

A DNA Vaccine to Protect Humans from Avian Influenza H5N1

Literature review

This investigation seeks to address a pervasive issue in global health, the pandemic threat of Avian Influenza A/H5N1, or “bird flu”. A global influenza pandemic could have a major effect on global economy and health. The current mortality rate of reported cases of H5N1 is estimated to be 57% (Thomas and Noppenberger 2007). So far the H5N1 reported cases have been contracted via direct contact with an infected bird. The concern is that this virus will mutate and be able to pass from human to human. Currently there are no vaccines available for the H5N1. However there are some antiviral medications on the market, such as Tamiflu and Relenza, but they are expensive and in short supply. A proposed DNA vaccine with a nasal booster would be a better solution because it would be more cost effective, manufactured faster and in large quantities. This would allow more people to be reached and gain protection from this deadly virus.

Influenza is a single-stranded RNA virus and a member of the Orthomyxoviridae family. The structure of the influenza virus has two receptors, hemagglutinin and neuraminidase. Hemagglutinin (HA) is an antigenic glycoprotein on the surface of the virus that is responsible for binding the virus to the cell that is being infected and it is cylindrical shaped. Neuraminidase (NA) is an antigenic glycoprotein enzyme on the surface of the virus and is mushroom shaped. The function of NA is to aid in the release of virions from the infected cell. In doing so, it promotes the spread of the virus. The antibody that is created for HA is a neutralizing type. In contrast, the antibody for NA works differently to modify the severity of the disease. There are 16 different HA antigens and 9 different NA, numbered H1 – H16 and N1 – N9 (International 2004). Influenza viruses are constantly evolving in that they can change in two different ways. One way is by antigenic drift, while the other is referred to as antigenic shift. Influenza viruses are changing all the time by antigenic drift, meaning that small gradual changes occur through point mutations in the genes that produce the surface proteins. Antigenic shift, on the other hand, is the process in which two different strains of influenza undergo recombination in pigs and form a new subtype.

Essentially, the HA of the virus has to attach itself to a cell. Then the envelope of the virus must fuse with the cell membrane to allow the protein-coated RNA virus to enter the cell. Once inside, the cell enzymes remove the protein coat and the viral RNA replicates into mRNA for protein synthesis. New viral RNA is synthesized and then surrounded by a protein coat. Once the synthesis is complete, the virus exits the host and surrounds itself in a plasma membrane for protection. At this point, the NA assists the virions in spreading by cleavage. If there are no antibodies to protect us from the virus, then the virus will be free to cause sickness.

The influenza virus is identified by a standard nomenclature. For example, a layout of a standard nomenclature would contain the name, such as A/USA/55/06 (H1N1), followed by the description. The description would include the virus type, geographical location where the virus was first isolated, sequential number of isolation, year of isolation, and HA/NA subtype (Services 2006). The distribution of the virus in nature is somewhat complex in that there are many strains of the influenza that have affected humans. These strains can come from pigs, chickens, wild waterfowl and quail. Antigens that have been seen in humans are: H1, H2, H3,

N1, N2 and N3. Other strains are in species such as cattle, ferrets, equine, whale and seals, but these strains do not affect humans.

Avian influenza is caused by avian influenza viruses and carried by wild birds in their intestines. The wild birds do not usually get sick from them, but the virus is very contagious between birds. These viruses not only make domesticated birds sick, but in some cases, can also be deadly (Services 2006). There are two forms of the avian influenza virus. One is low pathogenic (LPAI) and the other is high pathogenic (HPAI). In the LPAI form, symptoms are usually mild, hard to detect, and have low virulence. Some of the symptoms for the LPAI form are a noticeable drop in egg production and ruffled feathers. In contrast, the HPAI form spreads rapidly, affects multiple internal organs, and has a mortality rate of ninety to one hundred percent within forty-eight hours. Symptoms of the HPAI form are diarrhea, swelling of the head and face, and nervous disorders(Olsen, Munster et al. 2006).

There are many ways to spread avian influenza among birds. Some ways are by direct contact, contact with infected fecal matter, and contact with surfaces or materials that are contaminated. For example, a duck that is infected with HPAI migrates in the fall and lands in a rice field full of domestic ducks to forage for rice. While foraging, the duck defecates in the watery rice field, which the domestic ducks then drink and become infected (Davis 2005). Other examples of methods of contamination are through use of common areas, cages, water, and feed that have been contaminated with the virus. Humans can cause the spread of the virus by unknowingly selling infected birds in live poultry markets, called wet markets. The virus could possibly be transmitted to a human by living in close proximity with the animals or by the purchase an infected bird at a wet market. In a dense population of birds, it only takes one infected bird to infect the whole population(Ebrahim 2004).

The spread of H5N1 from human to human could occur the same way influenza spreads in respiratory droplets of coughs and sneezes, this is called droplet spread. In a paper written by Debby van Riel, a conclusion is made that it is highly unlikely that the virus will be spread from human to human, because the virus attaches in the lower respiratory tract. Influenza viruses are spread from the upper respiratory tract via coughing and sneezing. So unless a mutation occurs that would allow the virus to attach to the upper respiratory tract, transmission is unlikely(Van Riel, Munster et al. 2006). However, with the globalization of today if a mutation did occur the virus could spread much faster and be more devastating than the Spanish flu of 1819 (Crosby 2003). The 1918 Spanish flu was the first pandemic to occur causing the deaths of 500,000 people in the United States and 20 – 50 million people worldwide (Diseases 2006). The advancement of medicine and development of vaccines have reduced the death toll of pandemics since the Spanish flu.

According to HHS, there are some prescriptions that should work in treating avian influenza in humans. These medications are oseltamavir (Tamiflu) and zanamavir (Relenza). However, as with any virus, they can become resistant to these drugs and the medications may not work (Services 2006). The government plans to stockpile enough antiviral medication to treat 75 million people divided between Federal and State stockpiles and to establish and maintain a stockpile of 6 million treatment courses for domestic containment (Council 2006).

Roche, the Swiss company that manufactures oseltamavir cannot keep up with worldwide demands for the anti viral drug. The company has decided to work with other companies to allow for more of the drug to be made. By doing this they will be able to produce 400 million treatment courses a year starting in 2007. This is a large increase in production from the 6 million produced yearly. One course of treatment consists of 10 capsules. Even though the drug will become abundant it is still not cheap (Enserink 2006). There is much concern that developing countries will not be able to afford the treatment courses.

Description of vaccine

In a recent *Nature Immunology* review, it is stated that, “nearly 50% case-fatality rate among people who become infected with H5N1 viruses underscore the need for control strategies to prevent a potential influenza pandemic” (Subbarao and Joseph 2007). The profound and devastating potential of avian influenza is attributed to the fact that humans have little to no natural immunity to the various strains of influenza virus that circulate globally within wild bird populations. This leaves humans particularly susceptible to “bird flu,” and elicits the need of an accessible, stable, effective, and prophylactic Avian Influenza vaccine. Of particular interest to this study is the deadly H5N1 subtype of Influenza A virus. H5N1 is transmitted to humans via wild birds harboring the virus. Although Avian Influenza is not readily transmitted among infected humans, the present interest in developing an effective vaccine against the deadly H5N1 is necessitated by virtue of the virus’ mutability. The high frequency mutation of Influenza virus leads to heightened transmission among humans that may ultimately result in a catastrophic, pandemic outcome.

In order to address this concern, we propose the development of a DNA vaccine against the H5N1 subtype using stored samples of H5N1 virus strains that have had previous infection history in human populations. Two of the most notable H5N1 virus strains are the A/Hong Kong/213/03 (H5N1) and A/Vietnam/1203/04 (H5N1) influenza viruses that can be obtained from World Health Organization collaborating laboratories. These viral strains will be used in conjunction with other WHO isolated strains from Hong Kong 1997, Thailand 2004, Cambodia 2005, Indonesia 2005, and Turkey 2005 – all of which resulted in human infection and, in some cases, fatality. We predict that the broad range in H5N1 sample strains will allow for a more comprehensive DNA vaccine and confer a larger degree of protection.

DNA vaccination utilizes the science of reverse genetics, whereby a custom vaccine can be assembled using various genetic features of the aforementioned H5N1 viruses. By using sequence data from the isolated viruses, one can generate a DNA vaccine against any particular antigenic target, and even multiple viral targets. The emphasis of our vaccine is placed on isolating the genes for the viral hemagglutinin (HA) and neuraminidase (NA) membrane proteins, H5 and N1. In addition, the vaccine will also target highly conserved viral proteins such as nucleoprotein (NP), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA), all of which are specific for activation of cell mediated immunity by CD8 T lymphocytes (Subbarao and Joseph 2007).

However, H5 and N1 are primary targets because they are the implicated in binding host cells and aid in viral release from the host cells, respectively. In a study published by the World Health Organization it was found that there were conserved regions among human and avian HA

and NA genes. “The HA genes from H5N1 viruses isolated from human specimens were closely related to HA genes from H5N1 viruses of avian origin; human HA gene sequences differ from the nearest gene from avian isolates from the same year in 2–14 nucleotides (<1% divergence)” (Aubin, Azebi et al. 2005). The high degree of sequence homology between the avian versions that made their way into the human host population makes H5 and N1 suitable targets for vaccination, and may even confer a degree of cross protection for other HA and NA subtypes.

The concept of cross protection is a key benefit to the DNA vaccine model. Because there are a variety of viral samples at our disposal, the overwhelming benefit of DNA vaccine technology is that each of the genes corresponding to variations in H5N1, NP, PB2, PA can be cloned and inserted into the plasmid vectors for vaccine administration. The host then expresses the viral genes and manufactures the proteins, with the end result of highly specific antigen recognition without virulence. Also, by virtue of the function of each of these viral protein, there is a certain degree of sequence homology across the various strains to maintain the fidelity and functionality of the viral proteins throughout the normal course of infection by the Influenza virus.

The revolutionary development of reverse genetics makes it possible to also target components of the viral genome and incorporate them into the plasmid vectors used in the vaccine. The proposed viral targets for this vaccination are primarily the H5 and N1 genes, although the more ubiquitous NP, PB2, and PA proteins that are present in pathogenic Avian Influenza will also be incorporated in the plasmid construction of the DNA vaccine. This is significant to the vaccine cross protectivity because each Influenza virion has a genome that encodes similar proteins that are essential for proliferation and transmission. Generating an immune response to these genomic components of pathogenic influenza would be favorable in combating and preventing a possible pandemic. While the focus of this proposal is on the H5 and N1 components of the virus, selection of the viral genetic components would be derived from the aforementioned isolated strains from Hong Kong, Vietnam, Thailand, Cambodia, Indonesia, and Turkey.

The genetic components of the pathogenic Avian Influenza would be evaluated and verified using polymerase chain reaction to ensure vaccine integrity – that the viral genetic components are identical to the HA and NA genes derived from the source viruses (i.e. A/Hong Kong/213/03 (H5N1) and A/Vietnam/1203/04 (H5N1)). Plasmids containing these antigenic target genes in the “coding region” are constructed and are flanked by regions of transcriptional control, which is necessary for the action of the DNA vaccine. A eukaryotic promoter region precedes the coding region so that the viral genes can be expressed in humans. The plasmid preparations are also equipped with termination sequences and polyadenylation sequences to ensure transcriptional control and that the mRNA messages are able to “survive” without degradation intracellularly. The plasmids would then be tested for integrity and functionality (in human cell lines) before being used for vaccination purposes.

The plasmid vectors corresponding to the genetic components of the viral protein H5 and N1 are injected intramuscularly. After delivery, the plasmids containing the viral protein DNA are taken up by muscular or dendritic cells, which then express the plasmid DNA, resulting in the manufacture of the specified pathogen target proteins. The manufactured antigenic components generated by the host cell then undergo intracellular processing as they would during host

infection. The digested antigenic peptides are then transported to the cell surface as viral antigen bound to MHC Class I. The target antigens complexed to MHC I elicit a response via T cell mediated immunity and confer host protection. Generation of antibody is also observed meaning that the plasmid DNA products also reach and stimulate B-cell in the lymphatic organs. This activation may occur by secretion of antigen by the host cells or antigenic release after host cell death.

After vaccination, the generation of viral antigen by host cells elicits the concomitant generation of memory B and T cells, which confers protective immunity to the patient. This immunity includes the secretion of neutralizing antibody, and generation of memory cytotoxic T-cells and Th1 cells upon later challenge by the virus.

In addition, the plasmid DNA encoding antigen is coupled to a CTLA-4 compound, which enhances selective binding of the antigen to antigen presenting cells of the immune system. Moreover, use of an adjuvant like cytokine interleukin 12 adjuvant, has been shown to catalyze more robust immune responses in proposed HIV vaccination studies (Barouch, Santra et al. 2000). Our proposed H5N1 DNA vaccine elicits both development of antibodies and subsequent neutralization, as well as cell mediated response (antibodies and cytotoxic T cells), which provides active immunity against the virus. The vaccine confers protective immunity via secretion of neutralizing antibody and production of memory CTLs. Thus the proposed vaccine offers protection against later challenge by the virus.

The relative advantages of using a DNA vaccine are numerous. Using reverse genetics techniques, the target of the vaccine is very specific, in this case the various viral proteins on the H5N1 viruses. In addition, the vaccine generates active immunity, which is long lived. The DNA virus does not pose the threat of infection as a live attenuated virus does. Also, the DNA vaccine would be a simplification of current vaccine processes and has the potential for relatively low cost, and mass production. The DNA viruses are relatively stable, which simplifies delivery and enhances the accessibility of the avian flu vaccination to remote areas around the world.

Conversely, there are certain disadvantages to the use of DNA vaccines. As a relatively new technology, DNA vaccinations are not necessarily tried and true in terms of clinical application and the efficacy of DNA vaccines is being determined. In addition, there are possible long term, unintended consequences such as the potential for incorporation of the viral gene into the host genome (cellular DNA), oncogenesis, etc. that remain relatively unknown.

In three to four weeks, the Avian influenza vaccine will be booster with a different type of vaccine than the first two of the series of three. The second vaccine will be administered as an intranasal attenuated live vaccine. By stimulating the immune system locally, the body can develop local protection to areas like the respiratory tract where the disease first invades the body. The virus enters the body and binds to receptors in the eyes and lungs (Skowronski, Li et al. 2007). This vaccine will stimulate the body to create a response from the antibody IgA, which are found in the mucus membranes, very similar to having an actual infection. The cellular immunity developed by the initial DNA vaccine will work together with the attenuated live vaccine to protect the body from any poultry-human transmission of the disease.

The subunit type H5N1 has been found to have a greater the fifty percent chance of fatality and has become the target of this vaccine (Wong and Yuen 2006). The H5N1 virus has a lipoprotein membrane contains two subunits, H5 allows it to bind to the cell and N1 which helps the virus spread (Parham 2005). H5N1 virus will be injected in goats in order to produce a large sample of antibodies to produce a large number of vaccines. The goats will later have their blood drawn in order to obtain the antiserum. The antiserum will then be purified by antigen affinity chromatography. The vaccine will be an attenuated live vaccine containing 75µg of the anti-virus that will be administered intranasally in order to develop local and additional systemic protection. The intranasal vaccine proves to be more effective given this way and causes little discomfort administering it (Parham 2005). The vaccine will utilize Immune Stimulatory Complexes (ISCOMs) as the adjuvant to allow better stimulation of the cytotoxic T cells. By using the adjuvant ISCOMs we can stimulate the cytotoxic T cells to destroy the virus infected cells. ISCOMs consists of a matrix of lipid micelles the contain viral proteins. The micelles will fuse with the cell membrane delivering the peptide to the cytosol of the antigen presenting cell allowing it to bind MHC 1 and present on the cell surface attracting CD8 T cells (Parham 2005). Intranasal vaccines are affordable and easily administered making it a vaccine that almost anybody could give.

Description of immunity assessment

Upon administration of the DNA vaccine and intranasal booster, serological tests would be conducted to assess the immune response. Appropriate timing for each test would allow the immune system to respond to the vaccines. Antibodies would be generated and allowed for detection using immunological assays. Microneutralization and hemagglutination-inhibition (HAI) assays would be used to determine if neutralization and binding of antibodies has occurred. In addition, Flow cytometry would be used to quantify the frequency of H5 and N1 binding B cells.

The DNA vaccine contains plasmids with genes for the viral HA and NA membrane proteins and will be administered intramuscularly into the deltoid muscle. According to studies, an intramuscular route is more beneficial than intradermally for patients 18-60 years of age. The level of HA antigen in the intradermal dose was reduced to 40 percent of that in the standard intramuscular dose, with equivalent HAI antibody responses in younger persons; less vigorous responses were observed in older persons in both the intradermal and intramuscular groups...Relatively high efficacy is observed in healthy young people, who mount vigorous immune responses to 15µg of antigen given intramuscularly (Belshe, Newman et al. 2004). According to John J. Treanor's group, on the basis of these preliminary data, a two-dose schedule of 90µg of subvirion H5 vaccine would probably have an acceptable tolerability profile and could be effective in preventing H5 influenza in healthy adult recipients (Treanor, Campbell et al. 2006). Serological tests would confer if an immune response is generated.

The microneutralization assay is sensitive and specific for detecting antibody to Avian Influenza A H5N1 virus in adult human sera (Rowe, Abernathy et al. 1999). This assay is important because it is quantitative and specific, which holds several advantages for its use. For example, it has been used to successfully screen several thousand human serum samples in BSL2 conditions at the Department of Health Government Virus Unit in Hong Kong. In addition, it detects functional anti-HA antibody which is highly specific for H5N1 and can be developed

quickly upon recognition of a novel virus (Rowe, Abernathy et al. 1999). The H5 antibody would be crucial to protect the body against the H5N1 virus.

The microneutralization assay is used to detect if infection has occurred and is performed in two steps. First, the virus is mixed with antibody reagents. The next step is inoculation where the mixture is mixed with human sera. The human sera must be heat inactivated to stop complement at 56°C. If neutralization has occurred, there would be HA and NA antibodies present, therefore blocking the virus and indicating successful protection. If there was no neutralization, non-specific antibodies would be present and massive cell death would occur.

The HAI assay has been used to detect H5 antibody as a standard test. The test usually failed because it was not specific and less sensitive for detecting antibody responses. Although it may be less sensitive, according to Treanor, patients receiving lower doses of 45µg of vaccine had lower mean titers for hemagglutinin antibody. Doses at 90µg produced higher titers of antibody and were detected using the HAI assay. His group was able to demonstrate antibody responses to H5 using this assay (Treanor, Campbell et al. 2006).

The HAI assay is used to identify if antibodies are binding to the virus. The virus is mixed with red blood cells to see if they clump together. If binding occurs, there would not be any clumping. If binding did not occur, there would be clumping of red blood cells. Human sera must be collected at days 0, 28, and 56 days after vaccine has been administered. Antibodies must have a four-fold or greater rise in antibody titer and can be determined using different concentration of antibodies.

Flow cytometry will be used to quantify the frequency of H5 and N1 binding B-cells in the vaccinated test population of individuals 18-64. This assay is of particular interest because the specific mechanism underlying the initiation of B-cells is relatively unknown. Although the immune response to the DNA vaccine is relatively rapid, there is an expected lag time before there is a significant cell mediated response and rise in IgG production.

The assay will be conducted on a time course where day zero corresponds to the day the patient is given the vaccine. The patients will be baselined on day zero. Fluorescence labeled H5 and N1 antigen will be added directly to a sample of patient lymphocytes, or purified B-cells. The antigen that is removed in subsequent wash steps will not adversely affect the measured frequency of H5 and N1 binding B-cells. The patients' serum will be tested on days 0, 7, 14, and 21 following vaccination to mark the fluctuation in antigen specific B-cells.

Similarly, flow cytometry will be employed to assess the relative number of H5 and N1 antigen binding cytotoxic T-cells. Increase in frequency of H5 and N1 specific cytotoxic CD8 cells is presumed to increase after infection and primary defense. H5 and N1 antigen will be bound to an MHC I tetramer and fluorochrome tagged in order to elucidate the frequency of cytotoxic CD8 T-cells due to the fact that the CTL don't bind antigen directly. Higher mean fluorescence intensity indicates more specific MHC-peptide binding. This assay is generally carried out with populations of memory T cells where the frequency of specific cells has been increased by infection or immunization. Test subjects will be evaluated on a similar time scale as with the B-cell test – baseline on day 0, and test on days 0,7,14, and 21 following vaccine administration.

The most effective method to find out if the vaccine is providing cell mediated immunity is to measure T cell memory. Memory T cells are a subset of antigen-specific T cells that persist long after an infection has been resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their related antigen, thus providing the immune system with memory against past infections. Memory T cells comprise two subtypes: central memory T cells (TCM cells) and effector memory T cells (TEM cells). Memory cells may be either CD4+ or CD8+. T cell immunity activates macrophages, natural killer cells, antigen specific cytotoxic T lymphocytes, and the release of cytokines in response to an antigen. The most significant measurement is the activation of antigen specific cytotoxic T lymphocytes. To measure T cell memory the hemoglobin levels, total white blood cell count would be determined before vaccination and the seventh day after vaccination (Treanor, Campbell et al. 2006). Most cytotoxic T cells express T-cell receptors that can recognize a specific antigenic peptide bound to Class I MHC molecules and a glycoprotein called CD8. The cytotoxic T lymphocytes (CTLs) typically express CD8 and induce apoptosis of cells on which they recognize foreign antigens presented by MHC class I molecules, providing a defense against intracellular pathogens such as viruses (Thomas, Keating et al. 2006).

Activated T cells perform their effector functions when they encounter MHC-presented peptide on their target cells. The affinity between CD8 and the MHC molecule keeps the T cell and the target cell bound closely together during antigen-specific activation. CD8+ T cells are recognized as cytotoxic T (Tc) cells once they become activated and are generally classified as having a pre-defined cytotoxic role within the immune system. A microneutralization or a H5 specific ELISA, each followed by confirmation with a Western blot assay, would be used to detect antibody and T cell response for Influenza A (Katz, Lim et al. 1999).

The mechanism of an intranasal infection is a complex process but is simplified by breaking it down in stages. After intranasal infection, priming, activation, and the expression of influenza-specific CD8+ T cells take place in the mediastinal lymph node three to four days after infection. The influenza A virus-specific CD8+ T-cell response has been distinguished by using intracellular cytokine staining and MHC class I tetramer labeling. These techniques give us the opportunity to analyze and measure each stage of the reaction. The antiviral capacity of these virus-specific CD8+ cells is dependent on their capability to localize to the lungs and infected airway epithelium. The CD8+ cells initiate a response 5–7 days after infection. Because viral replication is restricted to cells in the respiratory epithelium CD8+ T cells put forth their effector functions at this location, resulting in antiviral cytokines and lysing of target cells presenting surface elements such as a specific T-cell receptor. The lysis of infected epithelial cells is intervened by exocytosis granules containing perforin and granzymes. The release of perforin and granzymes from influenza-specific CTLs is strictly controlled, occurring instantly after activation at or in close proximity between CTLs and the infected target cell (Thomas, Keating et al. 2006). This is significant because it demonstrates the antigen specificity of the response via intranasal infection.

A known test that is effectively used throughout the industry is the Flow Cytometry. Flow cytometry is generally used to count tetramer MHC-peptide-binding T cells. Flow cytometry can be used to count the number of cells in a suspension having specific molecules on their membrane. We can measure fluorescence intensity (amount of fluorescence) to interpret the results of the antigen specificity of the response. Fluorescence intensity for each cell is shown by

a dot whose vertical and horizontal position indicates fluorescence intensity for each fluorochrome-tagged antibody. CD8 T cells use TCR to bind peptide on Class I MHC. If we put a fluorochrome like FITC on the tetramer MHC Class I or on the anti-idiotype and pass the cells through the flow cytometer, it will count the number of MHC-peptide-specific binding T cells. The binding molecules only attach to CD8 T cells so we don't also need to use anti-CD8, although we could do so (PE-anti-CD8) and count CD8+ cells that also bound antigen or anti-idiotype (Rowe, Abernathy et al. 1999). Higher mean fluorescence intensity indicates more specific MHC-peptide binding thus the flow cytometry would be a very capable test to measure T cell memory. This assay is generally carried out with populations of memory T cells where the frequency of specific cells has been increased by infection or immunization.

Criteria for a successful trial include a safety review board to monitor all procedures and methods to ensure the safety of the workforce and the patients. In addition the findings should be consistent, specific, and replicable in different localities that all lead to the same conclusion, a strong association between the dose-response relationships of the vaccine with the adverse effect. The association should be distinctive; the adverse event should be linked uniquely or specifically with the vaccine concerned, rather than it occurring frequently, spontaneously or commonly in association with other external stimuli or conditions. The association should be coherent and credible according to known facts of the disease. As of 2007, DNA vaccination is still experimental, but shows promising results. The DNA vaccine requires insertion and expression of viral or bacterial DNA into human cells triggering immune system recognition thus it should be strictly monitored and regulated in stages to eliminate unwanted consequences such as oncogenesis. In addition of an intranasal booster three to four weeks after the initial two dose intramuscular DNA vaccine, human sera levels should be monitored to ensure full immunity. The estimated duration of the study will be 150 days to allow the vaccine to be fully incorporated into the immune system. Before and after the vaccination we will record the clinical signs and symptoms of the healthy recipients to analyze and measure adaptive immunity and possible adverse side effects of the vaccine.

References

- Aubin, J. T., S. Azebi, et al. (2005). "Evolution of H5N1 avian influenza viruses in Asia." Emerging Infectious Diseases **11**(10): 1515-1521.
- Barouch, D. H., S. Santra, et al. (2000). "Control of Viremia and Prevention of Clinical AIDS in Rhesus Monkeys by Cytokine-Augmented DNA Vaccination." Science **290**(5491): 486-492.
- Belshe, R. B., F. K. Newman, et al. (2004). "Serum antibody responses after intradermal vaccination against influenza." New England Journal of Medicine **351**(22): 2286-2294.

- Council, H. S. (2006). "National Strategy for Pandemic Influenza." from <http://www.pandemicflu.gov/>.
- Crosby, A. W. (2003). America's forgotten pandemic : the influenza of 1918. Cambridge ; New York, Cambridge University Press.
- Davis, M. (2005). The Monster at our door : the global threat of avian flu. New York The New Press.
- Diseases, N. I. o. A. a. I. (2006). "Timeline of Human Flu Pandemics." Retrieved May 8, 2006, from <http://www3.niaid.nih.gov/news/focuson/flu/illustrations/timeline/>.
- Ebrahim, G. J. (2004). "Avian flu and influenza pandemics in human populations." J Trop Pediatr **50**(4): 192-4.
- Enserink, M. (2006). "Oseltamivir Becomes Plentiful--But Still Not Cheap." Science **312**(5772): 382-383.
- International, W. (2004). "Human Infections with Avian Human Infections with Avian Influenza Viruses and Influenza Viruses and Pandemic Potential Pandemic Potential." Retrieved February 18, 2006, from http://www.who.int/vaccine_research/about/en/.
- Katz, J. M., W. Lim, et al. (1999). "Antibody response in individuals infected with avian influenza A (H5N1) viruses and detection of anti-H5 antibody among household and social contacts." Journal of Infectious Diseases **180**(6): 1763-1770.
- Olsen, B., V. J. Munster, et al. (2006). "Global Patterns of Influenza A Virus in Wild Birds." Science **312**(5772): 384-388.
- Parham, P. (2005). Manipulation of Immune Response The Immune System Garland Science: 381-389.
- Rowe, T., R. A. Abernathy, et al. (1999). "Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays." Journal of Clinical Microbiology **37**(4): 937-943.
- Services, U. S. D. o. H. a. H. (2006). "Epidemiology and Prevention of Vaccine-Preventable Diseases." 9th. from http://www.cdc.gov/NIP/publications/pink/def_pink_full.htm.
- Skowronski, D. M., Y. Li, et al. (2007). "Protective measures and human antibody response during an avian influenza H7N3 outbreak in poultry in British Columbia, Canada." Canadian Medical Association Journal **176**(1): 47-53.
- Subbarao, K. and T. Joseph (2007). "Scientific barriers to developing vaccines against avian influenza viruses." Nat Rev Immunol **7**(4): 267-278.
- Thomas, J. K. and J. Noppenberger (2007). "Avian influenza: A review." American Journal of Health-System Pharmacy **64**(2): 149-165.
- Thomas, P. G., R. Keating, et al. (2006). "Cell-mediated protection in influenza infection." Emerging Infectious Diseases **12**(1): 48-54.
- Treanor, J. J., J. D. Campbell, et al. (2006). "Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine." New England Journal of Medicine **354**(13): 1343-1351.
- Van Riel, D., V. J. Munster, et al. (2006). "H5N1 virus attachment to lower respiratory tract." Science **312**(5772): 399-399.
- Wong, S. and K. Yuen (2006). "Avian influenza virus infections in humans." Chest **129**(1): 156-68.