

**Comparison of Codon Optimized and Non-Optimized
Influenza HA DNA Vaccines**

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

Christopher Parker

January 11, 2006

APPROVED:

Shan Lu, M.D., Ph.D.
Department of Medicine
UMass Medical Center
Major Advisor

David Adams, Ph.D.
Dept of Biology and Biotech
WPI Project Advisor

ABSTRACT

An effective antibody response is essential for immunity against influenza (Flu) virus infection and is the primary goal for Flu vaccines. In this study, codon optimized and wild type DNA vaccines expressing hemmagglutinin (HA) antigens of human flu viruses A/H1N1/NewCal/20/99 (H1 serotype) were compared. Expression levels of HA antigens were increased in 293T cells transiently transfected with codon optimized as compare to the wild type HA gene construct. The codon optimized vaccine was able to elicit a greater immune response in rabbits and mice as measured by ELISA testing of the temporal response and end antibody titers.

TABLE OF CONTENTS

Signature Page.....	1
Abstract.....	2
Table of Contents.....	3
Acknowledgements.....	4
Background.....	5
Project Purpose.....	17
Methods.....	18
Results.....	26
Discussion	38
Works Cited	41

ACKNOWLEDGEMENTS

First and foremost I want to express my deep gratitude and respect to Shan Lu, M.D., Ph.D, Director of the Laboratory of Nucleic Acid Vaccines at the University of Massachusetts Medical School. Dr. Lu facilitated valuable and exciting learning experiences for me. His cutting edge instruction has given me a unique and solid foundation in the nascent technology of DNA vaccine research. I also extend thanks to every scientist at the Laboratory of Nucleic Acid Vaccines, especially Shixia Wang, Hong Cao, Scott Coley, Siyuan Shen, Xiaoyun Huang and Te Hui Chou. They extended their professional expertise, resources and support to me. In addition, I extend sincere appreciation to my WPI advisor, Professor Dave Adams, for guidance with this project and thesis preparation.

BACKGROUND

Influenza related sicknesses have become a global commonality during the winter months. The influenza virus belongs to the Orthomyxoviridae, enveloped viruses with a segmented single-stranded RNA genome (Palese and Garcia-Sastre, 2002). It is a highly contagious virus that attacks the upper respiratory tract and has plagued the world since nearly the beginning of written history. The “flu,” as it is commonly known, primarily causes achy muscles, fever and digestive system symptoms in humans, although it can cause death in people with complications in hosts with weakened immune systems (Langley and Faughnan, 2004). Some strains, however, cause global pandemics of catastrophic proportions such as the Spanish influenza of 1918-1919 during which up to 50 million of people were killed worldwide, regardless of the status of their immune system (Palese and Garcia-Sastre, 2002). According to the World Health Organization (WHO), we are due for another pandemic with a human adapted form of the avian flu as the leading potential strain. Global pandemics of the past reached all continents in 6-9 months, but with easier travel, the next pandemic could infect all continents in less than 3 months (WHO, 2005).

Virological Features of Influenza Virus

Types of Influenza Virus

There are three types of influenza currently in existence, types A, B and C. Types A and B are morphologically indistinguishable, while type C is differentiated by its glycoprotein spikes. The morphological differences will be discussed later. Infectiously,

type A can spread among both humans and animals, while type B affects only humans, and type C affects predominantly humans, but recently also infected swine in China. The type A virion is also described by the subtypes of hemagglutinin (HA protein) 15 subtypes, and neuraminidase (NA protein) 9 subtypes, protein spikes on the virus surface (Lamb and Krug, 1996; Murphy and Webster, 1996). For example, the current avian flu strain (H5N1), which currently is not readily transmittable to humans, is an influenza A virus with a type 5 HA protein with a type 1 NA protein. New influenza virus strains result primarily from antigenic changes of the hemagglutinin and neuraminidase proteins. Antigenic drift results from point mutations that occur during viral replication within a major serotype, and antigenic shift occurs when genes from animal influenza viruses are captured by the human virus via reassortment usually resulting in high mortality (Palese and Garcia-Sastrem, 2002).

Morphology of Influenza Viruses

The influenza virus is comprised of RNA, proteins, lipids and carbohydrates. The most striking features of the influenza virion are the mushroom shaped neuraminidase proteins and rod shaped hemagglutinin proteins (Figure 1). The NA and HA protein extend through the lipid bilayer and into the M₁ matrix protein. Types A, B and C also encode transmembrane proteins M₂, NB and CM₂ respectively. The proteins are used as ion channels. The outermost layer of the virus is a lipid bilayer, and under that is M₁ matrix protein. The matrix protein is thought to interact with the ribonucleoproteins (RNPs) in the nucleus of the virus (Lamb and Krug, 1996).

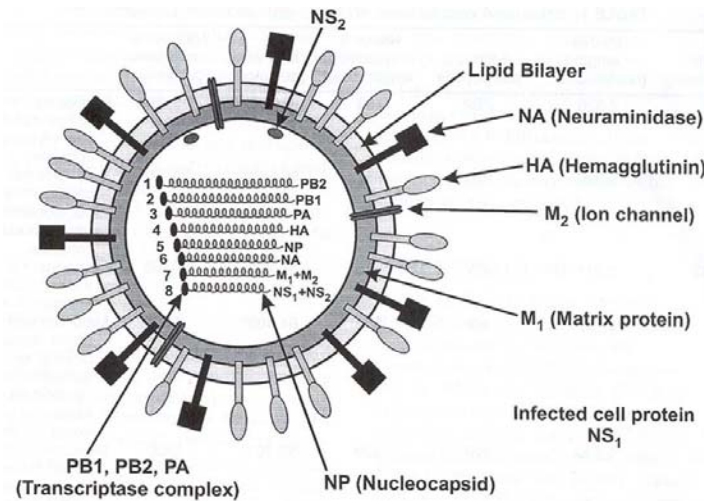


Figure 1: Morphology of the influenza virus. Note the Neuraminidase (black) and rod-like Hemagglutinin proteins (gray), the primary antigenic components of the virus.

The number of RNA segments in the types of virions differ; type A and B have eight strands, while type C has only seven strands. Type C lacks an RNA segment for NA, although the glycoprotein HEF mimics some of the missing NA functions which take place in type A and B viruses. Each of the eight strands has an associated transcriptase complex containing proteins PB1, PB2 and PA. Strand number one is longest, while strand number eight is shortest in length. The RNP is formed from the interaction of the nucleocapsid protein and an RNA strand (Lamb and Krug, 1996).

Protective Antigens

The hemagglutinin protein appears to be the most important surface protein. Its rod shape makes it ideal for penetration of the virus into the cytoplasm of the cell it is trying to infect. The penetration allows for the eventual release of the viral nucleocapsids into the cell (Lamb and Krug, 1996). Hemagglutinin is also the primary protein to which antibody responses are mounted against. The HA protein is expressed in the whole unit

form HA0 that is proteolytically cleaved into two subunits, HA1 and HA2, which are held together by a single disulfide bond. The HA1 subunit forms the orbicular head of the molecule, comprised of the receptor binding site and conformationally dependent epitopes to which neutralizing antibodies are primarily mounted against (Dowdle et al., 1974; Koopmans et al., 2004; Webster et al., 1975). The HA2 subunit anchors the molecule into the lipid membrane and is highly conserved among various influenza virus strains. Cleavage into HA1 and HA2 is required for the virus to become infectious making it a critical determinant in the ability of the virus to spread (Lamb and Krug, 1996).

Neuraminidase is mushroom shaped, dispersed throughout the membrane (Hay, 1974) and permits the transport of the virus through the mucus membrane of the respiratory tract allowing the virus to find its target epithelial cells. It is known that the NA polypeptide is not present in influenza type C viruses. This suggests that HA is involved in the budding process in viral replication. Some less common avian neuraminidases have a receptor binding site that causes hemagglutination (Murphy and Webster, 1996).

Replication

Entry of the virion into the target host cell is facilitated by the binding of the hemagglutinin spikes to the sialic acid of the target cell (Skehel and Wiley, 2000). This binding can be reversed by polysaccharide cleavage by neuraminidase spikes. It is advantageous for the virus to do this because it prevents virus from being concealed by inappropriate cell types or in mucus. Sialic acid is common on cell surfaces in mucus

and would neutralize the virus, rendering it useless. After binding, the cell engulfs the virus by endocytosis via endosomes. The vesicle is then acidified by the cell which allows for trypsin-like enzymes to cleave the HA (hemagglutinin) monomers. This process activates the membrane-fusion function and since the virus envelope and the endosome's membrane are so close to each other, they fuse together to form one membrane (Lamb and Krug, 1996).

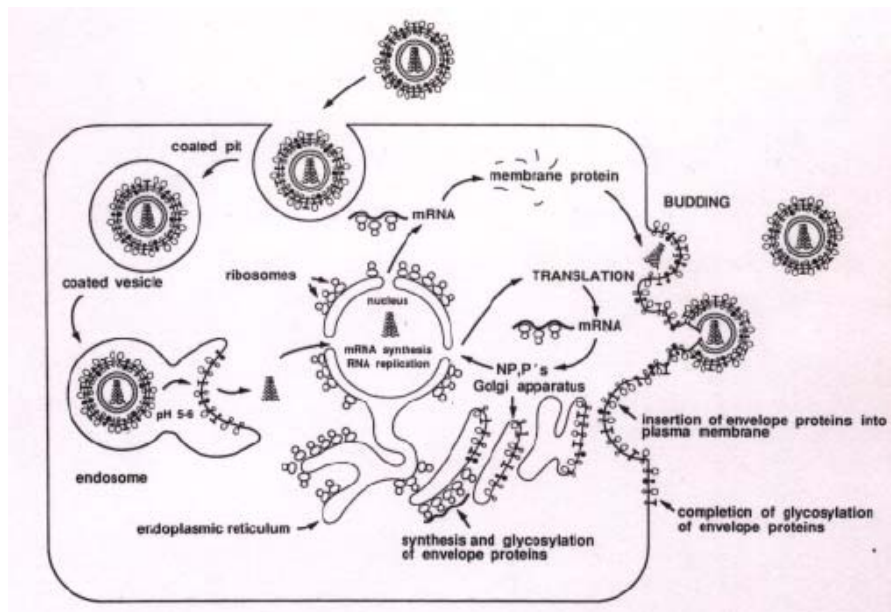


Figure 2: Influenza Virus Replication. The virus attaches itself to the outside of a host cell (upper part of diagram) and it enters the cell as a vesicle. The viral genes are transcribed and translated by the cell's enzymes and ribosomes. Now instead of producing only new cellular material, the cell produces many new virus particles. The new virus particles are eventually released via budding (diagram right side), and infect their own host cells. (Lamb and Krug, 1996)

Next, a target sequence begins in the nucleocapsid protein that results in passage of the nucleocapsid into the nucleus. When the virus first infects the cell, replication cannot occur because host cell DNA synthesis is required and virus replication is blocked by antibiotics like mitomycin C. Later, a protein is cleaved off of the RNA strand and a cap is exposed that acts as a primer. PB1 and PA complete the synthesis of the (+) sense

strand. There are two types of (+) sense RNA formed, incomplete which serve as mRNA, and cRNA which serve as templates for the synthesis of daughter (-) sense vRNAs. Most of the proteins created stay in the cytoplasm, but the NP protein drifts back to the nucleus where it interacts with the new vRNAs to form new nucleocapsids. Finally, the HA and NA proteins in the cytoplasm attach to a newly formed cell membrane which is exported from the host cell via budding (Murphy and Bang, 1952; Murphy and Webster, 1996).

Transmission and Prevention

Influenza virus is easily spread through the air when an infected person coughs or sneezes. The virus enters the body through the mouth or nose. It generally takes one to four days for a person to exhibit symptoms, and the person is infectious for up to seven days following the first signs of symptoms. The virus survives most easily in cold and dry weather, making the population most susceptible during the winter months (Center for Disease Control, 2005).

Prevention techniques include frequent hand washing, avoid touching eyes, nose and mouth, and coughing and sneezing into a tissue that is promptly thrown away. There are currently three antiviral drugs on the market (amantadine, rimantadine, and oseltamivir) approved for flu treatment and prevention. The foremost prevention measure is vaccination. There are currently two different methods of administering flu vaccines. The most common is made of an inactive type that is administered via intramuscular injection. The newest vaccine is administered through a nasal spray and is composed of live attenuated influenza virus (LAIV). Each year, a new vaccine is produced that contains three virus strains, two A strains and one B strain, strains

presumed to reach the United States during the next winter flu season. The attenuated version of the vaccine is still capable of replication so it is only recommended for healthy people ages five to forty-nine, while the inactivated virus vaccine can be given to anyone over the age of six months (CDC, 2005).

Current Influenza Vaccines

There are currently two different influenza vaccines used, inactivated and live attenuated viruses. The flu vaccines used in the United States are trivalent, consisting of three different strains forecasted to arrive in the U.S. by the World Health Organization. The strains present in the United States as of December 3, 2005 were two type A viruses H3N2 and H1N1, as well as a type B virus (CDC, 2005). The inactivated vaccines are grown in eggs, then purified and inactivated with chemicals to make it non-infectious. The effectiveness of the vaccine depends on the levels of similarity between the virus strains present in the population and the strains present in the vaccine, as well as the strength of a person's immune system and their age (Palese and Garcia-Sastre, 2002). The live attenuated vaccines are created from the same three strains predicted by the WHO. The vaccine is based upon a master strain which is grown in eggs at low temperature and over many growth cycles as a way to increase the antigen variability. The vaccine is administered via a nasal spray instead of a needle injection, but is only recommended for healthy people between the ages of 5-49 years old. The age restrictions are in place because the virus is only weakened, people with compromised immune systems because of age and/or disease can be susceptible to influenza viral replication and sickness (CDC, 2005). There are more vaccines in development such as a universal

flu vaccine based upon the antigenicity of the M2 protein (Fan et al., 2004; Neiryneck et al., 1999) and reverse genetics of the flu DNA plasmid (Palese and Garcia-Sastre, 2002).

DNA Vaccines

DNA vaccines are based upon the premise that only a portion of a virus' antigens delivered in the form of DNA is needed to mount a sufficient immune response to combat infection. Naked DNA is transfected into target cells and the cells produce the proteins. DNA vaccines are an effective way to create immunity because the antigens are produced within host cells and therefore are presented via both MHC-I and MHC-II pathways. DNA vaccines elicit a response in both the humoral (antibodies) and cellular (cell mediated) arms of the immune system (Lu et al., 1996; Lu et al., 1998; Robinson et al., 1999). Inactivated vaccines are not effective in generating strong immune responses, especially poor cell mediated immune responses (Johnson et al., 1999). Live attenuated vaccines are more effective but there is always safety concerns and people with compromised immune system can not use live attenuated vaccines.

The amount of DNA required to mount a sufficient immune response varies between inoculation methods although amounts are usually measured in micrograms. There are three most common methods of inoculation: particle bombardment (gene gun), intramuscular injection (i.m.) and intradermal injection (i.d.). In the current study, both the gene gun and intramuscular injections were used in the mouse study and only the gene gun was used in the rabbit study. There are advantages and disadvantages to both inoculation methods. Less DNA is required for gene gun inoculation than i.m. or i.d. injections, but i.m and i.d. may require more immunizations (Bohm et al., 1996; Hartikka et al., 1996). Intramuscular or intradermal injections are a variable technique because

experience and ability differs between researchers, whereas the gene gun method provides a more consistent result. The transfection efficiency of target cells is higher when using the gene gun, since the DNA is bombarded into the cells. The DNA dose required to elicit a good immune response in mice via i.m. injection ranges from 50 to 100 μg , while gene gun inoculation required only 6 μg at each immunization.

DNA is easy to engineer allowing for specific sequences to be designed as a way to increase immunogen variability. Different leader sequences, promoters and other subunits can be utilized to maximize the translational process as well as codon optimization which is discussed later. An example is the addition of a human tissue plasminogen activator (tPA) leader sequence which can accelerate the humoral antibody responses in rabbits against spike protein which is the protective antigen against the SARS associated coronavirus (Wang et al., 2005). Since only portions of viral DNA are used, there is no chance for subsequent infection after vaccination as there is when using live attenuated vaccines. Vaccines based on retroviral vectors always run the risk of becoming pathogenic or integrating into the host cell's genome with the possibility of carcinogenesis. However, adenoviral vectors are not pathogenic and are not integrative, but they do cause a strong immune response against the vector, hindering its effectiveness.

Influenza DNA Vaccines

Past DNA vaccines against the flu have yielded both positive and negative results. In a phase I clinical trial, Merck tested a DNA flu vaccine and assayed for cell mediated immunity. It had been hoped that cell mediated immunity (CMI) was the way to combat influenza (Donnelly et al., 1995), but the Merck trial failed to yield a detectable target

result for possibly two reasons: their assays were not effective enough at measuring CMI levels, or their DNA vaccine was not optimized enough via a method like codon optimization for example. On the other hand, it was demonstrated that there was complete protection from a lethal dose of influenza virus from as little as 1 µg DNA given in two doses (Deck et al., 1997). The DNA vaccines for influenza currently being developed in the laboratory of Shan Lu, University of Massachusetts Medical School, have shown in mouse and rabbit animal studies that a significant antibody response can be mounted (data in results).

An influenza DNA vaccine can be paired with other combinations of flu vaccines to create an increased immune response. It has been shown that a prime-boost method of immunization elicits a greater immune response as opposed to using one or the other modality alone (Richmond et al., 1998). Following this method, the DNA flu vaccine could be used as an immune primer, and an inactivated flu vaccine could be used to boost immunity. The inactivated flu vaccine does not create a lasting immune response unless there is a primer present. If the flu DNA vaccine is administered first then boosted with the inactivated vaccine, a smaller dose of the inactivated vaccine will be needed which hopefully could help prevent dose shortages as seen in the flu season of 2004.

Codon Optimization

Codon Optimization is performed as a way to increase protein expression in organisms by making the translation of a gene of interest more efficient from one organism to another, e.g. viral DNA sequence to be expressed in human cells. The amino acid sequence of the viral protein remains the same, while the triplet of base pairs (codon)

is altered to match the codon preferences in the new organism that are most frequent. Codons of low frequency in the original DNA are altered to codons of high frequency in the organism the DNA is being introduced to. It has been determined that there is a direct correlation between translational efficiency, which leads to high expression levels, and immunogenicity in host species. Nagata et al. (1999) showed that the amount of codon optimization of a gene directly correlated with the translational efficiency in mammalian cells.

The influenza A genome is codon biased meaning that some codons are more common than others, or that natural selection can distinguish between synonymous codons, or both even (Plotkin and Doshoff, 2003). The most commonly accepted explanation of codon bias states that codon appearance is optimized to match the amount of isoaccepting tRNAs available, allowing for an increased translational efficiency (Zuckerandl and Pauling, 1965; Ikemura, 1981). Other theories include that codon bias is a result of selection for regulatory function mediated by ribosome pausing (Lawrence and Hartl, 1991) or selection against codons that lead to early termination (Fitch, 1980; Modiano et al., 1981). Whichever the explanation may be, the determination of which codons occur most frequently can be used to increase the efficiency of mRNA translation. The codon usage is different from mutations at the epitopic regions of the HA1 antigen which has more frequent mutations than others (Plotkin and Doshoff, 2003).

André et al. (1998) created a HIV gp120 codon optimized DNA vaccine, syngp120, and performed cellular transfections and BALB/c mice studies. All wild type codons were replaced by codons found in human genes that are most highly expressed. The group found that codon optimized expression levels were significantly higher than

WT levels among various assays. In 293T cells, expression levels increased 10-50 fold in the optimized sequence, in BALB/c mice, the synthetic sequence produced greatly higher antibody titers from ELISA showing an increased humoral response.

Project Purpose

The purpose of this project was to use ELISAs and western blots to determine the immunogenic and expression differences respectively of wild type and codon optimized H1 influenza DNA vaccines. Mice, rabbits and 293T cells were used as model systems to fully understand and enumerate the ability to improve the immunogenicity under a variety of conditions. This research will provide useful information and lay groundwork for future experiments involving other influenza DNA vaccines as well as studies on B cell memory.

Materials and Methods

DNA Sequences

The influenza H1N1 strain A/H1N1/NewCal/20/99, GenBank accession number AJ344014, was used as a wild type baseline for experiments. Codon optimized constructs were created by Geneart (Regensburg, Germany) from the wt sequence into a G + C rich content found in humans. The gene inserts were ligated into vector pSW3891.

Recombinant DNA Techniques

Agarose Gel Electrophoresis

Agarose was dissolved in TAE buffer to a 1% (w/v) concentration. The mixture was then heated in a microwave oven until the agarose was completely dissolved. A Bio-Rad agarose gel casting system was used to pour the gel and after the agarose cooled down, samples were loaded with 6X loading buffer. The gel was run in a Bio-Rad gel box at approximately 50-70 Volts for one and a half to two hours. After that, the gel was stained in an Ethidium Bromide solution (25 ng/ml in TAE buffer) for 20-30 min. The stained gel was then photographed using an Olympus COMEDIA Master System 4.1™ digital camera under UV light.

Restriction Endonuclease Digestion

The final volume for restriction enzyme digestions was 15 µl for single digestions, and 20 µl for double digestions. The appropriate enzyme concentration and buffer, BSA, sterile H₂O, and DNA concentrations were estimated according to the New England

BioLabs catalogue. DNA was digested at 37°C for one and a half hours then analyzed on an agarose gel.

In vitro Expression of Influenza H1.HA0 Antigens

The expressions of H1 DNA vaccine constructs were examined by transient transfection of 293T cells (Pear, 1993). Transfection was performed when cells were at approximately 50% confluence on 100mm dishes by calcium phosphate coprecipitation, using 20 µg of plasmid DNA per dish. The supernatants and cell-lysates were harvested 72 h after transfection. The H1 protein expression was confirmed by Western blot. A quantitative enzyme-linked immunosorbent assay (ELISA) was used to measure the amounts of H1 produced from each DNA construct. A known amount of recombinant H1 protein was used to establish the standard curve.

DNA Vaccination Techniques

DNA Tubing Preparation

For all the procedures in this section, a Bio-Rad tubing prep station was used. Rislan tubing was dried for 1–2 hours by forcing N₂ at ~ 0.2 L/min into the tubing. The ends of the tubing were capped after drying to keep moisture out. Water was added to 1 µm gold beads (adjusted for each experiment) to achieve a final concentration of 100 mg/ml. The gold beads were centrifuged to the bottom of a microcentrifuge tube for ~10 seconds and the water was removed with a pipette, leaving a small amount of water (~50 µl) in the tube. A 100 µl aliquot of spermidine (100 mg/ml in water) was added to the gold bead pellet, and the mixture was vortexed thoroughly at high speed until the gold

was completely re-suspended. Plasmid DNA was added to the beads/spermidine suspension. The mixture was vortexed only briefly on high, as the DNA can shear easily. CaCl₂ (2.5 M in water) was added drop-by-drop into the mixture while vortexing at medium speed. The mixture was let stand at room temperature for 3-5 minutes to allow precipitation, and then centrifuged for 8 seconds, decanted and the supernatant was discarded. The coated gold beads were washed 5 times with 1ml dehydrated absolute ethanol. Each time, the ethanol-resuspended beads were centrifuged for 8-12 seconds and the supernatant removed. The beads were resuspended in the total volume of absolute ethanol needed for each preparation in a 20 ml glass scintillation vial and capped tightly. A piece of tubing 2 –3 inches beyond the right end of the tubing prep station was cut. A 10 ml syringe was attached to the right end of the cut tubing. The glass vial of gold beads was sonicated briefly to completely suspend the beads, and the suspension was drawn into the tubing with a syringe. The gold was allowed to settle out of the suspension in the tubing for 10 minutes. Then the ethanol was slowly drawn from the tubing with the syringe at a rate of about 2 inches per second. The tubing was connected to the nitrogen port and the prep station was turned on to rotate the tubing and spread the gold over the inside of the tubing for 1 minute. The nitrogen flow was turned on to 0.4 L/min to dry the remaining ethanol from the tubing for 5 minutes as it continued to rotate. The tubing was cut into half- inch cartridges using the tubing cutter, sealed in a scintillation vial with parafilm, and stored at –20°C.

Anesthesia, Immunization and Bleeding

Rabbits

A portion of skin was cleaned for the injection of anesthetic solution into the right

hind leg quadriceps or lumbar muscle of each animal. The anesthetic solution consisted of Ketamine (100 mg/ml)/Xylazine (100 mg/ml)/saline. The total volume of the anesthetic solution each animal received was calculated based on the animal's weight (see table V). Rabbits were then bled from the central ear vein. The backside of the ear was rubbed briskly with an alcohol prep. Then, using a 23-gauge butterfly needle, blood was removed with a syringe until the desired amount was collected. The blood was then transferred to a vacuum tube. The animal's abdomen was then shaved, and DNA-coated gold beads were inoculated to the shaved surface using the gene gun. Each animal received 36 µg of DNA per dose, and four doses were administered according to the schedule in table I .

Weight of rabbit (Kg)	Dose of mixed Ket/Xyl (ml)	Dosage/animal (mg)	
		Ketamine	Xylazine
2.0-2.5	0.60	50.0	10.0
2.5-3.0	0.65	54.2	10.8
3.0-3.5	0.70	58.3	11.7
3.5-4.0	0.75	62.5	12.5
4.0-4.5	0.80	66.7	13.3
4.5-5.0	0.90	75.0	15.0
5.0 & up	1.00	83.3	16.7

Table I: Dose and composition of the anesthetic solution injected to the rabbits

Mice

The abdominal fascia was rubbed with an alcohol prep, and 0.5-0.7 µl of anesthetic solution (Ketamine (100 mg/ml)/Xylazine (100 mg/ml)/Saline 4:1:10) was injected intraperitoneally using a tuberculin syringe. Once the animals were sedated, a glass capillary tube was used to draw blood from the periorbital cavity. The abdominal area was then shaved, and DNA-coated gold beads were inoculated using the gene gun. Each animal received 3 doses of 6 shots each according to the schedule in table IV.

*DNA Immunization
Mice*

Female Balb/C mice, 6–8 weeks old, were purchased from Taconic Farms (Germantown, NY) and housed in the animal facility managed by the Department of Animal Medicine at the University of Massachusetts Medical School in accordance with IACUC approved protocol. The animals received three monthly DNA immunizations by a Bio-Rad Helios gene gun (Bio-Rad, Hercules, CA). The original sequence or codon optimized H1-expressing DNA pSW-3891 plasmids were coated onto the 1.0 μm gold beads at 2 μg of DNA/mg of gold. Each shot delivered 1 μg of DNA and a total of six non-overlapping shots were delivered to each mouse at the shaved abdominal skin after animals were anesthetized. The serum samples were collected immediately before, and 2 weeks after each immunization.

Groups	# of Mice	DNA Vaccine
A	10	Saline I.M. (control)
B	10	wt I.M.
C	10	opt I.M.
D	10	wt G.G.
E	10	opt G.G.

Table II: Animal Groups and Immunizations Administered for the Mouse Experiment. The table shows the composition of the DNA vaccine used to inoculate the mice in each group. Each mouse was given 100 μg of DNA per immunization for I.M. injection while the gene gun mice received 6 μg of DNA per immunization.

Immunization	I	II	III	IV		V	
Bleed	I	II	III	IV	V	VI	VII
Week #	1	3	5	9	11	12	14

Table III: Immunization and Bleeding Schedule for the Mouse Experiment

Rabbits

Female New Zealand White rabbits weighing 2 kg were purchased from Millbrook Breeding Lab (Amherst, MA). Animals were housed in the facility managed by the Department of Animal Medicine at the University of Massachusetts Medical School following IACUC-approved protocols. Rabbits received four DNA immunizations by a Helios gene gun (Bio-Rad, Hercules, CA). A H1 original sequence or codon optimized DNA vaccine plasmid was coated onto the 1.0- μ m gold beads at 2 μ g of DNA/mg of gold so that each shot delivered 1 μ g of DNA. At each immunization, the rabbits were anesthetized, and a total of 36 nonoverlapping shots were delivered to the shaved abdomen.

Group	Rabbit #	DNA Vaccine
1	316-317	codon optimized
2	381-382	wt A/H1N1/NewCal/20/99

Table IV: Animal Groups and Immunizations Administered for the Rabbit Experiment. The table shows the composition of the DNA vaccine used to inoculate the rabbits in each group.

Immunization	I	II	III		IV		
Bleed	I	II	III	IV	V	VI	VII
Week #	1	3	5	7	9	11	13

Table V: Immunization and Bleeding Schedule for the Rabbit Experiments

Immunological Techniques

ELISA

Concanavalin A (ConA) Antigen Capture ELISA for Detection of Antibody

96-well flat bottom plates were coated with 100 μ l/well of Concanavalin A (ConA) at a concentration of 50 μ g/ml in PBS, pH 7.4, and incubated for 1 hour at room temperature (RT). The plates were washed with wash buffer 5 times: 3 times for 1 min, once for 15 min, and once fast. 100 μ l/well of PBS diluted H1.HA0.dTM antigen at a 1:10 or 1:5 dilution were added and allowed to incubate for 1 hour at room temperature. After that the plates were washed as above. Free ConA binding sites were blocked with 200 μ l/well of blocking buffer (5% non-fat dry milk in whey dilution buffer) and incubated overnight at 4°C. The plates were washed as above. 100 μ l of anti- H1 rabbit sera at 1:5000 dilution or mouse sera at 1:1000 dilution in whey dilution buffer were added to the wells, serial dilutions were performed, and the plates were allowed to incubate for 1 hour at RT. The plates were washed as before. 100 μ l of biotinylated anti-rabbit IgG at 1:5000 or anti-mouse at 1:1000 in whey dilution buffer were added to each well and allowed to incubate for 1 hour at RT. The plates were washed again. 100 μ l of streptavidin-conjugated horseradish peroxidase (HRPstreptavidin) at 1:10000 in whey dilution buffer were added to each well and allowed to incubate for 1 hour at RT. The plates were washed as above. Fresh TMB (1 TMB tablet/0.05 M phosphate/citrate buffer/0.006% H₂O₂) substrate was prepared and 100 μ l were added to each well allowing 3.5 min incubation time at RT. The reaction was stopped by adding 25 μ l of 2M H₂SO₄ to each well, and the OD was read at 450 nm using a Dynex Opsys MR™ plate reader.

SDS PAGE and Western Blots

A 10 % Acrylamide/bis-acrylamide minigel was run with the supernatant and or

lysate obtained from harvesting the transfected 293T cells, for approximately 1.5 hours at 50 mA. Before loading into the minigel, samples were mixed with loading buffer and boiled for 3-5 minutes. Using a semi-dry blotting apparatus, the protein was transferred to a PVDF membrane for 1.5 to 2 hours at approximately 80 mA per gel. The cut piece(s) of membrane was pre-treated by submerging it into methanol for 2 min, then it was transferred to ddH₂O and washed, and finally it was soaked in transfer buffer. A cut piece of 3MM Whatman™ paper (soaked in transfer buffer) was placed on the anode of the blotting apparatus then the membrane, then the gel, then another piece of Whatman™ paper. After the transfer was done, the membrane was incubated overnight in 10 ml of blocking solution (PBS, Tween-20 0.1-0.2% v/v, I-Block™ 0.1% w/v) at 4°C.

Antibody stocks were diluted to the desired concentration (1:200 for rabbit sera) in blocking solution. 5 ml of the diluted antibody were added to each membrane and they were allowed to incubate for 1 hour on the rotator at low speed. The membrane was then washed four times, 20 min each, with 20 ml of blocking solution, on the rotator at vigorous speed. The membrane was then incubated with the secondary antibody solution (diluted 1:5000 in blocking solution) for 1 hour, on the rotator at low speed. The membrane was then washed four times, for a total of 1.5 hours, with 20 ml of blocking solution, on the rotator at vigorous speed.

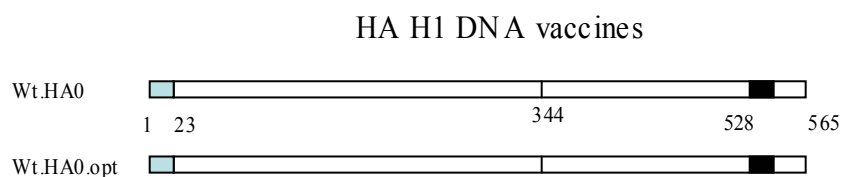
Chemiluminescence was performed using the Tropix Western Light™ protein detection kit. Two pieces of thin, transparent plastic, big enough to cover the membrane were cut. 1.5 ml of substrate was added per membrane. The “sandwich” was allowed to incubate for 5 min. The membrane was dried on a paper towel, and transferred to a new dry sandwich. This was exposed in the darkroom for variable times.

RESULTS

Codon Usage

Two different hemagglutinin based DNA vaccines were created to test variability in-vitro in 293T cells, and in-vivo in rabbit and mouse animal models. Both a wild type and codon optimized sequence were used (Figure 3A). Gene inserts were ligated into vector pSW3891 at BamH1 and Pst1 restriction enzyme sites (Figure 3B). The wild type sequence was codon optimized to increase the level of preferred codon usage in mammalian cells. The G + C content of the wild type gene was 41.24% while the codon optimized sequence was 60.13%, a dramatic improvement. A codon preference value of 1 indicates the same frequency of human preferred codons was used in this particular gene as would be expected from a human gene sequence. A value below 1 indicates a lower frequency usage of human preferred, G + C rich, codons. When a value greater than 1 is observed, it indicates a higher frequency of human preferred codons usage (Figure 4).

A



B

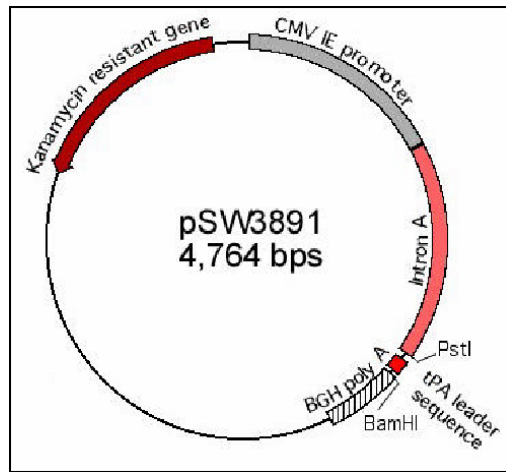
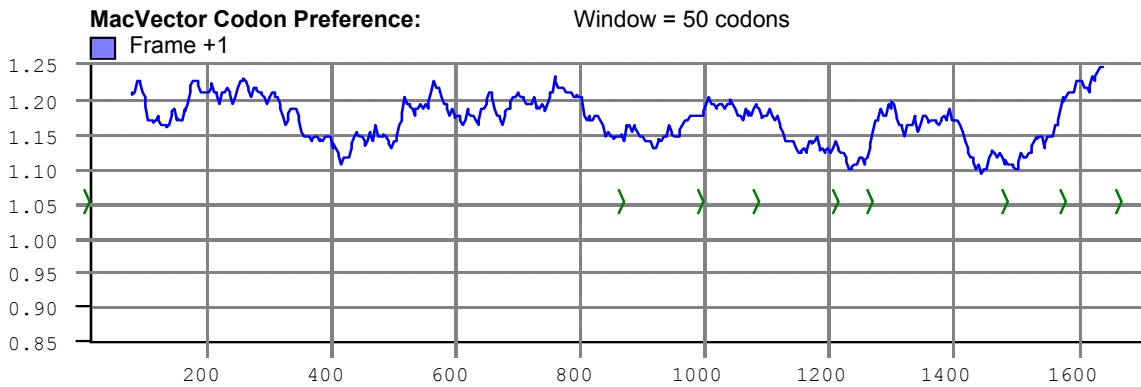


Figure 3: DNA Construct Inserts and Vector. Panel A: The wild type insert came from the influenza H1 strain A/H1N1/NewCal/20/99. The codon optimized insert was created with a greater G + C rich content to match human codon preferences. The blue box from 1-23bp is the HA natural leader sequence while the black box at 528bp location is the HA transmembrane domain. Panel B: Visualization of the pSW3891 vector to which the constructs were inserted between the PstI and BamHI restriction enzyme cut sites.

A



B

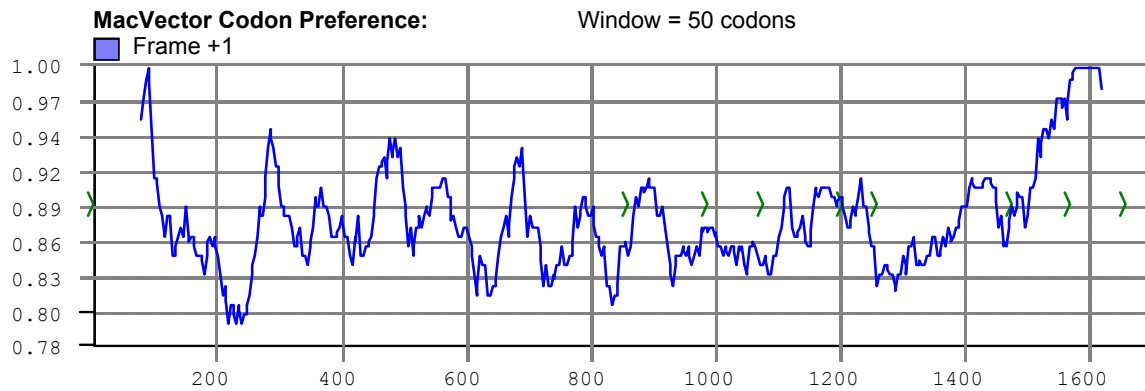


Figure 4: Influenza A H1 hemagglutinin (HA) gene codon preference. Sequences analyzed are optimized in Panel A versus the original sequence in Panel B and were those used in the study. Levels above 1.00 indicate strong codon preference for human environments and below 1.00 indicates the codons present are not preferred.

Protein Expression

In order to test expression levels of the optimized and wild type constructs, transient transfections and subsequent western blots were performed. 293T cells, a human embryonic kidney cell line, were inoculated with one of the two DNAs, and cell lysates and supernatants were collected. The cell lysates were used for western blot analysis. Antibody detection was performed using rabbit sera inoculated with H1-HA0.tPA.dTM. As seen in Figure 5, the codon optimized DNA sequence was expressed at a significantly higher level than the wild type sequence. At higher concentrations, the difference is not as noticeable so lower concentrations must be used to visualize the expression level difference.

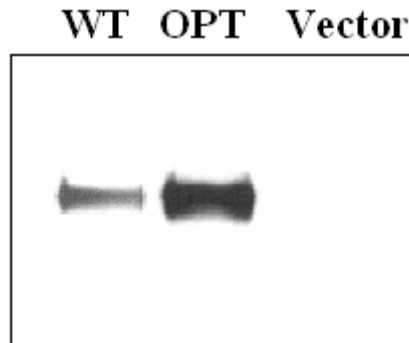


Figure 5: Western blot of wild type (WT) and codon optimized (OPT) H1 HA gene expressed in transfected 293T cells. A 0.1 $\mu\text{g}/\mu\text{l}$ transient transfection was used and a series dilution in loading buffer was performed on the H1 protein. A portion of the blot is shown as a representation of results. The pSW3891 vector was used as a negative control.

Immunogenicity

Animal Studies

Two animal models were chosen to test immune response. The mouse model used is more standardized and “genetically purer” than rabbits. Rabbits on the other hand allow for more sera to be harvested and their immune response more closely parallels a human response. The two specific animal models, Balb/c mice and White New Zealand rabbits were used because they are commonly used in vaccine studies and are highly documented in the literature. It is important to use multiple model systems in order to best qualify and quantify results.

Mice

Five groups of Balb/c mice were immunized as described in the methods. Two H1 DNA vaccines were tested, one codon optimized to a more G + C rich content and the other a non-optimized, wild type A/H1N1/NewCal/20/99. As a measure of H1 specific antibody response in mice after inoculation with the DNA vaccine, ELISAs were performed as a way to quantify the levels of antibodies. Each ELISA performed was

coated with the same H1.HA0.dTM antigen, although the antigen batch was changed on one occasion due to stock depletion.

In both the gene gun and intramuscular injection methods, the codon optimized sequences elicited higher antibody responses (Figure 6). The sera used were pooled from the 10 mice in each group. Overall, the gene gun method produced higher antibody levels. The gene gun (G.G.) immunized mice reached the linear phase sooner than the intramuscular (I.M.) mice because of the shortened lag phase associated with gene gun particle bombardment. It can also be seen that both codon optimized sequences fall off towards the final bleeding of this ELISA while the wild type sequences continue to grow (Figure 6).

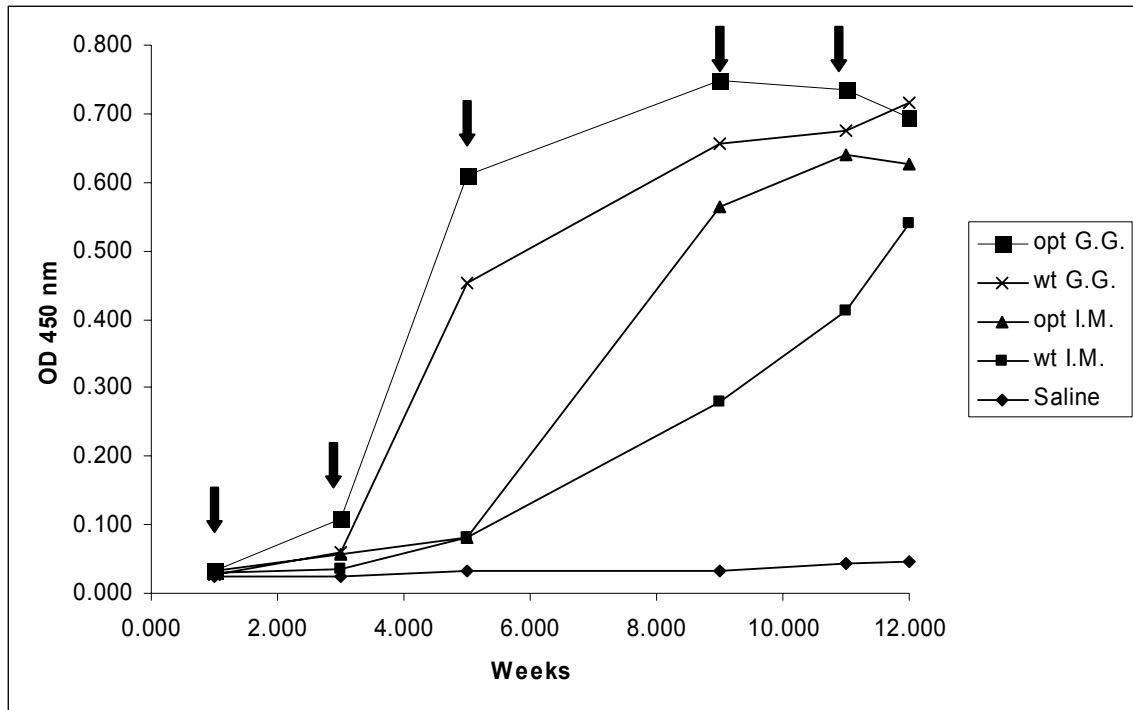


Figure 6: Temporal ELISA of mouse immune response to H1 DNA vaccine.

H1.HA0.dTM antigen was produced from DNA in 293 T cells, and used to coat ELISA wells to capture H1-specific IgG. Optimal results were seen from mice immunized with the codon optimized construct via gene gun. Samples used were pooled from the 10 mice in each group. Arrows indicate week at which an immunization was given.

A titration was performed to determine titer points as a way to quantify antibody levels (Figure 7). It is a method to assess whether an immune response to a specific antigen has occurred in immunized animals. As the serum is diluted, the optical density (OD) reading should continually decrease. Two primary results were expected and were found: 1. that the G.G. groups would have a higher titer than the I.M. groups, and 2. that the optimized groups would have a higher titer than the wild type groups. Both of these results were found and were quite distinguishable (Figure 7). A titer point is defined as the level at which during a titration, the OD value is twice the prebleed reading. A three fold series dilution was performed starting at an initial dilution of 1:300 in whey dilution buffer. The I.M. groups had a nine fold titer difference between the wild type and optimized groups while the G.G. groups had only a three fold difference (Figure 8).

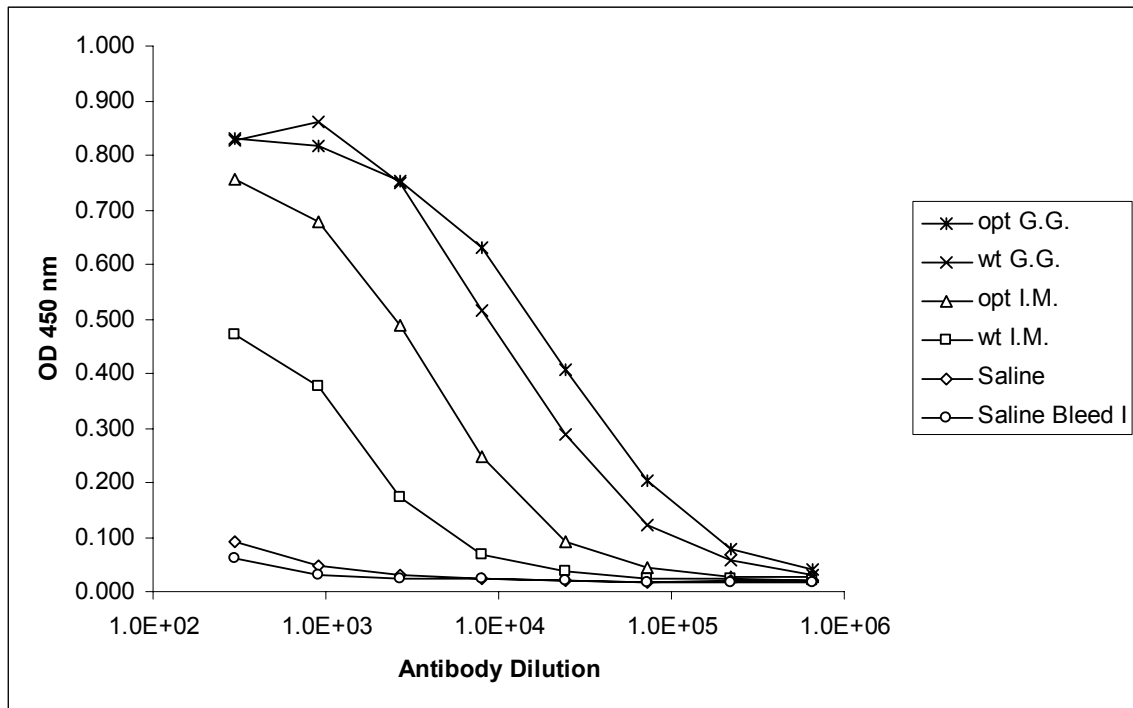


Figure 7: ELISA titration of mouse study pooled sera. Sera samples were from bleed V with a starting dilution of 1:300 then a 3x series dilution was performed. Gene gun (G.G.) immunization elicited a great immune response as compared to intramuscular

(I.M.) inoculation. The G.G. codon optimized sequence proved to be most immunogenic. Saline Bleed I was used as a pre-immunization control and background measure.

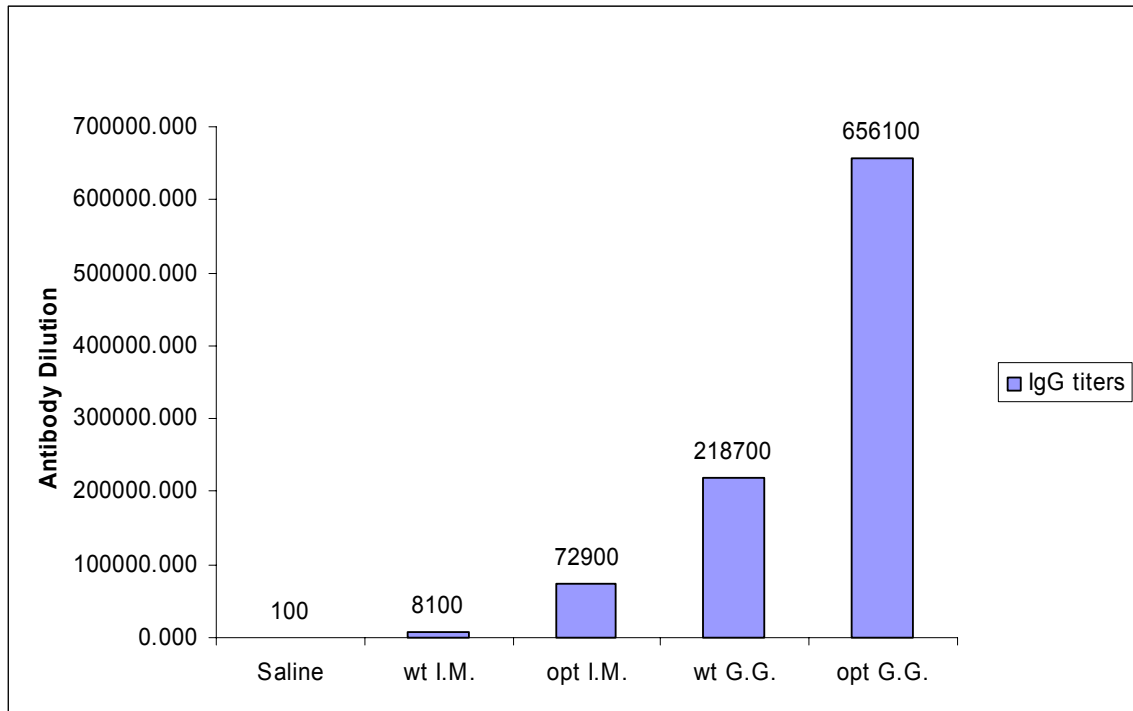
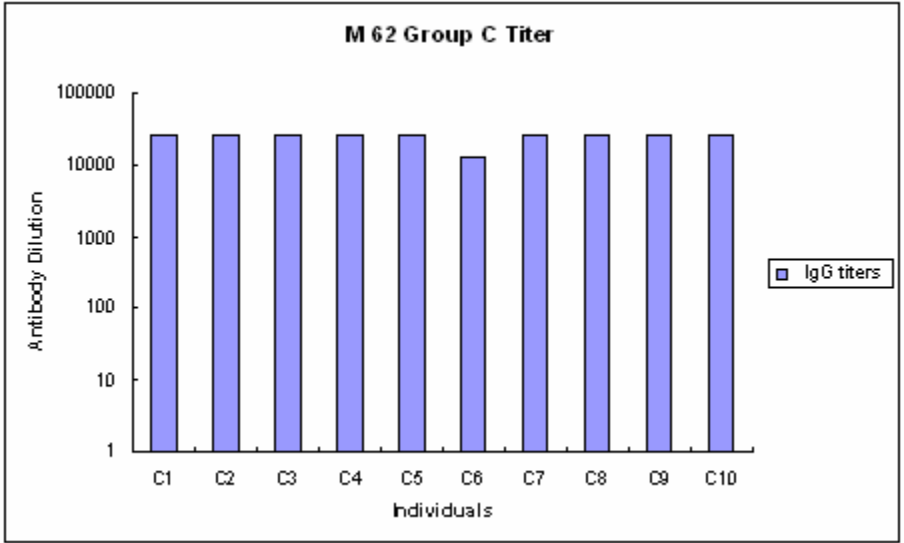
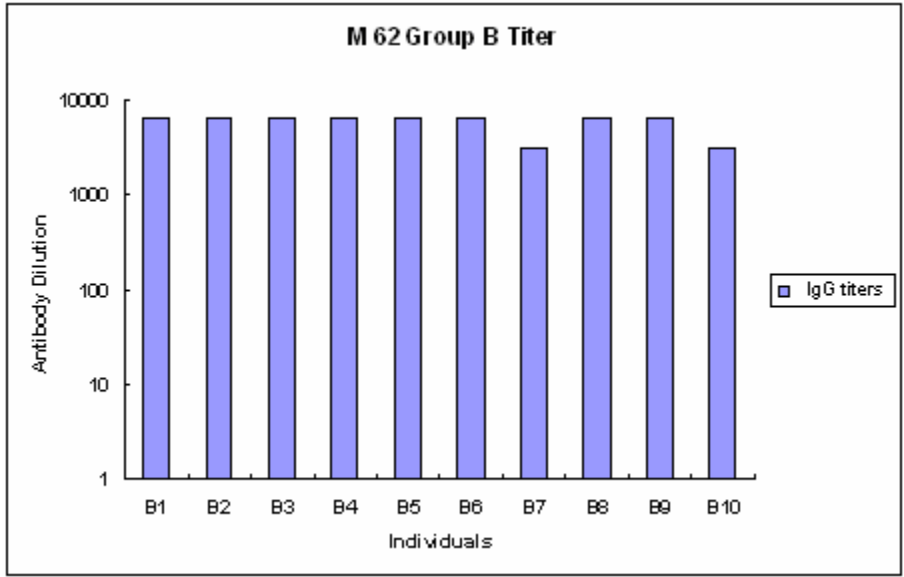


Figure 8: IgG titer points of mouse study pooled sera. Data based upon Figure 7. Highest points belonged to the codon optimized sequences and gene gun immunization.

When using pooled sera for ELISAs or other immunological assays, variability among individuals in a group is masked into an average. In order to show the true variation in titers among the ten mice in each group, a titration ELISA was performed and titer points are shown below (Figure 9). In each group, most mice had the same titers showing consistency in immunization technique, vaccine immunogenicity and animal health. Group B and C, immunized via the intramuscular injection method, showed lower titers as compared to groups D and E which were immunized with a gene gun. Also, the wild type groups, B and D showed lower titers than the codon optimized mice in groups C and E. Group E contains only nine animals (instead of ten) because one was prematurely terminated.



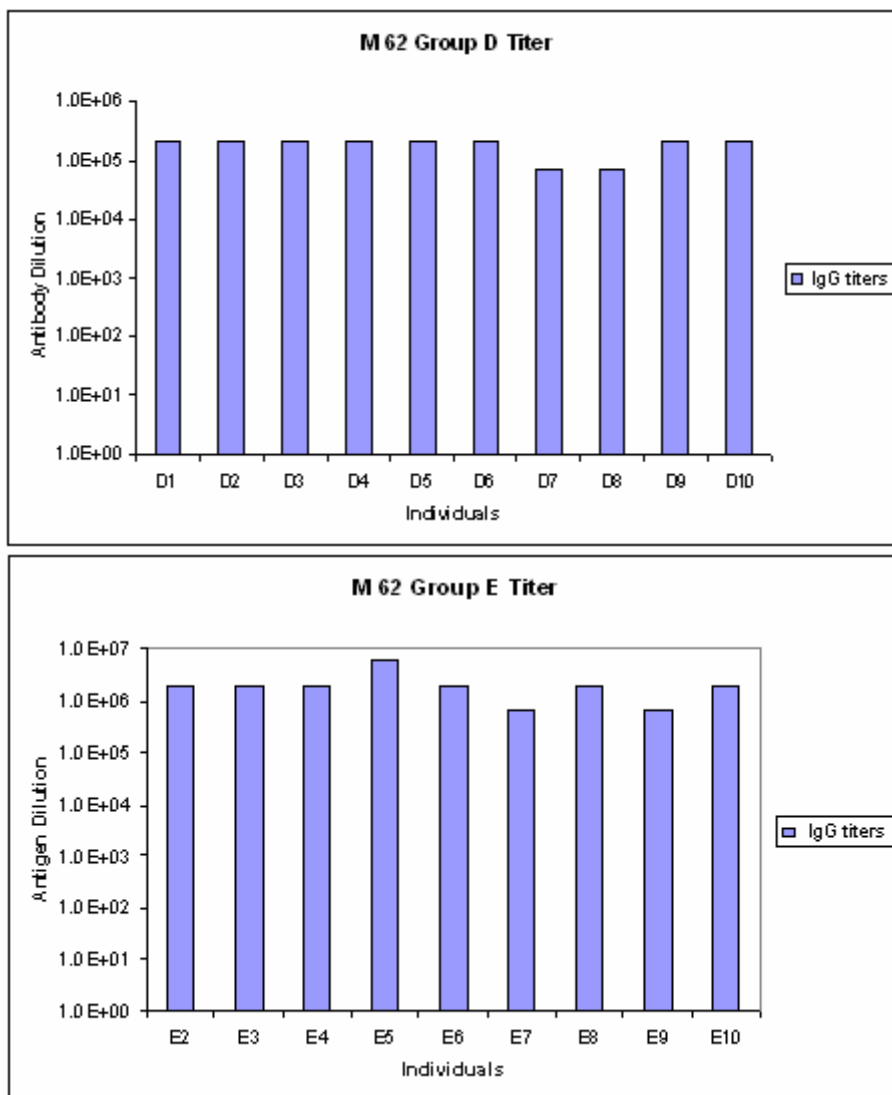


Figure 9: IgG titer points of mouse study individual mice. Variability among animals in each group was small. The highest points belonged to the codon optimized sequences (groups C and E) and gene gun immunization (groups D and E).

Rabbits

This experiment was designed as a way to compare codon optimized and wild type H1 gene sequences in rabbits. The two codon optimized construct rabbits, R # 316 and 317, and two wild type construct rabbits, # 381 and 382, were immunized via gene gun. Each rabbit was injected with 36 μ g of DNA. Immunizations were performed on weeks 1,3,5,9,14, and bleedings were performed on weeks 1,3,5,9,11,12,14,16. A time course plot was created from ELISA data using all the seven bleeds collected (Figure 10).

The drop in OD reading from week 7 to 9 was expected based upon the immunization schedule, Table V. The OD increased after week 9 because of the scheduled immunization. Codon optimized immunized rabbits showed a slight decrease in binding antibody from week 11 to 13, while in the wild type immunized rabbits, a much sharper decrease was noted over the same time period (Figure 10). This is because the codon optimized sequence elicits a higher immune response, creating more specific antibodies which will last longer in the animal's immune system.

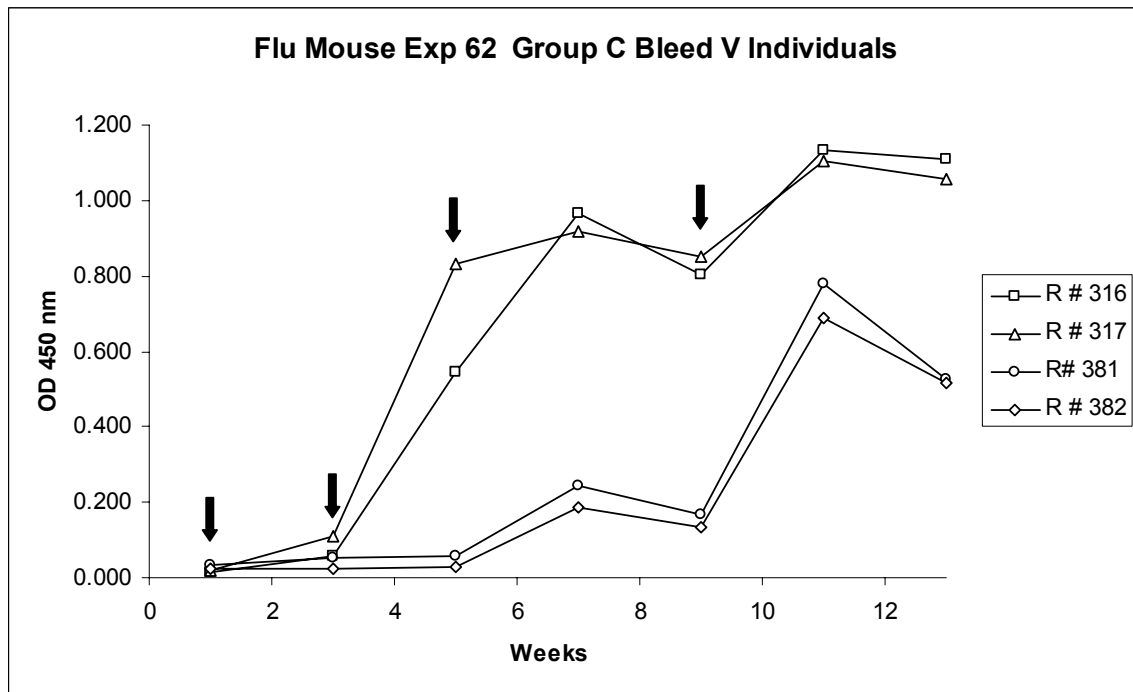


Figure 10: Temporal ELISA of rabbit immune response to H1 DNA vaccine. H1.HA0.dTM antigen were produced from DNA in 293 T cells. Rabbits # 316 and 317 were immunized with the optimized vaccine and rabbits # 381 and 382 were immunized with the wild type vaccine. Optimal results were seen in rabbits immunized with the H1 codon optimized vaccine. Arrows indicate week at which an immunization was given.

A titration was performed to observe titer points as seen in Figure 11. Rabbits immunized with the codon optimized sequence showed both higher initial OD readings (Figure 11), and higher titer points (Figure 12). The codon optimized animals reached a

9 fold higher titer point showing a much higher immunogenicity. Codon optimized animals reached a higher peak than wild type animals, and reached their peak at a quicker pace. This shows that not only can a higher antibody response be elicited using codon optimized DNA vaccines, but also that it is reached at a faster rate allowing for the potential of increased neutralization of the influenza virus.

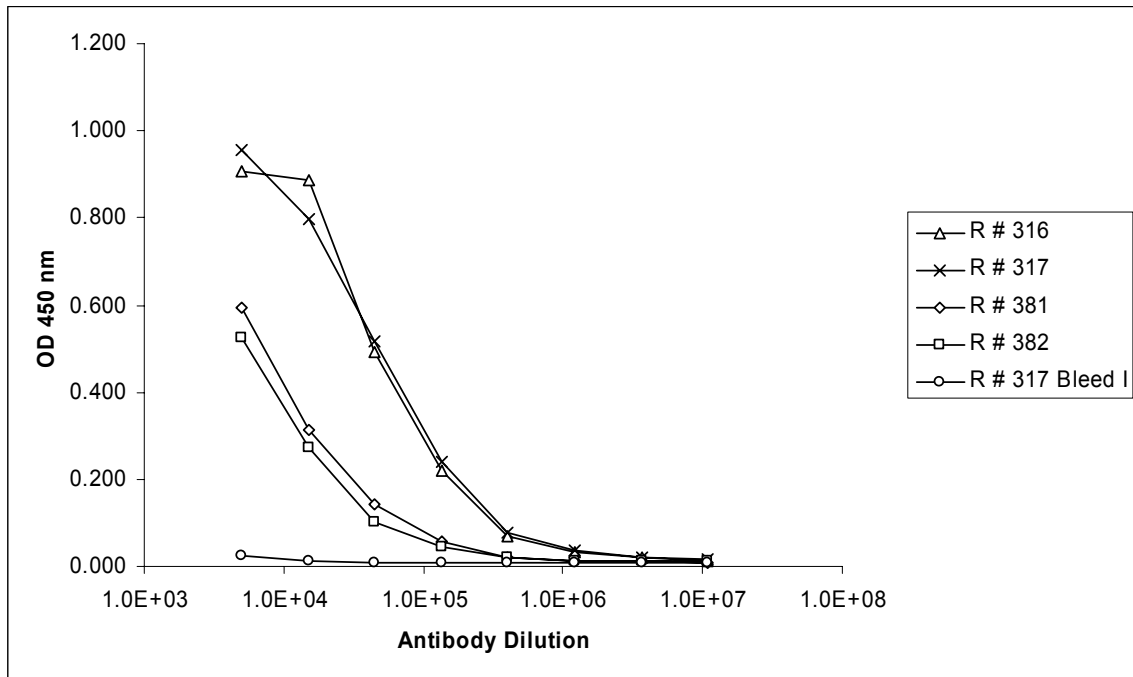


Figure 11: ELISA titration of rabbit sera. Sera sample were analyzed from bleed V with a starting dilution of 1:5000, then a 3x series dilution was performed. Rabbits R # 316 and 317 were immunized with codon optimized construct, while R # 381 and 382 were immunized with the wild type sequence. R # 317 Bleed I was used as a pre-immunization control and background measure.

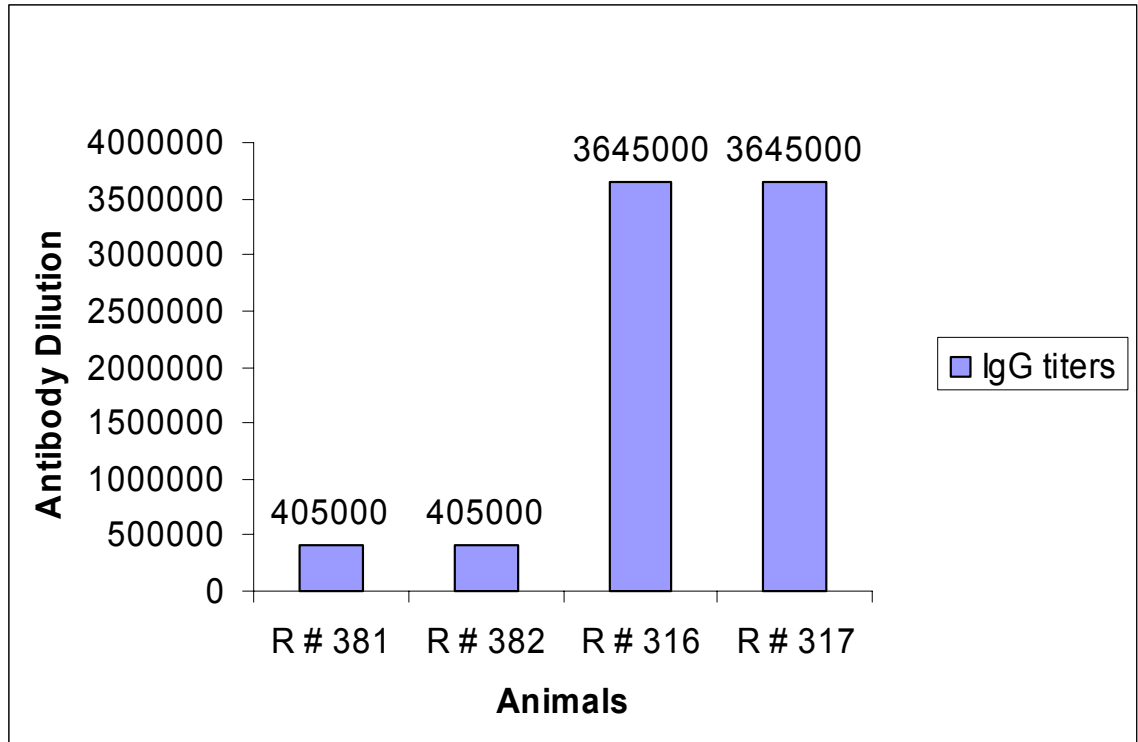


Figure 12: IgG titers of rabbit sera. Data based upon Figure 11. Codon optimized rabbits R # 316 and 317 had titer points 9 fold higher than wild type rabbits, R # 381 and 382.

DISCUSSION

The primary goal when creating a vaccine of any kind is to elicit the most antigen specific immune response. For DNA vaccines, this means optimizing the gene sequence to have the greatest translational efficiency inside the vaccinated animal or person. There are four primary steps for protein secretion: mRNA transcription from DNA, translation from mRNA to the amino acid sequence, protein modification on the endoplasmic reticulum, and lastly packing in the Golgi complex for exportation from the cell. Codon optimization, to a more G + C rich content, increases the mRNA to protein transition in mammalian cells increasing the exportation from the cell into its surroundings (Figure 5). In these in vitro experiments involving transfected 293T cells, the immunoblot membrane had to be exposed for a longer period of time in order to effectively visualize the weaker wild type expression levels.

Recently, codon optimization has become the method of choice for creating DNA vaccines, although it is a costly approach. There are still questions about codon optimized DNA vaccines which remain unanswered. First, it is not yet known to what extent codon optimization can improve already highly immunogenic DNA vaccines and some of these studies were performed with suboptimal vector components. Second, it isn't even known yet if codon optimized gene inserts require optimized vector components to reach optimal immunogenicity. Also, the mechanism to which codon optimization affects DNA vaccine immunogenicity is not well characterized and will need to be to create the safest and most effective influenza DNA vaccines. The addition of a tPA leader sequence would increase secretion levels of the protein, which when

coupled with codon optimization can increase the immunogenicity of DNA vaccines (Wang, 2005A).

Two primary conclusions can be reached from this study. The first is that codon optimization both increases expression (Figure 5) and elicits a higher immune response in animal models (Figures 8 and 12). The second is that gene gun immunization produces a greater immune response in animals as compared to intramuscular injection (Figure 6). This data supports reports by Andre et al. (1998) and shows that the result is the same for different proteins ranging from gp120 in HIV to HA in influenza. Andre et al. found a 10-50 fold higher expression level in 293T cells via western blot and in our studies, a relatively similar result was found (Figure 5).

In the influenza virus system, it has been reported that DNA vaccines stimulate a broader immunity than subunit or inactivated-virus vaccines (Donnelly et al., 1995; Montgomery et al., 1993). The introduction of cross-reactive immunity might be further augmented with a multigene DNA immunization strategy, which has been previously shown with malaria (Doolan et al., 1996) and mycoplasma (Barry et al., 1995). Future studies in the influenza project in the Nucleic Acid Vaccine Lab include multigene vaccines including H1, H3 and H5. An ongoing animal study is currently researching optimized H1 + H3 DNA vaccines. Various immunization schedules and procedures are being implemented, including the 'prime-boost' methodology, to find ways to create the greatest immune response.

The results of this Major Qualifying Project demonstrate the ability of modern science to create a vaccine which significantly increases the protective ability of "old science" vaccines. It is my hope that DNA vaccines will one day dominate the vaccine

industry because of both their efficacy and safety. Although DNA vaccine related immune responses may be most greatly increased with the prime-boost method (Richmond et al., 1998), not all people can be boosted with current flu vaccines, which would be used as boosts, because of egg allergies. In order to bypass this problem, future research must be completed as to prevent both seasonal flu illness as well as global pandemics.

REFERENCES

Andre S, Seed B, Eberle J, Schraut W, Bultmann A, Haas J. 1998 Increased immune response elicited by DNA vaccination with a synthetic gp120 sequence with optimized codon usage. *J Virol.* **72**(2):1497-503.

Barry, M. A., W. C. Lai, and S. A. Johnston. 1995. Protection against mycoplasma infection using expression-library immunization. *Nature* **377**:632-635

Bohm W, Kuhrober A, Paier T, Mertens T, Reimann J, Schirmbeck R. 1996 DNA vector constructs that prime hepatitis B surface antigen-specific cytotoxic T lymphocyte and antibody responses in mice after intramuscular injection. *J Immunol Methods.* **193**(1):29-40.

Center for Disease Control. Key Facts about Influenza and the Influenza Vaccine. <http://www.cdc.gov/flu/keyfacts.htm>. Updated September 28, 2005.

Deck, R.R., C.M. DeWitt, J.J. Donnelly, M.A. Liu and J.B. Ulmer. 1997 Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* **15**:71-78.

Donnelly, J. J., A. Friedman, D. Martinez, D. L. Montgomery, J. W. Shiver, S. L. Motzel, J. B. Ulmer, and M. A. Liu. 1995. Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. *Nat Med* **1**:583-7.

Doolan, D. L., M. Sedegah, R. C. Hedstrom, P. Hobart, Y. Charoenvit, and S. L. Hoffman. 1996. Circumventing genetic restriction of protection against malaria with multigene DNA immunization: CD8+ T cell-, interferon gamma-, and nitric oxide-dependent immunity. *J. Exp. Med.* **183**:1739-1746

Dowdle, W. R., J. C. Downie, and W. G. Laver. 1974. Inhibition of virus release by antibodies to surface antigens of influenza viruses. *J Virol* **13**:269-75.

Fan, J., X. Liang, M. S. Horton, H. C. Perry, M. P. Citron, G. J. Heidecker, T. M. Fu, J. Joyce, C. T. Przysiecki, P. M. Keller, V. M. Garsky, R. Ionescu, Y. Rippeon, L. Shi, M. A. Chastain, J. H. Condra, M. E. Davies, J. Liao, E. A. Emini, and J. W. Shiver. 2004. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. *Vaccine* **22**:2993-3003.

Fitch, W. M. 1980. Estimating the total number of nucleotide substitutions since the common ancestor of a pair of homologous genes: Comparison of several methods and three beta hemoglobin messenger RNA's. *J. Mol. Evol.* **16**:153-209.

Hartikka J, Sawdey M, Cornefert-Jensen F, Margalith M, Barnhart K, Nolasco M, Vahlsing HL, Meek J, Marquet M, Hobart P, Norman J, Manthorpe M. (1996). An improved plasmid DNA expression vector for direct injection into skeletal muscle. *Hum Gene Ther.* Jun 20;7(10):1205-17.

Hay, A. J. 1974. Studies on the formation of influenza virus envelope. *Virology* **60**:398-418.

Ikemura, T. 1981. Correlation between the abundance of Escherichia coli transfer RNAs and the occurrence of the respective codons in its protein genes *J. Mol. Biol.* **146**, 1–21

Johnson RP, Lifson JD, Czajak SC, Cole KS, Manson KH, Glickman R, Yang J, Montefiori DC, Montelaro R, Wyand MS, Desrosiers RC. 1999. Highly attenuated vaccine strains of simian immunodeficiency virus protect against vaginal challenge: inverse relationship of degree of protection with level of attenuation. *J Virol*, **73**:4952-4961.

Koopmans, M., B. Wilbrink, M. Conyn, G. Natrop, H. van der Nat, H. Vennema, A. Meijer, J. van Steenbergen, R. Fouchier, A. Osterhaus, and A. Bosman. 2004. Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. *Lancet* **363**:587-93.

Lamb, R.A. and Krug, R.M. Orthomyxoviridae: The Viruses and Their Replication. Fields Virology, Lippincott-Raven Publishers. (1996) p1353-1387.

Langley, Joanne M., Faughnan, Marie E., 2004. Prevention of influenza in the general population: recommendation statement from the Canadian Task Force on Preventive Health Care. *CMAJ* **171**: 1169-1170

Lawrence, J. G. & Hartl, D. L. 1991. Unusual codon bias occurring within insertion sequences in *Escherichia coli*. *Genetica* **84**:23–29.

Lu, S., J. Arthos, D. C. Montefiori, Y. Yasutomi, K. Manson, F. Mustafa, E. Johnson, J. C. Santoro, J. Wissink, J. I. Mullins, J. R. Haynes, N. L. Letvin, M. Wyand, and H. L. Robinson. 1996. Simian immunodeficiency virus DNA vaccine trial in macaques. *J Virol* **70**:3978-91.

Lu, S., R. Wyatt, J. F. Richmond, F. Mustafa, S. Wang, J. Weng, D. C. Montefiori, J. Sodroski, and H. L. Robinson. 1998. Immunogenicity of DNA vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with and without deletions in the V1/2 and V3 regions. *AIDS Res Hum Retroviruses* **14**:151-5.

Modiano, G., Battistuzzi, G. and Motulsky, A. G. 1981. Nonrandom patterns of codon usage and of nucleotide substitutions in human alpha- and beta-globin genes: an evolutionary strategy reducing the rate of mutations with drastic effects? *Proc. Natl. Acad. Sci. USA* **78**:1110–1114.

Montgomery, D. L., J. W. Shiver, K. R. Leander, H. C. Perry, A. Friedman, D. Martinez, J. B. Ulmer, J. J. Donnelly, and M. A. Liu. 1993. Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. *DNA Cell Biol.* **12**:785-789

Murphy, B.R., and Webster, R.G. Orthomyxoviruses. Fields Virology, Lippincott-Raven Publishers. (1996) p1397-1433.

Murphy, J. S., and F. B. Bang. 1952. Observations with the electron microscope on cells of the chick chorio-allantoic membrane infected with influenza virus. *J. Exp. Med.* **95**:259-267.

Nagata, T., Uchijima, M., Yoshida, A., Kawashima, M., Koide, Y. 1999. Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: Analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. *Biochem. and Biophys. Res. Comm.* **261**:445-451

Neiryneck, S., T. Deroo, X. Saelens, P. Vanlandschoot, W. M. Jou, and W. Fiers. 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. *Nat Med* **5**:1157-63.

Palese, P., and A. Garcia-Sastre. 2002. Influenza vaccines: present and future. *J Clin Invest* **110**:9-13.

Pear, WS., Nolan, G.P, Scott, ML., Baltimore D. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* **18**:8392-6.

Plotkin, JB, Doshoff, J. 2003. Codon bias and frequency-dependent selection on the hemagglutinin epitopes of influenza A virus. *Proc Natl Acad Sci USA* **100**:7152-7157

Richmond, J. F., S. Lu, J. C. Santoro, J. Weng, S. L. Hu, D. C. Montefiori, and H. L. Robinson. 1998. Studies of the neutralizing activity and avidity of anti-human immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein boosting. *J Virol* **72**:9092-100.

Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Manson, M. L. Kalish, J. D. Lifson, T. A. Rizvi, S. Lu, S. L. Hu, G. P. Mazzara, D. L. Panicali, J. G. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand, and H. M. McClure. 1999. Neutralizing antibody-independent containment of immunodeficiency virus challenges by DNA priming and recombinant pox virus booster immunizations. *Nat Med* **5**:526-34.

Skehel, J. J., and D. C. Wiley. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* **69**:531-69.

Wang, S., Farfan-Arribas, D.J., Shen, S., T.H. Chou, Hirsch, A., Feng, H., Lu, S. 2005A. Relative contributions of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of HIV-1 Env DNA vaccine. *Vaccine*: In Press.

Wang, S., T. H. Chou, P. V. Sakhatsky, S. Huang, J. M. Lawrence, H. Cao, X. Huang, and S. Lu. 2005. Identification of two neutralizing regions on the severe acute respiratory syndrome coronavirus spike glycoprotein produced from the Mammalian expression system. *J Virol* **79**:1906-10.

Webster, R. G., W. G. Laver, and B. Tumova. 1975. Studies on the origin of pandemic influenza viruses V. Persistence of Asian influenza virus hemagglutinin (H2) antigen in nature. *Virology* **67**:534-43.

World Health Organization. Ten things you need to know about pandemic influenza. <http://www.who.int/csr/disease/influenza/pandemic10things/en/> Updated October 14, 2005

Zuckerlandl, E. & Pauling, L. 1965. Molecules as documents of evolutionary history *J. Theor. Biol.* **8**:357–366.