

TEAM 3

THE SIN NOMBRE VIRUS IN HUMANS: GENE GUN THERAPY AND SUBUNIT VACCINE HANTAVIRUS PULMONARY SYNDROME: THE SIN NOMBRE VACCINE

Literature Review

Hantaviruses consist of one of the five genera of the family *Bunyaviridae*. There have been possible cases of hantavirus infection in China and England which date back to the first millennium and the Middle Ages. It was not until between 1951 and 1953 during the Korean War when hantavirus gained global attention: more than three thousand United States and United Nations soldiers acquired the illness, which was accompanied by acute renal failure and shock near a small river named Hantaan. The mortality rate of this illness was about 7%, and the infectious agent was finally identified in 1978 by H.W. Lee, who called it Hantaan virus. Today over 21 different species of Hantavirus have been found that infect certain areas of the globe. Hantaviruses can be roughly separated into two groups labeled Old World Hantavirus and New World Hantavirus, each causing a distinct illness in humans. Hemorrhagic fever with renal syndrome (HFRS) is caused by the Old World Hantaviruses, most notably the Amur, Seoul and the Hantaan strains, and affects almost 200,000 people a year with a mortality rate of about 15%. HFRS has an incubation period of about seven to 36 days and is characterized by systemic involvement of the venules and the capillaries and causes severe circulation disorders and various hemorrhagic manifestations. New World hantaviruses are associated with Hantavirus Pulmonary Syndrome (HPS) and the first strain of this to be discovered was the Sin Nombre strain in the early 1990s in the four corners region (Muranyi, Walter et al. 2005).

All members of the *Bunyaviridae* except Hantaviruses are transmitted by arthropod vectors. The Hantaviruses use rodents as their natural reservoir, and the virus is transmitted to humans via inhalation of aerosolized rodent excreta or, more rarely, by bites or scratches (Strauss, 2002). There are several different strains of Hantavirus that cause HPS, all of which are found in the New World. The strains found in the United States and Canada include Sin Nombre, New York, Bayou, and Black Creek Canal. Central and South America also contain several strains that cause HPS, more than in the US and Canada. There are Old World strains found in Europe and Asia, including Hantaan, Puumala, Seoul, and Dobrava, but they are not associated with HPS. Instead they cause HFRS, which is a much milder illness than HPS and has a much lower death rate.

Infection with Hantavirus can cause a mild, febrile illness or it can start as a febrile illness then progress to HPS. The symptoms of Hantavirus infection are initially fever, myalgia, cough, headache, nausea, and vomiting, and the time from infection to symptom onset is one to two weeks. In HPS, these symptoms are then followed by cough and shortness of breath, one to six weeks after infection. At this point, the disease quickly progresses to respiratory and cardiac failure. Laboratory findings associated with

Hantavirus infection include low platelet count, irregular immunoblasts, and elevated white blood cell count. The overall death rate associated with Hantavirus infection is 50%, but this number is higher if only HPS cases are considered (cdc.org).

Hantavirus is a negative sense, single stranded RNA virus (CDC). The virus is composed of an envelope and three linear RNA segments complexed with proteins to form the structural proteins of the virus. These segments are S (small-2,059 to 2,060 nucleotides), M (medium-3,696 nucleotides), and L (large-6,562 nucleotides). The small segment contains the gene for the nucleocapsid, the medium segment codes for a polyprotein that contains the envelope glycoproteins G1 and G2, and the large segment contains the L protein gene that codes for the viral transcriptase/replicase. The viral particle of hantavirus is a pleomorphic virion.

The different RNA segments in the viral particle form helical nucleocapsids with complementary interactions between the 5' and 3' terminal gene sequences of each segment. Virions attach to the host cell and fuse with the endosomal membrane, allowing for introduction of nucleocapsids to the host cell cytoplasm. It is also thought that the L protein cleaves cellular mRNA to aide in the transcription of viral mRNA. Heterodimers are formed by the glycoproteins and then transported to the Golgi complex for glycosylation. Virions are transported from the Golgi cisternae to the plasma membrane and leave the cell via exocytosis.

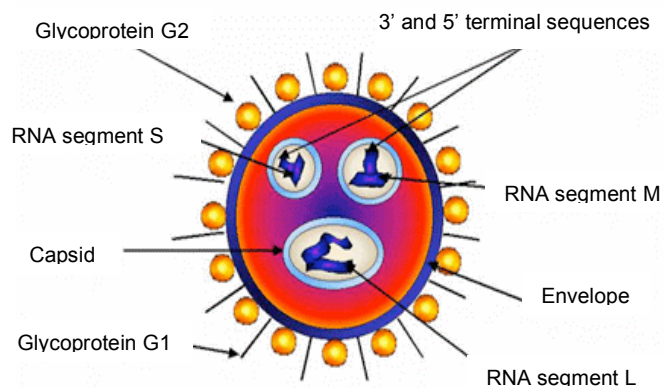


Figura 1
Estructura de los Hantavirus

Modificado de: Saz JV, Gegúndez MI, Beltran M. Hantavirus. Editorial Junta de Castilla y León. España, 1997.

As mentioned above, HPS is transmitted through the droppings, saliva or urine from its host, rodents. Other than a brief bout of viremia, the rodent host is not affected by the virus. After five to ten days, there is virus in all of the rodent's major organs, and the host begins to shed the virus through its excreta. There has been one case of human-to-human transmission with the Andes strain of HPS occurring in Argentina in 1996 (Padula, P.J. et al. 1997), but no other human-to-human transmission has been recorded for the other strains. This is perhaps due to when the patient dies, virus replication has already reached its peak, and the particles that are left behind are no longer infectious (Chizhikov, Vladimir et al. 1995). The outbreaks of HPS are usually seen in the spring and in the fall. The disease outbreaks in the spring can be tied to the increased rainfall and therefore, more food sources for the rodents, bringing them out and into contact with humans. This time also coincides with farmers working in the fields, increasing the possible contact with rodents. In the fall, the temperature changes bring the rodents from the fields into man-made structures, searching for warmth (cdc.org). The most important thing is for the at-risk individuals to be aware of the hantavirus symptoms.

The most common symptom stated by patients is a flu-like feeling. Patients will generally have a fever between 101°F and 104°F, headache, abdominal pain along with pain in joints and lower back, and mild coughing. Some patients report nausea and vomiting. As the infection becomes more severe, it can cause difficulty in breathing due to lungs filling with fluid, possibly causing death. The patient suffocates due to the airway being blocked with fluid. Other symptoms that may also be present are pneumonia, diarrhea, bleeding, low blood pressure, and in some cases, shock.

Symptoms will usually appear 3 days to 6 weeks after the initial infection, however the average is 2 weeks. Most people will not see a physician until the lungs are affected and breathing problems have already begun. Upon examination the physician observes vital statistics to determine if the patient is in acute distress and/or jaundiced. The next step would be to do a cardiac work up to make sure there are no epigastric distress, arrhythmia, heart disease or crackles and rales, which may lead to pneumonia. The abdominal examination checks for the presence of bowel sounds, and a rectal exam is also recommended to ensure there is no gastrointestinal bleeding (MedlinePlus).

Most pathogens take an opportunistic approach and infect people with weakened immune systems; hantavirus, however, infects mostly healthy people. The people who live, work or are active in closed spaces with no ventilation and who have an active rodent infestation are most at risk.

Currently there is no drug that will kill hantavirus. There have been some experimental drugs, such as ribavirin, recombinant human activated protein C (Xigris), and neuropeptide vasoactive intestinal peptide. However, the main treatment remains mechanical ventilation or use of a respirator. Most of the treated patients need to be in the intensive care unit while their lungs heal, until they can breathe easily on their own again. The standard therapy is mechanical ventilation, supplemental oxygen using a positive pressure mask. It is important to use tidal volumes lower than what is usually recommended because if the lungs are already injured, high pressure to them may only increase the injury. If there is organ failure, then proper organ support is addressed. Since there is no drug therapy at the current time the best treatment is prevention. People are encouraged to avoid inhaling dust, control mice by rodent-proofing the house, eliminating any nesting sites around the outside of the house, and keep lids on garbage cans and any food stored out doors. Also, disinfection with bleach is strongly recommended (MedlinePlus).

Vaccine Design and Protective Immunity

A hantavirus vaccine is of particular interest because of the high mortality rate among the infected. Due to the similarities in the symptoms to the common cold and/or influenza, the onset of HPS may progress without being given the necessary attention before the onset of irreversible pulmonary and cardiac damage, progressing to a less treatable stage. The relevance to develop an effective and easily accessible vaccine is great due to the geographic locations of the infections, being in the United States,

Canada, and Central and South America. The most at-risk populations are those who come into direct contact with rodents, which means that not only urbanites need to have access to this vaccine, but also those who dwell in agricultural areas. In its natural reservoir of rodents, the CDC has classified hantavirus as a Biosafety Level 4 (BSL 4) and in cell culture, as a BSL 3. Such classification is necessary because hantavirus poses a high risk of infection via inhalation and currently there are no effective vaccines or treatments for hantavirus (cdc.org).

In order to maintain the specificity and clarity of the vaccine, the focus is on one strain of hantavirus, called Sin Nombre virus (SNV) due to our geographic location in the Southwest. This strain was first discovered in the Four Corners area. SNV is one of the most virulent strains of HPS carried by the Sigmodontinae rodents (Lindkvist, Marie et al. 2007). However, due to their genomic similarities, developing a vaccine against one strain of hantavirus can very possibly protect the vaccinated from the other strains of hantavirus. There is some genomic conservation in the M segment between the different epitopes of hantavirus that would prove beneficial in terms of cross-protection (Lindkvist, Marie et al. 2007). This type of cross-protection proves very valuable because a geographic-specific vaccine will become one that can be used internationally, amplifying the number of the protected and minimizing a need for added efforts and funds to develop a specific vaccine for each of the New World hantavirus strains.

This vaccine is a combination of two techniques, inducing both cellular and humoral immunity. The technique that will induce a robust cellular immunity against SNV would be the use of the gene gun. A positive RNA strand must be made to SNV's negative RNA strand, then a double-stranded DNA plasmid, made using RT-PCR, made from the positive RNA strand is associated to a gold bead and effectively launched by a gene gun into the dendritic cells (DCs) of the epidermis of the patient (Huang, Bruce et al. 2007). Once the desired cDNA of the M segment is incorporated into the patients' chromosomal DNA, the patients' cells will begin to transcribe the G1 and G2 proteins of SNV. The glycoproteins will be recognized by the cell as foreign, and through proteosomal degradation, the SNV peptides will be presented on MHC Class I molecules of the cells. The gene gun approach with plasmid DNA associated beads has been proven to activate dendritic cells (DCs) and cause them to migrate to the closest draining lymph node, presenting them to CD8+ T cells (Gaffal, Evelyn et al. 2006). The second technique has been designed to also induce MHC Class II antigen presentation, leading to humoral response. G1 and G2 will be pre-made, with the use of the nonpathogenic E. Coli K-12 strain, and purified. Combined with an adjuvant, such as IL-12, the glycoproteins will be administered via a nasal spray, directly delivering the G1 and G2 to SNV's site of infection: the pulmonary system. It is understood that a nasal spray is not the most effective means of vaccine administration, as opposed to a subcutaneous injection. However, the desired Ig production is the production of IgA and IgM, the primary antibodies in cases of viral infection. With a subcutaneous injection, the predominate antibody produced would be IgG. Keeping in mind SNV is an intracellular pathogen, the most effective form of immunity is still cellular, but in terms of humoral, an antibody that specializes in the mucosal membrane is certainly more beneficial.

To further explain the triggered immune response by the vaccine, more details are needed. The G1 and G2 glycoproteins that are produced by self cells in their cytoplasm are bound by the MHC Class I in the endoplasmic reticulum. Then the MHC Class I are transported to the cell surface, signaling to the macrophages and DCs for phagocytosis. Cytokines and chemokines such as TNF- α , IL-1, and IL-6, produced by the macrophages and DCs will trigger more phagocytes to come to the site of antigen detection. MHC Class I presentation of the SNV antigen on the DCs triggers the activation of the CD8+ T cells. These antigen-presenting cells (APCs) migrate to the nearest draining lymph node, presenting the SNV antigen to naïve resting T cells, activating them to become cytotoxic CD8+ T effector cells. Memory T cells will also be made in this process. A robust cellular immune response is desirable because SNV is an intracellular pathogen, and the SNV-specific CD8+ memory T cells will be the dominant effector cell to phagocytise infected cells in the case of actual infection. However, a humoral response is also essential for further protection from an SNV infection.

The nasal spray technique of the vaccine is designed to mimic SNV's route of entry in the body. The immune response in the respiratory system is quite similar to the general immune response to antigen uptaken by the mucosal membranes. In this case, G1 and G2 proteins are inhaled through the respiratory tract, becoming extracellular pathogens. Along with the glycoproteins, IL-12 will be used as an adjuvant. IL-12 is a cytokine normally produced by phagocytes and B cells to stimulate T cells and Natural Killer cells (NKs) to secrete IFN- γ , promoting a Th1 response. IL-12 is also a strong stimulator of DCs and macrophages. But due to the extracellular nature of this part of the vaccine, the phagocytes would recognize such foreign protein and uptake it, inducing an MHC Class II presentation, leading to the activation of CD4+ as well. The helper T cells, specifically Th1, will induce the SNV epitope-specific B cells to undergo clonal replication, becoming plasma cells. Memory B cells are also made. The effector B cells will then produce mainly produce IgA and IgM, due to the mucosal nature of the infection. Some IgG production is seen as well. SNV-specific B cells and the immunoglobulins will then bind to the free, extracellular antigen.

In order to produce large quantities of G1 and G2 without consistent, direct contact to SNV, the full-length M (medium) genome sequence of the Sin Nombre Virus genome will be spliced into the *Escherichia coli* K-12 plasmid. K-12 derived strains are the best-known, safest strains of *E. coli* to use in laboratory experiments. Derivatives of K-12 also appear to be completely nonpathogenic and have been shown to be unable to colonize the human gut. Since all of the pathogenic components have been removed from these strains, there is no possibility of mutation or reversion back to a virulent strain. These strains also lack the O (lipopolysaccharide) antigen. They work well for lab use because their genetic makeup is very well understood. They are effective for gene cloning due to their fast growth rate and simple genetic modification, and they come in a wide range of genetic and phenotypic variations that allow them to be specially chosen for the needed application. A simple PCR reaction can be used to identify a K-12 derivative; they have a distinct band at 970bp (BATS). The K-12 strain seems to be the most appropriate, low-risk bacteria to use for growing our hantavirus genetic material that will be used in the gene gun.

The M genome sequence of the negative strand codes for the two viral envelope proteins: G1 and G2 (Botten, Jason et al. 2003). Because the negative strand is the genomic strand, it cannot be directly spliced into the E. coli plasmid. The complementary double-stranded DNA is formed and then transduced into the E. coli's plasmid.

In order to increase the rate of production and lower cost for both the manufacturer and the consumer, less manual labor and fewer required steps would be ideal. The most conventional technique for RNA replication will be used for mass production of the SNV RNA. However, such as the case for the gene gun, the positive strand RNA must be made first. Then the reverse transcriptase polymerase chain reaction (RT PCR) will be run. The M strand would be isolated and then be reproduced millions of times in a short period of time. The DNA complementary strands will be made to this desired RNA piece. Then the polymerase chain reaction would be implemented. The growth of the E. coli K-12 would not pose as a problem. The replication of E. coli is quite rapid and this can also be done in large vessels as long as the optimum conditions for growth are maintained.

In order ensure of the purity of the final transduced product, we will attach to the end of the M segment an ampicillin-resistant gene and then grow the transduced E. coli on agar plates with ampicillin. The only E. coli that will grow are those that have the transduced plasmids with the Sin Nombre cDNA M segment and the ampicillin-resistant gene. That way only the desired E. coli will be reproduced in large quantities for the vaccine.

This technique of manipulating the E. coli plasmid and its replicative mechanics is used in producing hormones as well. Hormones such as somatostatin and insulin can be produced using different strains of E. coli transduced with the appropriate human genome segment (epa.gov). Biosynthetic insulin has been produced using this technique since the early 1980s (Heinemann, Lutz 2002), which adds credibility and the reliability to recombinant DNA synthesis of the SNV glycoproteins. The maintenance of the E. coli culture is very important. Strict rules of draining waste and replenishing nutrients is necessary in the optimization of production.

The main concern of this vaccine proposal is to invoke the production of antibodies specific for SNV without actually causing an infection in the patient, from neither the SNV itself nor the delivery vessel, E. coli. It is quite possible that upon uptake of antigen, the body will produce both antibodies for SNV and this specific E. coli strain that we are using. With the viral proteins being produced by bacteria, the body will recognize the foreign proteins and immediately initiate an immune response. But because no self-cells are producing more virus due to the lack of viral RNA, the symptoms of HPS will not appear. The E. coli also serves as another foreign antigen, inducing the body to produce an antibody against the bacteria as well.

Such a combination of two techniques is the most appropriate way to ensure immunity to SNV in patient. In studies of the SNV, it was found to be unattenuable. The virus underwent five passages through Vero E6 cells, which are isolated from the kidney epithelial cells of the African green monkey, and then re-introduced into a human cell line. The genomic sequence of this supposedly attenuated SNV compared to the RNA taken directly from an SNV-infected patient showed no differences (Chizhikov, Vladimir et al. 1995). This leads us to believe that SNV has a low mutation rate, which proves to be an advantage for our vaccine. With low mutation rate, one can assume that the potency of the vaccine will remain over long periods of time.

However, the use of a gene gun may be intimidating for the patients, especially children. The gene gun emits a loud sound due to the high pressure necessary for embedding the gold bead into the skin. The gene gun may need to be fired multiple times to ensure embedment. Such repetition will call for eardrum protection in the form of earplugs and protective headphones to filter out sound for both the patient and the administrator. Secondly, as mentioned before, the nasal spray administration is perhaps a less effective means of vaccine delivery. However, the dose will be optimized for sufficient antigen uptake and recognition, inducing a production of IgA and IgM for the mucosal surfaces. This method is specifically tailored to the actual inhalation of SNV-containing rodent excreta in the cases of actual infection.

Vaccine Efficacy

Studies have been done to observe the effects of HPS on hamsters, and they have proven that the infection progression of HPS in hamsters is very similar to the HPS progression in humans (Campen, Matthew et al. 2006). Following the analysis of the studies done on hamsters, if the results prove promising, then human patients will be vaccinated for further test of vaccine efficacy.

Before the description of the actual experiment, it is pertinent to have the appropriate facilities to conduct experiments with such a virulent pathogen. In tissue culture, SNV is BSL 3, but since we are using a rodent animal model, the facilities must be capable of housing BSL 4 pathogen experiments. The necessary safety measures must be taken to protect the researchers who will be conducting the tests.

Since successful studies have been done on Syrian golden hamsters, the same animal model will be used. Such as in the Campen study, radiotelemetry devices were embedded in the hamster's back, allowing records of heart rate and blood pressure (Campen, Matthew et al. 2006). Two waves of experiments will occur. In the first set of experiments, there will be four groups, with each group containing four hamsters. The first group will be the control group, receiving no vaccine. The second, third, and fourth groups will be receiving two shots from the gene gun each and will also receive differing concentrations of the G1 and G2 plus adjuvant nasal spray.

Group 1: Control (no vaccine)

Group 2: 2.0×10^8 proteins (mixture of G1 and G2)

Group 3: 2.0×10^{10} proteins (mixture of G1 and G2)
Group 4: 2.0×10^{12} proteins (mixture of G1 and G2)

These concentrations of proteins are chosen based on Campen study's use of 2.0×10^{10} virus particles for each of the hamsters. Concentrations of the G1 and G2 above and below such a concentration of virus particles will give insight into the amount of proteins necessary to provide immunity upon challenging the subjects with the SNV. Before challenging the hamsters, however, blood samples from the hamsters will be taken every two days until day 10. The blood samples will be analyzed for humoral and cellular immunity. To detect antibody production, a sandwich ELISA will be used, and for cellular immunity, flow cytometry will be implemented.

Assuming all the vaccinated hamsters show antibody production and SNV-specific T cells, the concentration of 2.0×10^{10} SNV particles will be used to challenge the hamsters 10 days after vaccination. Because the immunoglobulins of interest are IgA and IgM, and the mode of infection is through inhalation, the SNV particles will be aerosolized and sprayed into the nostrils of the hamsters in Groups 2, 3, and 4. The hamsters will be closely monitored for symptoms such as change in behavior or mobility and the heart rate and blood pressure will be closely monitored. Blood will be drawn from the hamsters every four days to monitor antibody production and T cell activation. Twenty days after challenge (day 30), the number of surviving and deceased/euthanized hamsters will be recorded. On day 30, blood samples will be taken from all surviving test subjects and analyzed with flow cytometry and sandwich ELISA (Enzyme-Linked ImmunoSorbent Assay).

The next experiment is designed to find the optimum number of cDNA particles that need to be embedded into the hamster. In the previous experiment, two shots were used. The experiment design is exactly the same, but Groups 2, 3, and four will receive different numbers of shots from the gene gun.

Group 1a: Control
Group 2a: 1 shot
Group 3a: 2 shots
Group 4a: 3 shots

The number of shots used is based on the assumption that the previous experiments worked with only two shots from the gene gun. The most effective concentration of protein in the nasal spray will be known by this experiment due to the data from the previous experiment, which is revealed by the amount of production of antibodies, amount of SNV-specific T cells, and the number of survivors per group, and physical observations of the subjects. The same timeline will be used for this experiment as well.

To measure humoral immunity, an effective, yet relatively simple assay to use is the sandwich ELISA. The concept of this assay is to detect the presence of an antigen-specific antibody, and in this case, a SNV-specific antibody. For this ELISA, both

purified SNV and purified antibody from the hamster is needed. A microtiter plate is commonly used for this technique. The plate is coated first with the purified antibody from the hamster. Then purified SNV will be added to the plate, and if SNV-specific antibodies are present at the bottom of the plate, then the SNV will attach to them. Then an enzyme-attached antibody will be added to the plate, “sandwiching” the SNV. After adding a chromogenic substrate to the plate, the enzyme will react with the substrate, turning the wells yellow. Varying degrees of yellow mean varying concentrations of antibody. The more SNV-specific antibodies there are on the bottom of the plate, the more yellow the wells become.

However, an ELISA is not the only way to test for humoral immunity. The blood from the hamster is separated to remove all cellular components of the blood. Two tissue culture dishes are set up using hamster cells. Live hantavirus is added to dish 1, and PBS is added to dish 2 to serve as the negative control. The subject’s plasma is also added to each dish. If anti-hantavirus antibodies are present, they would prevent the live hantavirus from infecting and killing all of the cells in the tissue culture. Only if the vaccine is successful in evoking an adaptive immune response would there be anti-hantavirus antibodies present in the serum. The antibodies could not have been synthesized *de novo* because there are no B cells present in the plasma to be activated to produce the antibodies.

Measuring cellular immunity is not quite as straightforward as the humoral immunity detection. Because effector T cells are not as easy to test for, the preferred method is using MHC:peptide tetramers. A streptavidin molecule binds to four biotinylated (attached to biotin) MHC:peptide tetramer molecules. The biotinylated MHC:peptides are made from recombinant MHC molecules with the specific peptide sequences. Next the sample from the hamster is added to these MHC:peptide tetramers. The tetramers will be bound by the T cells (CD4+ and CD8+) which express the specific antigen being tested. Usually the streptavidin molecule is marked with fluorochrome (FITC) so that the results can be measured by flow cytometry.

The blood from the hamster is separated by Ficoll-Paque separation to separate PBMNC from other blood products. The PBMNCs are plated into two separate dishes. To one dish, hantavirus is added. Phosphate-buffered saline (PBS) is added to the second dish to serve as the negative control. The samples are then incubated at 37° C overnight. Two aliquots were removed from each dish. Tubes A and B contain hantavirus-stimulated PBMNC, and tubes C and D contain the control. The necessary FACS solutions were added to all tubes. To tubes A and C, which are to check for CD4+ cells, anti-CD4 FITC-conjugated antibody is added. To tubes B and D, which were to check for CD8+ cells, anti-CD8+ FITC-conjugated antibody is added. All four are then run on a FACS machine to check for the fluorescence that would be given by the conjugated antibodies. Tube A is compared to tube C, and tube B is compared to tube D. The comparisons should show statistically significant elevations of both T cell types.

The addition of live hantavirus to a vaccinated individual’s blood, if the vaccine was successful in eliciting an immune response, would cause any memory T cells to

become activated to divide, producing armed effector cells. Only an individual who has immunological memory would be able to produce the clones of effector cells that showed up in the FACS analysis because naïve T cells found in the blood cannot be activated *de novo*. There is no peripheral lymphoid tissue for the naïve T cells to differentiate into effector cell. Memory cells, however, can proliferate in response to its specific antigen immediately (Zeman, 2007).

Conclusion

If there are promising results from the animal-model experiments and all of the possible side-effects are identified, more tests and experiments must be run in order to progress to clinical trials. Studying such a high-risk virus poses many obstacles concerning the safety of the research team, which means the process of understanding this pathogen is a slow and arduous process. One hopes that this vaccine will provide some answers and elucidate such an elusive virus. The development of a vaccine, in general, is meant to benefit the human race, lessening the suffering and prolonging life. Especially with such a devastating virus, taking the lives of over one-third of the infected, an effective and safe vaccine needs to be found.

References

- Botten, Jason, Katy Mirowsky, Donna Kusewitt, Chunyan Ye, Keith Gottlieb, Joseph Prescott, and Brian Hjelle. "Persistent Sin Nombre Virus infection in the deer mouse (*Peromyscus maniculatus*) model: sites of replication and strand-specific expression." *Journal of Virology*. Volume 77: 1540-1550. 2003.
- Calisher, Charles H., William Sweeney, James N. Mills, and Barry J. Beaty. "Natural history of Sin Nombre Virus in western Colorado." *Emerging Infectious Diseases*. Volume 5: 126-134. 1999.
- Campen, Matthew J., Mary Louise Milazzo, Charles F. Fulhorst, Chrys J. Obot Akata, Frederick Koster. "Characterization of shock in a hamster model of hantavirus infection." *Virology*. Volume 356: 45-49. 2006.
- Center for Disease Control and Prevention*. Updated Oct. 2006.
<<http://www.cdc.gov/ncidod/diseases/hanta/hps/index.htm>>.
- Chizhikov, Vladimir E., Christina F. Spiropoulou, Sergey P. Morzunov, Martha C. Monroe, Clarence J. Peters, and Stuart T. Nichol. "Complete genetic characterization and analysis of isolation of Sin Nombre Virus." *Journal of Virology*. Volume 69: 8132-8136. 1995.
- Gaffala, Evelyn, Dirk Schweichela, Damia Tormoa, Julia Steitza, Julia Lenza, Etiena Basner-Tschakarjana, Andreas Limmerb, Thomas Tütinga. "Comparative evaluation of CD8+CTL responses following gene gun immunization targeting

- the skin with intracutaneous injection of antigen-transduced dendritic cells.”
European Journal of Cell Biology. Volume N/A: N/A. 2006.
- Hart, C.A., Bennett, M. “Hantavirus infections: epidemiology and pathogenesis.”
Microbes and Infection. Volume 1: 1229-1237. 1999.
- Heinemann, Lutz, Renata Linkeschova, Klaus Rave, Beate Hompesch, Martin Sedlak,
and Tim Heise. “Time-action profile of the long-acting insulin analog glargine
(H0E901) in comparison with those of NPH insulin and placebo.” *Diabetes Care:
Emerging Treatments and Technologies*. Volume 5: 644-649. 2000.
- Kim, Jong-Il and Michael M. Cox. “The RecA proteins of *Deinococcus radiodurans* and
Escherichia coli promote DNA strand exchange via inverse pathways.”
Proceedings of the National Academy of Sciences. Volume 99: 7917-7921. 2002.
- Kuhnert, Peter, and Joachim Frey. “Tools for safety assessment: Identification and
monitoring of *Escherichia coli* K-12 safety strains.” *Centre for Biosafety and
Sustainability*. Updated 01/10/1996.
<http://www.bats.ch/bats/publikationen/1996-1_e.coli/96-1_e-coli_k12.php>.
- MedlinePlus: A Service of the U.S. National Library of Medicine and the National
Institutes of Health*. Updated Nov. 2007.
<<http://www.nlm.nih.gov/medlineplus/ency/article/001382.htm>>.
- Muranyi, Walter, Udo Bahr, Martin Zeier, and Fokko J. van der Woude. “Hantavirus
infection.” *Journal of the American Society of Nephrology*.
- National Center for Infectious Diseases Special Pathogens Branch: All About
Hantaviruses*. Updated Oct. 2006.
<http://www.cdc.gov/ncidod/diseases/hanta/hps/index.htm>.
- Padula, P.J., A. Edelstein, S. D. L. Miguel, N. M. López, C. M. Rossi, and R. D.
Rabinovich. “Hantavirus pulmonary syndrome outbreak in Argentina: Molecular
evidence for person-to-person transmission of Andes Virus.” *Virology*. Volume
241: 323-330. 1998.
- Strauss, J. and E. Strauss. *Viruses and Human Disease*. Academic Press. 2002.

***Vasquez, Clovis, R.S. Nuris de Manzione, Hector Paredes, Lorenzo Basile, and Victor Alarcon. "Fiebres hemorragicas por Hantavirus en Venezuela." VITAE Academia Biomedica Digital. Volume 23. 2005.**

****Vasquez, Clovis, R.S. Nuris de Manzione, Hector Paredes, Lorenzo Basile, and Victor Alarcon. "Hemorrhagic fevers by hantavirus in Venezuela." VITAE Biomedical Academics Digital. Volume 23. 2005.**

Zeman, Alenka M., Tyson H. Holmes, Shaye Stamatis, Wenwei Tu, Xiao-Song He, Nancy Bouvier, George Kemble, Harry B. Greenberg, David Lewis, Ann Arvin, and Cornelia Decker. "Humoral and cellular immune responses in children given annual immunization with trivalent inactivated influenza vaccine." *Pediatric Infectious Disease Journal*. Volume 26: 107-115. 2007.

*Actual Article

**English Translation