Immunogenicity and Safety Assessment of Hantaan and Puumala Virus DNA Vaccines in Rabbits

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ABSTRACT

A repeat dose study was performed to characterize the immunogenicity and safety/target organ toxicity of Hantaan virus (HTNV) and Puumala virus (PUUV) DNA vaccines in rabbits. Six groups of 10 NZW rabbits/sex received saline only or HTNV and PUUV vaccines alone or in combination on days 1, 15, 29, and 57. Vaccines were administered by intramuscular (IM) injection with electroporation or intradermal (ID) injection with electroporation; saline was administered by IM + ID injection without electroporation. Toxicology evaluations included survival; clinical observations; physical examinations; injection site reactogenicity; body weights; food consumption; body temperature; clinical chemistry; hematology; coagulation; ophthalmology; organ weights; immunoassay analysis (pseudovirion neutralization assay); gross pathology; and microscopic pathology. Five rabbits per group were necropsied on day 59 (two days after the final vaccine dose); remaining animals were necropsied on day 85 (after a 28-day recovery period). Both vaccines induced robust immunogenic responses regardless of route of exposure, as all rabbits developed neutralizing antibodies against the target virus. Both vaccines were well tolerated by both routes: no treatment-related deaths were observed, and no clinical evidence of toxicity was seen in any animal. Body weights, food consumption, body temperatures, ophthalmology, and clinical chemistry parameters were comparable in all groups. A higher incidence of injection site reactogenicity was seen in groups receiving vaccines by the IM and ID routes; reactogenicity was attributed to the effects of electroporation and not to effects of the vaccines themselves. Vaccine toxicity was limited to increased monocyte counts, increased fibrinogen levels, and increased spleen weights in several groups; these effects were reversible, as changes were no longer present after the 28-day recovery period. No gross pathology was identified in any animal at either the Main Study or Recovery necropsies. Microscopic changes were limited to lesions at the injection site that were interpreted as typical effects of IM or ID dosing with electroporation. Both HTNV and PUUV DNA vaccines induced robust immunogenic responses in rabbits with very limited accompanying toxicity. [Funding provided by The Geneva Foundation through NIAID contract HHSN272201200019C]

INTRODUCTION

Hantaviruses, like the Hantaan (HTNV) and Puumala (PUUV) viruses, are single-stranded, enveloped, negative sense RNA viruses. Infection with hantavirurses is through contact with hantavirus-infected rodents or their urine, saliva, or feces. Infection generally occurs through airborne transmission when fresh urine, feces, or nesting materials are disturbed and tiny droplets containing the virus get into the air and are breathed in by the person. Transmission may also occur when infected urine or other materials are directly introduced into broken skin, through contact with mucous membranes of the eyes, nose, or mouth, or by rodent bites of infected animals. Infection with HTNV and PUUV may be fatal, resulting in a hemorrhagic fever with renal syndrome. Early infection is associated intense headaches, back and abdominal pain, chills, fever, nausea, and blurred vision. Later symptoms may include low blood pressure, acute shock, vascular leakage, and acute kidney failure that may cause severe fluid overload. Infection with HTNV causes more severe symptoms compared to infection with PUUV, with fatality rates ranging from 5-15% following HTNV infection compared to < 1% following PUUV infection.

One way to decrease the incidence of the hemorrhagic fever with renal syndrome caused by hantavirus infection is the development of vaccines to prevent infection. Under its prime contract with the National Institute of Allergy and Infectious Diseases (NIAID), The Geneva Foundation partnered with the United States Army Institute of Infectious Diseases (USAMRIID) investigators Drs. Connie Schmaljohn and Jay Hooper for development of their HTNV and PUUV DNA vaccine candidates. This program has promoted preclinical development of the vaccine candidates for the prevention of these serious infections of military and public health concern. The current study employed a rabbit animal model to evaluate the nonclinical toxicology of these vaccines alone or in combination following repeat-dose intramuscular or intradermal administrations with electroporation to generate experimental toxicology and immunogenicity data sets that are sufficient to meet regulatory requirements for preclinical safety assessments. The ultimate goal for this study is to gain regulatory approval through the submission of an Investigational New Drug (IND) application to the U.S. Food and Drug Administration (FDA) for the entry of these vaccines into Phase I and ideally Phase II clinical trials.

Experimental endpoints included moribundity/mortality observations, physical examinations, clinical signs of toxicity, injection site (Draize) reactogenicity scoring, body weights, body weight changes, food consumption; body temperatures, ophthalmology, clinical pathology (clinical chemistry, hematology, coagulation), organ weights, immunoassay analysis (pseudovirion neutralization assay), gross pathology, and microscopic pathology.

STUDY OBJECTIVE

The objective of this study was to evaluate the immunogenicity, safety and potential target organ toxicity of Hantaan virus (HTNV) and Puumala virus (PUUV) DNA vaccines alone or in combination in New Zealand White rabbits when administered intramuscularly (IM) or intradermally (ID) with electroporation (EP) on Study Days 1, 15, 29, and 57, and to evaluate toxicity and reversibility of effects after a four-week recovery period.

MATERIALS AND METHODS

Animal Welfare: Prior to the initiation of experimentation, the animal protocol was reviewed and approved by the IIT Research Institute Animal Care and Use Committee. This study was performed in full compliance with NIH Guidelines for the Care and Use of Laboratory Animals.

Animals: New Zealand White (NZW) rabbits (62/sex) were obtained from Covance Research Products (Greenfield, IN). The rabbits were approximately 20 to 21 weeks old and weighed 2.38 to 3.37 kg at dosing initiation. 60 rabbits/sex were randomized into the study.

Animal Husbandry: Rabbits were individually housed in stainless steel cages suspended over excrement pans lined with disposable polypads. Rabbits were fed certified rabbit diet *ad libitum*, except during designated periods of fasting for clinical pathology and necropsy. City of Chicago water was supplied *ad libitum* by an automatic watering system.

Test Articles: Hantaan virus (HTNV) DNA vaccine, pWRG/HTN-M(co) and Puumala virus (PUUV) DNA vaccine, pWRT/PUU-M(s2)

Control Article: Saline

Table 1. Experimental Design

Group	Treatment [a]	Route	Target Dose (mg)	Dose Volume (mL)	No. of Rabbits (M + F) Sacrificed on	
					Day 59 [b]	Day 85 [c]
1	Control (saline)	IM & ID (no EP)	0.0	1 x 1.0 (IM); 2 x 0.2 (ID)	5 + 5	5 + 5
2	HTNV	IM-EP	3.0	1 x 1.0	5 + 5	5 + 5
3	HTNV	ID-EP	1.2	2 x 0.2	5 + 5	5 + 5
4	HTNV + PUUV	IM-EP	6.0 [d]	1 x 1.0	5 + 5	5 + 5
5	HTNV + PUUV	ID-EP	2.4 [e]	2 x 0.2	5 + 5	5 + 5
6	PUUV	ID-EP	1.2	2 x 0.2	5 + 5	5 + 5

[a]Rabbits were administered the test and control articles on Days 1, 15, 29, and 57.
[b]Five rabbits/sex/group to be sacrificed on Day 59 (2 days after the last dose).
[c]Five rabbits/sex/group to be sacrificed on Day 85 (28 days after the last dose).
[d]Total target dose of 6.0 mg, with 3.0 mg of HTNV and 3.0 mg of PUUV.
[e]Total target dose of 2.4 mg, with 1.2 mg of HTNV and 1.2 mg of PUUV.

Administration

The TriGrid™ Delivery System (TDS) electroporation device (Ichor Medical Systems, Inc., San Diego, CA), plus the appropriate TDS IM or TDS ID probe was used to administer the vaccines either IM or ID with EP, respectively. Animals were anesthetized with ketamine (~30-38 mg/kg) + xylazine (~5 mg/kg) IM prior to dosing. For IM dosing, the injection site alternated between hind limbs with the left hind limb receiving the first dose. Both hind limbs were the sites for ID dosing (0.2 mL/hind limb).

Evaluations and Observations

Mortality/Moribundity Observations: Twice daily

Body Weights: Prior to dosing and at least once weekly throughout in-life study

Clinical Observations / Physical Examinations: Once daily / Once weekly

Food Consumption: Daily

Body Temperature: Prior to dosing and at approximately 6, 24, and 48 hours post-dose

Injection Site Reactogenicity (Draize scoring): Prior to dosing, one hour post-dose, and once daily for the next six days after each administration for erythema and edema.

Ophthalmic (Indirect funduscopic) Examinations: Pre-test and prior to the Day 59 necropsy

Clinical Pathology (Hematology, Clinical Chemistry, Coagulation): Standard battery of clinical pathology endpoints performed on blood samples collected pre-test and on Days 2, 4, 58, and 84

Pseudovirion Neutralization Assay (PsVNA; Immunoassay Analysis): HTNV and PUUV neutralizing antibody titers were measured in blood samples collected pre-test and on Day 57. The PsVNA measures the capacity of antibodies to prevent the entry of HTNV and PUUV pseudovirions into host cells. PsVNA50 and PsVNA80 titers were determined which are defined as the highest serum dilutions causing 50% (PsVNA50) and 80% (PsVNA80) inhibition of virus cell entry.

Gross Pathology and Organ Weights: Complete necropsy with tissue collection (approximately 50 tissues) on Days 59 and 85. The adrenals, brain, epididymides, gallbladder, heart, kidneys, liver, lungs, ovaries, spleen, testes, thymus, thyroids/parathyroids, and uterus were weighed at necropsy.

Microscopic Pathology: The following tissues were evaluated microscopically from animals euthanized on Day 59: adrenals, bone (femur with marrow), brain, kidneys, liver, lungs, lymph nodes (mandibular, mesenteric, inguinal, axillary), ovaries, skeletal muscle (injection site), skin (injection site), spleen, testes, and thymus. Target tissues (injection sites) were evaluated microscopically in the animals euthanized on Day 85.

Statistical Evaluations

Continuous data were compared via analysis of variance with *post-hoc* comparisons made using Dunnett's test; nonparametric data were compared via Kruskal-Wallis analysis of variance with *post hoc* comparisons using Dunn's test. Incidence data were evaluated using Chi-square analysis and/or Fisher's Exact test. A minimum significant level of $p < 0.05$ was used in these comparisons.

For the immunogenicity data, data are presented for groups as geometric means and lower and upper 95% confidence intervals (CI). Differences between groups were analyzed using ANOVA; $p < 0.05$ was considered significant.

RESULTS

Injection Site Reactogenicity (Draize Scoring): There was a higher frequency of positive reactogenicity scores (scores of 1 to 4) for erythema and edema formation in both sexes in the ID-treated vaccine groups (Table 2). Positive scores of 1 and 2 were significantly increased in incidence in male ID-treated vaccine groups (Groups 3, 5, and 6), and a score of 1 was significantly increased in incidence in female ID-treated groups (Groups 3, 5, and 6). These findings were considered to be related to treatment (most likely related to ID electroporation dosing device and not the vaccines). These findings tended to resolve within two weeks after the last dose; all animals in these groups were noted to be normal (scores of 0 for both erythema and edema) at the end of the recovery period. Due to the reversibility of these effects, the toxicological significance was considered to be minimal.

For the IM-treated vaccine groups (Groups 2 and 4), positive scores of reactogenicity were also seen; however, these positive findings often resolved shortly after dosing, and most animals were normal at their scheduled sacrifice time point. As the findings were mostly similar in incidence to the control group ($p > 0.05$) and the reactogenicity was reversible following cessation of dosing, this was considered to be of minimal toxicological significance.

Table 2. Injection Site Reactogenicity Scores (Draize Scoring)*

	Edema Scores					Erythema Scores				
Male Group	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
1 (control)	10/10	1/10	0/10	0/10	0/10	10/10	1/10	3/10	1/10	1/10
2 (HTNV IM-EP)	10/10	0/10	0/10	0/10	0/10	10/10	5/10	1/10	1/10	2/10
3 (HTNV ID-EP)	10/10	**10/10[b]**	**8/10[b]**	1/10	0/10	10/10	**10/10[b]**	**10/10[a]**	7/10	3/10
4 (HTNV+PUUV IM-EP)	10/10	0/10	0/10	0/10	0/10	10/10	3/10	2/10	1/10	0/10
5 (HTNV+PUUV ID-EP)	10/10	**9/10[b]**	**9/10[b]**	3/10	2/10	10/10	**10/10[b]**	**10/10[a]**	7/10	2/10
6 (PUUV ID-EP	10/10	**8/10[a]**	5/10	0/10	0/10	10/10	**10/10[b]**	**10/10[a]**	6/10	3/10
Female Group	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
1 (control)	10/10	0/10	0/10	0/10	0/10	10/10	3/10	0/10	1/10	1/10
2 (HTNV IM-EP)	10/10	1/10	1/10	0/10	0/10	10/10	3/10	0/10	1/10	0/10
3 (HTNV ID-EP)	10/10	**7/10[a]**	4/10	0/10	0/10	10/10	**10/10[a]**	5/10	0/10	1/10
4 (HTNV+PUUV IM-EP)	10/10	0/10	0/10	0/10	0/10	10/10	3/10	0/10	2/10	0/10
5 (HTNV+PUUV ID-EP)	10/10	**8/10[b]**	**7/10[a]**	1/10	0/10	10/10	**10/10[a]**	5/10	2/10	2/10
6 (PUUV ID-EP	10/10	**9/10[b]**	4/10	0/10	0/10	10/10	**10/10[a]**	6/10	1/10	2/10

*Data represent the number of animals for which the reactogenicity score (of 0 to 4) was observed at any point during the given study period.
[a] $p < 0.05$; [b] $p < 0.01$

Definitions of Injection Reactogenicity Scores:
0 = no edema or erythema (normal);
1 = very slight edema (barely perceptible), very slight erythema (barely perceptible);
2 = slight edema (raised edges), well-defined erythema;
3 = moderate edema (raised ~1 mm), moderate to severe erythema;
4 = severe edema (raised > 1 mm and extending beyond area of exposure), severe erythema (beet redness)

Clinical Observations/Physical Examinations: During the weekly physical examinations, transient findings of either scab or swelling on the hind limbs were noted in the ID-treated vaccine groups during the treatment period but resolved shortly after being noted; all animals were noted to be normal at their scheduled sacrifice time point. These were not toxicologically significant findings.

Body Weights, Food Consumption, and Body Temperature: Administration of the vaccines had no effects on mean body weights, mean body weight changes, mean food consumed, and mean body temperatures when compared to the controls.

Mortality: 1 control male died just prior to dosing likely due to the administration of the anesthesia mixture. All other animals survived to their scheduled sacrifice time point (Day 59 or 85).

Ophthalmology: No ophthalmic findings were noted.

Clinical Pathology (Hematology, Clinical Chemistry, Coagulation): No statistically significant, treatment-related, or toxicologically significant findings were noted for any clinical pathology endpoint evaluation performed on samples collected on Days 2, 4, 58, and 84.

Immunoassay Analyses: The vaccines were biologically active and immunogenic in this study. All rabbits that were dosed by either the IM or ID route with the HTNV vaccine (Groups 2 and 3), the PUUV vaccine (Group 6), or the HTNV + PUUV vaccines (Groups 4 and 5) developed neutralizing antibodies (Table 3 and Figure 1) against the respective viruses, as tested in the PsVNA performed at USAMRIID. No gender differences in viral titers were seen.

Table 3. Viral Titers

Titer	Results
HTNV Titers	Generally no differences noted in anti-HTNV titers when the HTNV vaccine was dosed as a single vaccine by either route (Groups 2 and 3)
	When co-administered with the PUUV vaccine (Groups 4 and 5), anti-HTNV titers were comparable when dosed by either route, but the titers were lower compared to the titers in the HTNV vaccine alone groups (Groups 2 and 3)
PUUV Titers	Highest anti-PUUV titers were seen when the PUUV vaccine was administered ID alone (Group 6)
	When co-administered with the HTNV vaccine (Groups 4 and 5), comparable anti-PUUV titers were seen regardless of dosing route

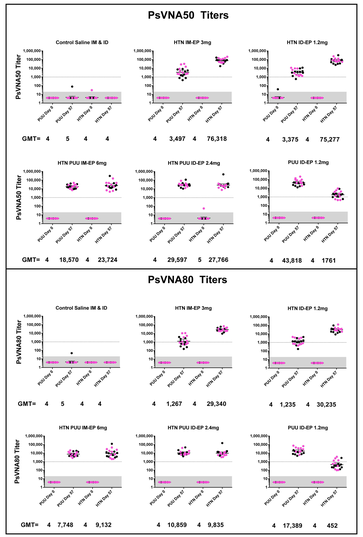

Figure 1. Day 0 (Pre-test); Day 57 PsVNA50 and PsVNA80 titers for HTNV and PUUV for each group.

Anti-HTNV and PUUV PsVNA50 titers (upper graphs) and PsVNA80 titers (lower graphs) for each vaccine group are shown (log_{10} scale). Symbols are color-coded by gender: males are black, females are magenta. Geometric mean titers (GMT) and 95% CI are shown, and the numeric GMT is presented below the graph. The limit of quantitation is 20 (grey shading). Samples with titers <20 were given a value of 14.1. The dashed line at 1,000 is an arbitrary reference point to aid in visualizing data.

Gross Pathology and Organ Weights: For the control male that died on Day 57 (prior to dosing), no gross or microscopic findings were seen to explain this death – as indicated above, it is believed this animal died as a result of the anesthesia. No test article-related gross findings were seen at scheduled necropsies and there were no toxicologically significant differences for any of the organs measured at necropsy compared to the controls.

Microscopic Pathology: Microscopic findings were seen at the left and right injection sites. On Day 59 (2 days after the last dose; Table 4), the following microscopic lesions were noted:

- Serocellular crusts in the skin
- Mixed cell infiltration in the skin, subcutaneous tissue, and/or muscle
- Ulceration of the skin
- Necrosis of the muscle and/or skin
- Muscle degeneration

These findings were often seen in both the right and left injection sites and were generally seen in most or all of the vaccine treated groups regardless of route and/or vaccine type(s) administered.

Some findings (mixed cell infiltration in skin, subcutaneous tissue, and/or muscle) were still present at the end of the recovery period (on Day 85; Table 5); however, these findings were generally less in incidence and/or severity. A new finding of muscle fibrosis was also seen at the right injection site in three animals (1 male and 1 female in Group 4 and 1 female in Group 6) and at the left injection site in one animal (1 female in Group 6) during this time point; fibrosis was an expected response to muscle damage.

Table 4. Summary of Microscopic Findings, Day 59

	Males						Females					
Group	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
No. Animals Examined	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Right Injection Site (No. Examined)	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Skin:												
Serocellular crust	0	2	1	0	2	0	0	4	2	1	1	0
Mixed cell infiltration	0	5	5	4	5	5	0	2	3	0	4	5
Ulceration	0	2	1	0	2	3	0	0	1	1	2	1
Subcutaneous tissue, mixed cell infiltration	0	3	3	4	5	5	3	2	3	1	4	3
Muscle:												
Mixed cell infiltration	1	4	3	4	4	2	3	3	3	4	1	3
Necrosis	1	4	2	1	2	2	3	3	1	2	1	2
Left Injection Site (No. Examined)	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Skin:												
Serocellular crust	1	0	0	0	1	1	0	0	0	0	3	1
Mixed cell infiltration	0	0	2	1	4	4	1	0	4	0	5	4
Necrosis	0	0	1	1	0	1	0	0	1	0	1	4
Ulceration	0	0	0	1	2	1	0	0	0	0	2	1
Subcutaneous tissue, mixed cell infiltration	0	0	5	1	5	5	0	0	4	0	4	5
Muscle:												
Mixed cell infiltration	1	0	5	1	5	3	5	1	2	0	3	5
Necrosis	3	0	4	0	4	2	5	0	3	0	2	5
Degeneration	3	2	3	0	5	2	1	0	3	0	3	1

Table 5. Summary of Microscopic Findings, Day 85

	Males						Females					
Group	**1**	**2**	**3**	**4**	**5**	**6**	**1**	**2**	**3**	**4**	**5**	**6**
No. Animals Examined	**4**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Right Injection Site (No. Examined)	**4**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Skin, mixed cell infiltration	0	0	0	0	0	1	0	1	0	2	0	3
Subcutaneous tissue, mixed cell infiltration	0	0	1	2	3	1	1	2	2	1	1	1
Muscle:												
Mixed cell infiltration	0	1	0	2	1	0	0	1	3	2	2	2
Fibrosis	0	0	0	1	0	0	0	0	0	0	1	0
Left Injection Site (No. Examined)	**4**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Skin, mixed cell infiltration	0	1	0	0	1	1	0	0	0	2	0	1
Subcutaneous tissue, mixed cell infiltration	0	0	4	1	4	3	0	1	0	1	2	2
Muscle:												
Mixed cell infiltration	0	1	0	2	1	0	0	1	0	1	2	2
Fibrosis	0	0	0	0	0	0	0	0	0	0	0	1

CONCLUSIONS

1. Treatment with the HTNV vaccine, PUUV vaccine, or HTNV + PUUV vaccines administered with electroporation either IM or ID with four doses was well-tolerated as there were no treatment-related or toxicologically significant effects seen for clinical observations, physical examinations, body weights, body weight changes, food consumption, body temperatures, ophthalmology, clinical pathology, organ weights, and gross pathology.
2. A higher frequency of positive injection site reactogenicity scores (of 1 to 4) for erythema and edema was noted in both the IM and ID vaccine-treated groups. These findings were likely attributed to the electroporation dosing device and not to treatment with the vaccines and were reversible. Therefore, the increased incidence in positive injection site reactogenicity scores was considered to be of minimal toxicological significance.
3. The vaccines, either administered alone or in combination, were biologically active and immunogenic. Vaccine administration resulted in the development of neutralizing antibodies against the respective HTNV or PUUV virus as measured by pseudovirion neutralization assay in 100% of the animals that were vaccinated either IM or ID with HTNV, PUUV, or HTNV + PUUV DNA vaccines. This finding indicated that the doses were adequately administered on the study, as well as being highly immunogenic to the rabbits.
4. Microscopic findings were noted at the injection sites (both left and right) on Days 59 and 85. These findings consisted of serocellular crust on the skin, mixed cell infiltrations (in the skin, subcutaneous tissue, and/or muscle), skin ulceration, necrosis (of skin and/or muscle), muscle degeneration, and/or muscle fibrosis. There was complete, partial, or no recovery of these lesions at the end of the recovery period. No other treatment-related microscopic lesions were noted.
5. The histopathologic findings at the injection sites were considered to be typical responses seen following either IM or ID administration with electroporation – none of these lesions resulted in any limiting toxicity.

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