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A Hantavirus Pulmonary Syndrome (HPS) DNA Vaccine Delivered Using a Spring-powered Jet Injector Elicits a Potent Neutralizing Antibody Response in Rabbits and Nonhuman Primates

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Abstract: Sin Nombre virus (SNV) and Andes virus (ANDV) cause most of the hantavirus pulmonary syndrome (HPS) cases in North and South America, respectively. The chances of a patient surviving HPS are only two in three. Previously, we demonstrated that SNV and ANDV DNA vaccines encoding the virus envelope glycoproteins elicit high-titer neutralizing antibodies in laboratory animals, and (for ANDV) in nonhuman primates (NHPs). In those studies, the vaccines were delivered by gene gun or muscle electroporation. Here, we tested whether a combined SNV/ANDV DNA vaccine (HPS DNA vaccine) could be delivered effectively using a disposable syringe jet injection (DSJI) system (PharmaJet, Inc). PharmaJet intramuscular (IM) and intradermal (ID) needle-free devices are FDA 510(k)-cleared, simple to use, and do not require electricity or pressurized gas. First, we tested the SNV DNA vaccine delivered by PharmaJet IM or ID devices in rabbits and NHPs. Both IM and ID devices produced high-titer anti-SNV neutralizing antibody responses in rabbits and NHPs. However, the ID device required at least two vaccinations in NHP to detect neutralizing antibodies in most animals, whereas all animals vaccinated once with the IM device seroconverted. Because the IM device was more effective in NHP, the Stratis® (PharmaJet IM device) was selected for follow-up studies. We evaluated the HPS DNA vaccine delivered using Stratis® and found that it produced high-titer anti-SNV and anti-ANDV neutralizing antibodies in rabbits (n=8/group) as measured by a classic plaque reduction neutralization test and a new pseudovirion neutralization assay. We were interested in determining if the differences between DSJI delivery (e.g., high-velocity liquid penetration through tissue) and other methods of vaccine injection, such as needle/syringe, might result in a more immunogenic DNA vaccine. To accomplish this, we compared the HPS DNA vaccine delivered by DSJI versus needle/syringe in NHPs (n=8/group). We found that both the anti-SNV and anti-ANDV neutralizing antibody titers were significantly higher (*p*-value 0.0115) in the DSJI-vaccinated groups than the needle/syringe group. For example, the anti-SNV and anti-ANDV $PRNT_{50}$ geometric mean titers (GMTs) were 1,974 and 349 in the DSJI-vaccinated group versus 87 and 42 in the needle/syringe group. These data demonstrate, for the first time, that a spring-powered DSJI device is capable of effectively delivering a DNA vaccine to NHPs. Whether this HPS DNA vaccine, or any DNA vaccine, delivered by spring-powered DSJI will elicit a strong immune response in humans, requires clinical trials.

Keywords: DNA vaccine, hantavirus, jet injection.

INTRODUCTION

Several rodent-borne hantaviruses, family *Bunyaviridae*, are pathogenic in humans. The endothelium-leak disease caused by these viruses can result in severe pulmonary and/or renal disease. Hantavirus disease in the Americas usually involves severe lung pathology and is known as hantavirus pulmonary syndrome (HPS); whereas hantavirus disease in Europe and Asia usually involves severe kidney pathology and is known as hemorrhagic fever with renal syndrome (HFRS). Here, our focus is on the development of a vaccine to prevent HPS. According to the Centers for Disease Control and Prevention, from 1993-2013, there have been 593 reported cases of HPS in the U.S. with 96% of those cases in the western states [1]. In the same time frame there have been approximately 4,000 HPS cases in South America, mostly in Chile, Argentina, and Brazil [2]. Sin Nombre virus (SNV) is the leading cause of HPS in North America and Andes virus (ANDV) is responsible for the vast majority of HPS cases in South America. Although rare, HPS is notorious because onset is sudden, progression to severe disease can be rapid, and there is an extraordinarily high case-fatality rate (~35%) regardless of age, health status, or access to advanced medical care. There are no FDA approved vaccines or specific drugs to prevent or treat HPS.

Hantaviruses are tri-segmented (S, M, and L segments), negative sense RNA viruses. The nucleocapsid protein (N) and the G_n/G_c envelope glycoproteins are encoded by the S and M genome segments, respectively. The L segment en-

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codes the polymerase protein. Both N and G_n/G_c can contribute to protective immunity via molecular vaccine studies [3]. However, neutralizing antibodies target the envelope glycoproteins exclusively. These neutralizing antibodies are capable of conferring protection as shown by passive transfer experiments using G_n/G_c-specific monoclonal and polyclonal antibodies [4-6].

We are interested in using molecular vaccine technology to develop active and/or passive vaccines to protect against hantavirus disease. We have found that DNA vaccines containing the full-length M gene open reading frame delivered by particle mediated epidermal delivery (PMED, gene gun) or intramuscular (IM) electroporation are capable of eliciting high-titer neutralizing antibodies against hantaviruses associated with HFRS (i.e. Seoul virus, Hantaan virus, and Puumala virus), and against hantaviruses associated with HPS (i.e. ANDV and SNV) [6-10]. Here, we have investigated the possibility of effectively delivering a DNA vaccine using PharmaJet's spring-powered needle-free disposable syringe jet injection (DSJI) technology. Conventional vaccine delivery via jet injection has a long, successful history, best documented in Chapter 61 of Vaccines (6^{th} ed.) [11] and by the exhaustive bibliography maintained by Dr. Bruce Weniger, US Centers for Disease Control (retired). In brief, best estimates are that hundreds of millions of doses of vaccines have been delivered by jet injectors over the past 60 years, including mass campaigns against smallpox, yellow fever, measles, influenza and many others [11]. The capacity of jet injectors, and especially spring-powered DSJI, to effectively deliver DNA vaccines is a relatively new area of research.

The two intramuscular (IM) and intradermal (ID) spring-powered DSJI used in this study are FDA 510(k)-cleared for use in humans. The devices have a general-use clearance, so they can be used to deliver appropriately labeled FDA-approved vaccines. The Stratis® IM device is available commercially. These devices have been used to deliver conventional vaccines including live-attenuated, inactivated, and subunit vaccines [12-15]. The use of DSJI eliminates needles from the process of administering vaccines and eliminates the costs and dangers associated with sharp-needle waste. Energy stored in a spring provides the power for the device and is replenished by human power using a reset station; therefore, no outside gas cartridges or electricity is required. These injectors create a coherent stream of pressurized liquid that penetrates tissues (13-40 mm for muscle and 2 mm for skin) at high velocity resulting in a distribution of inoculum that is similar, but not identical, to the distribution of inoculum from a conventional needle and syringe.

In this report, we evaluated SNV and ANDV DNA vaccines, alone and in combination, delivered by PharmaJet IM and ID spring-powered DSJI devices. These studies include, for the first time, the evaluation of the SNV and HPS DNA vaccine in nonhuman primates (NHP). Our findings demonstrate that the PharmaJet devices can effectively deliver these hantavirus DNA vaccines and elicit high-titer (titers >1,000) neutralizing antibodies in rabbits and NHP. Importantly, we demonstrate that the PharmaJet IM device elicits significantly higher levels of neutralizing antibodies than needle and syringe in NHPs.

MATERIALS AND METHODS

Viruses, Cells, Medium

SNV strain CC107 [16] and ANDV strain Chile-9717869 [17] were propagated in Vero E6 cells (Vero C1008; ATCC CRL 1586). Vero, Vero E6 and HEK 293T were maintained in Eagle's minimal essential medium with Earle's salts (EMEM) containing 10% fetal bovine serum (FBS), 10 mM HEPES pH 7.4, and antibiotics (penicillin [100 U/mL], streptomycin [100 μg/mL]) (cEMEM) at 37°C in a 5% CO_2 incubator. The VSVΔG*rLuc pseudovirion is a recombinant VSV derived from a full-length cDNA clone of the VSV Indiana serotype in which the G-protein gene has been replaced with the Renilla luciferase gene [18].

DNA Vaccine Plasmids

The SNV DNA vaccine plasmid, pWRG/SN-M(opt), has been described previously [7]. Three versions of the ANDV DNA vaccine were used in this study: pWRG/AND-M, pWRG/AND-M(opt), and pWRG/AND-M(opt2). pWRG/AND-M also known as pWRG/AND-M(1.1) has been described previously [6]. pWRG/AND-M(opt) is identical to pWRG/AND-M except the M gene open reading frame (ORF) was codon optimized for *Homo sapiens*. Gene optimization and synthesis were performed using a contract service (Genewiz). During the course of this work, it became apparent that pWRG/AND-M(opt) inadvertently lacked a stop codon resulting in an additional 24 amino acids added to the Gc C-terminus. This error was corrected by the insertion of a stop codon after the M gene ORF to produce pWRG/AND-M(opt2). Both pWRG/AND-M(opt) and pWRG/AND-(opt2) expressed the hantavirus Gn/Gc envelope glycoproteins when transfected into COS or HEK293T cells as measured by flow cytometry using a polyclonal anti-ANDV antibody produced in geese (data not shown).

Animals

Female New Zealand white rabbits (*Oryctolagus cuniculus*) aged approximately 11 weeks were used in the DNA vaccination studies. Female Syrian hamsters (*Mesocricetus auratus*) aged 6-8 weeks were used in the passive transfer/challenge study. Female rhesus macaques (*Macaca mulatta*), heavier than 5.0 kg, were used in these studies. Female cynomolgus macaques (*Macaca fascicularis*) between 2.5 and 3.0 kg were also used. All monkeys were anesthetized with Telazol at a dosage of 2-6 mg/kg body weight prior to handling.

Vaccination

Four vaccine delivery technologies were used in this study: 1) PharmaJet IM v1.0 DSJI, 2) PharmaJet ID v1.0 DSJI, 3) PharmaJet IM Stratis® DSJI, 4) 1 mL syringe and 25G 5/8 needle. IM injection sites for rabbits were the muscles of the lateral thigh. ID injection sites were the skin overlying the lateral thigh muscles. IM injection sites for NHPs were the right and left triceps. ID injection sites for NHPs were the skin overlying the scapular region. The injection volume for IM injections was 0.5 mL, and for ID injections was 0.1 mL. DNA was diluted in PBS pH 7.4. Fur at all injection sites was removed using electric clippers prior to vaccination.

PRNT

Plaque-reduction neutralization tests (PRNT) were performed using Vero E6 as previously described [17]. The 50% (or 80%) PRNT titer ($PRNT_{50}$ or $PRNT_{80}$ titer) was the reciprocal of the highest serum dilution reducing the number of plaques by 50% (or 80%) relative to the average number of plaques in control wells that received medium alone. The limit of detection in the PRNT is a titer of 20.

Pseudovirion Production

Pseudovirions (PsV) were prepared using a modified version of previously described methods [18, 19]. HEK293T cells were seeded in T75 tissue culture flasks and transfected with pWRG/SN-M (opt) or pWRG/AND-M(opt2) using Fugene 6 (Promega) at ~80% confluency. The pWRG/SN-M(opt) and pWRG/AND-M(opt2) were used to produce SNV PsV and ANDV PsV, respectively. After ~18 hr the transfection media was removed and the cells were infected with VSVΔG*rLuc at a multiplicity of infection of ~0.02 for 1hr at 37°C. The media was removed and fresh media was added, the flasks were then incubated at 37°C for 72 hr. The supernatant from infected cells was collected and clarified by low speed centrifugation and filtration through a 0.22 μm filter. The pseudovirions were concentrated further by pelleting the virus at 40,000 rpm in a SW41 rotor for 2 hr through a 30% sucrose cushion prepared in TNE buffer (10 mM Tris, 135 mM NaCl, 2 mM EDTA, pH 8.0). The pellet was resuspended overnight in 500 μL TNE buffer, aliquoted and stored at −70°C.

Pseudovirion Titration

An aliquot of the PsV was thawed and used for titration. Serial 5-fold dilutions were made in cEMEM. Fifty microliters of each dilution were added to 90-100% confluent Vero cells in a 96-well plate. The cells were incubated for 18–24 h at 37°C. The cells were fixed with a cold methanol/acetone, and then blocked with 5% non-fat milk. Luciferase expression was detected with the primary antibody anti-Renilla Luciferase (MBL) this was followed by the addition of secondary anti-rabbit 488 antibody (Invitrogen). Wells that contained between 20-200 green cells were counted under an inverted fluorescent microscope. Titers were calculated taking into account dilution factors and volumes used for inoculation and expressed in fluorescent focus units per mL.

Pseudovirion Neutralization Assay (PsVNA)

An initial 1:10 dilution of the heat-inactivated sera was made followed by five-fold serial dilutions that were mixed with an equal volume of cEMEM containing 4,000 fluorescent focus units of PsV with 10% guinea pig complement. This mixture was incubated overnight at 4°C. The next day, 50 μL was used to inoculate Vero cell monolayers on a clear bottom black 96-well plate (Corning) in triplicate. The plates were incubated at 37°C for 18–24 hr. The media was discarded, and the cells were lysed according to the luciferase kit protocol (Promega #E2820). A Turner Biosystems modulus microplate reader was used to read the flash luciferase signal for (Figs. **1,2,3**), while a Tecan M200 Pro was used to acquire the rest of the data. The values were graphed using Prism software (Graphpad Version 5) to calculate the % neutralization and then interpolated to obtain the PsVNA 80% titers.

N-specific ELISA

The ELISA used to detect hantavirus nucleocapsid (N)-specific antibodies was previously described [10]. End-point titers were determined as the highest dilution that had an optical density (O.D.) greater than the mean O.D. of serum samples from negative control wells plus three standard deviations. The Puumala virus N was used to detect SNV N-specific antibodies as previously published [17].

Challenge with Hantaviruses

Anesthetized (isoflourane) Syrian hamsters were exposed to SNV or ANDV by intramuscular (IM) injection of the caudal thigh. A dose of 2,000 PFU SNV (1,000 ID_{50}) or 200 PFU ANDV (25 LD_{50}) was diluted in sterile phosphate-buffered saline (pH 7.4) and administered in a volume of 0.2 mL. SNV infects hamsters as measured by seroconversion to the N protein as determined with N-specific ELISA, but does not cause disease. In contrast, ANDV not only infects hamsters but also causes an endothelium-leak disease that resembles human HPS [17, 20]. The mean day-to-death following a 200 PFU challenge with ANDV is 11 days.

Statistical Analysis

Differences in anti-N ELISA titers post-challenge were analyzed by ANOVA with Dunnett's post-hoc comparisons between Groups. Survival analyses were done using the log-rank test conducted with GraphPad Prism (Version 5). Differences in neutralizing antibody titers were determined by either Wilcoxon or non-parametric Savage Test (see Figure legends).

Ethics

Animal research was conducted under an IACUC approved protocol at USAMRIID (USDA Registration Number 51-F-00211728 & OLAW Assurance number A3473-01) in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

RESULTS

SNV DNA Vaccine Delivered by PharmaJet IM or ID Needle-free Jet Injection Device Elicits High-titer Neutralizing Antibodies in Rabbits

Recently we reported the development of an SNV DNA vaccine [7]. In that study, the vaccine was delivered to hamsters using a gene gun, or to rabbits using IM electroporation. Here, we were interested in determining if the same vaccine could be delivered effectively using DSJI. The PharmaJet v1.0 IM and v1.0 ID devices were evaluated for a capacity to effectively deliver the SNV DNA vaccine in rab-

bits (Fig. **1**). The vaccine was administered three times at 1-month intervals. Sera were collected on the indicated days and evaluated for neutralizing antibodies by PRNT (Fig. **1B**). All of the rabbits were negative for neutralizing antibodies on day 0 (data not shown). After a single vaccination with either device, all rabbits produced neutralizing antibodies with $PRNT_{50}$ titers of at least 160 and three rabbits in the ID group exhibited titers >1,000. After two vaccinations, the titers in the IM group continued to rise, whereas the ID group remained within 2-fold. After three vaccinations, the IM group was further boosted and all four animals developed titers >1,000. The animals in the ID group all had titers >2,560 after the third vaccination. In our studies, we consider $PRNT_{50}$ titers >1,000 to be high-titer.

The PRNT uses authentic virus and must be performed in high containment laboratories and it takes approximately two weeks to generate results. In order to more rapidly, efficiently, and safely obtain neutralizing antibody titers, we developed and refined a hantavirus pseudovirion neutralization assay (PsVNA). The same sera evaluated by classic PRNT were evaluated by SNV PsVNA assay (Fig. **1C**). The results were similar to the PRNT in that all rabbits were positive after the first vaccination and the titers ranged between ~3,000-~15,000 after the third vaccination. Geometric mean titers (GMT), maximum and minimum titers, and seroconversion rates for both PRNT and PsVNA after the third vaccination are shown in (Table **1**).

Sera from the IM vaccinated rabbits were also evaluated for a capacity to cross-neutralize ANDV. There were low levels of anti-ANDV neutralizing antibodies detected in the rabbits vaccinated with the SNV DNA vaccine; however the PRNT and PsVNA GMT remained <1,000 (Table **1**). This finding suggested that, in order to produce high-titer neutralizing antibodies against both SNV and ANDV, an ANDV DNA vaccine component would need to be included in a universal HPS DNA vaccine.

SNV Immune Sera From Rabbits Vaccinated with PharmaJet IM Device is Sufficient to Protect Against SNV Infection, But Not Against Lethal HPS Caused by ANDV

To look at the protective efficacy of the antibodies generated using the PharmaJet IM device, we tested antibodies produced in rabbits using the IM device in the SNV hamster infection model. SNV infects Syrian hamsters (ID_{50} is 2 PFU) but does not cause disease [17].

Infection with SNV is determined using an ELISA that detects antibodies to the hantavirus N protein 1-month after exposure to SNV. Four groups of eight hamsters were

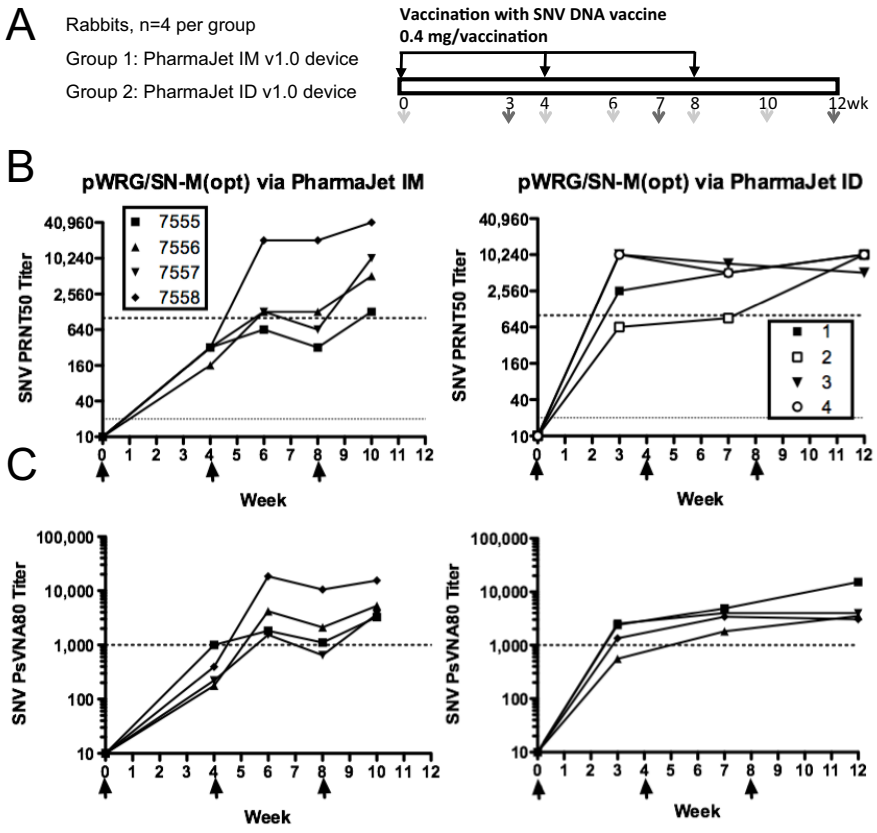

Fig. (1). Neutralizing antibodies in rabbits after vaccination with SNV DNA vaccine delivered by PharmaJet IM or ID device. A) Experimental design. Groups of four rabbits were vaccinated with 0.4 mg of pWRG/SN-M(opt) plasmid DNA using the indicated PharmaJet device. For IM, a single injection of 0.5 mL of vaccine was delivered per vaccination. For ID, a single injection of 0.1 mL was delivered per vaccination. Sera from the IM-vaccinated rabbits were collected on weeks 0, 4, 6, 8, and 10 (light grey arrows) and sera for the ID-vaccinated rabbits were collected on weeks 0, 3, 7, and 12 (dark grey arrows). **B**) Neutralizing antibodies were measured by PRNT. $PRNT_{50}$ titers are shown. **C**) Neutralizing antibodies were also measured by PsVNA. $PsVNA_{80}$ titers are plotted.

Table 1. GMT, titer ranges, and seroconversion rates in rabbits and NHPs after three vaccinations with SNV, ANDV, or HPS DNA vaccine.

DNA Vaccine Group												
		Fig. 1 Exp (rabbits)		**Fig. 3 Exp (rhesus)**		**Fig. 4 Exp (rabbits)**				**Fig. 5 Exp (cynomolgus)**		
	Vaccine:	SNV	SNV	SNV	SNV	HPS	ANDV	SNV	HPS	HPS	HPS	HPS
	Device:	IM v1.0	ID v1.0	IM v1.0	ID v1.0	IM Stratis®	IM Stratis®	IM Stratis®	IM Stratis®	IM v1.0	IM Stratis®	IM N/S
	Dose/vacc	0.4 mg	0.4 mg	1 mg	1 mg	2 mg each1	2 mg	2 mg	2 mg each2	1 mg each1	1 mg each	1 mg each1
Assay	**Results**	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 1**	**Group 2**	**Group 3**	**Group 4**	**Group 1**	**Group 2**	**Group 3**
SNV PRNT50	GMT	**7,241**	**8,611**	**2,032**	640	**1,560**	<	**4,695**	**2,153**	987	**1,974**	87
	max titer	40,960	10,240	10,240	1,280	40,960	20	40,960	40,960	10,240	20,480	320
	min titer	1,280	5,120	640	160	320	<	640	160	80	320	<
	seroconv.	100%	100%	100%	100%	100%	13%	100%	100%	100%	100%	88%
SNV PsVNA80	GMT	**5,607**	**5,068**	**3,917**	**1,957**	**3,169**	489	**4,630**	**3,005**	**1,476**	916	43
	max titer	15,384	15,344	15,963	4,415	24,845	1,801	9,563	11,663	4,361	2,866	265
	min titer	3,285	3,056	899	586	1,036	112	1,799	749	284	306	<
	seroconv.	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	75%
ANDV PRNT50	GMT	538	nd	nd	nd	**2,560**	761	<	538	415	349	42
	max titer	2,560	nd	nd	nd	20,480	5,120	20	5,120	2,560	2,560	1,280
	min titer	20	nd	nd	nd	640	160	<	80	40	<	<
	seroconv.	100%	nd	nd	nd	100%	100%	13%	100%	100%	88%	38%
ANDV PsVNA80	GMT	864	nd	265	93	**3,486**	871	190	**1,067**	562	433	31
	max titer	1,729	nd	1,077	159	18,389	4,192	424	4,051	1,803	918	170
	min titer	354	nd	55	32	867	174	95	314	164	175	<
	seroconv.	100%	nd	100%	100%	100%	100%	100%	100%	100%	100%	75%

[1]plasmids mixed 1:1 prior to injection.

[2]plasmids delivered to separate sites.

IM= intramuscular; ID= intradermal; N/S= needle and syringe; nd= not done; < less than 20; seroconv. = seroconversion rate.

GMT= geometric mean titer (bold if over 1,000).

injected subcutaneously with anti-SNV sera, anti-ANDV sera, normal rabbit sera, or untreated. One day after antibody injection the hamsters were challenged with SNV by the IM route. Anti-SNV sera injected one day before challenge significantly ($p < 0.0001$) reduced the level of infection relative to the anti-ANDV sera, normal sera, or no treatment group (Fig. **2A**). This finding demonstrated that the anti-SNV response produced using the SNV DNA vaccine delivered using the PharmaJet IM DSJI can confer protection *in vivo*.

We were also interested in testing the possibility that the anti-SNV antibody might cross-protect against a lethal challenge with ANDV. Unlike SNV, ANDV causes a lethal disease in hamsters that closely resembles HPS in humans [17].

Four groups of eight hamsters were injected with anti-SNV antibody, anti-ANDV antibody, normal rabbit sera, or no treatment. All of the hamsters injected with the anti-SNV antibody succumbed by day 15 indicating that the anti-SNV antibody cannot cross-protect against ANDV (Fig. **2B**). All but one of the rabbits treated with normal sera, or no treatment, also succumbed. In contrast, the anti-ANDV rabbit sera (positive control) completely protected the hamsters against lethal HPS caused by ANDV. ELISA on day 28-post challenge sera indicated that 6 of the 8 survivors in the anti-ANDV group had been infected and two had been protected against, not only lethal disease, but also against infection (data not shown).

Hantavirus DNA Vaccine Delivered by PharmaJet IM or ID Needle-free Jet Injection Device Elicits High-titer Neutralizing Antibodies in Nonhuman Primates (NHPs)

Having found the needle-free jet injection devices could effectively deliver the SNV hantavirus DNA vaccines to rabbits, we were interested in determining if these devices would also be effective in NHP. Groups of three rhesus macaques were vaccinated with the SNV DNA vaccine using either the PharmaJet IM or ID device three times at 1-month intervals

(Fig. **3A** arrows). Each vaccination consisted of two administrations of 0.5 mg of DNA/administration (right and left triceps). Sera were collected on week 0, 4, 8, and 11 and evaluated for neutralizing antibodies by both PsVNA and PRNT (Fig. **3B**, **C**). The IM device elicited neutralizing antibodies in all animals after a single vaccination. One animal (0660) produced exceedingly high neutralizing antibodies as measured by both PsVNA and PRNT. The ID device was less effective in NHP. Only 1 of 3 animals produced neutralizing antibodies after the first vaccination. After the second vaccination, 100% (n=6) of the NHPs developed neutralizing antibody responses. Titers remained >1,000 in the PsVNA through the last time point tested (1-month after the last vaccination). The group GMT, maximum and minimum titers, and seroconversion rates after three vaccinations are shown in (Table **1**).

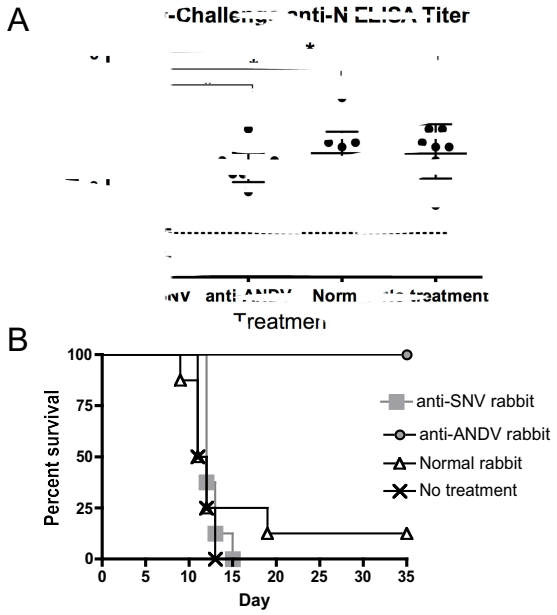

Fig. (2). Testing protective efficacy of passively transferred sera from rabbits vaccinated with SNV DNA vaccine using the PharmaJet IM v1.0 in hantavirus animal models. A) Passive transfer of 12,000 NAU/kg anti-SNV sera from a rabbit (#7558, wk 10. Fig. **1**) vaccinated with pWRG/SN-M(opt) inhibited SNV infection in hamsters following a 1,000 ID_{50} challenge with SNV as measured by development of post-challenge anti-nucleocapsid (N) antibody response. Symbols represent endpoint ELISA titers for individual hamsters (GMT from three assays). The group GMT and 95% confidence level is shown for the anti-SNV, anti-ANDV (rabbit vaccinated with ANDV DNA vaccine, intramuscular electroporation), Normal rabbit sera, and No treatment groups. * indicates the difference in post-challenge titer were significant. **B**) Passive transfer of 12,000 NAU/kg of anti-SNV sera did not cross-protect hamsters against a lethal challenge with ANDV; whereas transfer of 12,000 NAU/kg of anti-ANDV positive control sera completely protected against lethal disease. The anti-ANDV rabbit serum was described previously [6].

HPS DNA Vaccine Delivered As Separate Plasmids, Or As a Plasmid Mixture, by PharmaJet IM Stratis® Device is Immunogenic in Rabbits

To develop an HPS vaccine that elicits high-titer neutralizing antibodies against both the North and South American Hantaviruses, our approach has been to administer a combination of both the SNV and ANDV DNA vaccines. We performed an experiment in rabbits to determine whether mixing the SNV and ANDV DNA vaccine plasmids prior to DSJI vaccination was as effective as delivering the same plasmids to separate sites. The goal of this experiment was to determine if the HPS vaccine used in future clinical trials should be delivered as two separate injections (i.e. SNV and ANDV DNA vaccines delivered to separate sites) or as a plasmid mixture. Groups of 8 rabbits were vaccinated three times at 1-month intervals with a 1:1 mixture of the ANDV and SNV DNA vaccine plasmids, the ANDV DNA vaccine plasmid alone, the SNV DNA vaccine plasmid alone, or the ANDV and SNV DNA vaccines administered to separate sites (Fig. **4A**). The PharmaJet IM Stratis® was used for all injections. One rabbit in Group 1 was euthanized due to reasons unrelated to the vaccine. The SNV and ANDV neutralizing antibody $PsVNA_{80}$ mean titers after 1, 2, or 3 vaccinations are shown (Fig. **4B**). All Day 0 samples were negative by both ANDV and SNV PsVNA (data not shown). After 1 vaccination (Week 4), both combination vaccines (Groups 1 and 4) resulted in 100% seroconversion against both SNV and ANDV. After 1 vaccination, ANDV DNA vaccine and SNV DNA vaccine delivered alone resulted in 63% and 88% homotypic seroconversion, respectively. The SNV DNA vaccine, but not the ANDV DNA vaccine, exhibited some heterotypic cross-neutralizing activity after 1 vaccination. After 2 vaccinations (Week 8), 100% seroconversion was observed for all groups. The Group 1 GMT for both anti-SNV and ant-ANDV was >1,000. One month after the third vaccination (Week 12), both Groups 1 and 4 exhibited GMT against both SNV and ANDV >1,000. The SNV DNA vaccine alone also resulted in a GMT >1,000 against SNV, and the ANDV DNA vaccine alone resulted in a GMT just under 1,000. There was no significant difference between Groups 1 and 4 at any timepoint indicating the HPS vaccine was as effective when delivered as a mixture, or if each plasmid was delivered to a separate site.

The week-12 samples were also evaluated by PRNT. The PRNT data demonstrated that there was essentially no cross-neutralization between the SNV and ANDV DNA vaccines, whereas the HPS vaccine via either formulation elicited neutralizing antibodies against both SNV and ANDV. As was observed for the PsVNA on week-12 samples, the HPS DNA vaccine delivered as mixture elicited high-titer (>1,000) neutralizing antibodies against both SNV and ANDV. The group GMT, maximum and minimum titers, and seroconversion rates after three vaccinations are shown in (Table **1**).

PharmaJet IM v1.0 and PharmaJet IM Stratis® Needle-free Delivery Devices are Significantly More Effective at Delivering HPS DNA Vaccine to NHPs Than Needle/syringe

The NHP data shown in (Fig. **3**) demonstrated that the PharmaJet IM v1.0 device could be used to produce robust anti-SNV neutralizing antibodies using the SNV DNA vaccine, and the rabbit data shown in (Fig. **4**) demonstrated that the SNV and ANDV DNA vaccines (i.e. HPS vaccine) delivered using the Stratis® device could be mixed without loss of potency. To determine if an HPS DNA vaccine (i.e., 1:1 mixture of SNV and ANDV DNA vaccine) could be deliv-

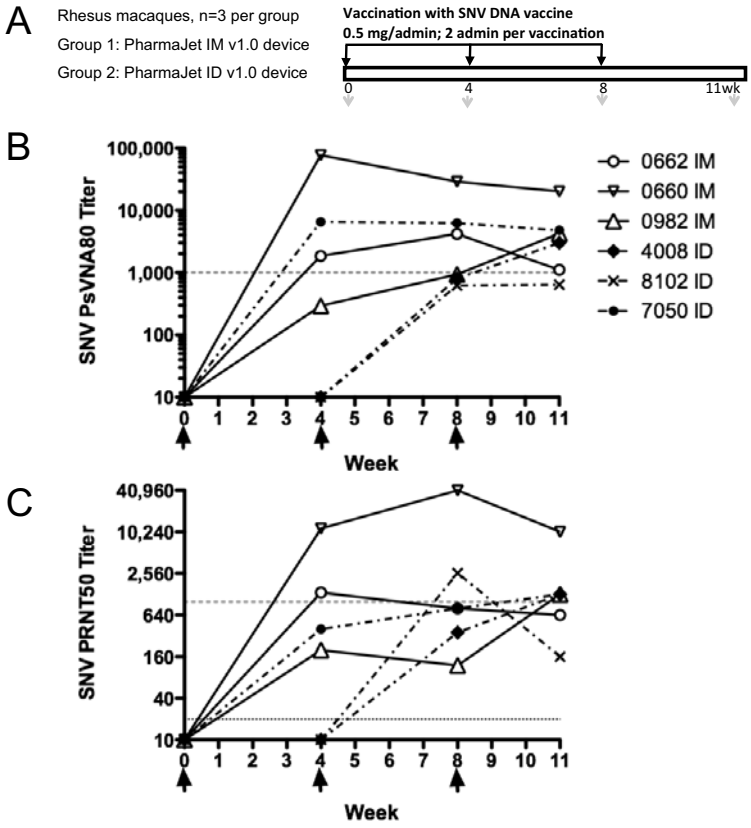

Fig. (3). Hantavirus DNA vaccine delivered to NHP using PharmaJet IM v1.0 or ID v1.0 needle-free jet injection device. A. Groups of three NHP were vaccinated with the SNV DNA vaccine (pWRG/SN-M[opt]) using either the PharmaJet IM v1.0 or ID v1.0 device three times at 4-week intervals (arrows). Each vaccination consisted of two administrations of 0.5 mg of DNA/administration, one to each triceps muscle. **B**. SNV $PsVNA_{80}$ titers were determined and plotted. The animal ID numbers and device (IM or ID) are shown in legend. **C.** SNV $PRNT_{50}$ titers were determined and plotted to quantify the capacity of the sera to neutralize authentic virus.

ered effectively to NHPs, and to compare the PharmaJet IM v1.0 and Stratis® devices with needle/syringe, an experiment involving 24 cynomolgus macaques was performed (Fig. **5A**). The SNV DNA vaccine was pWRG/SN-M(opt) and the ANDV DNA vaccine was pWRG/AND-M(opt). Three groups of eight NHPs were vaccinated with the HPS DNA vaccine using one of the three methods described above three times at 1-month intervals. Sera were collected and evaluated for the presence of neutralizing antibodies by PRNT and PsVNA. Week 0 (prebleed) titers were below detection for both SNV and ANDV in all but two samples. Those two samples that gave low titer (<50) in either PRNT or PsVNA, but not in both, and likely represented background activity (data not shown). After one vaccination, anti-SNV and/or anti-ANDV neutralizing antibodies, as measured by PRNT or PsVNA, were detected in 13 of 16 (81%) animals vaccinated with either PharmaJet needle-free devices, and 4 of 8 (50%) animals vaccinated with needle/syringe (Fig. **5B-C**). The anti-SNV GMT as measured by both PRNT and PsVNA were greater than the anti-ANDV GMT (Table **1**). Anti-ANDV antibodies, as measured by PRNT, were not detected after one vaccination with needle/syringe. After two vaccinations, a significant difference between both PharmaJet devices versus needle/syringe became apparent. Specifically, the anti-SNV $PRNT_{50}$ GMT in the PharmaJet IM v1.0 group was significantly higher (*p*-value 0.0461) than the needle/syringe group; and both the anti-SNV and anti-ANDV $PsVNA_{80}$ GMT were significantly higher (p-values ≤0.0172) in both the PharmaJet groups versus needle/syringe. After three vaccinations, the levels of anti-SNV neutralizing antibodies as measured by PRNT for both PharmaJet devices were significantly higher (p-values ≤0.02) than those produced by needle/syringe (Fig. **5B**). Likewise, both the anti-SNV and anti-ANDV $PsVNA_{80}$ GMT were significantly higher (*p*-values 0.0115) in the PharmaJet groups than the needle/syringe group (Fig. **5C**). The group GMT, maximum and minimum titers, and seroconversion rates after three vaccinations are shown in (Table **1**).

DISCUSSION

Clinical investigations have shown DNA vaccines, including hantavirus DNA vaccines, to be safe and well tolerated in Phase I and II trials for many diseases [21-23]. Nevertheless, licensure of any DNA vaccine for human use remains elusive. Our search of ClinicalTrials.gov, revealed one plasmid DNA vaccine currently in phase 3, for CMV trial # NCT01877655 [24]. The main challenge in human DNA vaccine development is the production of sufficiently high levels of protective immune responses (e.g., neutralizing antibodies). Attempts to increase the potency of these relatively simple nucleic acid-based vaccines usually include the addition of ancillary components or technologies that add complexity to the vaccine. Examples of some of the most

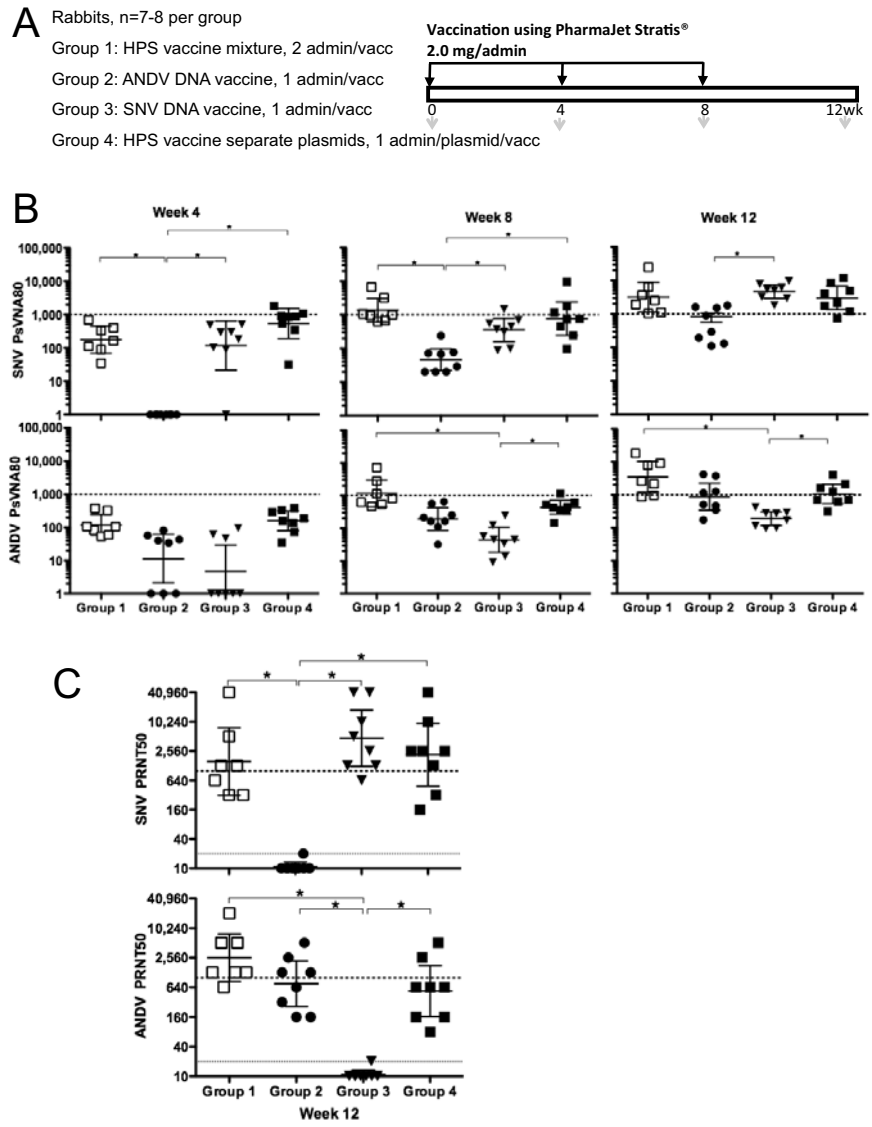

Fig. (4). Delivering the HPS DNA vaccine as a mixture, or to separate sites. A) Experimental design. Rabbits were vaccinated on week 0, 4, and 8 with: Group 1: HPS vaccine (1:1 mixture of SNV DNA vaccine, pWRG/SN-M(opt); and ANDV DNA vaccine, pWRG/AND-M(opt2)) delivered to two sites; Group 2: the ANDV DNA vaccine alone (1 site); Group 3: the SNV DNA vaccine alone (1 site); or Group 4: the ANDV and SNV DNA vaccine to two separate sites. Sera were collected on indicated weeks (grey arrows). In all groups, 2 mg of DNA was injected in a 0.5 mL volume per administration. Groups 1 and 4 received 2 administrations per vaccination, one to the right and left lateral thigh muscle. Groups 2 and 3 received a single administration per vaccination. **B**) $PsVNA_{80}$ titers for SNV and ANDV are shown for weeks 4, 8, and 12. Geometric mean titers +/- 95% confidence intervals are shown. Significant differences between delivery devices are shown $p < 0.05$ *, as determined by Wilcoxon Test. Titers above 1,000 (large-dashed line) are considered high-titer. Admin= administrations, vacc= vaccination, wk= week.

effective approaches at enhancing DNA vaccine potency are the addition of plasmids encoding immune stimulating molecules and/or the delivery of the DNA using electroporation technology [23, 25]. In the present study, our aim was to advance preclinical testing of a candidate HPS DNA vaccine using a spring-loaded, FDA-cleared DSJI device. Our rationale was that the logistical and regulatory advantages of such a device might compensate for a limited reduction in potency of the vaccine relative to a more complex DNA vaccine.

Delivery methods that impart plasmid translation efficiency above that typically provided by needle and syringe delivery include DSJI, gene gun, and electroporation. From this list DSJI is the least cumbersome and the most pragmatic for reasons including: 1) liquid vaccine can be directly administered without the need for special reformulation; 2) spring powered DSJI devices eliminate the need for electricity or compressed gas, making field use very straight forward; 3) devices are available on the market that are FDA-cleared, WHO-prequalified and ISO 21649-compliant, greatly increasing access while decreasing the regulatory burden and overall developmental cost.

Here, we found that the PharmaJet IM and ID devices could elicit high-titer neutralizing antibodies (titers >1,000) in rabbits and, more importantly, in NHPs. The IM devices

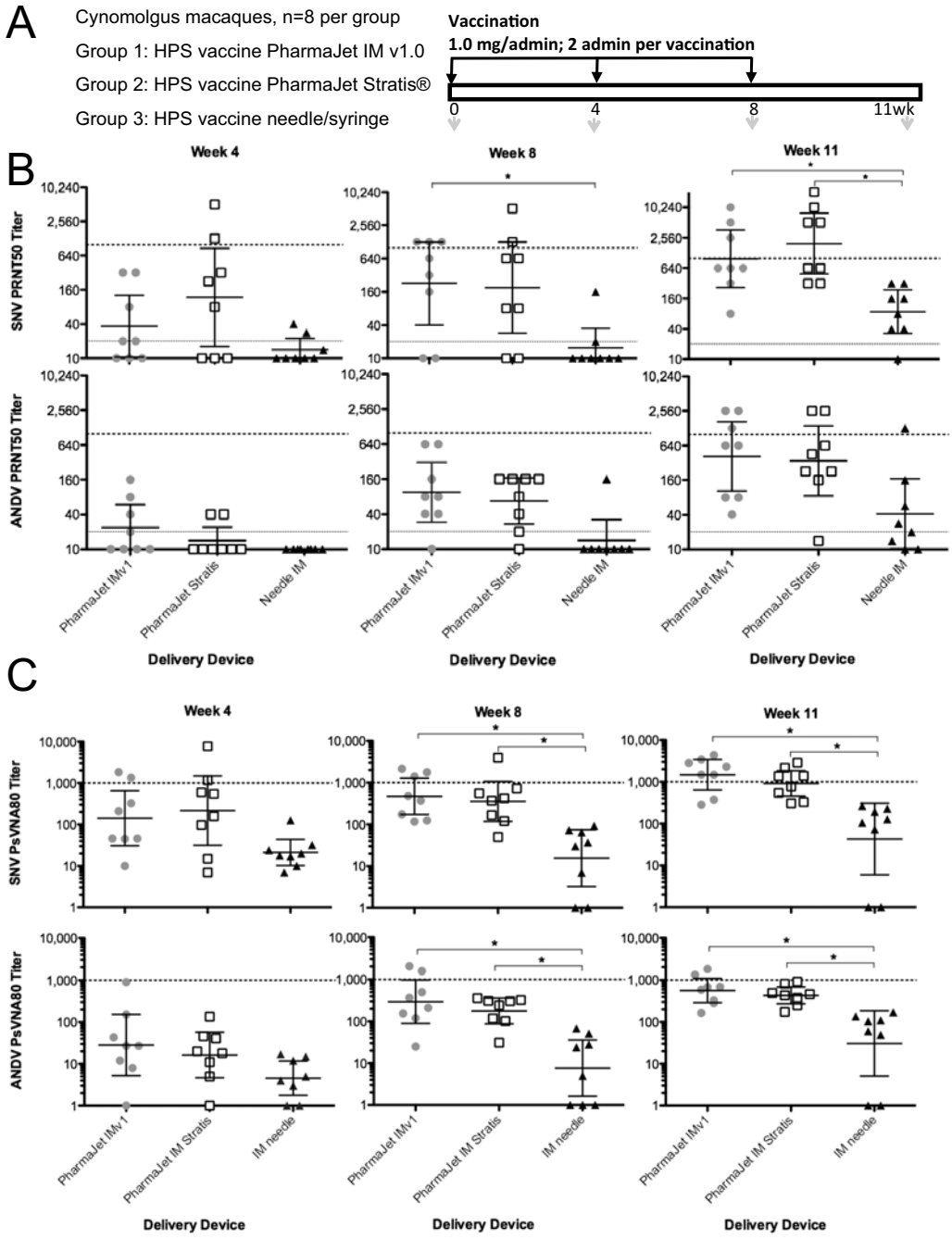

Fig. (5). Comparing immunogenicity of HPS DNA vaccine in NHPs when delivered by two PharmaJet IM devices versus needle/syringe. A) Experimental design. NHP were vaccinated at 1-month intervals with HPS vaccine (equal mixture of SNV DNA vaccine, pWRG/SN-M(opt) and ANDV DNA vaccine, pWRG/AND-M(opt)). Sera were collected on indicated weeks (grey arrows). Group 1 was vaccinated with the PharmaJet IM device, version 1(v1.0) (grey circle). Group 2 was vaccinated with the PharmaJet Stratis® device (open square). Group 3 was vaccinated with a needle/syringe (black triangle). In all groups, 1.0 mg of DNA was injected in a 0.5 mL volume to the right and left triceps muscle. **B**) $PRNT_{50}$ and **C**) $PsVNA_{80}$ titers for SNV and ANDV are shown for weeks 4, 8, and 11. Geometric mean titers +/- 95% confidence intervals are shown. Significant differences between delivery devices are shown $p < 0.05$ *, as determined by non-parametric Savage test.

appeared to be more effective than ID in the NHP because fewer vaccinations were needed to achieve high titers. We suspect that difference in skin properties between species is the most likely reason for the observed species difference between rabbits and NHP with the ID device. The skin of the rabbit seemed to permit consistently good ID accumulation of injected material, but the skin of the NHP used in this experiment did not (i.e., as measured by bleb size and the amount of fluid remaining on the skin after injection). We have observed that the skin of NHPs is more difficult to deliver an ID injection of conventional vaccines to than the skin of rabbits, Guinea pigs, pigs, or humans (unpublished observations). An important finding of this study was that the PharmaJet v1.0 and Stratis® devices resulted in an ap-

proximate 10-fold increase in neutralizing antibody titer in NHP when compared to needle and syringe in the same experiment (Fig. **5** and Table **1**). These results are not consistent with the findings of groups reporting that DSJI do not significantly improve the immunogenicity of the plasmid DNA vaccine over needle and syringe [26], but are consistent with other groups reporting enhanced immunogenicity [27-29].

From a hantavirus vaccine perspective, this is the first time that an SNV vaccine of any kind has been shown to be immunogenic in NHP. The neutralizing antibody responses that were produced in NHP vaccinated with the SNV DNA vaccine using the spring-powered DSJI IM device ranged from $PRNT_{50}$ titer of 640 to 10,240. Importantly, we found that there was no interference between the SNV and ANDV DNA vaccines when combined into the HPS DNA vaccine. This is most clearly shown in the rabbit data (Fig. **4**, Groups 1 and 4) where the same neutralizing antibody responses were achieved whether the plasmids were mixed together or delivered independently. The anti-ANDV response was lower than the anti-SNV response in the NHP experiment shown in (Fig. **5**). One possible explanation for the reduced potency of the ANDV DNA vaccine in that experiment is that the pWRG/AND-M(opt) plasmid was used. This plasmid was retrospectively found to have additional amino acids at the c-terminus due to the absence of a stop codon. Nevertheless, there was still a potent neutralizing antibody response against both SNV and ANDV in that experiment. The fact that the SNV and ANDV plasmids can be mixed and delivered in the same injection allows flexibility for future vaccine development.

Our immune readout in these HPS vaccine studies was the production of neutralizing antibodies. Neutralizing antibodies are known to play an important role in hantavirus immunity. For example, we have demonstrated that hantavirus neutralizing antibodies produced using DNA vaccines are sufficient to protect animals in passive transfer experiments [5, 6, 30]. Here, we provided additional passive protection data demonstrating that the sera from a rabbit vaccinated with the SNV DNA vaccine administered with the PharmaJet IM device could protect hamsters against infection with SNV (Fig. **2**). In most of the experiments reported here, we measured the neutralizing antibody response to the vaccines using two methods: the PsVNA and the classic PRNT. The PRNT measures hantavirus neutralizing antibodies against authentic hantavirus performed in BSL-3 containment, whereas the hantavirus PsVNA involves defective VSV expressing a luciferase reporter pseudotyped with the hantavirus glycoproteins [18, 19, 31]. These assays can be performed rapidly and do not require containment. This is the first time a SNV PsVNA has been described and it is the first time PsVNA have been used to evaluate hantavirus vaccines. In general, the PsVNA and PRNT provide similar information on the level of neutralizing antibodies in the sera of animals vaccinated with the DNA vaccines. There were instances where the PsVNA appeared to detect cross-neutralizing antibodies in samples that were not detected by PRNT. For example, rabbits vaccinated 1x with the ANDV DNA vaccine were negative for SNV neutralizing antibodies by PRNT, but were positive by PsVNA (Fig. **4**). After the second vaccination, cross-neutralizing antibodies were detected by both assays. There were other instances, albeit rare, where the PsVNA for a specimen was negative and PRNT positive. The relationship between the PsVNA, PRNT, and protective immunity will require further study. Although we acknowledge that a cellular immune response likely contributes to protective immunity conferred by these M gene-based hantavirus DNA vaccines, we do not have an assay to measure specific T cell responses at present.

Previously we reported that the HPS DNA vaccine delivered by IM electroporation (2 mg/vaccination) to rabbits n=3 could elicit very high levels of hantavirus neutralizing antibodies: SNV $PRNT_{50}$ ranging from 5,120 to 40,960, GMT=20,480; and ANDV $PRNT_{50}$ ranging from 640-40,960, GMT=6,451 [7]. Here, the HPS DNA vaccine delivered by Stratis® (4 mg/vaccination) to rabbits n=8 elicited titers that were not as high: SNV $PRNT_{50}$ ranging from 320-40,960, GMT=1,560; and ANDV $PRNT_{50}$ ranging from 640-20,480, GMT=2,560. There were several differences in the experiments other than delivery technology (e.g., dose, schedule, number of animals) so this is not a direct comparison of delivery technology. Thus, although the GMT achieved using Stratis® in rabbits were lower than those produced using IM electroporation, the titers in some animals were as high and our impression was that the observed response using the Stratis® device warrants further testing. For perspective, many HPS survivors have neutralizing titers ($PRNT_{80}$) < 1,000 [32, 33], but titers can be as high as 10,240 [5].

In summary, this report demonstrates the usefulness and efficiency of a spring-powered, needle-free DSJI device for delivering DNA vaccines. The data reported herein demonstrate, for the first time, that a spring-powered DSJI device is capable of effectively delivering a DNA vaccine to NHPs. The possibility that spring-powered DSJI devices might also be capable of effectively delivering a DNA vaccine to humans is of great interest because these devices are relatively inexpensive, practical, and already in commercial use for delivery of conventional vaccines around the world.

CONFLICT OF INTEREST

MR was a PharmaJet employee during the conduct of these experiments.

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PATIENT CONSENT

Declared none.

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