MQP-BIO-DSA-9759

DESIGN OF VIRAL VECTORS FOR EXPRESSING INFLUENZA PROTEINS

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

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January 14, 2011

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ABSTRACT

Influenza virus is a major cause of human illness and death, and as such requires tremendous attention. Current vaccine research shows that adult human sera have influenza virus-specific antibodies which can mediate the complement-dependent lysis (CDL) of viral infected cells, and these antibodies may be more cross-reactive than conventional neutralizing antibodies so they might serve as a broad vaccine against multiple types of influenza. This project focused on the development of a protein expression system that would allow human A549 epithelial cells to express individual influenza proteins that could be used in analyzing the specificity of CDL antibodies. The results show that a lentiviral expression system is not the best method of expression for this particular project. Out of all the hemagglutinin protein genes that were transfected, only H3 protein expression was confirmed by immunoblotting. Several influenza gene constructs were then successfully constructed in preparation for future vaccinia virus expression experiments.

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ACKNOWLEDGEMENTS

This project was completed in the laboratory of Dr. Francis Ennis at the Center for Infectious Disease and Vaccine Research (CIDVR) at the University of Massachusetts Medical School. I would like to thank Masanori Terajima for serving as my Major Advisor, and giving me the opportunity to work on this project. His advice and knowledge provided a strong learning environment that I will always be grateful for. I would also like to thank Anita Leporati for all of her assistance and expertise; her help was crucial in the completion of the project. Thanks to John Cruz for providing the influenza strains for my project. I also appreciate the help of Jenny Babon and the rest of the staff at CIDVR. Lastly, I would like to thank Dave Adams for guiding me not only through this project but in my school career as well; without his help I never would have made it this far.

BACKGROUND

Influenza

Influenza, or the flu, is a serious respiratory illness caused by the influenza virus. This contagious disease can range from mild to severe, and can even cause death especially in the very young or old. Several influenza outbreaks throughout human history were especially devastating, with the Spanish flu being the worst. In 1918, Type A H1N1 influenza, "Spanish flu", infected approximately one third of the world's population, killing about 50 million. It has been assumed that this was the ancestor to all other Type A infections since, however the pathogenicity has never been as extreme (Taubenberger and Morens, 2006, pg 15-22). Since 1918, there have been other serious outbreaks of influenza: Asian, Hong Kong, Russian, and most recently H1N1 "Swine flu". The H1N1 swine flu of 2009 was quite different from previous H1N1 variants, and caused the first pandemic since the mid 20th century, with about 18,000 deaths worldwide (H1N1, 2010).

It is estimated that 5% to 20% of the population in the United States becomes infected with the flu virus annually and that roughly three million people are infected worldwide (Influenza, 2010). The number of deaths caused by influenza varies from year to year, but according to the Centers for Disease Control and Prevention anywhere from 3,000 to 49,000 people worldwide will die from flu related incidences annually. Anybody can become infected, but the most susceptible are the very young and the elderly; these age groups are also the ones who suffer the greatest risk of death due to infection; about 90% of flu related deaths occur in people 65 years and older (Seasonal Influenza, 2010).

Influenza viruses infect the respiratory tract, and due to the target of infection it is best to notice signs early before complications develop. There are many symptoms related to the flu, but not all symptoms are present during infection. The symptoms may include fever and/or chills, cough, sore throat, headaches, body aches, runny/stuff nose, fatigue, and vomiting/diarrhea. The flu usually lasts from a couple of days to 2 weeks, and most recover without serious issue. Some people however develop other complications, such as pneumonia and bronchitis, which can increase the chance of death.

Influenza Virus

Nomenclature

The influenza virus is classified into three main types: A, B, and C, based on the type of nucleocapsid found within virus. Viral types A and B are responsible for the annual epidemics that occur each winter, while Type C only causes mild illness and is not believed to cause an epidemic. Type A is further divided into *subtypes* based on the multiple combinations of viral surface proteins hemagglutinin and neuraminidase (Seasonal Influenza, 2010). According to the World Health Organization, the current subtypes affecting humans are influenza A-(H1N1) and A-(H3N2). Type C influenza happens at a lower frequency than types A and B, and is thus not normally included in vaccines.

There are 16 hemagglutinin (HA) subtypes, three of which are found in human infections (Kimball, 2010). The hemagglutinin protein provides the flu virus with the ability to agglutinate red blood cells and allows virus binding to sialic acid residues present on the surface of host epithelial cells. This action allows viral entry into host cells (Staley et al., 2007, pg 949-952).

There are also 9 neuraminidase (NA) subtypes, with only two found in infecting viruses (Kimball, 2010). Neuraminidase glycoproteins allow newly synthesized virus to be released from the cell to spread infection (Goodsell, 2010). NA protein accomplishes this by cleaving the sialic acid groups that were originally used during attachment of the virus. It is also assumed that NA plays a role in preventing self-aggregation, which also lets virus export infected cells to infect other areas (Lamb and Choppin, 1983, pg 467-506).

It is the combination of these two HA and NA glycoproteins that give the influenza strain names such as H1N1 or H3N2. The different combinations allow for infection in specific species, for example H5 is known to mostly infect avian species, though there has been a few cases reported in humans (Seasonal Influenza, 2010). This species-specific attack occurs due to the structure of the HA and NA proteins which are tuned to binding species-specific proteins on the cell surface (Squires and Scheuermann, 2006).

Influenza Viral Structure

Influenza is an orthomyxovirus with a fairly simple structure. It is an envelope type virus, containing an RNA genome surrounded by a matrix protein, a lipid bilayer, and glycoproteins on the surface (Staley et al., 2007, pg 949-952). The virions are roughly 80-120 nm in diameter, usually taking a spherical or pleomorphic morphology (ICTV, 2006). The lipid bilayer derives from the host cell's plasma membrane. Within this membrane is another layer made from viral membrane protein M1 (Lamb and Choppin, 1983, pg 467-506). After infection, an ion channel is formed in the viral envelop by the M2 protein, which allows hydrogen to enter the cells and alter the pH. The pH change is related to virus' ability to release RNPs and replicate.

The genome of the influenza A virus is about 14kb long and made of eight segmented, linear negative-sense single stranded RNA; the segment sizes range from approximately 3000 to 1000 nucleotides. There are seven structural proteins encoded by the RNA: PB1, PB2, PA, HA, NA, NP, and M1. There are also three nonstructural proteins found only in infected cells: NS1, NS2, and M2 (Lamb and Choppin, 1983, pg 467-506). Each RNA segment lies in its own nucleocapsid, which is then surrounded by an envelope; these structures are referred to as ribonucleoproteins (RNPs) (ICTV, 2006). The segmented genome allows for the re-assortment of RNA genes when multiple viruses have infected a cell; this antigenic shift can be very dangerous because the newly altered surface proteins are not targets of the immune system or vaccines and can lead to pandemics (Staley et al., 2007, pg 949-952).

Influenza Viral Infection and Replication

Once the influenza virus has entered its human host, the HA on the viral surface attaches to the sialic acid portion of host cells in the upper respiratory tract; the virus then enters the cells through endocytosis and releases its genome into the cell. The viral genomic RNPs are then transported into the nucleus by host machinery. At this point, proteins PB1, PB2, and PA combine to form the viral polymerase, and along with NP the viral RNA is synthesized into mRNA and cRNA; the polymerase is crucial in this process because it copies the viral genome and directs the production of its proteins (Squires and Scheuermann, 2006). The cell's host machinery is again used during translation of the viral genome; while at the same time normal host cell activity is being prohibited. NS1 prevents the posttranscriptional processing of cellular pre-mRNAs that are responsible for antiviral activity (Krug et al., 2003, pg 181-189). NS1 accomplishes this without interfering with the translation of viral mRNAs. NS1 also binds to

host PKR (protein kinase R) to inactivate it. While NS1 is synthesized in infected cells, it is not incorporated into virions (Squires and Scheuermann, 2006).

The newly synthesized viral RNA is reformed into RNPs, and is exported out of the cell using host cellular exportation pathways. New M1 proteins assist in the interaction of RNPs and the NEP/NS2 protein; the NEP/NS2 protein then interacts with the nucleoporins of the cell to export the RNPs. Once back in the cytoplasm, the virus assembles and buds from the cell, taking part of the plasma membrane with it to form the outer envelope (Squires and Scheuermann, 2006). Once established, the virus moves to the lower respiratory tract, destroying epithelial cells along the way; NA is assumed to take a key part in this process (Lamb and Choppin, 1983, pg 467-506). The loss of epithelial cells allows secondary infection to occur, mostly bacterial infections, which usually causes death in the infected individual (Seasonal Influenza, 2010).

Influenza Vaccines

Due to the virus' high infectivity and death rates, it is important that a vaccine is prepared to fight the viral attack each year; however, due to influenza's frequent antigenic drift/shift the vaccine needs constant modification. There are two methods of vaccination: a trivalent inactivated vaccine, known as the "flu shot", and a live cold-adapted vaccine (FluMist) which comes in the form of a nasal spray. The nasal spray composed of live virus is of very low titer, and the virulence is non-existent. Both vaccines are made up of the three currently circulating viruses: two A strains and a B strain. The major difference between the two vaccines is the method of delivery and this characteristic determines the immune response induced in humans. The inactivated trivalent vaccine elicits strong serum antibody responses, but provides poor induction of mucosal IgA antibody and cell-mediated immunity. The live viral nasal spray,

however, has the ability to produce a long-lasting, broader immune response, both humoral and cellular, which comes close to replicating natural immunity (Cox et al., 2004, pg 1-15). But despite the spray's advantage of inducing both types of immune responses, the trivalent inactivated vaccine continues to prove more effective (Monto et. al., 2009, pg 1260-1267).

The vaccines are further divided into different subtypes, based solely on the content of the vaccine. According to the WHO, the flu shot can be either: i) whole, inactivated virus, ii) split, or partial disrupted, inactivated particles, or iii) subunit vaccine, containing mainly HA and NA surface proteins (Influenza, 2010). Each vaccine elicits a specific immune response depending on the component of the vaccine and the delivery system (Cox et al., 2004, pg 1-15). The protein-based vaccines are significant due to their ability to induce strong humoral responses (Vaccine, 2006). However, DNA-based vaccines induce *both* humoral and cellular (CD4+ and CD8+) responses in mice. In 2002, the plasmid-based vaccines expressed influenza proteins HA, NA, M, and NP (Ulmer, 2002).

Vaccine Production Problems

As mentioned before, the influenza virus A-strain has the ability to change genetically at a very frequent rate, this poses a problem during vaccine production. This means that each year a new vaccine needs to be made to match the three viruses predicted to likely infect the world's population; this new vaccine synthesis costs the U.S. anywhere from $70 to $170 billion a year.

Another related issue is the production of the vaccine itself. Currently, the vaccine is developed in chicken eggs, which can take months before a workable vaccine is ready for distribution. This becomes a problem when the virus suddenly mutates and the current vaccine is ineffective. This was the case during the 2009 pandemic of "swine flu", where the current

vaccine was not suited for preventing the outbreak and a new vaccine had to be made. This leaves ample time for the virus to take hold on the human population and cause major devastation (Cox et al., 2004, pg 1-15). The WHO meets twice a year to discuss the current influenza viruses that are circulating and to re-evaluate the vaccine to adjust it accordingly; this however does not ensure total protection should a sudden mutation occur again (Influenza, 2010).

Vaccine Priorities

It is usually recommended that every person gets vaccinated against influenza annually, but some people take precedence over others. Those considered high risk are most strongly recommended for vaccination, they include: young children, the elderly, pregnant women, and individuals with serious health conditions which would be further complicated by influenza. Those that work in healthcare or interact with a large number of people should also consider vaccination. Due to the severity of the recent H1N1 pandemic, especially for the very young and old, the CDC recommends that all individuals become vaccinated to help prevent the spread of disease to these at risk individuals (Influenza, 2010).

Complement Cascade Activation by Influenza

The complement cascade is an immune response that occurs in mammals during infection. The cascade is part of the innate immune system and is activated by the presence of antibodies, in basic terms the complement system helps in the elimination of the infection. There are three ways in which the cascade can be activated: the classical pathway, alternative pathway, and the MBL-MASP pathway. The classical pathway begins with C1 protein, which becomes

activated when it is bound by an antibody that has been released due to an infection. This binding causes the activation of a protease which cleaves another protein that is present in the serum. This second cleavage creates proteins which also become proteases that continue to amplify the cascade process of cleavage and activation. The final result is a complex that breaches the membrane of the cell and causes the infected cell to lyse. There is a benefit to the long cascading process aside from lysis; the creation of certain proteins/proteases attracts many other components of the immune system which are also used to fight the source of infection. For example, macrophages and neutrophils have receptors for one of the intermediate proteins (C3b) during the cascade, and as such are activated and move to their location to devour (by phagocytosis) any cells showing this particular protein. Basophils and mast cells are also activated when the C3a component is created; these cells are responsible for anaphylaxis. The complement system, when activated, provides useful protection during most infections; the key is the production of an antibody that allows the cascade to begin (Prince, 2006).

The other two remaining pathways result in the same mode of protection, however their activation is different. The alternative pathway is brought about by spontaneous activation when C3 changes to C3b without cleavage by its preceding protease. Since the alternative pathway is not dependent on antibody activation, it does not provide much use in influenza studies. The MBL-MASP pathway is similar to that of the classical, the main difference being the proteins that begin the cascade process. Instead of C1 being activated, opsonin, mamman-binding lectin, and ficolins are the initiators; which will bind to HA and NA proteins and mediate CDL of infected cells (Prince, 2006).

The complement cascade has been shown to be activated during influenza infection, and its activation during this particular infection enhances beneficial influenza virus-specific CD4

and CD8 T-cell responses; it also aids in the retention of long-term memory to influenza (Gonzales et. al., 2008, pg 186-193). Antibodies produced to fight influenza activate the *classic* cascade, so it is important to discover which antibodies cause this activation in the hope of creating more efficient influenza vaccines.

CDL Assay

In response to this inquiry, the complement-dependant lysis (CDL) assay was created. This assay uses influenza virus-infected cells as target cells. These cells are then exposed to serially diluted sera or monoclonal antibodies mixed with a known amount of complement (endogenous complement in sera are inactivated by heating prior to the serial dilution) to quantitate the specific lysis of the target cells. The purpose of this project was to modify our CDL assay to determine which specific viral proteins are targets of antibodies that activate complement. Further experimentation by modifying the viral genes will reveal the specific regions of those viral proteins that are targeted by these influenza virus-specific CDL antibodies (Ennis et al., 1977, pg 893-898).

The determination of epitopes these CDL antibodies target in influenza proteins is important because some CDL antibody epitopes maybe subtype-cross-reactive. For example, it was discovered that some healthy young adults who were only exposed to H2N2 and H3N2 viruses also showed a low level CDL response against H1N1. A hypothesis was developed that the antibodies that were generated against the H2N2 and H3N2 subtypes of influenza HA may target conserved regions in the HA or other viral proteins (Quinnan, 1980). This assumption that the HA glycoproteins share a common region, and there are antibodies specific for this region, could provide a strong single vaccine strategy against multiple subtypes of influenza. Recently

two groups reported neutralizing human monoclonal antibodies which were subtype-cross-reactive but did not inhibit hemagglutination. These antibodies bound to the stem region of the HA, which is more conserved than the head region, which conventional hemagglutination-inhibiting/ neutralizing antibodies bind to (Ekiert et. al,. 2009, pg 246-251).

Lentiviral Expression System

In order to perform the CDL assay, a stock of cells is needed that can express the influenza genes of interest. Lentiviral expression systems allow for the creation of replication-incompetent/ HIV-1-based lentivirus which can deliver and express genes of interest in both dividing and non-dividing mammalian cells. The benefits gained from this expression system include the long-term expression that extends beyond that of the traditional adenoviral-based systems, the broad host range of infectable cells, and the efficient delivery of genes into mammalian cells either *in vitro* or *in vivo* (Yee et. al., 1994, pg 9564-9568).

PROJECT PURPOSE

Recent publications have shown that the classic complement cascade of the innate immune system is activated during influenza infection. This MQP focused on the development of an *in vitro* protein expression system in which human lung epithelial cells (A549) are used to express individual influenza viral proteins which can be used in the complement-dependant lysis (CDL) assay to help identify specific viral proteins that are targets of CDL antibodies. The long range goal of the Terajima lab is to identify antibodies capable of lysing target cells infected with multiple subtypes of influenza-A viruses, which may include antibodies binding to the conserved regions of the HA or other viral proteins. To achieve this expression goal, a lentiviral transduction system was developed to transduce A549 cells with plasmids containing various influenza HA cDNAs. As a backup expression system for influenza viral proteins, recombinant vaccinia viruses are also being created.

METHODS

Cell Maintenance

Three cell lines were used during the course of the project: a human lung cancer cell line (A549), and two types of human embryonic kidney cell lines (293 and 293FT), all of which were obtained from ATCC. The 293 cells lines were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with fetal bovine serum (to 10%), 5 ml sodium pyruvate (100 mM), 5 ml nonessential amino acids (0.01 mM), 5 ml streptomycin (100 µg/ml), and L-glutamine (2mM) (all from Gibco); 293FT cells received an additional 1.5 ml of G418 (10 mg/ml). The A549 cell line was maintained in F-12K Nutrient Mixture Kaighn's Modification supplemented with fetal bovine serum (10%), 5 ml sodium pyruvate (100 mM), and 5 ml nonessential amino acids (0.01 mM). The cells were grown in T-175 flasks at 37°C in 5% carbon dioxide. As cells approached 90% confluence, the medium was aspirated, and 5 ml of 0.05% Trypsin in 0.53 mM EDTA (Gibco) was added to detach the cells. The cells were passaged in a ratio of 1:15, using the appropriate media. The cell lines were frozen in culture media/DMSO mixture, and stored in liquid nitrogen.

Transfection to Produce Lentiviral Particles

293FT cells were transfected using the Invitrogen ViraPower™ Lentiviral Expression System; the protocol was modified from 100mm plates to T-175 flasks. It was important to prevent the cells from reaching confluence, and to limit the number of passages before performing the transfection. On the first day of transfection, the DNA/Lipofectamine complexes were prepared and added to a T-175 flask of 12×10^6 cells. The complexes were composed of 27

µg of ViraPower Packaging Mix, 9 µg of DNA (either pLenti6/V5-GW/lacZ expression plasmid, or plasmids containing single influenza HA genes), 108 µl of Lipofectamine, and 9 ml of growth media without serum; the mixture was incubated for 20 minutes at room temperature. The maintenance flasks of 293FT cells were trypsinized and counted to obtain 12 x 10^6 cells, which were placed in a new T-175 flask along with the DNA/Lipofectamine complex and medium with serum but lacking antibiotics; the flasks were allowed to incubate overnight at 37°C in a humidified 5% CO_2 incubator. A total of nine flasks were created: a negative control without DNA or Lipofectamine, the positive control pLenti6/V5-GW/lacZ, and 5 HA constructs: *H1 from A/Puerto Rico/8/34, H1NC from A/New Caledonia/20/99, H2 from A/Japan/305/57, H3W A/Wisconsin/67/2005, and H5 from A/Hong Kong/156/97*. The media was changed the next day, replaced with complete culture medium without antibiotics, and then allowed to incubate for 72 hours. At the end of the incubation, the supernatants were harvested and centrifuged at 3000rpm for 15 minutes at 4°C, and then concentrated by further centrifugation using 50ml Amicon Ultra Centrifugal Units at 2500 rpm for 20 minutes at 4°C. The concentrated samples were placed in cryovials in 1ml aliquots, and stored at -80°C.

Virus Titration

Lentivirus titration was performed on transduced A549 and 293 cells. The cells were plated in separate 6-well plates the day before to obtain a 30-50% confluence. The pLenti6/V5-GW/lacZ lentiviral stock was thawed and diluted 10-fold ranging from 10^{-2} to 10^{-6} using complete culture medium to a volume of 1 ml. Each dilution, including a negative control virus free solution, was added to one well of cells, and incubated overnight at 37°C in 5% CO_2. The following day, 4 µg/ml Blasticidin was added to all of the wells to select for only transduced

cells; the media were changed every 3 days along with new Blasticidin. After 12 days, the plates were stained with crystal violet solution to allow for counting.

Transduction and Immunoblot Assay

It was essential to confirm that the delivery and expression system was working correctly, so to monitor the system the cells were transduced and analyzed by Western Blot. A549 and 293 cells were seeded on T-25 flasks at a concentration of 1.5 x 10^6 cells, and incubated overnight at 37°C in 5% CO_2. The next day, the plates were aspirated, and 600 µl of concentrated lentivirus was added (H1, H1NC, H2, H3W, and H5) along with 1µl of Polybrene. Two positive controls were created as well, infecting cells with 200 µl live virus, one A/Wisconsin (H3N2) sample and one A/New Caledonia (H1N1) sample and 400 µl of MEM 2.5%. To create a positive control for the H2 set, 2 µl of H2 HA protein was mixed with 2 µl of 2x dye:DTT (5:1) and added directly to the gel. A positive control was created for H5 in the same manner as H3 and H2, however instead of live influenza virus the H5 protein was cloned into a vaccinia construct; infecting cells and harvesting cell lysate was handled by UMass staff. In addition to the virus and constructs, plates were created for the negative control and pLenti positive control generated during the lentivirus transfection. The plates containing the lentiviruses were incubated for 48 hours at 37°C in 5% CO_2; the live virus infected plates were incubated for 24 hours at 37°C in 5% CO_2. The cells were harvested after their incubation period by removing the media, washing with cold PBS, and adding 100 µl of 2X loading dye: DTT (5:1). The cells were scraped from their flasks, and removed to 1.5ml Eppendorf tubes. A 15% polyacrylamide gel was made for the Western Blot analysis composed of: 7.5 ml Tris pH 8.8, 15 ml of 30% acrylamide (29:1), 7.5 ml dH_2O, 150 µl APS, and 30 µl of TEMED. 50 µl of each sample was loaded along with 10 µl

of Benchmark Pre-Stained Proteins Ladder (Invitrogen) and 7.5 µl of MagicMark XP Western Protein Standard. Following electrophoresis, the proteins were transferred to a PolyScreen PVDF Transfer Membrane and stained with the primary antibodies for one hour; each antibody was diluted in PBS supplemented with Tween from Sigma-Aldrich (1:1000) and BSA (1%). The following were the primary antibodies used: Influenza A Virus Hemagglutinin (c102) mouse antiserum, lot #c1208 (H1N1) diluted 1/2500 in PBS/Tween/BSA, Anti HA High Affiinity (clone 3F10) Roche # 11867 423 001, rat monoclonal (H3N2) diluted 1/1000 in PBS/Tween/BSA, goat antiserum to A/Singapore/1/57 (H2N2) diluted 1/2000 in PBS/Tween/BSA and NR:661 sheep antiserum to A/Hong Kong/156 (483)/97 (H5N1) diluted 1/2000 in PBS/Tween/BSA. Solomon and Wisconsin antibodies obtained from the Centers of Biologies Evaluation and Research; Japan and Hong Kong antibodies obtained from BEI Resources. The membranes were then washed 5 times with PBS/Tween (1:1000) and stained with the secondary antibodies for another hour. The following secondary antibodies were used, and all were diluted in PBS/Tween/BSA: rabbit anti-sheep IgG-HRP diluted 1/2000, goat anti-mouse diluted 1/2000, donkey anti-goat diluted 1/2000 and goat anti-rat diluted 1/2000; all from Santa Cruz Biotechnology. The membranes were then washed another 5 times in PBS/Tween. Chemiluminescense signals were detected by staining the membranes with HyGLO Chemiluminescent HRP Antibody Detection Reagent (Denville Scientific) for one minute, placing them in an autoradiography film cassette, and developing the signals on photographic film.

Recombinant Vaccinia Virus

Multiple vaccinia viruses were created, each one with a single influenza protein. The creation of the recombinant virus involved modifying fragments of various HA cDNAs and inserting them in transfer vectors, transforming *E. coli* cells, growing large volumes of the transfer vector plasmids, and then transfecting the transfer vector plasmids into vaccinia virus-infected cells for homologous recombination. Selection and purification of recombinant vaccinia viruses and large scale preparation of the recombinants are the final stages of the experiment; however due to biosafety concerns, this part of the project had to be handled by the Ennis Lab Staff.

Restriction Enzyme Digestion

The HAs from four subtypes were prepared to make recombinant vaccinia virus: H1, H2, H3, and H5. This project focused on producing H1 from a New Caledonia strain, and H3 from a Wisconsin strain. HA cDNAs from these two strains, which were synthesized by Bio Basic Inc (Markham, Ontario, Canada) based on the GenBank sequence data, were gifts from Cheryl Pikora of Division of Infectious Diseases, Department of Pediatrics. For homologous recombination, both cDNAs were subcloned in pSC11MJ transfer vector plasmids by introduction of restriction enzyme recognition sites suitable for subcloning (only for the Wisconsin HA), restriction enzyme digestion, and ligation. The New Caledonia strain HA cDNA along with its vector was cut by Kpn I and Nhe-HF (NewEngland Bio Labs); The Wisconsin strain HA cDNA with its vector was cut by Sac II and Nhe-HF (NewEngland Bio Labs). The plasmids were digested overnight before purification.

Vector and DNA Fragment Purification

After the restriction digestion, the transfer vector plasmid and influenza inserts were run on 1.4% Low Melting Point agarose gels and extracted. The insert and vector bands were cut with a razor blade and purified using MPbio's GeneClean Turbo Kit.

Ligation/Transformation and Miniprep/Maxiprep

Once the vector and inserts were purified, they needed to be ligated together to form a plasmid and used to transform *E. coli* cells. All inserts and vectors were ligated using the Fast-link DNA Ligation Kit from Epicenter Biotechnologies. Once the plasmids were formed, 2.5 µl of the ligation mixture containing the Wisconsin HA cDNA insert was added to TOP10 Competent Cells following the One Shot TOP10 protocol from Invitrogen; CopyCutter™ EPI400™ Competent cells from Epicenter Biotechnologies was used for the New Calendonia transformation due to the toxicity of H1 HA cDNA to the TOP10 cells. The cells were grown in 250 µl of S.O.C, and placed in a floor shaker at 37°C for 1 hour. After the hour incubation the samples were added to ampicillin agar plates at concentrations of 20 µl and 200 µl, and grown at 37°C overnight. The next day, colonies were picked and placed in 5 ml LB Broth and grown for 24 hours at 37°C in the floor shaker. After incubation, the tubes were centrifuged at 2500rpm for 10 min at room temperature. The supernatant was poured off, and the remaining cell pellet was used to isolate the plasmids. The plasmids were isolated using Qiaprep Spin Miniprep Kits and run on a 0.8% agarose gel. The presence of correct insert fragment was confirmed by restriction enzyme digestion. Hi-Speed Plasmid Maxi Kits from Qiagen was used for large scale preparation of plasmids for transfection experiment in the generation of the recombinant vaccinia viruses.

RESULTS

Data Overview

The goal of this project was to generate human lung epithelial cells (A549 cells) that express influenza proteins. These cells would be used in the CDL assay to analyze human sera for their specificity. To achieve this goal, two delivery systems were used to produce influenza protein expression in the human cell lines. The first method made use of the lentiviral expression system, which was used to infect A549 cells to get them to express influenza proteins. We also used 293 cells as a control for lentivirus transduction. Although 293 cells are frequently used to express exogenous proteins, our preliminary experiment found that these cells are too sensitive to lysis by complement (lysed by complement alone without specific antibodies). Unfortunately, the A549 cells were found to be difficult to transduce, and expression was very poor. Therefore, we decided to try another protein expression system currently being used in our laboratory, recombinant vaccinia viruses. We are generating recombinant vaccinia viruses with H1, H2, H3 and H5 HA cDNAs using vSIGK1 strain of vaccinia virus, which lacks C3L gene encoding complement inhibitor protein (the C3L gene product wild type vaccinia virus can inhibit the complement-dependent lysis of infected cells).

Lentivirus Generation and Titration

The process began with the cotransfection of 293FT cells with lentiviral vector plasmids containing five different influenza genes for HA (H1, H1NC, H2, H3W, and H5), which were previously constructed by Dr Terajima. The cells were allowed to grow, and the lentivirus-containing supernatants were harvested. The lentiviral supernatants were used to transduce

A549 and 293 cells, which were then harvested and analyzed on immunoblots to detect the presence of HA proteins; the supernatant collected from the positive control (containing pLenti6/V5-GW/lacZ plasmid) was also used for viral titration. The cells were plated in 6-well plates and transduced by 10-fold serial dilutions; each well then received 4 µg/ml of Blasticidin. The media/antibiotic was aspirated, and replaced every three days for 14 days. Plates were treated with crystal violet to make infected colonies visible; non-infected cells were unable to produce colonies due to Blasticidin treatment. The "0" wells contained cells without lentivirus, the remaining were all infected with a lentivirus. **Figure 1** shows the titration plates for 293 (top half) and A549 (bottom half) cells that were transduced with the lentiviral positive control. The number of cells infected decreased as the dilution of virus increased; as seen by the decreasing number of crystal violet-positive colonies on each plate. The titer was very low: 2.38 x 10^6 TU/ml for the 293 cells, and only 6.01 x 10^4 TU/ml for the A549 cells. But despite the low titers, the cells still showed signs of successful transduction.

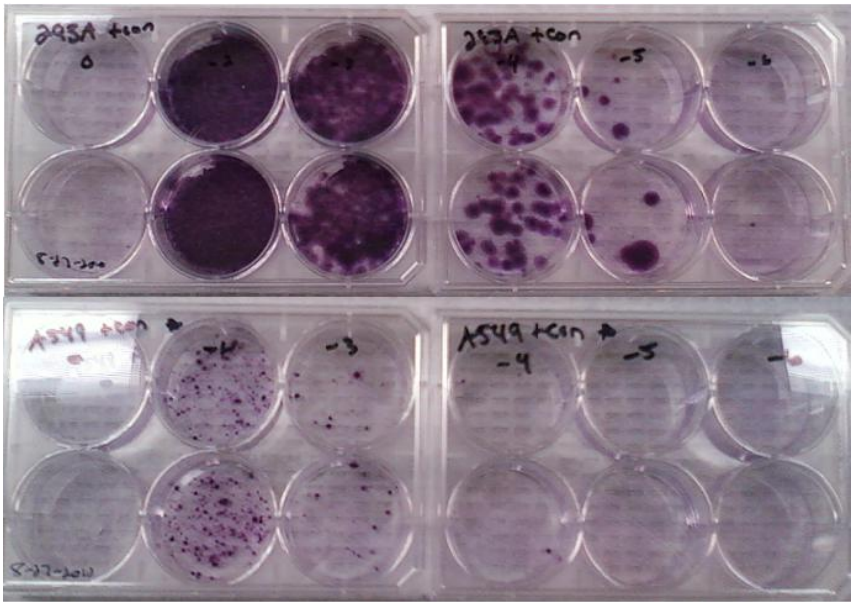

Figure 1: Lentivirus Titration Using the Positive Control Virus. The top half of the figure shows 293 cells, and the bottom half A549 cells. The "0" column (left side) contained cells with no virus, the remaining wells contained diluted virus starting at 10^{-2} to 10^{-6}. The positive control virus had the pLenti6/V5-GW/lacZ plasmid to prove that the cells could be transduced.

Two influenza constructs were made and titered during the same transfection experiment: New Caledonia H1 HA and Wisconsin H3 HA. Transducing the target cells with the HA cDNA-containing lentivirus also had low viral titers. **Figure 2** shows the titration plates for the Wisconsin H3 lentivirus transduction. For both H3 transduced 293 and A549 cells, the number of transduced cells was less than the positive control plates (shown in the previous figure for each cell type). The viral titers for the 293 cells had 1.14 x 10^6 TU/ml, and the A549 cells had 2.6 x 10^4 TU/ml.

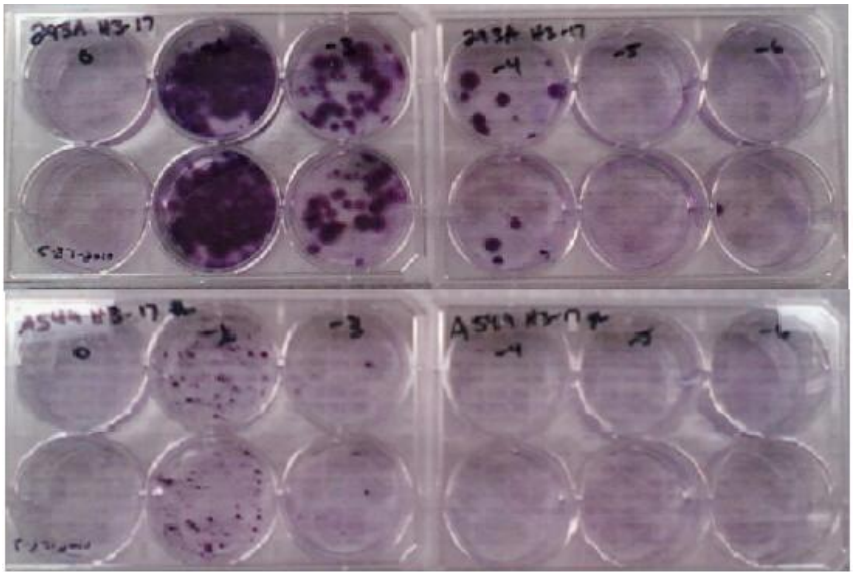

Figure 2: Lentivirus Titration With Wisconsin H3 HA Gene. Top half of the figure represents the transduced 293 cells, and bottom half denotes transduced A549 cells. Cells were transduced with lentivirus containing a Wisconsin influenza H3 gene. Dilutions of the virus are the same as the positive control plates in the previous figure.

The New Caledonia H1-transduced plates (**Figure 3**) provided the best results of all the viral titers; the 293 cells had a titer of 3.95 x 10^6 TU/ml and the A549 cells had 2.22 x 10^5

TU/ml. The same characteristic held true for all trials, that very small colonies were produced during A549 transduction, while the 293 cells formed much larger colonies.

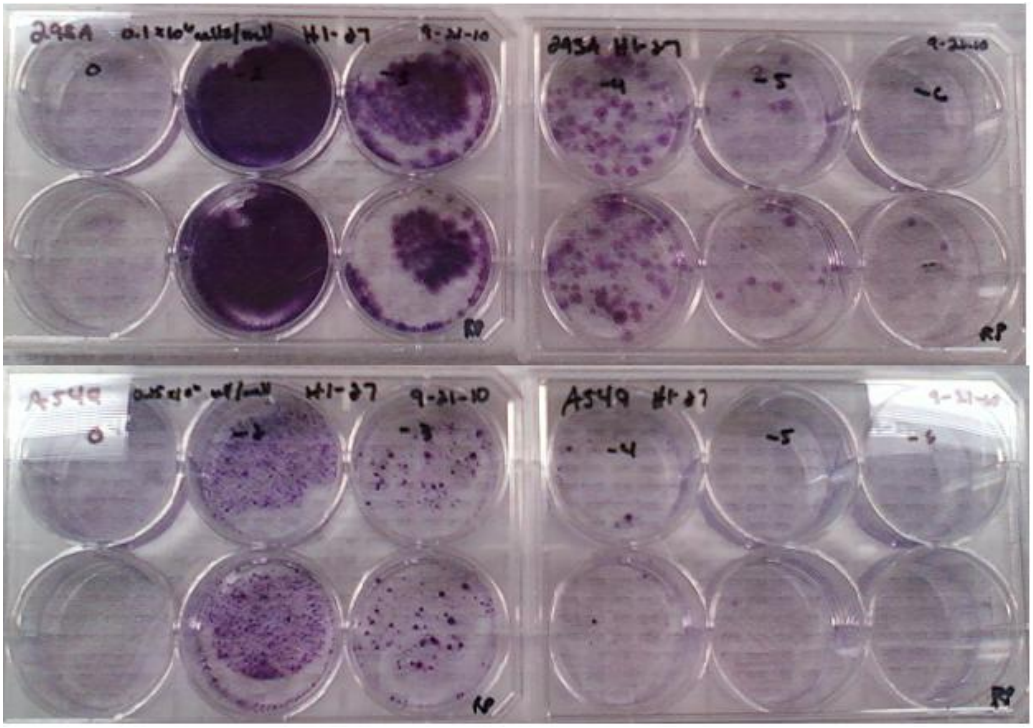

Figure 3: Lentivirus Titration With Virus Containing the New Caledonia H1 HA Gene. Top half of figure represents 293 cells, and bottom half represents A549 cells. Cells were transduced with lentivirus containing the New Caledonia H1 HA gene. The virus was diluted in the same manner as the positive control plates (shown in Figure 1).

The transfection/viral production experiment was repeated to try to produce higher viral titers, but the results remained unchanged (data not shown); the titers still did not reach the standard set by the lentiviral protocol. Due to the low viral titers, all other lentiviral constructs were concentrated after harvesting from the 293FT cells. Titration experiments were not performed on the concentrated samples; instead all concentrated constructs were immediately used in transduction and immunoblot analysis.

Immunoblot Analysis of Transduced 293 and A549 Cells

In order to test for the expression of influenza viral proteins from the lentiviral constructs, lysates were prepared from 293 cells transduced by various viral constructs, and the lysates were analyzed for the expression of HA proteins by immunoblots (**Figure 4**). A549 cells were prepared and analyzed in the same way as 293, however even after three trials a positive result was never produced (data not shown). Negative controls consisted of cells transduced by the pLenti6/V5-GW/lacZ plasmid containing no influenza genes. Cells infected with live influenza virus (H1 and H3) and vaccinia virus (H5) were used as positive controls. For H2 protein, the positive control was purified H2 protein (Biodefense & Emerging Infections Research Resources Repository) not live virus, because live H2N2 virus requires an enhanced BSL-3 facility. The experimental set consisted of 293 cells transduced by lentiviral constructs containing influenza glycoprotein genes. Out of the four lentiviral constructs, only one (H3 construct) produced positive results (Figure 4C). Both of the H1 constructs (H1 and H1NC) were identical to the negative control (Figure 4A), missing the two H1 protein bands. The positive control showed the two H1 bands that should have appeared, one around 63kb (full length) and the other around 23kb (cleaved). The H1 gel also failed to produce a ladder, which should have been to the right of the H1NC lane. The immunoblot for H2 also did not produce any influenza protein bands in the lentiviral construct (Figure 4B). The H2 antibodies picked up several bands on the H2 positive control, making it difficult to determine where the H2 target proteins should be. And both the H2 lentiviral lane and negative control failed to express H2. The H5 immunoblot developed similar to the H2 immunoblot (Figure 4D), making it difficult to see the H5 target bands in the positive control lane; there were 4 noticeable bands present in the positive control lane: ~30kb, ~50kb, ~60kb, and ~80kb. And like the H2 immunoblot, the H5 lentiviral and

negative control were showed no H5 expression. The one positive expression result was found in the H3 immunoblot (Figure 4C); the H3 lentiviral construct produced a band that aligned with the uncleaved full length H3 band in the positive control lane, ~90kb. This band was not found in the negative control lane which consisted of 293 cell lysate that was prepared the same way as the lentiviral construct not encoding an influenza protein.

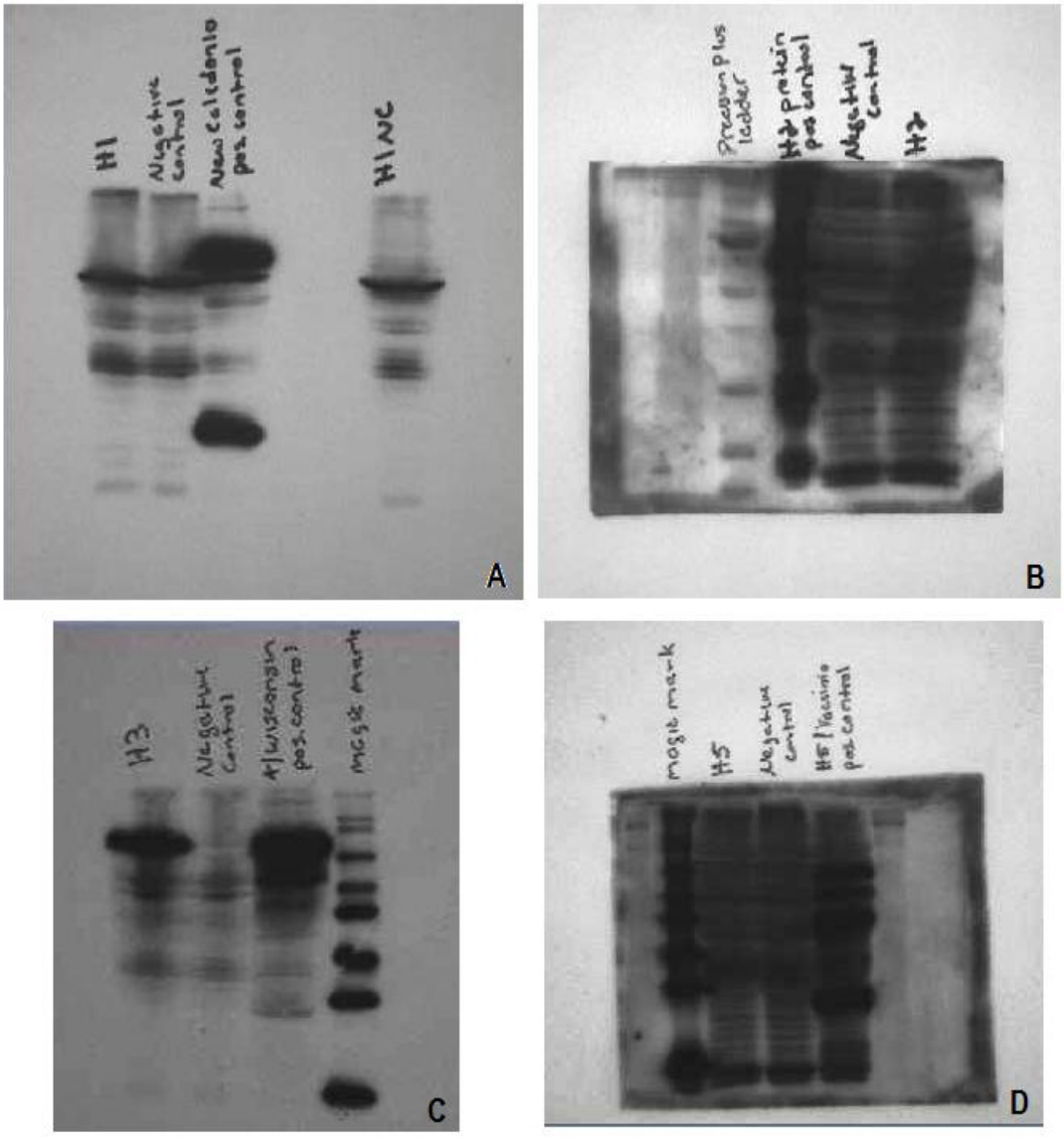

Figure 4: Immunoblot Analysis of Four Lentiviral Constructs. Panels A-D show the immunoblots for H1, H2, H3, and H5 proteins, respectivelly. Lanes labeled as H1, H2, H3, and H5 are the lentiviral constructs. Positive controls are live influenza virus (for H1 and H3), pure influenza protein (H2), and vaccinia constructs (H5). Negative controls are cell lysates from 293 cells containing lentivirus not encoding any influenza viral proteins.

Recombinant Vaccinia Virus Construction

Due to the lack of expression that arose from the lentiviral system it was decided that another delivery method should be attempted. This involved the use of vaccinia virus modified to include plasmids containing influenza genes. This recombinant virus would then be used to infect the A549 cell line. Before the virus could be used, the viral construct containing the influenza genes of interest needed to be built. This included cutting out the HA genes from other plasmids (H1NC), or introducing suitable restriction enzyme recognition sites by PCR (H3W), and inserting them into vectors that could be incorporated into vaccinia. The same HAs used in the lentivirus experiment were used here: H1, H1NC, H2, H3,H3W, and H5; this project however consisted only in the construction of H1NC (H1N1 New Caledonia) and H3W (H3 Wisconsin) plasmids.

Dr Terajima performed the PCR for this portion of the project, while insert removal from cloned plasmids was performed by the author of this report. The HA cDNAs were ligated with the pSCllMJ transfer vectors, and grown to large quantities by transforming *E. coli* cells and preparing maxi/mini preps. **Figure 5** shows the electrophoresis of the plasmid pSC11MJ which was cut with both sets of enzymes to accommodate the H3 and H1 inserts. Lane 2 is a 1 kb ladder used to confirm target molecular weights. Lane 3 contained the uncut pSC11MJ vector which has a molecular weight of 7883 bp, showing two strong bands for supercoiled and linear bands. One band appeared near the 8 kb marker, and another around the 5 kb marker. Lane 5 is the vector cut with enzymes Kpn I and Nhe-HF; Lane 6 is the vector cut with Nhe-HF and Sac II. Both cut lanes produced a ~8 kb digested vector.

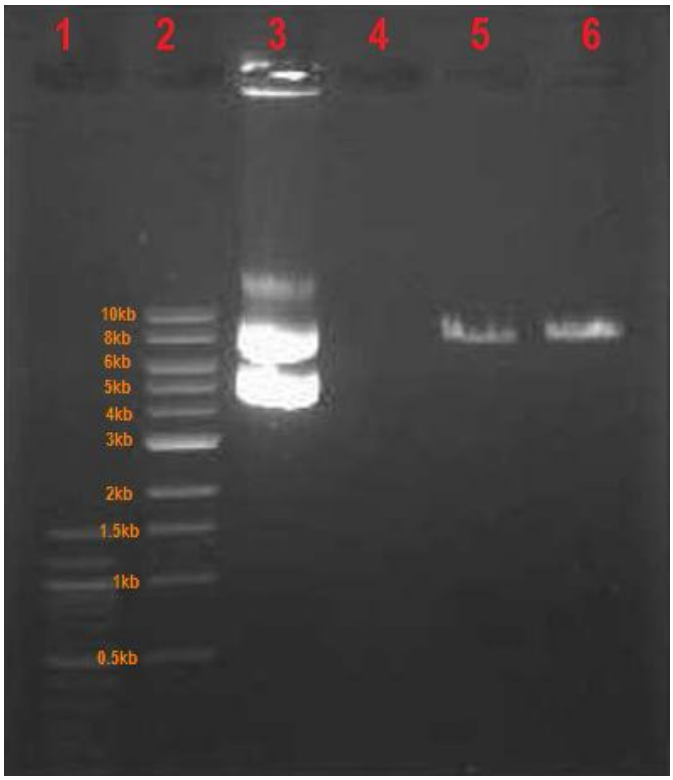

Figure 5: pSC11MJ Restriction Digest. Digestion of pSC11MJ vector to accommodate H1 and H3 inserts. Lane 1 is 100 bp marker; lane 2 is l kb marker; lane 3 is uncut vector; lane 5 is vector cut with KpnI and Nhe-HF; lane 6 is vector cut with Nhe-HF and SacII.

After digesting the vector, the two inserts needed to be prepared by cutting with the corresponding enzymes. The PCR product containing the Wisconsin H3 gene was cut with Kpn I and Nhe-HF, and the plasmid with the H1 gene was cut with Nhe-HF and Sac II. **Figure 6** is the gel electrophoresis run after the restriction digestion. The 1 kb ladder used (lane 2) is the same used during the vector electrophoresis. Lane 3 is the uncut PCR product containing H3 Wisconsin, and Lane 4 is the digested product with A/Wisc insert located just above the 1.5 kb mark. The insert is known to be ~1.7 kb so the correct insert was produced during digestion (Terajima, 2010). Lane 5 contains the uncut plasmid with H1 HA; Lane 6 is the digested product

with the target band (extremely faint on this gel) located around the 1.5 kb mark. The size indicates that the correct insert was created (Terajima, 2010).

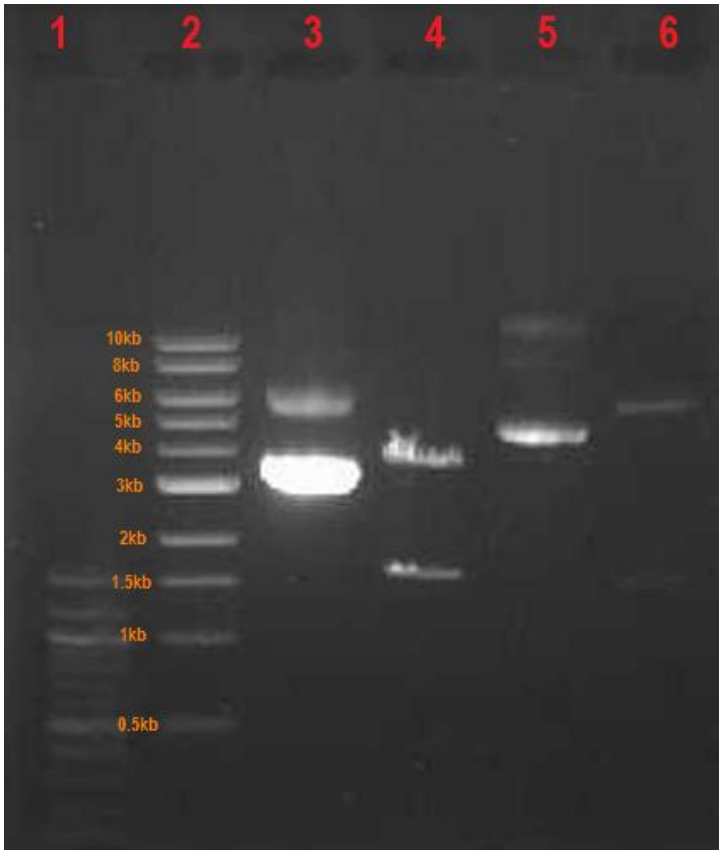

Figure 6: A/Wisc and H1N1 Restriction Digestion. Inserts created by digesting with the same enzymes used in vector digestion. Lane 1 100 bp ladder; lane 2 is 1 kb ladder; lanes 3 and 5 contain uncut H3 Wisconsin and H1N1, respectively; lanes 4 and 6 contain cut H3 Wisconsin and H1N1 respectively.

After restriction digestion of the vector and inserts, each sample was run on 1.4% LMP gels, and the appropriate bands were extracted and purified (**Figure 7**). The end product consisted of two pSC11MJ vectors (one for each insert), an H3 insert, and an H1 insert; the final volume of each sample after purification was 30 µl. Figure 7A is the electrophoresis for the pSC11MJ vector and H1N1 insert. Lanes 3-5 produced one band which was the cut pSC11MJ vector at the correct size; lanes 6-8 contain the entire H1N1 digest. The bands that were extracted

and purified included the middle bands located at 1.5 kb for the insert and the only existing bands for the vector. Figure 7B is the gel for the pSC11MJ vector and H3 insert. Lanes 3-5 contain the vector, and lanes 6-8 contain the H3 digestion. The insert bands located at 1.5 kb were extracted and purified; all bands were extracted and purified for the vector.

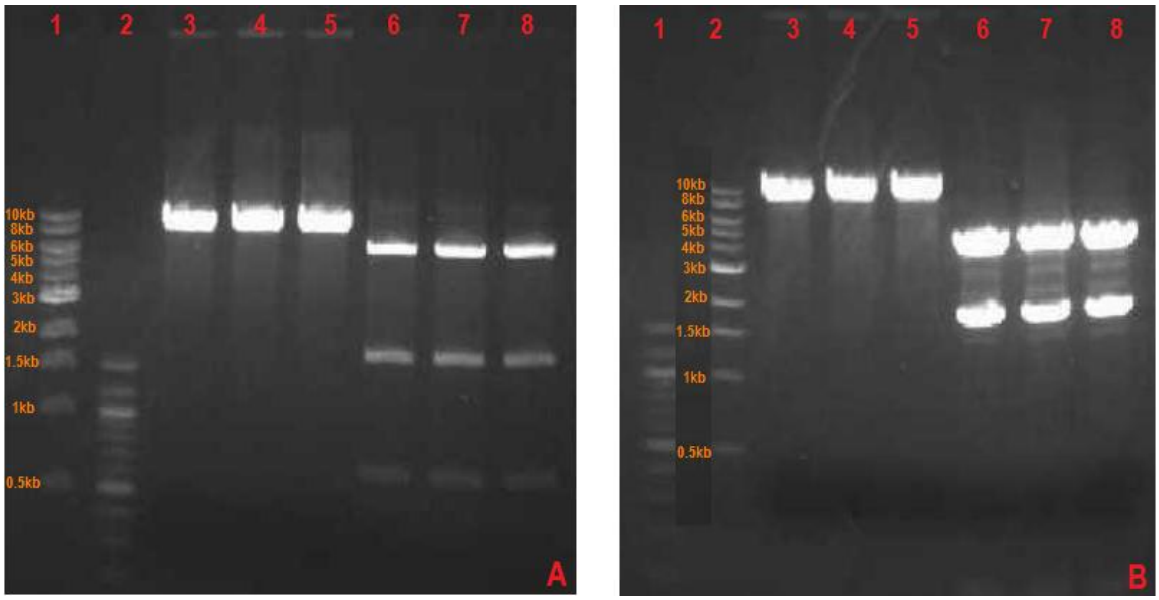

Figure 7: Gel Extraction of Transfer Vectors and Influenza Inserts. Panel A is the low melting point gel for the H1N1 insert and pSC11MJ vector cut by Sac II and Nhe-HF. The H1 target insert is located at 1.5 kb. Panel B is the LMP gel for the H3 insert and pSC11MJ vector cut by Kpn I and Nhe-HF. The target H3 insert is located at 1.5 kb.

The purified DNA bands were then ligated and transformed in *E. coli* cells. After ligation, each appropriate vector/insert pair was seeded at 20 µl and 200 µl on ampicillin agar plates, and incubated overnight at 37°C. The following day, both the pSC11MJ/H3 plates and pSC11MJ/H1 plates showed visible colonies. Six colonies were picked from each set and grown using Qiaprep Miniprep kits. Each miniprep sample (12 in total) was run on a 0.8 % agarose gel to confirm that proper ligation was achieved (**Figure 8**). A successful ligation would result in

bands present just above the 6 kb band in the ladder lane representing uncut supercoiled DNA with insert (Terajima, 2010). The gel confirmed that five of the six H3 Wisconsin minipreps, and three of the six H1N1 minipreps, had plasmids of the correct size. Lanes with bands smaller than 6 kb indicate failed ligation of vector and insert.

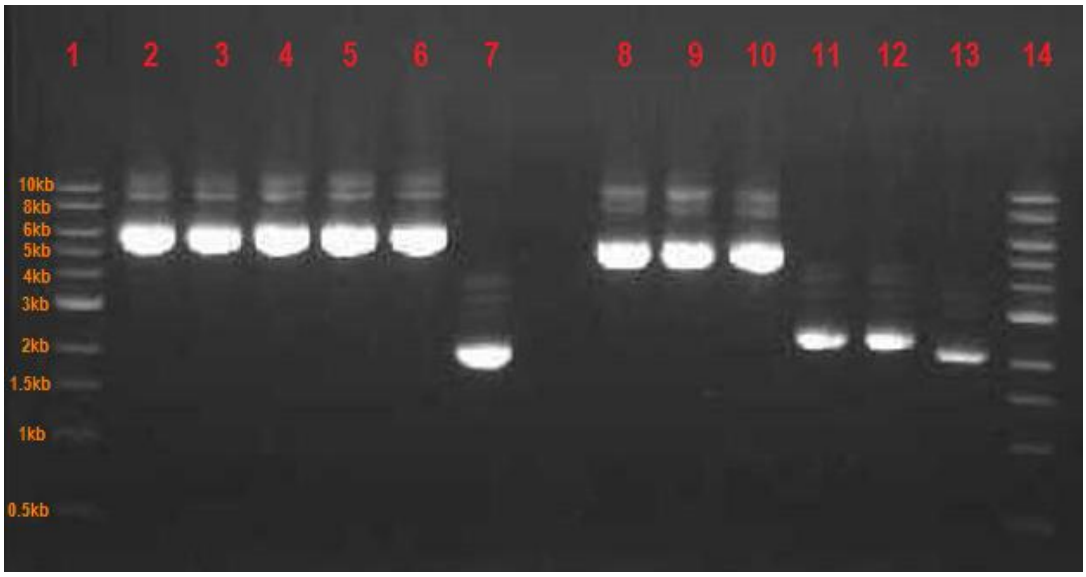

Figure 8: Screening of Uncut Supercoiled DNA from Minipreps.
Confirmation of successful ligation of vector and insert was determined through electrophoresis. Lanes 1 and 14 are 1 kb ladders, lanes 2-7 contain the six minipreps with the plasmid constructed by the pSC11MJ vector and H3 insert. Lanes 8-13 contain the six minipreps with the pSC11MJ vector/H1 insert plasmid. Lanes 7, 11-13 did not contain target plasmid.

To confirm that the constructed plasmids were composed of the proper vector and insert, each sample was digested to remove insert, and run on a 0.8% agarose gel (**Figure 9**). Confirmation was achieved when an 8 kb vector and 1.5 kb insert were seen (Terajima, 2010). Restriction digestion was performed on the first five miniprep samples for the pSC11MJ/H3 plasmid that appeared to be the correct size in the preliminary uncut DNA screening. Digestion was done in an identical manner as the first restriction digestion, using the Kpn I and Nhe-HF

restriction enzymes. The same was done to the first three pSC11MJ/H1 minipreps using Sac II and Nhe-HF. The data confirms that all of the H3 plasmids contain the correct vector and insert, however only two of the three H1 plasmids contained the H1 insert.

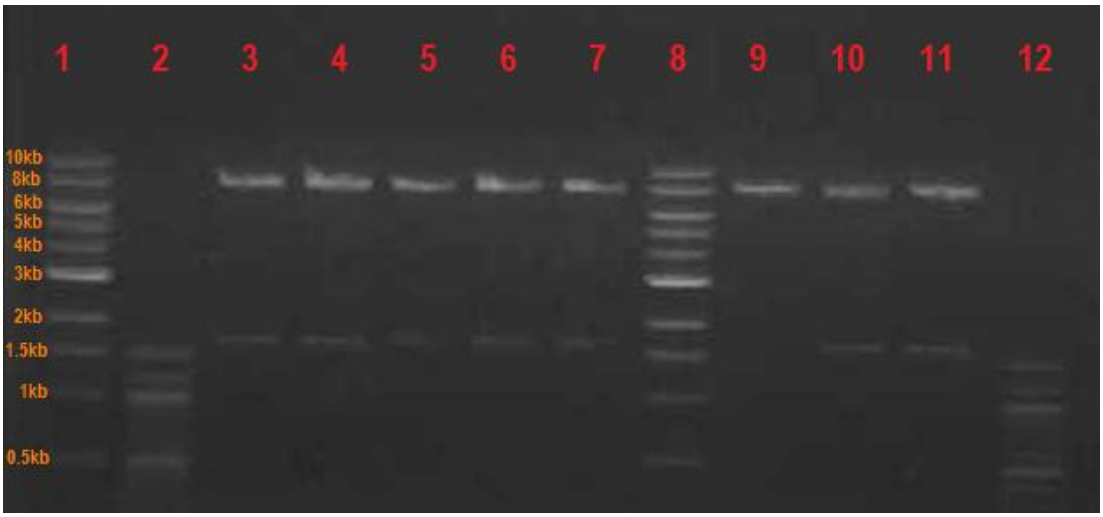

Figure 9: Screening of Cloned Positives by Restriction Digestion. Plasmids that appeared to be of the correct size in the preliminary uncut DNA screening (previous figure) were digested to confirm that each plasmid was composed of the correct insert and vector fragments. Lanes 1 & 8 are 1 kb ladders; lanes 2 & 12 are 100 bp ladders; lanes 3-7 are digested pSC11MJ/H3 plasmids; lanes 9-11 are digested pSC11MJ/H1 plasmids.

DISCUSSION

The goal of this project was to design viral vectors that could be used to express influenza genes in human lung epithelial cells (A549). The results indicate that the lentiviral expression system tested in this project was not suitable for this type of experiment. After 7 months of trial and error experimentation, the lentivirus failed to generate transduced A549 cells that could express influenza genes. Although the lentiviral system did express influenza H3 HA in the 293 cell line (Figure 4), this cell line can not be used for the complememt-dependent lysis (CDL) assay, so the 293 cell line acts only as a type of positive control. The lentiviral transduced A549 cells did not produce virus to high titer. The 293 cells transduced with the H3 lentiviral construct produced higher amounts of lentivirus, and lysates were analyzed on an immunoblot, along with a negative control and a live A/Wisconsin virus as a positive control. In both the positive control and H3 lentiviral construct lanes, a strong band of around 85 kb was present, which is exactly where an uncleaved full length HA protein should travel (Weis et. al., 1988, pg 426-431). The negative control did not contain the H3 band, indicating the detected protein is not normally found in 293 cells. Based on the positive expression result of the H3 immunoblot, it was determined that the lentiviral system is capable of transducing 293 cells to express viral proteins; however it was not able to transduce A549 cells required for the CDL assay, so the lentiviral system did not meet the demands of the project.

One possible reason behind the failed lentiviral expression in A549 cells could be due to incompatibility between the cell line and the lentivirus. From the very beginning, issues arose when trying to titer the lentivirus in both A549 and 293 cells (Figures 1-3). It is possible that the original source of the influenza cDNAs were prepared incorrectly, which would lead to lentivirus

generation but lacking influenza protein expression. However, after speaking with Dr. Terajima I was assured that all the influenza cDNAs and their corresponding proteins were functional.

Producing A549 cells that express foreign proteins in not an impossibility, as other laboratories have used these cells to produce other types of proteins (Rimmelzwaan et. al., 2004), so an alternate expression method was tested. The Institute of Virology located in The Netherlands successfully created an A549 cell line transduced with the HLA-A*0201 gene; this particular gene is involved with presenting influenza proteins to $CD8^+$ cells (Rimmelzwaan et. al., 2004, pg 2769-2775). It is possible that our A549 cells were not incorporating the influenza genes, and that the only remedy would be the addition of a gene that allows for viral presentation. Instead of using this approach, the project focused on the creation of plasmids containing single HA genes which would then be incorporated into a different type of expression virus, vaccinia virus. The vaccinia virus would then be used as the delivery system to transduce A549 cells and hopefully express influenza genes. As the author of this report has not been vaccinated against the vaccinia virus, this project was only allowed to go as far as plasmid creation.

During the plasmid generation process a few minor inconveniences occurred. Occasionally ligation of the pSC11MJ vector and H3 Wisconsin insert would result in no plasmid generation, or when plasmid was generated colonies failed to grow on the agar plates (data not shown). On one occasion, the digested miniprep resulted in a decrease in vector size (data not shown). It was determined that the wrong vector sample was being used during ligation, confusing purified samples with other vectors that were in various stages. Once the error was discovered, the proper plasmids were created (Figure 9) and verified for recombinant

vaccinia generation. Hopefully these constructs will successfully express influenza proteins using the vaccinia viral system.

Based on the information gathered during this project, it would be worth the effort in determining why the lentiviral expression system failed (except for H3) in producing mammalian cells capable of expressing the influenza proteins. The expression kit claims that this delivery system is on par with that of the adenovirus delivery system, and that it actually provides a broader range of host mammalian cells. If that is the case, there should not have been an issue in transducing A549 cells; especially since this project was able to successfully transduce 293 cells with at least one influenza protein.

While the project did not meet the initial goal of generating influenza expressing A549 cells for eventual use in a CDL assay, it was successful in determining the steps that should be taken to develop these cells. Once created, the cells will be used to locate specific CDL antibodies which will hopefully lead to the creation of a multi-valent cross-reactive vaccine against influenza viruses.

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