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The immunogenicity of recombinant vaccines based on modified Vaccinia Ankara (MVA) viruses expressing African horse sickness virus VP2 antigens depends on the levels of expressed VP2 protein delivered to the host

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ABSTRACT

African horse sickness (AHS) is a lethal equine disease transmitted by *Culicoides* biting midges and caused by African horse sickness virus (AHSV). AHS is endemic to sub-Saharan Africa, but devastating outbreaks have been recorded periodically outside this region. The perceived risk of an AHS outbreak occurring in Europe has increased following the frequent epidemics caused in ruminants by bluetongue virus, closely related to AHSV.

Attenuated vaccines for AHS are considered unsuitable for use in non-endemic countries due bio-safety concerns. Further, attenuated and inactivated vaccines are not compatible with DIVA (differentiate infected from vaccinated animals) strategies. All these factors stimulated the development of novel AHS vaccines that are safer, more efficacious and DIVA compatible.

We showed previously that recombinant modified Vaccinia Ankara virus (MVA) vaccines encoding the outer capsid protein of AHSV (AHSV-VP2) induced virus neutralising antibodies (VNAb) and protection against AHSV in a mouse model and also in the horse. Passive immunisation studies demonstrated that immunity induced by MVA-VP2 was associated with pre-challenge VNAb titres in the vaccinates. Analyses of the inoculum of these MVA-VP2 experimental vaccines showed that they contained pre-formed AHSV-VP2.

We continued studying the influence of pre-formed AHSV-VP2, present in the inoculum of MVA-VP2 vaccines, in the immunogenicity of MVA-VP2 vaccines. Thus, we compared correlates of immunity in challenged mice that were previously vaccinated with: a) MVA-VP2 (live); b) MVA-VP2 (live and sucrose gradient purified); c) MVA-VP2 (UV light inactivated); d) MVA-VP2 (UV light inactivated and diluted); e) MVA-VP2 (heat inactivated); f) MVA-VP2 (UV inactivated) + MVA-VP2 (purified); g) MVA-VP2 (heat inactivated) + MVA-VP2 (purified); and h) wild type-MVA (no insert). The results of these experiments showed that protection was maximal using MVA-VP2 (live) vaccine and that the protection conferred by all other vaccines correlated strongly with the levels of pre-formed AHSV-VP2 in the vaccine inoculum.

1. Introduction

African horse sickness (AHS) is an arthropod-borne viral disease of solipeds transmitted by haematophagus insects of the genus *Culicoides*, the horse being the most severely affected species. The disease is caused by African horse sickness virus (AHSV), a member of the genus *Orbivirus*, family *Reoviridae*, closely related to bluetongue virus (BTV). The non-enveloped 55–70 nm AHSV spherical virion consists of a triple-layered capsid surrounding ten double-stranded RNA segments and three proteins involved in viral replication (VP1, VP4 and VP6, encoded by segments 1, 4 and 9 respectively) (Roy et al., 1994; Manole et al., 2012). The inner capsid protein VP3, encoded by segment 3, forms the icosahedral scaffold to which trimers of the conserved VP7 protein bind. The outer capsid is formed by two major structural proteins, VP2 and VP5 (encoded by segments 2 and 6 respectively), involved in cell attachment and entry. VP2 is the most variable antigen of AHSV, determines serotype formation (Burrage et al., 1993) and contains most of the virus neutralising antibody (VNAb) epitopes identified so far (Bentley et al., 2000; Burrage et al., 1993; Martinez-Torrecuadrada et al., 1999).

To date no effective treatment exists for AHS and, consequently, control of the disease relies on vaccination, control of animal movements and prevention of bites by *Culicoides* midges. Live attenuated AHS vaccines (LAV) have been in use in Africa for almost 100 years and

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permitted the subsistence of horses in that part of the world (Coetzer and Guthrie, 2004; Mellor and Hamblin, 2004; von Teichman and Smit, 2008). There are nine different serotypes of AHS virus (AHSV) and protective immunity is long-lived but serotype-specific. Despite their apparent efficacy, the use of LAV presents a series of bio-safety concerns, especially in non-endemic countries, which arise from the ability of vaccine viruses to replicate *in vivo* and to exchange their genome segments with other vaccine or field AHS viruses. Recent studies indicate that outbreaks of AHS occurring in the Western Cape Province of South Africa between 2004 and 2014 may have resulted from re-assortment events involving vaccine strains of serotype 1 (Weyer et al., 2016).

Over the last 30 years much attention has been given to the development of safer alternative AHS vaccines and a number of different approaches have been explored. These included the use of inactivated AHSV (House et al., 1994; Lelli et al., 2013), baculovirus-expression of AHSV capsid proteins (Roy, 1996), plasmid DNA vaccines (Romito et al., 1999) or poxvirus expression vectors (Alberca et al., 2014; Castillo-Olivares et al., 2011; de la Poza et al., 2013; Guthrie et al., 2009). In previous studies we showed that recombinant modified Vaccinia Ankara (MVA) viruses expressing AHSV-VP2 induced VNAb and complete clinical protection in a mouse model and in the equine species (Alberca et al., 2014; Castillo-Olivares et al., 2011; de la Poza et al., 2013).

There is evidence suggesting that cell-mediated immune responses play an important role in AHS immunity. Cell-mediated immune responses have been detected in horses immunized with live attenuated vaccines (Pretorius et al., 2012) or recombinant Canarypox viruses expressing VP2 and VP5 (El Garch et al., 2012) and, more recently, in interferon alpha receptor gene knock-out mice (IFNAR −/−) after vaccination with MVA VP2/NS1 (de la Poza et al., 2013). However, we demonstrated that the effector mechanisms of immunity of MVA-VP2 vaccination in mice are mediated mainly by antibodies (Calvo-Pinilla et al., 2014, 2015).

Recombinant MVA vaccine viruses are replication-deficient in mammalian cells and clearance of MVA and its genes occurs rapidly following inoculation (Altenburg et al., 2014). Despite the transient expression of MVA encoded proteins within the vaccinated host, recombinant MVA vaccines efficiently induce cellular and humoral immunity (Drexler et al., 2004; Sutter et al., 1994). Thus, induction of AHSV-VP2-specific antibody responses after MVA-VP2 vaccination is thought to depend on expression of VP2 protein from MVA-VP2 infected cells.

In our previous studies, MVA-VP2 vaccines were administered as MVA-VP2-infected DF-1 cell lysates. Thus, pre-formed VP2 was believed to be present in the inoculum of these vaccines. In this paper, we describe the results of experiments aimed at determining the role that both pre-formed VP2, and VP2 synthesised *de novo* from MVA-VP2 infected host cells, play in the immunogenicity of MVA-VP2 vaccines.

2. Materials and methods

2.1. Viruses and cells

Vero cells (ATCC, Cat. No. CCL-81) and Chicken embryo fibroblast (DF-1) (ATCC, Cat. No. CRL-12203) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% foetal calf serum (FCS). AHSV serotype 4 (AHSV-4, Madrid/87) was grown in Vero cells and MVA viruses (wild type-MVA and MVA-VP2) grown in DF-1 cells. Virus stocks were generated by infection of sub confluent cells using a multiplicity of infection (MOI) of 0.1. When a total cytopathic effect (CPE) was visible, the cells and supernatants were harvested and centrifuged. The virus was released from the cells by three freeze/thaw cycles, sonication and then titrated by plaque assay.

2.2. MVA-VP2 vaccine preparations

Various MVA-VP2 vaccine preparations were used:

a) MVA-VP2 (live): MVA-VP2, expressing AHSV-4 VP2, was previously described (Castillo-Olivares et al., 2011; Chiam et al., 2009) and was bulked up for this study in DF-1 cells and subsequently used as a cell lysate.
b) MVA-VP2 (UV) was obtained by exposure of 1 ml aliquots of MVA-VP2 dispensed on a p100 tissue culture plate to 254 nm wavelength UV light for 40 min. UV irradiation was achieved by placing the plates at 3 cm below a TUV T8 lamp (Philips).
c) MVA-VP2 (HI), was heat-inactivated by heating 100 µl MVA-VP2 aliquots in a thermal block for 15 min at 56 °C.
d) MVA-VP2 (SGP) is a sucrose-gradient-purified MVA-VP2 preparation obtained as follows. A volume of 20 ml of MVA-VP2 was overlaid carefully on top of 20 ml of a sterile 36% (w/w) solution of sucrose in PBS using 40 ml ultra-clear centrifuge tube (Beckmann). The samples were centrifuged for 1.5 h at 300000 g and 4 °C in a SW 28 rotor. The pellets were re-suspended thoroughly in 0.5 ml sterile PBS and sonicated briefly before titration.

2.3. MVA-VP2 inactivation test

MVA-VP2 (HI) and MVA-VP2 (UV) were tested for infectivity using MVA-VP2 (live) as a positive control. All three preparations were inoculated (MOI of 1) into DF-1 cell cultures. After an adsorption period of 1.5 h cells were washed and medium was replaced. After 48 h, infected cells were washed three times with PBS and RNA was extracted from the cells using Trizol reagent (Invitrogen) according to the method recommended by the manufacturer.

Reverse transcription-PCR (RT-PCR) was used to detect mRNA from AHSV-4 genome segment 2 (coding for VP2 protein) in DF-1 cells incubated with live and inactivated MVA-VP2. Oligonucleotide primers used to amplify a segment of 900 bp were: a) forward primer: 5′-CGC CCGGGATGGCGTCCGAGTTTGGAATATTG-3´; and b) reverse primer: 5′-CGCCCGGGCTACCCCTGCTTATCACCTGCTGA-3´.

RNA was denatured in the presence of a reverse VP2 gene-specific primer and dNTP mix by heating to 65 °C for 5 min. A mix of RT buffer, $MgCl_2$ and Reverse Transcriptase (200 U/µL) was added and the reaction was incubated for 1 h at 50 °C. Amplification of the VP2 gene was performed by PCR using PCR Buffer II (Invitrogen), dNTPs, specific primers (forward and reverse primers), $MgCl_2$ solution, AmpliTaq DNA Polymerase (Invitrogen), and cDNA template. Amplification cycle parameters were: 94 °C for 2 min (1×); 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min (30×); 94 °C for 15 min (1×).

2.4. Western blot

Immunoblotting was performed as described previously (Chiam et al., 2009). Samples were mixed 1:1 with 2× Laemmli sample buffer and 12 µL were loaded in each well of polyacrylamide gels. Three immunogenic AHSV-4 VP2-derived KLH-conjugated peptides (NH_2-KKKEE-GEDDTARQEIRKAWC-COOH; NH_2-NKGKWKEHIKEVTEKLKKA-COOH; NH_2-DMNEKQKPYFEFEYDDFKPC-COOH) were selected to obtain a VP2-specific rabbit polyclonal antibody from a commercial source (GenScript). This antibody was used at a 1:400 dilution. A goat anti-rabbit peroxidase (Bio-Rad) was used at a dilution of 1:10000.

2.5. Mice

Seven-week-old, female, Type I interferon receptor KO A129 IFNAR (−/−) mice were purchased from B&K Universal. The animals were rested for about a week before the experiments were performed in the animal facilities of the Centro de Investigación en Sanidad Animal (INIA-CISA). All protocols for animal use were approved by the Ethical