GENE GUN-DELIVERED DNA VACCINES FOR HEMORRHAGIC FEVER WITH RENAL SYNDROME: ADVANCEMENT TO CLINICAL TRIALS

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ABSTRACT

We developed a DNA vaccine to protect against hemorrhagic fever with renal syndrome (HFRS). The vaccine consists of two plasmid DNAs that produce immunogenic proteins of two of the four etiologic agents of HFRS, Hantaan, and Puumala viruses. The DNA is precipitated onto micrometer-sized gold beads and delivered to the skin of animals or human volunteers using a hand-held disposable "gene gun." We showed that these DNA vaccines elicited high levels of antiviral neutralizing antibodies and protected animals from challenge with all four HFRS-causing viruses. We assessed the safety of the gene gun-delivered DNAs in animals using Good Laboratory Practices (GLP). Rigorous statistical analyses indicated that there were no significant differences between vaccinated and control animals. To demonstrate potency and stability of the vaccines, we developed a rapid, reliable, and sensitive flow-cytometric assay. The assay was adapted to fit within a standard operating procedure (SOP) for regulation under GLP guidelines. Safety and potency results were submitted to the U.S. Food and Drug Administration and after their review, a Phase 1 clinical study in 27 volunteers was initiated. This DNA-gene gun vaccine study is the first of its kind to be performed by the U.S. military, and if successful, will introduce a technology that could be applied for developing vaccines for many endemic diseases or bioterrorism pathogens that threaten troops.

1. INTRODUCTION

Hantaviruses are RNA viruses belonging to the family *Bunyaviridae*, and are the etiologic agents of hemorrhagic fever with renal syndrome (HFRS) in the Old World and hantavirus pulmonary syndrome in the New World. The viruses are carried by persistently infected rodents and are found worldwide. There are no licensed vaccines for hantaviruses; thus, they continue to threaten military personnel who come into contact with rodents during field operations or training. Because hantaviruses replicate poorly in standard culture systems, and because they must be handled under high containment, traditional vaccine development methods are dangerous, expensive, and inefficient. To overcome these obstacles, we developed a DNA vaccine to protect against HFRS.

The ability of DNA plasmids encoding antigenic proteins to induce immune responses *in vivo* was first reported in the early 1990s (Tang et al. 1992). After almost two decades of active research, DNA vaccines are just entering the commercial market, with three veterinary vaccines recently approved for preventing West Nile virus in horses (Powell 2004), infectious hematopoietic necrosis virus in salmon (Lorenzen; LaPatra 2005), and melanoma in dogs (Bergman et al. 2006). Human vaccines have been slower to gain acceptance, in part due to the poor immunogenicity of the vaccines when they are delivered by needle injection into muscles. Newer delivery methods are now available, which render the vaccines much more immunogenic. One such method is particle-mediated epidermal delivery (PMED) also known as "gene gun" delivery. This method involves the use of a needle-free device that delivers gold particles coated with DNA plasmids encoding vaccine antigens into the epidermal layer of the skin. PMED differs from intramuscular or intradermal injection of DNA with a needle and syringe in that it results in direct delivery of the vaccine into the intracellular environment rather than into extracellular spaces. With PMED, the DNA is delivered into both non-professional antigen-presenting cells (APCs) (i.e., keratinocytes) and professional APCs (i.e., Langerhans cells) of the viable epidermis (reviewed in (Fuller et al. 2006). Because of the efficiency of this delivery, only very small amounts of DNA are required to generate a strong immune response.

A recent advancement in PMED technology is the production of hand-held disposable devices that contain the DNA-coated gold in plastic cassettes with membranes on both sides (Fig. 1). The devices also contain a small helium canister. When the device is actuated, the helium is released through the membranes, propelling the gold out of the tip of the device and into the skin of the vaccine recipient (Fig. 2).

To produce a DNA vaccine for HFRS, we engineered plasmids containing the genes of two hantaviruses, Hantaan

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
DEC 2008	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Gene Gun-Delivered Dna Vaccines For Hemorrhagic Fever With Renal Syndrome: Advancement To Clinical Trials	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Medical Research Institute of Infectious Diseases Fort Detrick, MD 21702**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
See also ADM002187. Proceedings of the Army Science Conference (26th) Held in Orlando, Florida on 1-4 December 2008, The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**7**	

(HTNV) and Puumala (PUUV) viruses. We showed that these vaccines could protect animals from challenge with all four hantaviruses known to cause HFRS (Hooper et al. 2001; Spik et al. 2006). To advance the HFRS DNA vaccine to the clinic, it was necessary to perform extensive safety testing and to develop assays that would reliably measure their potency and stability. Here we report the results of those studies, which have led to the initiation of a Phase 1 clinical assessment.

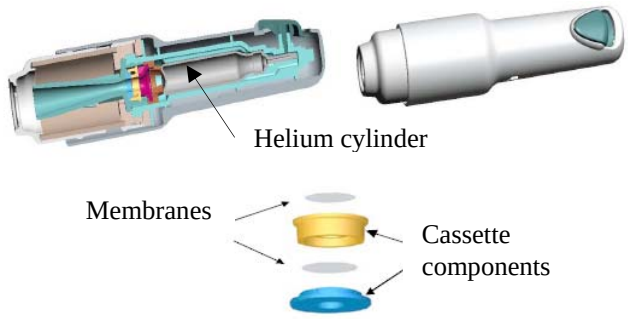

Fig. 1. Particle-Mediated Epidermal Delivery (PMED) device. A molded plastic casing surrounds a rigid cassette with membranes on each side. The cassette is filled with the DNA-coated gold and placed in the device. The device also contains a small helium cylinder, which is connected to an actuation pin. Upon actuation, the helium is released, ruptures the membranes, and propels the gold coated with the DNA vaccine out of the device and deposits the DNA in the epidermis of the vaccine recipient.

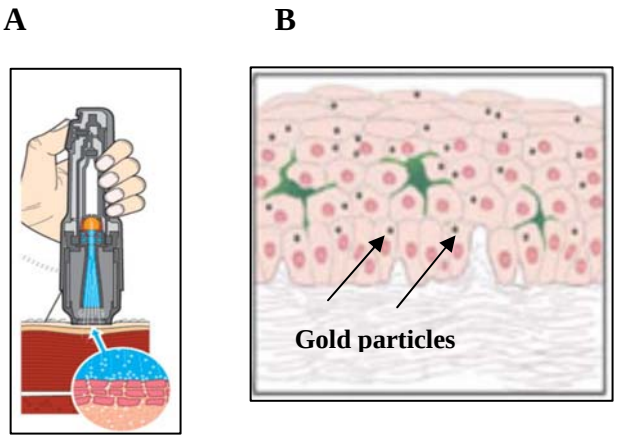

Fig. 2. PMED vaccination. The hand-held disposable device, which is filled with the HFRS DNA vaccine coated onto gold, is placed firmly against the skin of the vaccine recipient and is actuated (A). Gold beads are delivered to the cells of the epidermis (B) where the DNA elutes and is transcribed and translated by the host cells' machinery to yield immunogenic hantavirus proteins.

2. METHODS

2.1. Preclinical Safety Studies in Hamsters

Two preclinical safety studies of the hantavirus DNA vaccines delivered to Syrian hamsters (*Mesocricetus auratus*) by PMED were conducted at Charles River Laboratories, Redfield, Arkansas. Both studies were performed in compliance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations (21 Code of Federal Regulations [CFR] 58). Research was performed in compliance with the Animal Welfare Act and other federal statues and regulations relating to animals and experiments involving animals and adheres to principles stated in the guide for the Care and Use of Laboratory Animals. For each study, equal numbers of males and females were randomly assigned to groups by a computer-generated, weight-ordered method such that group mean body weights did not exceed ± 20% of the overall mean weight for each sex. The study design included three phases of sample and data collection. The first phase consisted of an in-life stage in which the systemic response to treatment and local skin reactivity was evaluated, and samples for clinical chemistry, hematology, and serology were collected. In the second phase, euthanized animals were subjected to full necropsy during which protocol-specified organs were examined, weighed, and sampled for histopathology. In the third and phase, clinical chemistry analyses, hematological analyses, serological testing, and histopathology of selected tissues were completed. With the exception of the serology (immunogenicity assessment) component, all stages of the studies were performed under GLP regulations. All testing of sera for HTNV and PUUV antibodies was performed using standard operating procedures (SOPs) and qualified methodology.

2.2 Quantitative and Qualitative Assays of Biologically Active DNA delivered by the PMED Devices

The quantitative portion of the potency test measured expression of the hantavirus genes. For this, a PMED device was actuated into 0.5 ml of ethanol that had been placed in a single well of a 12-well cell culture plate. The DNA remained attached to the gold in this non-aqueous environment. To ensure that all of the expelled gold was captured, a neoprene washer was centered over the well before discharge (Fig. 3). Contents of the well were transferred to a micro-centrifuge tube and the DNA-coated gold was recovered by centrifugation. The DNA was then eluted by adding 20 µl of buffer. The gold was removed by centrifugation and the supernatant containing the DNA was recovered for assay. Dilutions of a reference plasmid DNA were prepared to construct a standard curve against which the eluted DNA was measured. The eluted hantavirus DNA vaccine and the reference DNA were introduced into mammalian cells by

using a commercial liposomal transfection reagent (FuGene).

The qualitative portion of the potency test sought to determine whether active plasmid DNA could be recovered after discharging the PMED device directly into cultured mammalian cells. Post-discharge, the plate was rocked gently back and forth to distribute the gold particles evenly. The transfected cells from both the quantitative and qualitative assays were incubated at 37° C with 5% CO_2 for ~44 hours before processing for flow cytometry.

2.3. Preparation of Cells and FACS Analysis of the Hantavirus DNA Vaccines

Transfected cells were proteolytically detached from the plates by adding 100 µl per well of trypsin-EDTA and allowed to incubate at room temperature for ~15 minutes until cells began to "round up" and separate. Detached cells were recovered by centrifuging and were fixed by adding buffer containing 4% w/v paraformaldehyde. After washing, cells were permeabilized by using a saponin-based buffer to allow cellular access to the primary and secondary antibodies that were used to detect the hantaviral proteins. After washing, cells were probed with a 1:100 dilution of a HTNV-specific mouse monoclonal antibody. After 30-minute incubation, excess antibody was removed by washing, and then a secondary anti-mouse antibody tagged with fluorescein isothiocyanate (FITC) was added. Excess antibody was removed by washing; cells were then suspended in buffer and kept on ice until analysis by flow cytometry (Fig. 3A).

2.4 Flow Cytometry

Stained cells were analyzed on the four-color, dual-laser, bench-top FACSCalibur (Becton Dickenson) flow cytometer equipped with a filter setting for FITC (530 nm, Fl-1 channel) according to standard procedures. Before detecting intracellular viral protein expression, permeablized cells transfected with the empty plasmid vector were used to establish forward and side scatter parameters. To ensure standardized conditions, FITC-fluorescent polystyrene beads were used to calibrate the flow cytometer before each experiment. Cells staining positive for intracellular viral proteins were shown as a percentage of total cells enumerated (10,000). Histograms and dot plots were produced using Stanford University's FlowJo flow cytometry analysis software (Fig. 3 B, C).

2.5 Data Analysis

The quantitative amount of plasmid DNA that results in protein expression from test samples was calculated from the standard curve using GraphPad Prism 4 Software: A four-parameter logistic (4PL) equation: y = (a-d)/(1+ (x/c)b) + d was used for curve fit (where y was the expected response; x, concentration; a, response at zero concentration; d, response at infinite concentration; c, concentration resulting in a response halfway between a and d (EC50); b, slope parameter).

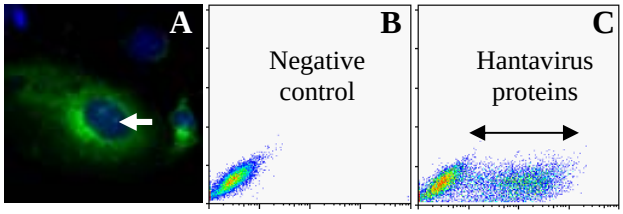

Fig. 3. Fluorescent antibody detection of viral proteins produced by the DNA vaccines. Cells were probed with a hantavirus-specific mouse monoclonal antibody and a FITC-tagged anti-mouse antibody (A, green staining). A gold bead is visible in the nucleus of the cell (A, arrow). A flow cytometer was used for detecting the cells containing the fluorescent proteins. Scatter plots were generated to provide a graphic view of the amount of fluorescent cells transfected with a negative control plasmid DNA (B) or the DNA vaccine plasmid (C).

3. RESULTS

3.1 Preclinical Toxicological Safety Studies

The general safety and immunogenicity of multiple administrations of the HTNV and PUUV DNA vaccines were tested in GLP-compliant animal studies. After consultation with the U.S. FDA, hamsters were chosen for these studies because they are used routinely for infectivity and vaccine protection determinations, thus safety could be assessed with an immunologically relevant animal model.

The initial safety study was performed before the hand-held disposable devices had been fully developed; therefore, this study was performed with a laboratory-based device connected to a helium tank and electrical outlet (Eisenbraun et al. 1993; Pertmer et al. 1995). The second study was performed with the PMED disposable devices. For both studies 10 male and 10 female hamsters were assigned to each group. In the first study, animals in a vaccine group were given 1 µg of HTNV DNA vaccine/0.5 mg gold at six skin sites on days 1, 22, 43, 64. A control group received a placebo vaccine consisting of 0.5 µg of gold with no DNA.

For the second study, two vaccine groups were assessed. Both groups received 2 µg of DNA/1 mg gold at four skin sites per dose at days 1, 22, 43, 64. One group received only the PUUV DNA vaccine and the other group received both the HTNV and PUUV DNA vaccines (two administrations of each DNA per animal

per dose). A third group received a placebo vaccine consisting of 1 mg of gold without DNA.

In both studies half of the animals in each group were necropsied on day 66 and half on day 92 and tissues and organs were collected for pathology testing. General safety observations and analyses were performed on all animals and included (1) physical and ophthalmologic examinations (pre-study and before necropsy); (2) clinical observations (including twice daily mortality and moribundity checks); (3) food intake (recorded but not reported); (4) body weights; (5) hematology; (6) clinical chemistry; (7) dermal scoring (modified Draize evaluation) at each site of vaccine administration at each dosing interval (within 1 hour of dosing and at post-dose days 2, 4, and 7, and then weekly thereafter until site resolution); (8) macroscopic pathology observations of major organs at necropsy; (9) organ weight; (10) histopathology of skin tissue taken at necropsy from sites of vaccine administration; and (11) histopathology of all tissues collected at necropsy.

Skin effects after vaccination, which typically included erythema and edema, were rapid in onset (i.e., within 3 days), transient, and resolved completely without adverse biological sequelae. No meaningful or biologically significant differences were observed between placebo and vaccine-treated groups for clinical observations, body weight or body weight gain, hematology, clinical chemistry, or gross or microscopic anatomic pathology.

Immunogenicity was measured by incubating sera from the vaccinated hamsters with infectious HTNV or PUUV and determining the amount of viral inactivation by a plaque reduction neutralization test (PRNT) in cultured cells. This method is similar to one reported earlier (Chu et al. 1995), but was adapted to a SOP format for regulatory compliance. Analysis of sera collected from hamsters at day 85 of the first study showed that 90% of them developed neutralizing antibodies to HTNV. Analysis of sera collected in the second study from hamsters vaccinated three (day 57 sera) or four times (day 78 sera) with the PUUV DNA vaccine or with both the HTNV and PUUV DNA vaccines showed that between 70-90% of them developed neutralizing antibodies to PUUV. Hamsters vaccinated four times with both of the DNA vaccines also produced HTNV-specific neutralizing antibodies (sera collected after three vaccinations were not tested for HTNV-specific neutralizing antibodies). Additional PRNTs and a more exhaustive evaluation of the immunogenicity data are in progress.

These data indicate that the DNA vaccines used were immunologically active. Based on the toxicology and the immunogenicity findings from these two GLP studies, we concluded that the DNA vaccines were safe and immunogenic in this preclinical animal model. Consequently, these data were submitted to the U.S. FDA as part of an Investigational New Drug application for human testing.

3.2 Development of Potency and Stability Assays

In order to provide reliable measures of potency and stability of the DNA vaccines before initiating clinical studies, we developed both quantitative and qualitative flow-cytometric assays. These assays were designed to detect biologically active DNA delivered by the PMED devices. For qualitative analysis, we introduced the DNA-coated gold directly into cultured cells by actuating the gene gun devices above plated cells (Fig. 4).

Fig. 4. Recovery and detection of the DNA vaccines contained in the PMED devices. The HTNV and PUUV DNA vaccines were manufactured in single use hand-held disposable PMED devices and packaged in sealed pouches containing a desiccant (A). For potency and stability tests, a PMED device was discharged into one well of a 12-well plastic plate containing either ethanol (for the quantitative assay) or into mammalian cells (for the qualitative assay). To ensure complete delivery, a neoprene washer was placed over the well before activation of the device (B). DNA was recovered and expression assessed.

For the quantitative assay, we eluted the DNA from the gold before transfecting it into cell cultures. For both assays, we enzymatically removed the DNA vaccine-containing cells from the plastic culture vessels, fixed, and permeabilized them, then probed them with a virus-specific mouse monoclonal antibody followed by a fluorescent anti-mouse antibody. Flow cytometry was used to detect expression and measure the amount of DNA recovered. The amount of plasmid DNA that resulted in expression to yield hantaviral proteins was calculated by comparing flow cytometry measurements of test samples to standard curves that were generated from flow cytometer readings taken on cells transfected with reference standard DNAs. Standard curve readings obtained at different times were highly reproducible, and signal to noise ratios were low (Fig. 5). This method was adapted to fit within a standard operating procedure (SOP) format for regulation under GLP guidelines.

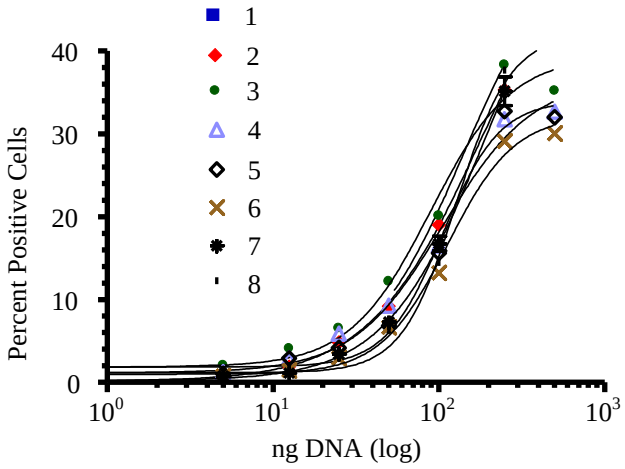

Fig.5. Standard curves from eight separate experiments. Overlay of multiple standard curves demonstrates the reproducibility of this method.

3.3 Potency and Stability of the DNA Vaccines

The purpose of the *in vitro* potency and stability tests was to determine if the HFRS DNA vaccines formulated in PMED devices retained the ability to generate immunogenic proteins at various times after manufacture. Both the amount of DNA delivered by the PMED device and the expression of the hantavirus genes were measured. We assessed potency and stability of the DNA vaccines just after manufacture, 1-month later and thereafter at 3-month intervals. A histogram was used to define positive cells containing proteins produced by the hantavirus DNA vaccine plasmids relative to cells that were transfected with a negative control plasmid (Fig. 6). Statistics were derived from 10,000 events collected with the flow cytometer and are noted as the percent positive subset.

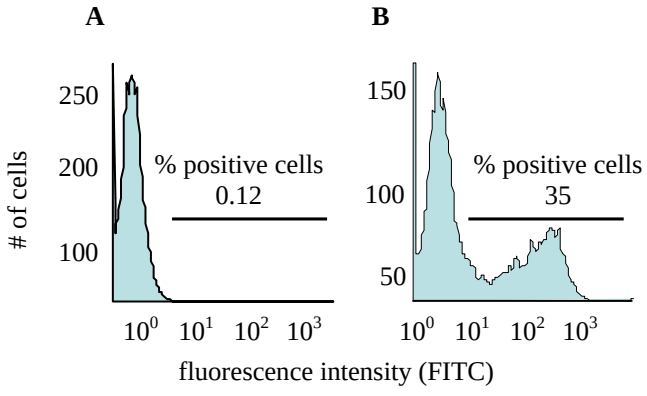

Fig. 6. Generation of positive cell subsets. Histogram depiction of flow cytometer results from control plasmid (A) and hantavirus DNA vaccine plasmid (B) analysis. Statistics were derived from 10,000 events collected and noted as the percent positive subset.

To date, the vaccine and devices have been shown to be both potent and stable for at least 9 months after manufacture (Fig. 7). They will continue to be assessed until the human clinical study has concluded.

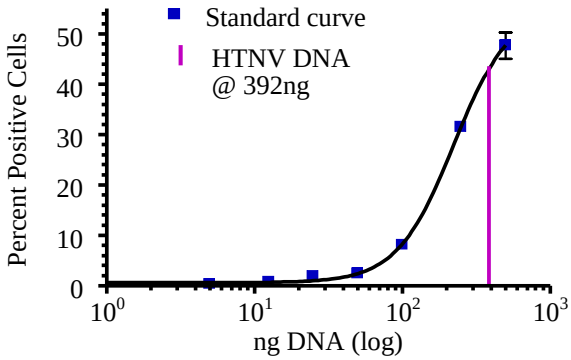

Fig. 7. Quantitative assay example. Comparison to the reference standard indicated that 392 ng of HTNV DNA was expelled from a PMED device**.**

3.4 Design and Initiation of a Phase 1 Clinical Study of the HFRS DNA Vaccine

The Phase 1 clinical study of the HFRS DNA vaccine is an open-label, single-center study with three vaccination groups. The study was designed to include 27 active participants. Vaccination and monitoring are being performed at the USAMRIID Clinical Research Unit, Fort Detrick. The study initiated in August, 2008 and is currently ongoing.

Study subjects are being enrolled without regard to gender or race and are assigned to groups in the order in which they sign a consent form and complete the required screening procedures. All subjects receive four, single, sequential administrations (2 µg DNA/1 mg gold) of the DNA vaccine on each day of the dosing schedule (days 0, 28, and 56). The total dose per vaccination is 8 µg DNA/4 mg gold. The four vaccine administrations are spaced approximately 1 cm apart on the inner surface of the upper right or left arm (Fig. 8). The total amount of vaccine administered over the course of the study will be 24 µg DNA/12 mg gold. Subjects in Groups 1a and 1b are being vaccinated with the HTNV DNA vaccine, subjects in Groups 2a and 2b with the PUUV DNA vaccine, and subjects in Groups 3a and 3b are being co-vaccinated with the HTNV and PUUV DNA vaccines.

To minimize risk to the volunteers, three subjects in each of Groups 1a and 2a were vaccinated first, with at least 1 hour between each subject. These subjects will be monitored for 14 days for the development of serious adverse events (SAEs) before the remaining six subjects in Groups 1b and 2b receive their vaccinations. After an additional 14 day monitoring period, the three subjects in Group 3a will be vaccinated. Assuming there are no vaccine-related SAEs observed within 14 days after the

first vaccination in Group 3a, the six subjects in Group 3b will be vaccinated.

Fig. 8. Vaccination of a volunteer with the HFRS DNA vaccine with a PMED device.

All subjects will be monitored for 4 months after receipt of their third dose of vaccine. The volunteers will also be invited to participate in a minimal risk study at 12 months after their first vaccination to evaluate the endurance of the immune response.

3.5 Study Endpoints

The primary purpose of the Phase 1 clinical study is to assess the safety of the HFRS DNA vaccine. A secondary purpose is to assess immunogenicity. Safety is measured through observation by medical staff and by self reporting on diary cards. Systemic reactions such as fever, headache, fatigue, and local reactions such as tenderness at the vaccination site, redness and pain will be recorded.

Immunogenicity will be assessed by measuring the development of virus-specific antibodies in the subjects' sera, which will be collected on days 28, 56, 84, 140, and 180 after vaccination. Antibody titers will be determined by using PRNT and those in excess of 1:20 will be considered as positive.

CONCLUSIONS

Hemorrhagic fever with renal (HFRS) was first recognized as a military threat when more than 3000 United Nations Forces developed renal failure associated with hemorrhagic fever during the Korean War. Since then HFRS has continued to threaten troops deployed to endemic disease regions. Four different hantaviruses can cause HFRS, and to date, there are no U.S. licensed vaccines to any of them. Here we report the advancement of a DNA vaccine for HFRS to clinical studies.

The vaccine that we developed consists of 2 plasmid DNAs expressing genes of HTNV and PUUV. We showed that the combination of these plasmids can induce neutralizing antibodies and can protect animals from infection with all four HFRS-causing hantaviruses.

To deliver the DNA vaccines, we precipitated the plasmids onto microscopic gold beads, which were placed into single use, hand-held, disposable, particle mediated epidermal delivery (PMED) devices, also known as "gene guns." With PMED delivery the DNA vaccines are deposited directly into intracellular compartments of skin cells, rather than into extracellular spaces, as occurs with methods using needle injection. This eliminates the need for the DNA to cross cell membranes, thus only small amounts of DNA are required to elicit strong immune responses.

Before we could test our DNA vaccines in humans, we needed to demonstrate that they were safe and effective in animals and we needed to develop assays that would guarantee that they remained potent during storage. To show safety and efficacy, we performed two GLP-compliant preclinical toxicology studies with the DNA vaccines. The results of these studies demonstrated that the HFRS DNA vaccines caused no serious adverse events in the recipient animals but that they did elicit neutralizing antibody responses. Therefore, the DNA vaccines were shown to be safe in an immunologically relevant animal model.

To ensure the potency and stability of the vaccines we developed sensitive and reliable quantitative and qualitative flow cytometric assays that measure the amount of biologically active DNA that is present in the devices. The assays were adapted for use under GLP regulatory guidelines. Using these assays, we have demonstrated potency and stability at numerous times after vaccine manufacture. Based on these findings, we submitted an IND application to the U.S. FDA to further test the DNA vaccines in human volunteers.

A Phase 1 Clinical Study was initiated in August, 2008 with 27 volunteers expected to receive three doses of the HTNV, PUUV, or both DNA vaccines. This study concludes almost a decade of research aimed at development of a gene-based vaccine for HFRS and is the first of its kind to be conducted by the U.S. military.

ACKNOWLEDGEMENTS

We are indebted to the Military Infectious Disease Research Program (MIDRP) for funding these studies. We gratefully acknowledge the support of our MIDRP liaison, Dr. Rudy Kuppers. We thank Dr. Ellen Boudreau for the enormous amount of work that she performed to obtain human use approval and that she will continue to perform as the Clinical Principal Investigator of the Phase 1 study. We thank Ms. Diane Ullman for regulatory advice, Ms. Julie Newhart for efficient project management, and Mr. Matt Josleyn for excellent technical assistance. Finally, we thank our many colleagues and collaborators at PowderMed Vaccines for their continued support of our vaccine development efforts.

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