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Induction of neutralizing antibodies to Hendra and Nipah glycoproteins using a Venezuelan equine encephalitis virus *in vivo* expression system

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ABSTRACT

The emergence of Hendra Virus (HeV) and Nipah Virus (NiV) which can cause fatal infections in both animals and humans has triggered a search for an effective vaccine. Here, we have explored the potential for generating an effective humoral immune response to these zoonotic pathogens using an alphavirus-based vaccine platform. Groups of mice were immunized with Venezuelan equine encephalitis virus replicon particles (VRPs) encoding the attachment or fusion glycoproteins of either HeV or NiV. We demonstrate the induction of highly potent cross-reactive neutralizing antibodies to both viruses using this approach. Preliminary study suggested early enhancement in the antibody response with use of a modified version of VRP. Overall, these data suggest that the use of an alphavirus-derived vaccine platform might serve as a viable approach for the development of an effective vaccine against the henipaviruses.

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1. Introduction

Hendra Virus (HeV) and Nipah Virus (NiV) are important human pathogens that have emerged only recently. HeV and NiV are the prototype members of a new genus, *Henipavirus*, in the *Paramyxoviridae* family, and are also zoonotic biological safety level-4 (BSL-4) select agents (reviewed in Eaton [1]). NiV was first recognized in 1998 during an outbreak in Malaysia and was primarily transmitted to humans from infected pigs. The outbreak was responsible for 265 cases of encephalitis in people, with a nearly 40% mortality rate [2–4]. There have been more than a dozen occurrences of NiV since its initial recognition, most appearing in Bangladesh and India (reviewed [5]) and in March 2008 [6] and January 2010 [7]. Among these spillover events of NiV, the human mortality rate has been higher (~75%) along with evidence of person-to-person transmission [8–10] and direct transmission of virus from flying foxes to humans via contaminated food [11]. HeV emerged in Australia in 1994 and was identified as the cause of fatal respiratory disease in horses, which in turn was transmitted to humans causing fatal pulmonary disease [12,13], and HeV has also repeatedly caused fatal infections in horses with documented human illness and seroconversion [14]. There have been 14 recognized occurrences of HeV in Australia since 1994 with at least one occurrence per year since 2006, the most recent in May 2010. Every outbreak of HeV has involved horses as the initial infected host, causing lethal respiratory disease and encephalitis, along with a total of seven human cases arising from exposure to infected horses, among which four have been fatal and the most recent in 2009 [5,15].

NiV and HeV have been classified as category C select agents, and both can be readily isolated from natural sources [16–18], and readily grown in cell culture [19]. Being newly described, there is limited but growing knowledge about the biology of these viruses, and there are currently no approved therapeutic regimens or vaccines available for henipaviruses making them a biodefense concern. Efforts to date to develop vaccines have included the use of both recombinant poxviruses and soluble glycoprotein subunits. A recombinant vaccinia virus expressing the NiV attachment (G) and fusion (F) glycoproteins [20,21] has been shown to induce NiV-neutralizing antibodies in mouse and hamster animal models [20,21]. A canarypox virus-based vector encoding F and G glycoproteins of NiV has also been shown to protect animals against NiV challenge in a pig model [22]. Finally, a subunit vaccine approach utilizing purified soluble versions of the G glycoproteins (sG) from HeV and NiV protected cats from subsequent NiV challenge [23].

In vivo expression systems derived from Venezuelan equine encephalitis (VEE) virus have been shown to elicit protective mucosal and systemic immunity against a variety of viral diseases [24–28]. In this study we have employed a VEE-based vector, which

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packages genomic VEE replicon expressing a transgene into virus replicon particles (VRPs). These VRPs were used to induce immune responses to HeV and NiV in a murine model. Our primary objective was to determine the effectiveness of VRP for induction of antibodies that neutralize HeV and NiV. In addition, we also compared the immunogenicity of the wild-type VEE vector and a modified VEE replicon capable of prolonged expression that we constructed. The VEE-based vaccine approach takes advantage of the vector's inherent ability to deliver immunologic proteins to immune cells as well as its potential for induction of mucosal and systemic immunity. The results demonstrate the induction of potent immune responses against both HeV and NiV glycoproteins using as expression vectors two VRP variants that differed with respect to duration of transgene expression. Taken together, these findings suggest that an alphavirus-derived vaccine platform could serve as a viable approach for the development of an effective vaccine against the henipaviruses.

2. Materials and methods

2.1. Cell cultures

The baby hamster kidney cell line, BHK-21 (ATCC, Manassas, VA) and human embryonic kidney cell line, 293T (ATCC, Manassas, VA), used in this study were maintained in Dulbecco's minimal essential medium (Gibco) supplemented with 10% fetal bovine serum, L-glutamine, penicillin–streptomycin (Gibco) and tylosin (Sigma). The HeLa (ATCC CCL 2) cell line was maintained in Dulbecco's modified Eagle's medium (Quality Biologicals, Gaithersburg, MD) supplemented with 10% cosmic calf serum (HyClone, Logan, UT) and L-glutamine (DMEM-10). The human head and neck carcinoma PCI 13 cells were maintained in DMEM-10 supplemented with 10 nM HEPES (Quality Biologicals). All cell cultures were maintained at 37 °C in humidified 5% CO_2 atmosphere.

2.2. VEE replicon construct

The VEE constructs pRepX (VEE replicon vector) and pCV have been previously described [24]. They were kindly provided by J. Smith, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD. The pGPm has been previously described [29]. It is a modified version of the pGP [24] with two back mutations: E1 (272A/T) and E2 (209E/K) that facilitate lymphoid trafficking and immunogenicity of VRP [29]. All PCR amplified genes from donor plasmids that were cloned into pRepX were amplified by primers designed to introduce a ClaI recognition sequence followed by a 16-nucleotide VEE promoter sequence at the 5′ end of the gene, and a ClaI recognition sequence at the 3′ end [24,29]. All PCR amplifications were performed using rTth DNA polymerase (Applied Biosystems, Foster City, CA). A mutation was introduced into the NsP2 region of pRepX by site-directed mutagenesis (Stratagene Quick Change) according to manufacturer's instructions. Proline (P)^{713} was replaced by glycine (G). Replicon constructs with the resulting G mutation are designated starting with mV. The pRepX-R2gp160 (also named V-R2gp160) and its corresponding R2 envelope gene has been previously described [29,30].

The V-HeVF, V-HeVG, V-NiVF and V-NiVG were constructed as VEE replicon vectors encoding the corresponding fusion (F) and attachment (G) glycoproteins of HeV and NiV. The constructs were made by ClaI restriction cloning of PCR amplified coding sequences of the corresponding envelope glycoprotein into pRepX. The original vaccinia virus promoter-driven expression vector, pMC02, with the corresponding F and G coding sequences has been previously described [31]. The modified VEE replicon construct carrying a single G mutation in the NsP2 region was generated by replacing an ApaI/NotI digested fragment of the mutant pRepX with a similarly digested fragment from a wild-type VEE replicon construct encoding the Hendra G glycoprotein (HeVG) transgene. The modified VRP has shown a more sustained expression of transgene compared to wild-type based on laboratory experience.

2.3. Generation of packaged VEE replicon particles

The two-helper system developed by Pushko et al. was employed in the preparation of VRP. Replicon particle preparation has previously been described [29]. Replicon plasmids V-R2gp160, V-HeVF, V-HeVG, mV-HeVG, V-NiVF, and V-NiVG together with helper plasmids pGPm, and pCV were linearized with NotI and *in vivo* transcribed using T7 RNA polymerase (Ambion, Austin, TX) according to manufacturer's instructions. The resulting transcripts of individual replicon constructs were separately combined with the transcripts of pGPm and pCV, and each mixture was used to transfect 2×10^7 BHK-21 cells by electroporation using a Bio-Rad Gene Pulser (Bio-Rad, Hercules, CA). The cells were seeded into 75-cm^2 tissue culture flasks and fed with medium. Cells transfected with wild-type constructs were maintained at 37 °C for 27 h in 5% CO_2 atmosphere. Those transfected with replicon constructs carrying a single NsP2 mutation were maintained at 35 °C. Medium from each flask was harvested and then clarified by centrifugation at 10,000 rpm for 30 min at 4 °C in a Beckman L5-5E ultracentrifuge. The clarified medium was transferred to a 35-ml centrifuge tube, underlayered with 5 ml of 20% sucrose in phosphate-buffered saline (PBS), and ultracentrifuged at 24,000 rpm for 3 h to pellet the particles. The medium and sucrose were removed, the pellet was covered with 0.5 ml Dulbecco's phosphate-buffered saline containing Ca^{2+} and Mg^{2+} (D-PBS) and 0.1% fetal bovine serum (FBS) at 4 °C overnight and then scraped off into D-PBS, and aliquots were stored at −70 °C until needed.

Infectivity of replicon particle preparations and infectious particle concentrations was determined by an immunofluorescence assay (IFA), as has been previously described [29]. BHK-21 cells were seeded at a density of 2×10^4/well into wells of 16-well LabTek tissue culture slides (Nalge Nunc International), incubated overnight at the appropriate temperature of either 35 °C or 37 °C in 5% CO_2 atmosphere. Wells were inoculated with 50 μl aliquots of serial 10-fold dilutions of replicon particle preparations in D-PBS with 0.1% FBS. Medium was then added in 150 μl aliquots. After 24 h, the cells were fixed with cold acetone at −20 °C for 20 min, air dried, and stored at −20 °C. For IFA, the cells were rehydrated in PBS with 0.1% bovine serum albumin (BSA), and blocked with PBS containing 7.5% BSA for 15 min. Cells were probed with the appropriate anti-serum (either globulin fraction of human HIV-1 immune serum [32], soluble HeVG (sHeVG)-specific rabbit immune serum [31], NiVF- or HeVF-specific rabbit anti-sera [31]) or negative serum, each diluted 1:200 in PBS containing 0.1% BSA. Sera were applied for 1 h at room temperature. The wells were washed twice and the assays were developed by using the corresponding goat anti-human or goat anti-rabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). The numbers of infected cells per well were determined by fluorescence microscopy, and the volume inoculated and dilution of replicon particles applied to the particular well were used to calculate the concentration of infectious particles in the starting inoculum.

A safety test was performed on each preparation to test for replication competence. Confluent BHK-21 cell culture in six-well plates was inoculated with 1:10 dilutions of replicon particle suspensions, allowed to adsorb for 1 h, washed three times with PBS, fed with medium, and incubated at 37 °C for 24 h. The medium was