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DNA vaccination regimes against Schmallenberg virus infection in IFNAR$^{-/-}$ mice suggest two targets for immunization

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ABSTRACT

Schmallenberg virus (SBV) is an RNA virus of the *Bunyaviridae* family, genus *Orthobunyavirus* that infects wild and livestock species of ruminants. While inactivated and attenuated vaccines have been shown to prevent SBV infection, little is known about their mode of immunity; specifically, which components of the virus are responsible for inducing immunological responses in the host.

As previous DNA vaccination experiments on other bunyaviruses have found that glycoproteins, as well as modified (i.e. ubiquitinated) nucleoproteins (N) can confer immunity against virulent viral challenge, constructs encoding for fragments of SBV glycoproteins G_N and G_C, as well as ubiquitinated and non-ubiquitinated N were cloned in mammalian expression vectors, and vaccinated intramuscularly in IFNAR$^{-/-}$ mice. Upon viral challenge with virulent SBV, disease progression was monitored. Both the ubiquitinated and non-ubiquitinated nucleoprotein candidates elicited high titers of antibodies against SBV, but only the non-ubiquitinated candidate induced statistically significant protection of the vaccinated mice from viral challenge. Another construct encoding for a putative ectodomain of glycoprotein G_C (segment aa. 678–947) also reduced the SBV-viremia in mice after SBV challenge. When compared to other experimental groups, both the nucleoprotein and G_C-ectodomain vaccinated groups displayed significantly reduced viremia, as well as exhibiting no clinical signs of SBV infection.

These results show that both the nucleoprotein and the putative G_C-ectodomain can serve as protective immunological targets against SBV infection, highlighting that viral glycoproteins, as well as nucleoproteins are potent targets in vaccination strategies against bunyaviruses.

1. Introduction

Schmallenberg virus (SBV) is a member of the *Bunyaviridae* family (genus *Orthobunyaviridae*). Discovered in Germany in 2011 (Wernike et al., 2014; Gibbens, 2012; Hoffmann et al., 2012), SBV has spread throughout the European continent, spanning from Ireland to Turkey (Yilmaz et al., 2014). Initially characterized in domesticated sheep, goat and cattle, further epidemiological studies have linked SBV to wild ruminants such as deer, mouflon (i.e. wild sheep) and bison (Larska et al., 2013a, 2014; Wernike et al., 2015b). In affected herds, SBV infection has been associated with diarrhea, fever and decreased milk production (Hoffmann et al., 2012; Wernike et al., 2013b; Peperkamp et al., 2015; Lievaart-Peterson et al., 2015). Although symptoms are generally mild and short-lived in adult ruminants, SBV infection has been linked to widespread abortions and developmental malformations in newborn domestic ruminant livestock (Bayrou et al., 2014).

SBV is an arthropod-borne pathogen that has been shown to be transmitted by biting midges (i.e. Culicoides) (Balenghien et al., 2014; Larska et al., 2013b, 2013c). Structural information on the whole virion is lacking and only the tridimensional crystal structure of the nucleoprotein is known (Dong et al., 2013; Ariza et al., 2013). Phylogenetic studies have shown the L, M and S-segments of the SBV genome to be most similar to viruses of the Simbu serogroup of orthobunyaviruses, with viruses of the species *Sathuperi virus* being the closest relatives (Goller et al., 2012).

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Currently, inactivated vaccines against SBV are available for use in ruminant animals, and have been shown to be effective in inducing neutralizing antibodies and completely inhibiting viral replication upon experimental viral challenge (Hechinger et al., 2014; Wernike et al., 2013c). While effective, the cost-benefit ratio of the inactivated SBV vaccine may prevent its widespread use, possibly due to the fact that SBV outbreaks appear to be endemic, e.g. in Germany (Wernike et al., 2015c). Therefore, a more practical approach to SBV vaccination may involve the development of lower-cost subunit vaccines or DNA vaccines (Babiuk et al., 2014; Kutzler and Weiner, 2008). However, the efficacy of such as strategy depends on the ability of individual recombinant viral proteins to induce a potent immune response.

Previously, the use of DNA vaccination against components of Rift Valley fever virus (RVFV) with particular emphasis on studying the protective role of glycoproteins (encoded by the M segment), as well as the nucleoprotein was successfully evaluated (Lagerqvist et al., 2009; Boshra et al., 2011a). Using interferon α/β knockout mice (IFNAR$^{-/-}$), it was demonstrated that the glycoprotein-encoding segment of RVFV could confer complete protection against viral challenge (Lagerqvist et al., 2009; Lorenzo et al., 2010); and that nucleoprotein, when expressed as a fusion protein with ubiquitin, can also confer near-complete protection against RVFV infection (Boshra et al., 2011b). In the case of SBV, monoclonal antibodies generated in mice inoculated with whole virus demonstrated a high degree of antigenicity against the N-terminal portion of glycoprotein C (G_C; aa 468–702 of the M-segment), as well as the nucleoprotein (Wernike et al., 2015a), with the former being associated with neutralizing activity (Roman-Sosa et al., 2016). However, whether or not these antigenic sites are sufficiently immunogenic to induce a complete protective immune response *in vivo* is not known. Over the past two decades, IFNAR$^{-/-}$ mice have been used as animal models for the study of bunyaviral infection and immunity (Lorenzo et al., 2010; Boshra et al., 2011b; Hefti et al., 1999; Pavlovic et al., 2000; Schuh et al., 1999; Proenca-Modena et al., 2015; Oestereich et al., 2014; Zivcec et al., 2013). Recently, IFNAR$^{-/-}$ mice were also shown to be susceptible to SBV infection (Wernike et al., 2012; Ponsart et al., 2014; Sailleau et al., 2013). Although, unlike with RVFV, clinical signs were not as evident following infection with virulent strains of the virus. While IFNAR$^{-/-}$ mice infected with RVFV resulted in complete mortality, IFNAR$^{-/-}$ infected with virulent SBV displayed primarily decreased weight loss, ataxia, apathy and limited mortality (Kraatz et al., 2015). This model has already been used to validate deletion mutants of SBV for their potential use as attenuated strains for vaccination (i.e. mutants lacking NSm and/or NSs) (Kraatz et al., 2015).

Here, we report on the use of DNA vaccination, encoding for different components of SBV. These include: a putative ectodomain of glycoprotein N (G_N) (aa 23–181), and two putative ectodomain segments of glycoprotein C (aa 468–1403), G_C-ecto1 (aa 678–947) and G_C-ecto2 (aa 866–1323) as well as ubiquitinated and non-ubiquitinated forms of the nucleoprotein (N).

We show that, when expressed separately, cDNAs encoding for the G_C–ecto1 as well as the nucleoprotein N can render mice asymptomatic following SBV challenge. These results demonstrate that individual components of SBV used here are sufficient to induce protective immunity; and serve as a basis for the creation of subunit vaccines against Schmallenberg virus infection in ruminants.

2. Materials and methods

2.1. Virus and mice

The BH80/11-4 strain of SBV was amplified once in cultured BHK cells in order to obtain sufficient titers for subsequent challenge studies. The mice used were Sv/129 IFN α/β −/− (B & K Universal Ltd, UK). All experiments were performed in the BSL3 facilities of INIA-CISA (Madrid), adhering to institutional and ethical guidelines for animal care and experimentation. Approval for the containment, vaccination, viral challenge and euthanasia were obtained prior to all *in vivo* experiments being performed. Throughout all steps of experimentation, the mice were monitored by staff veterinarians, and all animals that showed significant signs of morbidity were sacrificed by cervical dislocation.

2.2. Construction of DNA vaccines

Five DNA-vaccines were designed based on different components of Schmallenberg virus (Fig. 1A), using pCMV-based expression vectors (pOPIN vector suite; Oxford Protein Production Facility UK). The vectors either lacked a secretory signal (pOPINE for N and ubiquitinated-N), or possessed a secretory signal (pOPING for G_N-ecto, G_C-ecto1, G_C-ecto2). An empty vector was used as a negative control and all constructs possessed a carboxy-terminal histidine tag for *in vitro* confirmation of expression (Fig. 1B). All viral genes were synthesized (Genscript, USA), based on the Schmallenberg virus isolate Bovine Schmallenberg virus BH80/Germany/2011. PCR amplification steps were performed using Fusion HF DNA polymerase (New England Biolabs, USA).

The nucleoprotein alone as well as the ubiquitinated construct, was expressed as the complete 233-amino acid protein (Genbank accession number H2AM13). The modified Ubiquitin gene was designed as a 76-amino acid fusion protein, expressed at the N-terminus of the nucleoprotein, as previously described (Rodriguez et al., 1997, 2001).

Three glycoprotein constructs were also generated based on the same viral isolate. Primary sequences for G_N and G_C glycoproteins were also submitted to TMpred software (Krogh et al., 2001) for prediction of transmembrane helices and to Phyre2 protein fold recognition server (Kelley and Sternberg, 2009) to grasp possible structural homology with available sequences of known structure. The first, G_N-ecto, was designed based on the putative soluble ectodomain of glycoprotein N (amino acids 23–181 of the M-polyprotein). Similarly, the segments G_C-ecto1 (aa: 678–947 of the M-polyprotein) and G_C-ecto2 (aa: 866–1323 of the M-polyprotein) were designed, cloned and expressed, as putatively soluble globular portions. Interestingly, the G_C-ecto2 resulted to encompass the highly confident (96.2%) homology model (aa 953–1297) (Fig. 1A, inset) that Phyre2 returned in our recent sequences resubmission, as being similar to the severe fever with thrombocytopenia syndrome (SFTS) virus G_C glycoprotein [PDB ID 5G47; (Halldorsson et al., 2016)].

In all three glycoprotein constructs, an ATG codon was cloned to the 5'-end of the gene. For a negative control, an empty pOPING construct was used.

2.3. In vitro expression of DNA vaccine constructs

In order to verify the expression of all of the DNA vaccine candidates, each construct was transiently transfected in human embryonic kidney (HEK) 293T cells adapting protocols previously described (Aricescu et al., 2006). Briefly, 1 μg of each construct was incubated with 2 μg of polyethylenimine (PEI) in a volume of 100 μL Dulbecco's Modified Essential Medium (DMEM) for 30 min, followed by incubation for 2 h in 10^6 cells in a total volume of 500 μL of DMEM. The cells were then supplemented with 2 mL of DMEM/10% FBS and incubated at 37 °C for 24 h. Following transfection, the cells were washed with PBS, and lysed using 100 μL RIA buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris–Cl pH 8.0). Western