencies both in quiescent and

* Corresponding author. Tel.: +54 11 4686 6225; fax: +54 11 4686 6225.
E-mail address: nmattioncevan@centromilstein.org.ar (N. Mattion).

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proliferating cells from most mammalian species, including antigen-presenting cells in vivo, (iii) genetic stability, and (iv) strong adjuvant effects, very long-lived immune responses, and the capacity of generating both humoral and cellular immune response and mucosal immunity (Cuchet et al., 2007; De Silva and Bowers, 2009; Epstein, 2009; Hocknell et al., 2002; Kaur et al., 2007; Laimbacher and Fraefel, 2014).

In this study, genetic vaccines based on HSV-1 amplicon vectors are explored as vehicles to deliver DNA encoding *A. marginale* Major Surface Protein 5 (MSP5) to assess the immune response generated in mice, in homologous viral vector vaccination, or in combination with recombinant protein.

2. Materials and methods

2.1. Cells and viruses

Vero, MA104, Vero-7b (Krisky et al., 1998; Marconi et al., 1996) and human glioma Gli36 (Kashima et al., 1995) cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/ml), streptomycin (100 μg/ml). Vero-7b cell line provides the essential ICP4 and ICP27 immediate-early (IE) herpesvirus gene functions *in trans*, to support the in vitro propagation of replication defective HSV-1-LaLΔJ virus (Zaupa et al., 2003), used as helper in the production of the amplicon vectors. For culturing Vero-7b cell line, Geneticin® (500 μg/ml, Invitrogen, CA, Carlsbad, USA) was included in the medium.

2.2. Construction of amplicon plasmids

Amplicon DNA plasmids were constructed using the *pHSVs* plasmid system, as previously described (D'Antuono et al., 2010). The main genetic elements of *pHSVs* are: (i) a transcription unit including the HSV-1 IE 4/5 promoter to support transgene expression and an IRES element followed by EGFP as reporter gene for titration; (ii) a prokaryotic origin of DNA replication (*colE1*) and the ampicillin resistance gene (*AmpR*), to allow the propagation of the plasmids in *E. coli*; (iii) two functional HSV sequences (*ori* and *pac*) that support replication of the amplicon DNA and the packaging into HSV-1 particles.

The *msp5* open reading frame (Acc. num.: M93392.1) was PCR amplified from recombinant plasmid *pGEM/AmMSP5* using specific primers (sense: 5'-*GTCGAC*ATGAGAATTTTCAAGATTGT-3' and antisense: 5'-*TTCGAA*CTAAGAATTAAGCATGTGAC-3'). The resulting product was inserted into the *Sal*I and *Bst*BI restriction sites of $pHSV_S$, generating *pHSV/MSP5* amplicon plasmid. An amplicon vector expressing the EGFP gene, *pHSV-EGFP* (D'Antuono et al., 2010), was used as negative control in immunofluorescence assays and immunization experiments. *pHSV-EGFP* possesses a transcription unit composed by the HSV-1 IE4/5 promoter, the EGFP coding sequence, and the SV40 early region polyadenylation site.

2.3. Stocks of amplicon vectors

In order to generate stocks of amplicon vectors, the amplicon plasmid DNA was transfected into T75 flasks of 70% confluent Vero-7b cells, using Lipofectamine™ and Plus™ Reagent (Invitrogen), following manufacturer's instructions. Transfected cells were infected the next day with HSV-1-LaLΔJ helper virus, at a multiplicity of infection (MOI) of 0.3 PFU/cell in medium 199 (M199, Invitrogen) supplemented with 1% FBS. When total cytopathic effect was observed, cells were scraped off and centrifuged at 2000 × *g* 5 min at 4 °C and the pellets were resuspended in 400 μl of M199 1% FBS and subjected to three cycles of freezing/thawing. The suspended pellets were sonicated on ice for 10 s, and clarified at 3000 × *g*, 10 min at 4 °C. Released amplicon vectors were passaged once in Vero-7b cells, by adding HSV-1-LaLΔJ at an MOI of 0.1 helper virus per cell. Titers of helper virus in viral stocks were determined by plaque assay on Vero-7b cells (PFU/ml) and amplicon vectors titers were assessed on Gli36 cells by scoring the number of GFP positive cells under a fluorescence microscope, and expressed as Transducing Units per milliliter (TU/ml).

2.4. Immunofluorescence analysis

For immunofluorescence analysis, Vero or MA104 cells were seeded on round 12-mm cover glasses placed in 24-well plates at a density of 10^5 cells per well. After 24 h, the monolayers were washed with PBS, and infected at a MOI of 0.1 TU/cell with amplicon vectors diluted in DMEM. The particles were allowed to adsorb for 1 h at 37 °C, 5% CO_2, washed with PBS, overlaid with DMEM supplemented with 10% FBS, and incubated at 37 °C, 5% CO_2. At 24 h post infection (hpi), the cells were washed once with cold PBS, fixed with 3.7% paraformaldehyde in PBS at room temperature (RT) for 15 min, and neutralized for 5 min with 100 mM glycine in PBS. After being washed with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 15 min at RT, and blocked with PBS supplemented with 3% bovine serum albumin (PBS-B) for 30 min at RT. Fixed cells were first incubated during 1 h in a humid chamber with the anti-MSP5 monoclonal antibody (MAb) ANAF16C1 (VMRD, Inc.) 2 μg/ml in PBS-B. After three washes with PBS, the cells were incubated, with a rabbit anti-mouse IgG Alexa Fluor 594-conjugated secondary antibody (Molecular Probes), diluted at 2 μg/ml in PBS-B buffer. All incubations were carried out at RT. Cell nuclei were stained with 300 nM DAPI (4',6'-diamidino-2-phenylindole; Invitrogen) in PBS for 5 min. The monolayers were washed three times with PBS, once with distilled water, and mounted using FluorSave™ Reagent (CalBiochem®) to preserve the fluorescence-labeled samples. Images were acquired using a Nikon Eclipse E600 microscope equipped with the Nikon DS-Fi1 camera. Images were processed with ImageJ software (Schneider et al., 2012).

2.5. Expression of recombinant MSP5 protein

Two different recombinant MSP5 proteins were produced for different purposes: MSP5-Maltose Binding Protein (MBP) fusion and MSP5-6xHis (H_6) tagged. To obtain the recombinant proteins, *msp5* open reading frame was amplified by PCR (sense: 5'-*GGATCC* ATGAGAATTTTCAAGATTGT-3' and antisense: 5'-*CTGCAG*CTAAGA ATTAAGCATGTGAC-3') and subcloned into the *BamH*I and *Pst*I sites of *pMAL-c2x* (New England Biolabs, Inc., Ipswich, MA, USA) or *pRSET* (Invitrogen) plasmids, respectively.

The recombinant plasmid *pMAL/MSP5* was used to transform *Escherichia coli* Top10 (Invitrogen), and the rMSP5-MBP fusion protein produced was purified by affinity chromatography on an amylose resin (New England Biolabs, Inc., MA, USA), as described elsewhere (Knowles et al., 1996). This protein was used for animal immunization, and a competitive inhibition enzyme-linked immunosorbent assay (cELISA).

For lymphoproliferation and total antibody ELISA assay, the recombinant MSP5 protein with a 6xHistidine tag was used (MSP5-H_6). For this purpose, the *pRSET/MSP5* plasmid was used to transform *E. coli* BL21(DE3)pLysS (Invitrogen Corporation), and the recombinant MSP5-H_6 protein produced was purified by nickel metal affinity chromatography. Briefly, 3 ml of an overnight culture was inoculated into 300 ml of LB medium with 100 μg of ampicillin and 34 μg of chloramphenicol per milliliter, and grown at 37 °C to 0.5 $OD600_{nm}$. At that point, the expression of rMSP5-H_6 was induced by the addition of 1 mM of isopropyl-β-D-thiogalactopyranoside. The bacteria were incubated at 37 °C for 3 additional hours, and harvested by centrifugation at 3500 × *g* for 20 min and the pellet was