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Short Communication

In vivo assessment of equine arteritis virus vaccine improvement by disabling the deubiquitinase activity of papain-like protease 2

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ABSTRACT

Arteriviruses are a family of positive-stranded RNA viruses that includes the prototypic equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV). Although several vaccines against these viruses are commercially available there is room for improvement, especially in the case of PRRSV. The ability of arteriviruses to counteract the immune response is thought to decrease the efficacy of the current modified live virus vaccines. We have recently shown that the deubiquitinase (DUB) activity of EAV papain-like protease 2 (PLP2) is important for the inhibition of innate immune activation during infection. A vaccine virus lacking PLP2 DUB activity may therefore be more immunogenic and provide improved protection against subsequent challenge than its DUB-competent counterpart. To test this hypothesis, twenty Shetland mares were randomly assigned to one of three groups. Two groups were vaccinated, either with DUB-positive ($n = 9$) or DUB-negative ($n = 9$) recombinant EAV. The third group ($n = 2$) was not vaccinated. All horses were subsequently challenged with the virulent KY84 strain of EAV. Both vaccine viruses proved to be replication competent *in vivo*. In addition, the DUB-negative virus provided a similar degree of protection against clinical disease as its DUB-positive parental counterpart. Owing to the already high level of protection provided by the parental virus, a possible improvement due to inactivation of PLP2 DUB activity could not be detected under these experimental conditions. Taken together, the data obtained in this study warrant further *in vivo* investigations into the potential of using DUB-mutant viruses for the improvement of arterivirus vaccines.

1. Introduction

Arteriviruses are a family of animal viruses that includes the prototypic equine arteritis virus (EAV) and porcine reproductive and respiratory syndrome virus (PRRSV). Whereas outbreaks of equine viral arteritis (EVA) are only occasionally reported, infections with PRRSV pose a major threat to swine-farming industries worldwide. Especially the emergence of highly virulent strains of PRRSV in China since 2006 has been a major concern (Zhou and Yang, 2010). Although current vaccine-based control strategies for EAV are considered adequate, some concerns regarding efficacy (*i.e.* limited cross protection) and safety (*i.e.* shedding of vaccine virus) provide room for improvement (Balasuriya et al., 2013; McCollum et al., 1987). In contrast, whereas currently available vaccines against PRRSV generally provide protection against clinical disease, they do not consistently prevent replication of field strains and shedding of infectious virus, and especially the limited protection against heterologous field strains is a major issue (Kimman et al., 2009). The design of (novel) PRRSV vaccines that provide improved protection against both homologous and heterologous field strains is therefore of significant importance.

It has been suggested that the immune-evasive capabilities of PRRSV play an important role in reducing vaccine efficacy (Kimman et al., 2009; Wang et al., 2013b). One of the arterivirus protein domains that have been suggested to be involved in downregulating the innate immune response is papain-like protease 2 (PLP2) (Frias-Staheli et al., 2007; Sun et al., 2010; van Kasteren et al., 2012), which functions both as a deubiquitinase

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(DUB) and plays an essential role in the autoproteolytic maturation of the viral replicase polyproteins. Importantly, we have recently been able to show that the DUB activity of EAV PLP2 inhibits the innate immune response in infected cells, *via* the structure-based design of a viable mutant virus lacking this activity (van Kasteren et al., 2013). The aim of the current study was to determine *in vivo* whether this DUB-negative virus provides better protection against subsequent challenge infection than its parental DUB-competent counterpart. Since to date the separation of DUB and polyprotein processing functions of arterivirus PLP2 has only been successful for EAV, we have used this virus for the present animal study.

2. Methods

2.1. Cells and viruses

BHK-21 cells were cultured in Glasgow minimum essential medium (Lonza) supplemented with 5% foetal bovine serum (FBS), 10% tryptose phosphate broth, and 10 mM Hepes (pH 7.4). Primary equine lung fibroblasts (ELFs) were cultured in minimum essential medium (Lonza) supplemented with 10% FBS and grown on collagen-coated plastics for a maximum of 10 passages. Vero cells were cultured in proprietary cell culture medium (MSD Animal Health) supplemented with 1% FBS. All culture media contained 100 U/mL of penicillin and 100 mg/mL of streptomycin or neomycin.

The cloning and production of the DUB-competent (in our previous publication referred to as "wild-type") and DUB-negative (T312A/I313V/I353R, amino acid numbering based on polyprotein) viruses (strain Bucyrus; EAN551) that were used for vaccination were described previously (van Kasteren et al., 2013). Both viruses have been thoroughly characterized in cell culture experiments, revealing no differences in replication kinetics, yet a strongly decreased DUB activity and an enhanced induction of interferon beta mRNA expression (a hallmark of innate immune activation) of the mutant virus compared to its parental counterpart (van Kasteren et al., 2013). Viral titres were determined by standard plaque assay on ELFs. For experimental challenge, we used the virulent Kentucky 1984 (KY84) strain of EAV, which has been previously described (Zhang et al., 2012).

To confirm the use of the correct virus for vaccination, the presence or absence of PLP2 mutations was established, both before and after vaccination. Before vaccination, viral RNA was isolated from the produced virus stocks using the QIAamp viral RNA mini kit (Qiagen) and converted to cDNA using RevertAid H Minus reverse transcriptase (RT) (Fermentas) and random hexamer primers. The PLP2-encoding region was subsequently PCR amplified using *Pfu* DNA polymerase (Fermentas) and sequenced. After vaccination, viral RNA present in the blood of four horses from each of the vaccinated groups at 4 days post vaccination was subjected to sequencing. This was done essentially as described above with the exception that the RT reaction on whole blood total RNA (see below) was performed using a primer that recognizes the EAV genome and thus specifically converts the limited amount of viral RNA present in the samples to cDNA. Primer sequences are available upon request.

2.2. Experimental vaccination and challenge of horses

The experiment was performed in accordance with European Community guidelines and national laws on animal experiments. The design of the experiment was approved by the MSD Animal Health's Committee on the Ethics of Animal Experiments (*Dierexperimentencommissie*), which is required by national legislation to include both MSD Animal Health employees and independent members, prior to the start of the trial (Permit Number: EXP 12.059). All efforts were made to minimize animal discomfort.

Twenty female Shetland horses (*Equus ferus caballus*; average age 7.5 ± 5.1 years) that tested negative for EAV-neutralizing antibodies (antibody titres were determined as described previously, Zhang et al., 2012) before the start of the experiment were randomly assigned to one of three treatment groups. After a one-week acclimatization period, horses in Group 1 ($n = 9$) and Group 2 ($n = 9$) received an intramuscular (cervical muscle) vaccination of 1 ml phosphate-buffered saline containing 1×10^7 plaque-forming units (PFU) of parental or PLP2 DUB-negative EAV, respectively. Horses in Group 3 ($n = 2$) were not vaccinated and were included in the study one week before challenge. At 34 days post vaccination (dpv), all horses were challenged by intranasal inoculation with 1×10^5 PFU of EAV KY84 in a total volume of 5 ml phosphate-buffered saline.

Given the fact that the viruses used for vaccination qualify as genetically modified organisms (GMO), vaccinated horses were kept in vBSL3 containment during the entire experiment. All horses were housed in groups, but Group 1 horses were kept separate from Group 2 horses to prevent any cross-contamination. Horses from Group 3 were divided among the two stables upon inclusion, without having direct contact with Group 1 or 2 horses. Water was provided *ad libitum* and standard feeding procedures were applied.

The general health status of the animals was checked by a veterinarian before vaccination as well as before challenge, and daily by animal care-takers during the entire course of the experiment. In addition, clinical signs were recorded daily from 0 to 14 days post challenge (dpc) and scored according to Table 1. Rectal temperatures were taken daily from 0 to 14, and at 21 and 28 dpv, and daily from 0 to 14, and at 21 and 27 dpc. Blood samples for serum and total RNA isolation were taken every other day between 0 and 14, and at 21 and 28 dpv, and every other day between 0 and 14, and at 21 and 27 dpc. Animals were euthanized according to standard procedures at 61 dpv (27 dpc). For a schematic overview of the experimental set-up see Fig. 1.

2.3. Virus neutralization assay

Blood for serum neutralizing antibody analysis was collected in 8 ml Vacuette Serum Clot Activator Tubes (Greiner Bio-One) and incubated for at least 4 h at room temperature to allow for clotting. Serum samples were subsequently collected by centrifugation at

Table 1
Clinical signs scoring table.

Clinical sign	Score/day	Total score [average per animal/day]		
		Group 1	Group 2	Group 3
Depressed, normal appetite	1			1 [0.03]
Depressed, reduced appetite	2			12 [0.40]
Hives, legs (mild)	1			2 [0.07]
Hives, legs (marked)	2			4 [0.13]
Hives, neck	1			5 [0.17]
Mucopurulent eye discharge	2		6 [0.04]	22 [0.73]
Muscle tremors	2			2 [0.07]
Nasal serous discharge	1	38 [0.28]	40 [0.30]	6 [0.20]
Petechial haemorrhages	1			1 [0.03]
Red oral mucosa	1		2 [0.01]	4 [0.13]
Reduced appetite	1			1 [0.03]
Stiffness	1			1 [0.03]
Temperature 38.5–39.0 °C	1			1 [0.03]
Temperature 39.1–39.5 °C	2			6 [0.20]
Temperature 39.6–40.0 °C	3			9 [0.30]
Temperature > 40 °C	4			24 [0.80]
Total		38 [0.28]	48 [0.36]	101 [3.4]