

Multi-Epitope DNA Vaccine Provides Immunity Against H5N1 Influenza Virus in Humans

Introduction:

Influenza, commonly known as the “flu,” is a highly contagious, air-borne respiratory disease that is caused by the flu virus. Every year a mild form of the flu causes epidemics infecting 10-20% of the world’s population with up to 5 million serious illnesses and 500,000 deaths. Due to antigenic drift and shift, the flu virus can mutate and reassort its genes to resist neutralization from the host’s immune system. At times, new flu subtypes can emerge and can be so virulent that no immunity exists in the human population and pandemics can occur (Wang, et al., 2007).

Currently, the outbreak of the influenza A virus subtype, H5N1, is a future pandemic threat. This immensely lethal strain was responsible for the death of over 200 million birds in 10 countries in Asia and Europe (Jackson Allen) between late 2003 and early 2006. The continually expanding geographic distribution of avian influenza A (H5N1) indicate that greater amounts of human populations are at risk. At present, H5N1 can be transmitted bird to human, nonsustained human to human transmission, and possibly environment to human (NEJM 9-05). The fear is that H5N1 will achieve efficient human-to-human transmission allowing the virus to spread easily from person to person via respiratory droplets (Child). If the virus could be transmitted with ease through the human population, and the virus was to maintain the same high mortality rates as the current H5N1 virus, millions of people would be at risk due to their inability to make antibodies against it (Chest).

The first documented case of animal to human transmission occurred in Hong Kong in 1997. During this outbreak 18 cases of H5N1 human influenza were documented; 6 of the cases were fatal. The virus showed up again in Hong Kong in 2001 and 2002 in poultry, but there were no documented human cases.

In December of 2003, South Korea experienced an H5N1 outbreak among poultry, followed shortly thereafter by the largest known A/H5N1 poultry epidemic ever documented, with the disease infecting poultry in Vietnam, Japan, Thailand, Laos, Cambodia, China, Indonesia, and Malaysia. Poultry to human transmission caused 133 cases of human H5N1 infection throughout these countries and resulted in 68 deaths (Chest).

2004 brought about the first suspected case of direct human-to-human transmission in Thailand. An 11-year girl who became ill after being exposed to dying poultry is suspected of passing the virus to her mom. The mom lived in another province and became ill after caring for her sick daughter; the mother had no known exposure to infected poultry (Child). Since that episode, there have been several documented incidences of household clusters of infection. It was concluded that in these household cluster incidences, the virus was spread by means of intimate contact without

the use of precautions and no human-to-human transmission has been identified (NEJM 9-05).

Epidemiology:

There are three types of influenza viruses: A, B, and C. Influenza viruses B and C are normally found only in humans. The C type viruses cause mild disease and are not included in the annual flu vaccine. The B type viruses can cause severe disease and occasional epidemic due to antigenic drift in the strain; antigens of B type are the main component of the annual flu vaccine, as well as two influenza A subtypes: H1N1 and H3N2. All influenza A viruses originated in birds, although many influenza A subtypes are now common in humans. These subtypes are based on surface proteins: hemagglutinin (HA), of which there are 16 types, and Neuraminidase, containing nine types. The different arrangement of these surface proteins is the basis of naming influenza A subtypes, and confers varied virulence and host susceptibility (Jackson Allen).

Amongst the three flu virus family (A,B, and C), the FluA virus is the most virulent strain infecting birds, swine, and potentially humans; birds and swine can be reservoirs for the fluA virus strain and can increase persistence. Natural hosts for FluA viruses are wild or migratory birds. When wild birds come in contact with domesticated fowl, they can spread the virus to the flock exemplified by the H5N1 strain. Humans can then be accidental hosts to the virus when in close contact with infected poultry (Strauss, et al., 2002).

Pathogen Structure:

The type A influenza virus is an RNA virus that is further grouped into different subtypes based on the antigenic nature of the surface membrane glycoproteins: haemagglutinin (HA) and neuraminidase (NA) (Kristen). Their genome consists of eight single stranded RNAs with negative polarity. Most of the RNA molecules are single gene units that vary in size, ranging from 934 to 2341 nucleotides. The six largest RNA segments encode only one protein each, while the other two encode for two proteins each. They are referred to as minus-stranded RNA viruses, since they cannot be directly translated into protein, and thus, they have to be transcribed into plus-strand mRNAs (class notes). Furthermore, the RNAs are wound to a nucleoprotein center, forming a helical structure, surrounded by three polymerase proteins (Molecular Expressions).

The morphology of Influenza A viruses is not homogeneous and the filamentous-like structures that appear as a halo around the virus consist of the numerous HA and NA proteins found in the lipid bilayer. Hemagglutinin makes up approximately 80% of those proteins (Molecular Expressions). It is synthesized as a single peptide but it is found as a trimer in the envelope. This glycoprotein complex is cleaved into two subunits HA1 and HA2 to facilitate the fusion of the virus with the membrane of the cell upon infection (Chen). The other 20% correspond to the glycoprotein neuraminidase, which is involved in the release of newly synthesized viruses from the host cell. Moreover, the

ribonucleoprotein structures (RNP) described above are enclosed within an antigenic protein matrix called M1, which is chemically bounded to the RNPs (Molecular Expressions).

In general influenza viruses are thought to be very genetically unstable. One explanation for this is the segmentation of the genome into small RNA units, which make these viruses more prompt to genomic reassortment. During this process, genes from two distinct viral strains are exchanged upon co-infection of a host cell. This phenomenon, also known as antigenic shift, can result in the formation of new viral subtypes and create a H1N2 strain from reassortment of H1N1 and H2N2 viruses. Hence, antigenic drift gives rise to new viral subtypes. Another way of obtaining genetic variability is through antigenic drift, where point mutations within the coding regions of NA and HA proteins occur spontaneously and allow the virus to evade neutralization by the host's immune system (Unknown "General Information").

Disease Mechanism:

Infection occurs when there is transmission of respiratory droplets of one infected bird to another. Transmission from birds to human has also been found to occur. The virus binds to the cells of the respiratory epithelium. Attachment to cells occurs when the hemagglutinin subunits on the virion surface bind to the sialic acid on the surface of the cells. The virus enters the cell via endocytosis. The virus is placed in an intracellular vesicle called an endosome. The endosome membranes contain a proton pump that helps acidify the inside of the vesicle. The acidic environment promotes a conformational change of the cleaved hemagglutinin protein to promote fusion of the virion and endosome membranes. The HA₂ subunit unfolds to make a flexible spike that inserts itself into the endosomal membrane. The spike will then refold to fuse the endosomal and virion membranes together, therefore allowing the M2 protein in the matrix of the virion to pump protons inside the virus and release the ribonucleoprotein(RNP) portion into the cytosol of the infected cell (Strauss, et al., 2002).

The RNP is then transferred from the virus into the host cell nucleus via recognition of nuclear recognition sequences on the NP protein promoting mRNA synthesis. Protein NS1 then binds to capped cellular mRNAs that are in the nucleus and protein PB2 cleaves the cap by a process called "cap snatching." The snatched caps are used as primers for mRNA synthesis on each of the viral RNA segments. Synthesis is initiated by PB1 protein and ends at the sequence near the 5' end of each segment by PB1 and PA proteins. After mRNA synthesis, the mRNA is transported to the cytoplasm for viral protein synthesis (Strauss, et al., 2002).

Replication occurs when there is sufficient NP protein available. Nucleus mRNA synthesis stops and cRNA synthesis begins. cRNA copies the 5' terminal sequence that serves as a template for the synthesis of the progeny virus RNA segments. Replication of the viral segments produces intermediates used as templates to produce new copies of the viral RNA segments (Strauss, et al., 2002).

Newly synthesized hemagglutinin and neuraminidase proteins associate with the infected cell membrane. The nucleoprotein migrates back to the nucleus where it alters the direction of RNA synthesis and where it encapsidates newly synthesized vRNA molecules for transport to the cytoplasm. Budding of new virions occurs where concentrations of hemagglutinin and neuraminidase proteins are highest. RNP particles are included at random in the newly virions making only those with a complete set (about 3%) active in continuing the infection. Viral particles that adsorb onto the infected cell after budding are released by the action of neuraminidase. Infected host cells eventually die and infection spreads leading to possible death (Strauss, et al., 2002).

Clinical Manifestations:

Signs and Symptoms: After an incubation period of two to ten days, the majority of persons infected with H5N1 influenza experience fever and influenza-like illness with respiratory tract symptoms. Other presenting symptoms seen early in the course of the disease include diarrhea, vomiting, abdominal pain, pleuritic pain, and bleeding from the nose and gums. Upon clinical evaluation, almost all patients have pneumonia and radiographic abnormalities. Initial lab results typically show viral pneumonia with no underlying secondary bacterial infection. Other common laboratory findings include “leucopenia, particularly lymphopenia; mild to moderate thrombocytopenia; and slightly or moderately elevated aminotransferase levels (NEJM 9-05)”. Because the symptoms of A/H5N1 are not distinguishable from many other types of influenza, the only feature that raises the suspicion of avian influenza is recent contact with poultry (Chest).

Diagnosis: A throat swab or nasopharyngeal aspirate can be used for diagnosis by antigen detection or reverse transcription-PCR for influenza virus. Viral antigens can then be detected by means of immunofluorescence, enzyme immunoassays, or rapid immunochromatographic assays, but unfortunately these tests do not distinguish between human and avian viruses or their subtypes, and reliability for detecting H5N1 ranges from 33.3 to 85.7%. A definite diagnosis of influenza H5N1 is “a positive viral culture result from clinical specimens or demonstration of a fourfold rise in serum neutralizing antibody titer toward the presently circulating genotype of avian viruses” (Chest) which would then be confirmed by a recombinant H5 western blot test. These tests are neither rapid nor readily available, but will provide definitive diagnosis.

Population affected: The current disease predominantly affects children and younger adults. This is most likely due to the fact that in developing countries it is the responsibility of the children to care for the families’ poultry. However, if the virus were to reassort with a new HA and N combination, there may be little or no immunity among the human population. This problem could then be compounded if the virus were able to be transmitted efficiently from human to human.

Current Treatments/Vaccines:

Currently the only treatment for avian flu is the use of antiviral drugs—specifically oseltamivir, zanamivir, amantadine and rimantadine. However, resistance to these

medications is already becoming evident. Genetic sequencing has revealed cases in Vietnam and Thailand that are resistant to amantadine and rimantadine.

Given the highly pathogenic nature and emerging drug resistance of the virus, it would appear that the focus should be centered on prevention.

The most efficient method of preventing the spread of avian flu is vaccination. There are currently at least two types of vaccines in clinical studies- inactivated and DNA.

As of March 2005, the National Institute of Allergy and Infectious Disease (NIAID) granted contracts to two companies- Sanofi Pasteur and Chiron to provide vaccines made from the inactivated virus. For their part, Sanofi Pasteur's vaccine is using virus isolated from Southeast Asia in 2004. In January 2007, the Vaccine Research Center (VRC) of the NIAID announced that its DNA vaccine began human trials on December 21, 2006. The trial consists of 45 volunteers between the ages of 18 and 60. Of the volunteers, 30 will receive the vaccine over a 2-month period and will be followed for one year.

Vaccine Proposal

The common vaccines against Influenza viruses that have been developed are based on generating an antibody response through the use of surface, viral antigens. Theoretically, this is probably the most direct strategy in preventing infection, however, it has not been proven to be very efficient. Epitope-based humoral immunity relies on the fact that the viral strain used for the production of the vaccine has to be identical to the strain responsible for the infection of the host, thus requiring a high degree of specificity (Dominick et al, 2007). As previously discussed, Flu viruses are capable of antigenic drift and shift. Antigenic drift occurs when mutations in the flu virus alters the HA and NA sequences. Antigenic shift occurs when reassortment of two different flu RNA segments occurs creating a new flu virus strain resulting in various subtypes of HA(1-16) and NA(1-9 proteins). Due to the influenza virus susceptibility to antigenic shift and antigenic drift, creation of an effective vaccine is difficult because antibody response is highly specific and variation in the virus can make a vaccine ineffective. Particularly, HA and NA surface proteins have been exhaustively utilized in the creation of vaccines that could provide immunity to the H5N1 strain. Such vaccines have been tested in mice and they have been shown to elicit the appropriate IgA and IgG neutralizing antibodies. Although, the concentrations of such hemagglutination inhibition antibodies produced at the surface of the lung mucosa are found to be very low, virus replication is eradicated and thus, the infection is prevented (Shantha et al, 1999).

On the other hand, the potential of vaccines that emphasize the induction of a cellular immunity response rather than B-cell memory immunity has been neglected. Nevertheless, T-cell mediated response requires less antigen specificity due to the cross-reactive nature of CD4 and CD8 T cell receptors, which are able to recognize a variety of epitopes, even if mutations have occurred. In general, internal, viral proteins are good candidates for cellular immunity. Furthermore, they are not constrained to the interaction with antibodies, which makes them genetically more stable than surface proteins. Lastly, CD4+ helper T cells activated via a cellular response are capable of stimulating B cells

for the rapid production of antigen specific antibodies, thus, enhancing a humoral response (Dominick et al, 2007).

In order to ensure optimal efficacy of our vaccine through the stimulation of both humoral and cellular responses, a multi-epitope DNA vaccine is proposed. Gene sequences encoding HA and NA from two closely related H5N1 strains will be used. The most remarkable difference between the HA genes is the presence or absence of glycosylation sites. One clade circulates in Cambodia, Thailand and Vietnam and the second clade circulates in China, Indonesia, the Middle East, Europe, and Africa. Sub-clades of the second clade have also risen in countries of the Middle East, Europe, and Africa. Currently, vaccines targeting clade one uses reference virus Vietnam/1194/04 or Vietnam/1203/04 and vaccines targeting clade two use reference virus turkey/Turkey/1/05, Indonesia/CDC625/06, etc. By making a vaccine from clade one and two, this creates a more specific and therefore, effective means to combat genetic differences in flu strains that might arise through genetic shift or drift (Laddy, et al., 2007).

Additionally, DNA from SN1 gene will be incorporated in the vaccine construct in order to induce T-cell immunity. The NS1 gene product is involved in the replication of the virus inside the host, specifically on the regulation of the amount of mRNA coming out the nucleus. As it is transcribed, some will be loaded onto MHC I cells. Here, CD8+ T cells and NK cells will destroy some of the cells thereby activating these immune cell types. The SN1 sequence will be selected by looking at the most prevalent strains of H5N1 and constructing a phylogenetic tree for the NS1 gene. The lineages of NS1 that have acquired the most substitutions should be chosen for the vaccine type. Furthermore, it has being demonstrated that the SN1 has an antagonistic role to gamma interferon. Upon invasion of the Influenza virus, the host releases an IFN antiviral response, which is combated by the virus with the production of an anti-IFN protein. This opposing antiviral activity can be eliminated by altering the SN1 protein sequence. For instance, a research study has demonstrated that the N-terminal of the NS1 protein alone induces an intermediate IFN response in mice. Hence if only that part of the SN1 protein is included in the vaccine construct, a strong cellular response can be stimulated without compromising the IFN response of the host; in other words, our vaccine will be robust but attenuated.

Immune Response

To evaluate the potential of the H5N1 plasmids as DNA vaccines, plasmids carrying the three protein coding genes (H5, N1, and SN1) would be injected intramuscularly into participants, while another group of participants received the plasmid DNA without any inserts as a control. The use of multiple plasmids encoding for the three proteins was intended to provide greater protection than a single antigen. The more variable viral coating proteins (H5 and N1) are used to elicit an antibody response, while the more constant SN1 protein elicits a T-cell response. Intramuscular insertion was chosen over more esoteric methods such as nasal drops as such methods did not produce sufficient immune responses. Uptake by the cell allows incorporation of the

genes into the host cell DNA. The genes will then be expressed utilizing the host cell machinery; the antigen produced by gene expression elicits a quantifiable immune response (Whalen).

The very use of an injected DNA vaccine mimics an initial viral infection, as the injected plasmids require host cell machinery in order to be expressed. The immune response to the vaccine therefore mimics the actual infection of influenza H5N1.

Because of the methodology of DNA vaccines, no antigen is actually produced until the host cell expresses the transmitted DNA. Upon expression of the three protein-coding genes, the infected cell, sensing viral presence, begins to produce cytokines IFN-alpha and IFN-beta through a series of cascading signals. Induction of IFN-alpha and IFN-beta is also stimulated by the presence of viral RNA and DNA in the endosome through the action of Toll-like receptors (TLR). The production of these cytokines increases MHC class I expression and antigen presentation in all cells, as well as signals NK cells to kill 'virus infected' cells.

Dendritic cells that have taken up plasmids will process antigen and migrate to the secondary lymphoid tissue where they can present it to the T cells on their MHC class I and II. Naïve T-cells that interact with their specific antigen can then proliferate and differentiate: The CD8 T-cells bind antigen peptide on MHC class I while the CD4 T-cells bind antigen peptide on MHC class II. However, because of the large amounts of IFN-alpha and beta having been produced during the initial response, the majority of the T-cells will be CD8 cells, and therefore, when activated, will become cytotoxic T-cells. Upon activation, the Cytotoxic T-cells proliferate and differentiate, producing a clone of effector T-cells.

Vaccine Tests

To assess cellular and humoral immunity, FACS or cell flow cytometry and ELISA must be performed respectively. For the ELISA assay that detects humoral and antibody response, an RNA sequence complementary to the H5, N1, and NS1 gene products must be added to the well plates to prevent non-specific binding of antibody. Then the antigen must be bound to the anti-gene product antibody. A protein hapten attached to the antigen will then be bound by a second antibody labeled anti-hapten. This antibody will then be linked to an enzyme that will change a colorless precursor to a colored compound. This will signal to the researcher that the specific antigen being tested (H5, N1, or NS1) is present and is being bound by B cell antibody. For the NS1 system, B cells late in the infection will secrete primarily IgG, IgA and IgM antibodies. These antibodies will then be neutralizing for the NS1 proteins while promoting other effect or classes. As a consequence, if real influenza is administered, opsonizing as well as neutralizing antibodies should be made.

T cell function must also be estimated as an indicator of cellular immunity. To accomplish this feat, cell flow cytometry apparatus must be used. Markers bound to T cells such as CD4, CD8 and CD3 can be used to determine which population of cells has

matured to single positives from a pool of naïve T cells. H5, N1, and NS1 antigen are needed in all cases for T cell activation so the purpose of the cytometry machine would be to estimate the pool of functional adult T cells that have differentiated in response to each particular influenza antigen being tested. The T cells that have matured will fluoresce forming light intensity proportional to the area under the curve. In this way, T cell immunity will be determined as a consequence of vaccination because each mature T-cell bearing a specific receptor for the three types of antigens can be labeled with different fluorescence, allowing for easy quantification of T-cell response.

Current Influenza H5N1 strains are only capable of being transmitted between humans through close contact. The fear among infectious disease specialists is that the virus might undergo change through antigenic shift, antigenic drift, or reassortment, and allow the virus to be spread easily from person to person through respiratory droplets. If this were to happen, millions of people worldwide would be at risk, as we currently do not have the immune response capabilities to combat such an infection. Our design of a multi-epitope vaccine is a novel approach to a possible pandemic that would provide coverage at both the humoral and cellular level.

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