Vaccination of mice with a modified Vaccinia Ankara (MVA) virus expressing the African horse sickness virus (AHSV) capsid protein VP2 induces virus neutralising antibodies that confer protection against AHSV upon passive immunisation

Eva Calvo-Pinilla a, Francisco de la Poza b, Simon Gubbins a, Peter Paul Clement Mertens a, Javier Ortego b, Javier Castillo-Olivares a,∗

a The Pirbright Institute, Pirbright, Woking, Surrey, United Kingdom
b Centro de Investigación en Sanidad Animal, CSIC-INIA, Valdeolmos, Madrid, Spain

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ABSTRACT

In previous studies we showed that a recombinant Modified Vaccinia Ankara (MVA) virus expressing the protein VP2 of AHSV serotype 4 (MVA-VP2) induced virus neutralising antibodies in horses and protected interferon alpha receptor gene knock-out mice (IFNAR−/−) against challenge. We continued these studies and determined, in the IFNAR−/− mouse model, whether the antibody responses induced by MVA-VP2 vaccination play a key role in protection against AHSV. Thus, groups of mice were vaccinated with wild type MVA (MVA-wt) or MVA-VP2 and the antisera from these mice were used in a passive immunisation experiment. Donor antisera from (a) MVA-wt; (b) MVA-VP2 vaccinated; or (c) MVA-VP2 vaccinated and AHSV infected mice, were transferred to AHSV non-immune recipient mice. The recipients were challenged with virulent AHSV together with MVA-VP2 vaccinated and MVA-wt vaccinated control animals and the levels of protection against AHSV-4 were compared between all these groups. The results showed that following AHSV challenge, mice that were passively immunised with MVA-VP2 vaccinated antisera were highly protected against AHSV disease and had lower levels of viraemia than recipients of MVA-wt antisera.

Our study indicates that MVA-VP2 vaccination induces a highly protective humoral immune response against AHSV.

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1. Introduction

African horse sickness (AHS) is an arthropod-borne viral disease of solipeds transmitted by haematophagous insects of the genus Culicoides, the horse being the most severely affected species. Mortality rates of AHS outbreaks in immunologically naïve populations may exceed 90% (Mellor and Hamblin, 2004).

AHSV is a member of the genus Orbivirus, family Reoviridae, and is closely related to the virus causing bluetongue in ruminants. The non-enveloped 55–70 nm AHSV spherical virion capsid is composed of three concentric layers (Roy et al., 1994). 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 cell entry. VP2 is the most variable antigen of AHSV and is responsible for serotype formation (Burrage et al., 1993). The outer capsid rests on the outer core layer formed by the segment 8 encoded protein VP7. This is a highly hydrophobic protein and an antigenically conserved major antigen of AHSV that forms the basis of ELISA-based serological diagnostic tests. The inner core is formed by VP3, encoded by segment 3, an antigenically conserved protein providing theicosahedral framework of the virion. The inner core encloses VP1, VP4 and VP6 proteins, (encoded by segments 1, 4 and 5, respectively) comprising the viral replicate complex and the 10 double-stranded RNA genome segments. There are four nonstructural proteins (NS1, NS2, NS3/3A, and NS4), involved in virus and morphogenesis (Belhoucet et al., 2011; Manole et al., 2012; Roy et al., 1994; van Staden et al., 1991).

AHSV infection in horses most often results in severe clinical disease and death but those animals that survive exhibit a solid lifelong serotype-specific immunity. The humoral nature of AHS immunity has been associated with virus neutralizing antibodies (VNAb)
in passive immunisation experiments carried out in both horses and mice using colostrum and monoclonal antibodies, respectively (Blackburn and Swanepoel, 1988; Burrage et al., 1993). Consistent with this, vaccination and challenge experiments demonstrated a correlation between the presence of VNABs in serum and protection. The main target of VNABs is AHSV VP2 (Burrage et al., 1993) and several studies mapped neutralising epitopes to the first half of the VP2 protein (Bentley et al., 2000). AHSV VP5 appears to be involved in formation of virus neutralising epitopes by influencing the conformation of VP2 (Martínez-Torrecurauda et al., 1996).

Only very recently, information about cell-mediated immunity against AHSV has become available. Studies by Pretorius et al. (2012) demonstrated an in vitro recall cellular immune response in peripheral blood mononuclear cells (PBMC) of horses vaccinated with live-attenuated AHSV vaccines and El Garch et al. (2012) showed IFN-γ CD8+ T-cell responses in PBMC collected from horses vaccinated with a recombinant Canarypox virus co-expressing both VP2 and VP5 of AHSV-4. More recently, cell-mediated immune responses have also been observed in interferon alpha receptor gene knock-out mice (IFNAR−/−) after vaccination with MVA VP2/NS1 (de la Poza et al., 2013). Since cellular immunity has been associated with protection in bluetongue, it is generally assumed that the same applies to AHS.

Control of AHSV in endemic countries is primarily based on vaccination. Live-attenuated AHSV vaccines have been in use, with relative success, for more than five decades. However, concerns about reversion to virulence, gene segment re-assembly between outbreak and vaccine strains and the lack of a differential diagnostic test make the use of attenuated vaccines undesirable for non-endemic countries. Consequently, AHSV vaccine research focused in recent years on the development of DIVA (Differentiating Infected from Vaccinated Animals) vaccines based on selected proteins of the AHSV antigenic repertoire, in particular the capsid proteins. Thus, baculovirus expression systems, plasmid DNA and viral vector vaccines have all been used to induce protective immunity using any combination of VP2, VP5, VP7, NS1 and VP3 antigens (de la Poza et al., 2013; Guthrie et al., 2009; Martínez-Torrecurauda et al., 1996; Roy et al., 1996).

Recently, we have shown that vaccination of ponies with a recombinant modified Vaccinia Ankara (MVA) virus expressing VP2 of AHSV serotype 4 (MVA-VP2) induced VNAB (Chiam et al., 2009). In a follow up study, vaccination of IFNAR−/− mice with MVA-VP2 induced VNAB and complete protection upon challenge with a virulent AHSV-4 (Castillo-Olivares et al., 2011). Since viral vectors in general, and MVA in particular, are capable of inducing both humoral and cell-mediated immunity, we began to investigate whether the protective immunity against AHSV induced by MVA-VP2 vaccination is mediated by either of these effector mechanisms of immunity. In this study we evaluated the protective capacity against AHSV of MVA-VP2 vaccine-induced antibodies in a passive immunisation experiment. We have demonstrated that MVA-VP2 vaccination induces a highly protective humoral immune response.

2. Materials and methods

2.1. Viruses and cells

Chicken embryo fibroblast (DF-1) (ATCC, Cat. No. CRL-12203) and Vero cells (ATCC, Cat. No. CCL-81) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% fetal calf serum (FCS). AHSV serotype 4 (Madrid/87) (AHSV-4) was grown in Vero cells and MVA viruses grown in DF-1 cells. Virus stocks were generated by infection of confluent cells using a multiplicity of infection (MOI) of 0.1. At 48 h post-infection, or 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 and thaw cycles and then titrated by plaque assay. The MVA VP2 virus used in this work has been previously described (Chiam et al., 2009).

2.2. Mice

IFN α/β Ro/o IFNAR−/− mice on a 129 background were purchased from B&K Universal Ltd (UK). Eight-week old mice were used throughout. Mice were maintained under pathogen-free conditions and allowed to acclimatize to the biosafety level 3 (BSL3) animal facilities at the Centro de Investigacion en Sanidad Animal, INIA, Madrid (CISA-INIA), for 1 week before use. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the ethical review committee of CISA-INIA (CEEA 2010-034).

2.3. Murine immunisations with MVA-VP2 and MVA-wt and preparation of donor antisera (Stage 1)

Mice were randomly divided in four groups of six animals at the start of the experiments (Table 1). Vaccinations were performed on days 0 and 21 by administering, by the intraperitoneal route, two doses of 10⁷ pfu of MVA-VP2 (groups 1 and 2) or MVA-wt (groups 3 and 4). Two weeks after the second vaccination, groups 2 and 4 were euthanized by anaesthesia with 5% isoflurane followed by cervical dislocation, and splenocytes and blood samples collected from these animals. The blood was used to prepare donor antisera. Also, at two weeks after the second vaccination, groups 1 and 3 were challenged by subcutaneous inoculation of 10⁵ pfu/mouse of AHSV-4. Clinical signs and viraemia were evaluated from these animals. All mice from group 1 survived the challenge and blood from these animals was also used to prepare donor antisera. All donor antisera were prepared by pooling individual sera from mice of the same group. Thus, antisera D1, D2 and D4 were obtained from groups 1, 2 and 4, respectively.

2.4. Passive immunisation with donor antisera (Stage 2)

The donor antisera were inactivated (30 min at 56°C) and 100 µl used for intra-peritoneal injection of 4 groups of 10-week-old recipient IFNAR−/− mice: (a) group 5, received undiluted D2 antisera; (b) group 6 received D2 antisera diluted 1 in 10; (c) group 7 received undiluted D1 antisera; and (d) group 8 received D4 antisera. One hour following transfer, all recipient mice were infected with a dose of 10⁶ pfu of AHSV-4 administered subcutaneously. An MVA-VP2 vaccinated control group (group 9) and an MVA-wt vaccinated negative control group (group 10) were infected at the same time as the recipients (Table 2). Clinical signs and viraemia were evaluated in all the animals.

2.5. Evaluation of clinical signs after challenge with AHSV-4

Following sub-cutaneous injection of 10⁶ pfu of AHSV-4 animals were monitored twice daily from the start of the experiment and more regularly (at least three times per day) at the onset of any signs of morbidity. These included: changes in behaviour and activity, changes in water or food intake, alterations in the hair coat appearance, body weight loss, presence of ocular signs (conjunctivitis, ocular discharge, swelling), changes in hydration and presence of neurological signs (i.e. paresis, paralysis, ataxia). The humane end-points for euthanasia included: persistent hunching, severe conjunctivitis, signs of dehydration, loss of more than 20% of body weight, presence of any neurological signs, or any other condition that prevented food or water intake. Animals that displayed...
any of these clinical signs were humanely euthanized by cervical dislocation following anaesthesia with 5% isoflurane.

2.6. Virus neutralisation test

Serum samples from mice were heat-inactivated for 30 min at 56 °C and two-fold serial dilutions were incubated with 100 TCID₅₀ of AHSV-4 in triplicate wells of 96-well microtitre plates for 1 h and then overnight at 4 °C. The following day, 2 × 10⁴ Vero cells were added to each well and incubated for 3 days at 37 °C, 5% CO₂. The plates were checked for AHSV cytopathic effect, with the endpoint of the assay being taken as the highest dilution that prevented AHSV cytopathic effect in 50% of the wells. Antibody titres were then calculated by the Karber (1931) formula and expressed in log₁₀ units.

2.7. VP2-specific antibody detection by indirect enzyme-linked immunosorbert assay (ELISA)

Briefly, 96-well polystyrene plates (Nunc) were coated overnight with 160 ng/well of recombinant AHSV-4-VP2 expressed in a baculovirus expression system. Plates were washed four times with PBS with 0.05% Tween 20 and blocked with 5% milk diluted in PBS for 1 h at room temperature. Serum samples were diluted 1:50 in PBS 2.5% milk and allowed to incubate for 1 h at 37 °C. Plates were washed four times, and a horseradish peroxidase (HRP) conjugated rabbit antibody against total mouse immunoglobulins (Southern Biotech) was diluted (1:2000) in blocking buffer and added to plates. Plates were incubated for 1 h at 37 °C, washed four times and a colorimetric reaction developed after addition of 100 μL of TMB substrate (Sigma-Aldrich). Plates were incubated in the dark for 30 min, and the reaction was stopped with 2N H₂SO₄. Optical densities at a wavelength of 450 nm (OD 450) were read by a spectrophotometer (BioTek). The cut-off point between a positive and a negative result was set at an optical density of 0.2.

Table 1
Stage I: Preparation of donor antisera.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Day 0</th>
<th>Day 21</th>
<th>Day 35</th>
<th>Day 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MVA-VP2, 10⁵ pfu/mouse</td>
<td>MVA-VP210⁵ pfu/mouse</td>
<td>Challenge AHSV-410⁶ pfu s.c.</td>
<td>Collection of serum (D1)</td>
</tr>
<tr>
<td>2</td>
<td>MVA-VP2, 10⁵ pfu/mouse</td>
<td>MVA-VP210⁵ pfu/mouse</td>
<td>Collection of serum (D2) and spleen</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>MVA-wt, 10⁵ pfu/mouse</td>
<td>MVA-wt10⁵ pfu/mouse</td>
<td>Challenge AHSV-410⁶ pfu s.c.</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MVA-wt, 10⁵ pfu/mouse</td>
<td>MVA-wt10⁵ pfu/mouse</td>
<td>Collection of serum (D4) and spleen</td>
<td></td>
</tr>
</tbody>
</table>

* Sub-cutaneous.

2.8. Detection of AHSV-4 in blood

Whole blood from animals was collected in EDTA at regular intervals after inoculation for virus isolation. Volumes of 200 μL of blood were washed in PBS, mixed with 900 μL of water to lyse the cells and then 100 μL of 10× PBS was added to the sample. The amount of infectious virus was measured by standard plaque assay on Vero cells.

2.9. Isolation of splenocytes and antigen stimulation

Vaccinated mice were euthanized and their spleens aseptically collected. Spleen cells were then released into RPMI media by mashing the organs and filtering through 70 μm cell strainers (Falcon). After red blood cell lysis with NH₄Cl (0.83%), splenocytes were re-suspended in complete RPMI media supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine. Cells were stimulated with an AHSV-4 infected Vero cell extract that had been inactivated by a 30 min exposure to ultraviolet light (400 J/cm²). The effectiveness of virus inactivation was confirmed by failure of the extract to infect cell monolayers in a standard plaque assay. Positive control cells were stimulated with PMA/ionomycin (Sigma-Aldrich) (PMA at 100 ng/mL, ionomycin at 12.5 M), and negative control cells with an uninfected Vero cell extract. After 6 h of incubation, extracellular protein transport was inhibited by the addition of brefeldin A (Invitrogen) at a dilution of 1/1000 in RPMI complete medium. Cells were further incubated for 6 h more before staining.

2.10. Flow cytometry for intracellular IFN-γ detection

Following antigen stimulation, cells were recovered to perform surface staining with FITC-labelled anti-CD4 and PE-labelled anti-CD8 antibodies (Bioscience). Subsequently, permeabilizing buffer was added (PBS 0.02% saponin 4% FBS) and intracellular staining with PE-labelled anti-IFN-γ was performed. Finally cells were fixed in 4% paraformaldehyde in phosphate-buffered saline for flow cytometric analysis.

The analysis was performed using a FACScalibur flow cytometer (BD Biosciences, France) and analysis was done using CellQuest Pro software. The percentage of antigen specific IFN-γ producing cells of each mouse was calculated as the percentage of IFN-γ producing cells obtained with virus stimulation minus the percentage of IFN-γ producing cells obtained with cellular extract stimulation.

2.11. Statistical methods

Survival: Survival data (days post infection at which each mouse died) were analysed using a log rank test with mice grouped by immunisation strategy. Mice which survived the experiment were right-censored.

CD8+ T cells producing IFN-γ: The proportion of CD8+ T cells producing IFN-γ derived from vaccinated and unvaccinated mice (measured as the percentage of cells producing IFN-γ following virus stimulation minus the percentage of cells producing IFN-γ
following stimulation with cell extract; see Section 2.10) was compared using a Wilcoxon rank-sum test.

Viraeurna: Data on viraemia were analysed using a linear mixed model with log_{10} (pfu/ml + 1) as the response variable, immunisation strategy and days post infection (as a factor) as fixed effects and mouse as a random effect. Model selection proceeded by stepwise deletion of non-significant terms (as judged by the Akaike information criterion (AIC), with a difference in AIC of two or more taken to indicate significance), starting from an initial model including immunisation strategy and days post infection. Once the final model had been constructed, post hoc Tukey tests were used to identify significant (P < 0.05) differences between factor levels.

Virus neutralisation: Virus neutralising antibody titres at 17 days post infection were compared using a linear model with log_{10} titre as the dependent variable and immunisation strategy as a fixed effect. Post hoc Tukey tests were used to identify significant (P < 0.05) differences between factor levels.

All analyses were implemented in R (R-Core-Team, 2012).

3. Results

To investigate the protective role of the antibody response induced by MVA-VP2 vaccination against AHSV, a passive immunisation study was conducted whereby antisera from MVA-VP2 vaccinated mice was transferred to recipient mice that were subsequently challenged with AHSV-4. We conducted this study in two stages: (1) determination of protective immunity of MVA-VP2 vaccination and preparation of donor antisera; and (2) determination of protective immunity following passive immunisation with MVA-VP2 vaccination-induced antisera.

3.1. Determination of MVA-VP2 protective immunity and preparation of donor antisera (Stage 1)

To test the protective capacity of antisera from MVA-VP2 vaccinated mice it was necessary first to confirm that the MVA-VP2 vaccination protocol used induced protective immunity against AHSV-4. Thus, MVA-VP2 vaccinated and MVA-wt vaccinated mice (groups 1 and 3, respectively) were challenged with AHSV-4 and clinical signs and viraemia evaluated. In parallel, groups 2 (MVA-VP2) and 4 (MVA-wt), vaccinated in exactly the same manner as groups 1 and 3, were used for preparation of donor D2 and D4 antisera. Surviving mice from group 1 were the source of a positive control donor antisera D1.

3.1.1. Protective immunity of MVA-VP2 vaccination against AHSV-4 challenge (groups 1 and 3)

Following AHSV-4 infection, 3 mice from group 3 developed clinical signs such as weight loss, dehydration, ruffled hair, ocular discharges, periorbital swelling and ataxia and were euthanized between 6 and 10 days post-infection according to the humane endpoints described in Materials and Methods. Two mice displayed clinical signs but did not surpass the severity limit of the experiment and finally recovered by day 14 post-infection. The animals in this group were viraemic and the percentage of survival in the MVA-wt vaccinated mice following challenge was 33.33%. In contrast, all MVA-VP2 vaccinated animals (group 1) remained clinically normal throughout the study (Fig. 1). Furthermore, survival times differed significantly between the two groups (log rank test: \( \chi^2 = 5.5 \ df = 1; P = 0.02 \) (Fig. 1). No infectious virus was isolated from MVA-VP2 vaccinated animals. In contrast, MVA-wt mice presented viraemia for a number of days and peaked at day 5 post-infection with a mean value of 1358 ± 458 pfu/ml. All these data indicated that vaccination with MVA-VP2 completely protected IFNAR−/− mice in this experiment and is consistent with our previous observations (Castillo-Olivares et al., 2011).

![Fig. 1. Survival rates and viraemia after challenge with AHSV-4.](image)

The donor antisera D2, obtained from MVA-VP2 vaccinated mice (group 2), was derived from animals that had the same vaccination regime as those that were immune to AHSV-4 (group 1). This antisera is therefore adequate to address the question of whether antibody responses induced by MVA-VP2 vaccination played a role in protection. Group 3 mice, vaccinated with MVA-wt were fully susceptible to AHSV-4 infection. Therefore, antisera D4, obtained also from MVA-wt vaccinated mice was adequate as a negative control donor antisera. The positive control donor antisera D1 was collected from group 1 mice (MVA-VP2 vaccinated) 15 days after they had survived challenge with AHSV-4.

3.1.2. Antibody responses following immunisation with MVA-VP2 (groups 1–4)

Serum samples were collected at 35 days post-vaccination (at the time of challenge) from mouse groups 1–4 and analysed for viral antibodies by virus neutralisation test and VP2-specific ELISA. The results are summarised in Table 3. Mean VNAb titres were 1.9 ± 0.164 in MVA-VP2 vaccinated animals, whilst antisera from MVA-wt vaccinated mice did not neutralise the virus. The results

<table>
<thead>
<tr>
<th>Groups</th>
<th>log_{10} VNAb_{50}</th>
<th>OD ELISA VP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.9 ± 0.164</td>
<td>1.574 ± 0.334</td>
</tr>
<tr>
<td>2</td>
<td>1.9 ± 0.154</td>
<td>1.666 ± 0.222</td>
</tr>
<tr>
<td>3</td>
<td>0.322 ± 0.029</td>
<td>0.122 ± 0.029</td>
</tr>
<tr>
<td>4</td>
<td>0.107 ± 0.035</td>
<td>1.074 ± 0.334</td>
</tr>
</tbody>
</table>
of mice vaccinated with MVA-VP2 (group 2, V7-V12) and MVA-wt vaccinated mice (group 4, C7-C12) was analyzed by flow cytometry at 35 days post-vaccination. Black bars represent the percentage of IFN-γ CD8+ T-cells of splenocytes stimulated with cell extract. Grey bars represent the percentages of IFN-γ CD8+ T-cells of splenocytes stimulated with AHSV-4 antigen.

of the ELISA test showed that only vaccinated mice (groups 1 and 2) showed specific antibodies against VP2 with a mean OD value of 1.574 ± 0.334 (Table 3).

3.1.3. Recall cell-mediated immune responses measured by intracellular IFN-γ staining in MVA-VP2 (group 2) and MVA-wt (group 4) vaccines

Splenocytes from donor mice were isolated to perform intracellular cytokine staining of IFN-γ in T-lymphocytes. The results of these experiments are depicted in Fig. 2. In MVA-VP2 vaccinated mice, the average percentage of antigen-specific CD8+ IFN-γ responder cells (measured as the difference between bars in Fig. 2) was 1.01 ± 0.27%. In contrast, the average was 0.12 ± 0.06% in MVA-wt mice (Wilcoxon rank-sum test: P = 0.002). No significant difference in the expression of IFN-γ by CD4+ T cells upon re-stimulation in immunised mice (0.15 ± 0.21%) was observed compared with control mice (0.33 ± 0.15%). The difference in AHSV-specific IFN-γ response from CD8+cells between MVA-VP2 vaccinated and MVA-wt control mice indicated that vaccination with MVA-VP2 primes CD8+ T-lymphocytes thereby activating anti-AHSV-4 CD8+ T cell responses.

3.1.4. VP2-specific reactivity and neutralising antibody titres of donor antiserum

The reactivity by the VP2-specific ELISA and VNAb titres of the antiserum (D1, D2 and D4) used for passive immunisation was determined. Antiserum D1, had an OD value of 2.69 by the VP2 ELISA and a VNAb titre of 2.51 (log10) whereas antiserum D2 had an O.D. value of 1.58 in the VP2 ELISA and a VNAb titre of 1.95 (log10). As expected, D4 was negative by both assays.

3.2. Passive immunisation and AHSV-4 challenge (Stage 2)

To interrogate the protective capacity against AHSV-4 of antibodies induced by MVA-VP2 vaccination passive immunisation with antiserum was undertaken as indicated in Section 2.4.

3.2.1. Clinical signs, survival and viraemia of groups 5–10 after AHSV-4 challenge

The results of the AHSV-4 challenge of groups 5–10 are depicted in Figs. 3 and 4. By day 3 after AHSV-4 infection, MVA-wt vaccinated mice (group 10) and mice immunised with antiserum D4 (group 8) started to develop clinical signs. These were weight loss, dehydration, ocular discharges, periocular swelling, and in some cases, ataxia and paresis in the hind limbs. All mice from group 10 and 4 from group 8 were euthanised between days 5 to 11. High viral loads were detected in mice from both groups 8 and 10, reaching maximum values before death. Even at day 3 post-challenge viral titres up to 900 pfu/ml were detected in mice from group 10. In contrast and as expected, MVA-VP2 vaccinated animals (group 9) remained completely healthy and had no detectable viraemia until the end of the experiment.

Protection against AHSV-4 challenge in groups immunised with antiserum D2 (group 5), D2 (diluted) (group 6) and D1 (group 7) varied. Protection was high for groups 5 and 7 and partial for group 6. Thus, all mice in group 5 survived the challenge and showed minimal clinical signs (one individual showed ruffled hair and ocular discharge). In addition, no infectious virus was isolated from any mouse in the group at day 3 post-infection and although viraemia was found on following days, it was low and the virus was finally cleared. Similarly, mice from group 7 were completely protected against AHSV challenge. The levels of viraemia in these animals were similar to those in group 5, but virus was cleared earlier in some mice from group 7. In comparison to the control groups (8 and 10) the course of disease was slightly delayed in mice from group 6 but 50% of the animals did have to be euthanised by day 11. Viraemia data for group 6 showed that virus titres were higher and viraemia lasted longer than in groups 5 and 7.

The levels of viraemia seen at all time points differed significantly (P < 0.01) amongst groups of mice, and the groups could be divided into those that had high (groups 8 and 10), intermediate (groups 5–7) and low (group 9) levels of viraemia. The highest virus titres were observed in mice which were either MVA-wt vaccinated (group 10) or which received antiserum D4 (group 8). Intermediate titres were observed in mice which received antiserum D2 (undiluted or diluted) or D1 (groups 5–7). The lowest titres of viraemia were found in mice vaccinated with MVA VP2 (group 9) (no viraemia). Moreover, there were significant (log rank test: χ² = 40.3, df = 5; P < 0.001) differences in survival times for mice immunised according to different strategies (Fig. 4). In particular, survival was lowest in mice which received antiserum D4 (group 8) or which were vaccinated with MVA-wt (group 10), and was intermediate in mice which received antiserum D2 (diluted) (group 6).

These results indicate that the protection against AHSV induced by MVA-VP2 vaccination is mediated primarily by antibodies and that the level of protection observed correlated with antibody titre. However, it is also probable that other effector mechanisms of immunity induced by MVA-VP2 vaccination are important in protection against AHSV, since MVA-VP2 vaccinated mice (group 9) were better protected than any of the passively immunised groups.

3.2.2. Antibody responses of groups 5–10

Serum samples were collected from all animals of groups 5–10 immediately before and 17 days after challenge with AHSV and VNAb and AHSV-4-VP2-specific antibody responses were analysed (Fig. 5). Only MVA-VP2 vaccinated mice (group 9) were positive for VNAb prior to challenge. At 17 days post-challenge the levels of VNAb differed significantly (P < 0.001) amongst the different animal groups. The highest titres were recorded in mice vaccinated with MVA-VP2 (group 9) (log VNAb = 3.13 ± 0.132). Intermediate titres were observed in D2 (undiluted or diluted) or D1 recipients (groups 5–7). The lowest VNAb titres were observed in mice that received antiserum D4 (group 8) (log VNAb = 2.55).

The AHSV-4-VP2-specific antibody responses were determined by ELISA using pools of sera from each group of mice (Fig. 5B). All samples were negative at the time of challenge, except for those from MVA-VP2 vaccines (group 9). By day 10 post-infection maximum levels of VP2-specific antibodies were detected in all groups.
Fig. 3. Passive immunisation and AHSV-4 challenge. Survival. Each panel corresponds to a different mouse group (groups 5–10) and shows the survival curves, expressed as a percentage of surviving mice per day, for each individual group of mice.

Fig. 4. Passive immunisation and AHSV-4 challenge. Viraemia. Each panel corresponds to a different mouse group (groups 5–10) and shows individual virus titres of blood samples collected at different time points post-infection calculated by standard plaque assay. Each mouse is indicated by a different colour and symbol.
Some of these approaches have been successful in experimental conditions but are not commercially viable whilst others have not yet addressed important vaccination efficacy issues such as duration of immunity or cross-protection.

The use of replication deficient poxviruses expressing AHSV capsid proteins has yielded promising results. Thus, a canarypox virus expressing VP2 and VP5 of AHSV–4 protected horses against homologous challenge (Guthrie et al. 2009). In addition, previous studies in our laboratory showed that a recombinant MVA expressing AHSV-VP2 stimulated high levels of neutralising antibodies in ponies (Chiam et al., 2009) and provided protective immunity in the IFNAR–/– mouse model (Castillo-Olivares et al., 2011). These vaccination strategies are suited for differential diagnostics (DIVA compatible) since they are based on one or two antigens of AHSV.

Having demonstrated the capacity of MVA-VP2 to induce VNAb in horses and VNAb and full protection against AHSV in a mouse model (Castillo-Olivares et al., 2011), the primary objective of the present study was to determine, in a passive immunisation experiment, whether MVA-VP2 protection was mediated by antibodies. Thus, immunologically naïve mice received antisera from MVA-VP2 vaccinated or from MVA-VP2 vaccinated and AHSV infected mice and were then challenged with virulent AHSV and their protective immunity assessed. Our results showed that recipients of MVA-VP2 vaccinated mouse antisera (D2) were highly protected against clinical disease and death following AHSV challenge, suggesting that MVA-VP2 vaccine induced immunity is mediated primarily by antibodies. Passive immunisation was less effective when antisera D2 was diluted 1:10, and was more effective in mice that received D1 antisera obtained from MVA-VP2 vaccinated and subsequently infected mice. Antibodies directed against other AHSV proteins (D1 derived from MVA-VP2 vaccinated and AHSV-4 infected mice), such as VP5, or even to more natural forms of VP2, could have contributed to the slightly higher immunity exhibited by the D1 recipients. It is known that VP5 exerts some conformational effect on VNAb epitopes contained in VP2 (Martinez-Torrecuadrada et al., 1996). However, it is more likely that the levels of VP2-specific VNAb of the donor antisera were the main factors influencing the degree of protection observed in the passively immunised mice, since protection levels decreased in the following order: D1 recipients (VNAb titre 2.51), D2 recipients (VNAb titre 1.95) and 1:10 diluted D2 recipients.

The passively immunised mice showed a lower degree of viraemia despite being clinically protected, whereas the MVA-VP2 vaccinated mice were completely protected against both clinical signs and viraemia. It is possible that cell-mediated immunity played an additional protective role following MVA-VP2 vaccination. The capacity of recombinant MVA-based vaccines to induce cellular immunity has been documented in the literature and described before for BTV and AHSV (Calvo-Pinilla et al., 2012; Calvo-Pinilla et al., 2009; de la Poza et al., 2013). In the present study we observed that the level of IFN-γ producing CD8+ T-cells in MVA-VP2 vaccines was significantly higher than in MVA-wt vaccines after in vitro stimulation with inactivated AHSV, indicating that CD8+ T lymphocytes were primed in vivo by MVA-VP2. However, the VNAb titres of MVA-VP2 vaccines at the time of challenge (2.00) were higher than those of the passively immunised mice, considering the dilution effect of the administered volume of the donor antisera (100 μl) in the total blood volume of the recipients (total blood volume of a 25 g mouse is 1.46 ml). This could probably explain the higher protection observed in the MVA-VP2 vaccines. Further studies are necessary to discern whether MVA-VP2 vaccination induced immunity is exclusively mediated by antibodies and to determine what is the specific role of T cell responses in protection, but here we have provided clear evidence that antibodies induced by MVA-VP2 vaccination are highly protective against AHSV infection. This suggests that MVA-VP2 vaccination induced

4. Discussion

AHS is a lethal disease of horses transmitted by the same hematophagous biting midges (Culicoides sp.) that transmit blue-tongue virus, which causes a disease in ruminants that has spread through Mediterranean and Central Europe in the last two decades. The higher presence of insect vectors capable of transmitting AHSV at the present time in Europe has increased the risk of another outbreak. Current live attenuated AHSV vaccines have been used with relative success in Africa but there are concerns over their biosafety in the field. Killed vaccines have been used in the past, though on a limited scale (House et al., 1994; Ozawa and Bahrami, 1966), and both attenuated and killed vaccines are incompatible with DIVA (differentiating infected from vaccinated animal) control strategies and therefore are regarded as inadequate for use in non-endemic countries. This prompted the investigation of alternative vaccination strategies based mainly on the AHSV outer capsid proteins VP2 and VP5 which are the target of VNAb (Burrage et al., 1993; Martinez-Torrecuadrada and Casal, 1995). These strategies included the use of baculovirus-expressed AHSV antigens, plasmid DNA vaccines and recombinant vaccinia virus vector vaccines (Romito et al., 1999; Roy et al., 1996; Stone-Marschät et al., 1996). Some of these approaches have been successful in experimental

![Image](https://example.com/image.png)
antiserum could potentially be used as an emergency treatment of AHSV infection in the target species. We are currently exploring this possibility.

In conclusion, our study showed that MVA-VP2 vaccination induces both antibody and cell-mediated immunity and that the VP2-specific, VNAb response induced by MVA-VP2 vaccination plays a key role in the protection against AHSV.

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