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HUMAN IGG AND IGE ANTIBODY RESPONSE TO LOW-DOSE INTRADERMAL VERSUS STANDARD DOSE INTRAMUSCULAR INFLUENZA VACCINATION

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HUMAN IGG AND IGE ANTIBODY RESPONSE TO LOW-DOSE INTRADERMAL VERSUS STANDARD DOSE INTRAMUSCULAR INFLUENZA VACCINATION

By Jenea M. Sweeter

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Masters of Science in Biology, Microbiology Concentration.

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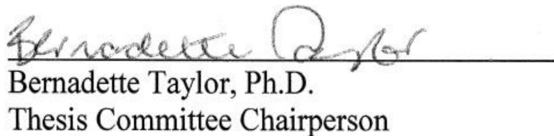

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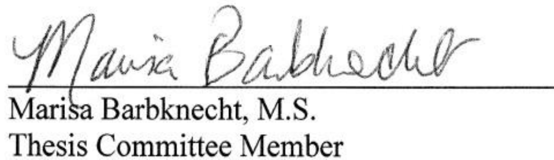

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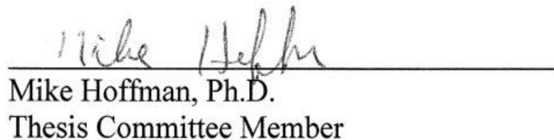

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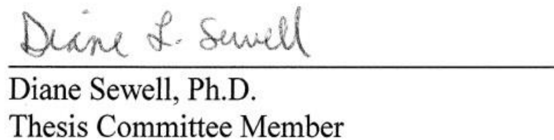

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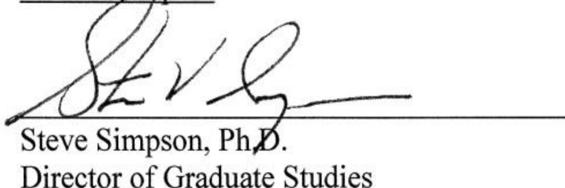

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ABSTRACT

Sweeter, J.M. Human IgG and IgE antibody response to low-dose intradermal versus standard dose intramuscular influenza vaccination. MS in Biology-Microbiology Concentration, December 2013, 67pp. (B. Taylor)

The influenza virus causes human respiratory disease and vaccination is the most reasonable approach for controlling viral infections. However, a shortage in annual vaccine production is a dangerous concern. Replacing standard dose intramuscular (IM) vaccination with low-dose intradermal (ID) vaccination is an attractive solution for sparing vaccine annually if low-dose ID vaccination can produce a similar immune response to standard IM vaccination. Unfortunately, low-dose ID vaccination may also provoke an increased IgE-mediated response. This study tested the ability of low-dose ID vaccination to produce a similar immune response to standard IM vaccination by measuring the amount of virus-specific whole molecule IgG antibody. Additionally, the potential for producing influenza-specific IgE antibody was measured. Serum from individuals vaccinated with a standard IM, 1/5 ID, or 1/25 ID vaccine dose were subjected to an ELISA specific for total A/New Caledonia/20/99(H1N1) virus-specific IgG and IgE antibodies. Results showed a dose-dependent IgG response with no significant difference between the three vaccine dose groups. No A/New Caledonia/20/99(H1N1) virus-specific IgE was detected. From this, we can conclude that low-dose ID vaccination does produce a response similar to standard IM vaccination. Additional studies are needed to determine if IgE is produced when low-dose ID vaccination is administered.

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INTRODUCTION

The influenza virus has been a source of human respiratory disease for centuries and influenza remains a serious health issue today. In the 1918 influenza pandemic alone, close to one third of the world population, totaling almost 500 million people, was infected with influenza virus and showed clinical symptoms (Taubenberger & Morens, 2006). The Center for Disease Control (CDC) estimates that approximately 5-20% (15.7 and 62.8 million) of Americans are infected during annual epidemics. Globally, the World Health Organization reports that 250,000-500,000 mortalities result, most commonly in individuals over the age of 65 (Organization, 2009). Influenza also poses a crippling economic burden of more than $11 billion in direct medical costs, $16 billion in indirect loss of earnings, and $88 billion in hospitalization costs and lost productivity each year in the United States (Molinari et al., 2007).

The influenza virus spreads from person to person easily within aerosol droplets that infect epithelial cells in the respiratory tract, causing a highly contagious acute respiratory disease (Hilleman, 2002). The infection has a short incubation period and sudden onset with characteristic fever and chills. This may be followed by headache, myalgia, a dry cough and diarrhea. The respiratory tract infection may lead to other complications such as primary pneumonitis or streptococcal, staphylococcal, and *Haemophilus influenzae* infection, leading to severe pulmonary complications and potentially death caused by pneumonia. At a less frequent rate, cardiac, muscle, central

nervous system and renal complications may also occur (Cox & Subbarao, 1999; Hilleman, 2002).

Influenza virus is difficult to treat and contain. There are four antiviral drugs used for treatment of influenza infection, with the trade names of Symmetrel, Flumadine, Relenza and Tamiflu. In order to be effective, these drugs must be administered before or within the first few days after infection. If a drug is administered on time, it merely dampens symptoms rather than completely eliminating the viral burden. Further, these drugs may also cause serious side effects, among them central nervous system damage. To complicate matters more, Symmetrel and Flumadine cannot be used on all virus strains as the influenza virus has become resistant to them (Organization, 2009). Due to the ineffective nature of these drugs, vaccination is the most reasonable approach for controlling influenza virus infections.

There are several challenges that need to be addressed concerning mass influenza vaccination, however. For example, children attending American public schools are mandated to have polio, measles, and mumps vaccinations (Lantos et al., 2010). Additionally, the viruses which cause these diseases are genetically stable, so one vaccine can be used to protect an individual for a long period of time. This also causes a more predictable demand for each vaccine. Conversely, influenza vaccine has never been mandated and the virus is genetically unstable (for reasons addressed later). This creates a need for annual vaccination, because the vaccine must be adapted to the viral strains circulating each year, and has caused an unpredictable vaccine demand. Unfortunately, this is only one of the many hurdles manufactures struggle to overcome every season.

Early each year, the World Health Organization (WHO) identifies the three to four strains they predict will be in highest circulation the upcoming winter season. The strains are then released to manufacturers in the spring. Consequently, manufacturing companies have only a few months to produce a safe and effective vaccine for the season (World, 2013).

Additionally, the process for producing vaccine demands viral growth in extremely large numbers of chicken eggs. The process can be daunting as not all strains adapt to egg replication easily. These strains must be crossed with a strain that has been egg-adapted and grows rapidly. However, this method does not always work as well as expected (Genzel, Rodig, Rapp, & Reichl, 2014). If demands are not met, there may be a vaccine shortage and high demand for the vaccine. Often though, many individuals choose not to be vaccinated and doses that are not used cannot be turned over the following season, causing a large loss for the manufacturer.

As manufacturers battle the many obstacles of vaccine production, the threat of an influenza pandemic becomes an increasing cause for concern. In 2009, an H1N1 swine strain emerged from Mexico that was highly contagious. The virus spread across the globe and peaked in October, which is atypical from the Northern Hemisphere influenza season that generally occurs during winter months and peaks in February. An estimated 44,000 deaths were directly attributed to the 2009 pandemic virus in the U.S. alone. The 2009 H1N1 virus caused an even larger rate of morbidity, particularly in the young who did not have cross-protection from previous exposure to a similar virus, and was a substantial health burden during circulation (Noah & Noah, 2013).

Moreover, in April of 2013 a new H7N9 avian influenza strain emerged in China. This strain caused great unease because it had a >20% human mortality rate and uncertain mode of transmission. The H7N9 virus contains a hemagglutinin (H7) and neuraminidase (N9) subtype that have not previously circulated in humans, meaning that the world population is immunogenically naïve. Because of these factors, the virus is considered to have the potential for evolving into a pandemic strain if it evolves to become more easily transmitted between humans. In the event of a pandemic of a highly pathogenic influenza strain, the CDC has predicted a three to seven fold increase in both hospitalization and mortality rates, with at least a 20-fold economic impact increase for the U.S. The impact would be even more overwhelming in global regions with less-advanced health care systems (Meltzer & Bridges, 2003; Meltzer, Cox, & Fukuda, 1999). Additionally, the total loss of domestic fowl and swine populations, because of pathogenic influenza or preventative culling, would have devastating impacts on worldwide markets (Agriculture., 2006; Noah & Noah, 2013; Phipps, Essen, & Brown, 2004).

Currently, the U.S. only produces enough vaccine for about one third of its citizens annually (Centers, 2013a). Furthermore, the global annual production of influenza vaccine is approximately one billion doses. While the WHO projects increased annual production to two billion doses by the year 2015, this still leaves five billion individuals un-vaccinated in the event of a pandemic (World, 2013).

In order to better understand the challenges of mass influenza vaccination, it is important to know more about the virus, immune response, and vaccination.

Influenza Virus Background

In order to fully understand and appreciate the low-dose vaccination, it is useful to first review the structure and replication cycle of the influenza virus. The influenza virus is in the family *Orthomyxoviridae*. There are three genera of human influenza viruses in this family, named type A, B, and C. Type A is the most prominent virus, causing outbreaks and epidemics in the winter seasons of the northern and southern hemispheres annually (Simonsen, 1999). Influenza A is a roughly spherical enveloped virus with a negative-sense, single stranded RNA genome (Figure 1). The RNA genome of the virus is composed of eight segments, and encodes 11 viral genes. These 11 genes code for hemagglutinin (HA), neuraminidase (NA), matrix 1 (M1), matrix 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein, also known as nuclear export protein (NS1 or NEP), polymerase acidic protein (PA), polymerase basic protein 1(PB1), polymerase basic protein 2 (PB2), and polymerase basic protein 1-F2 (PB1-F2) (Fields BN, 2007). Three of these proteins, HA, NA, and M2, are in the viral envelope, which is a lipid bilayer that the virus obtains as it buds from the host cell's plasma membrane (Nayak, Balogun, Yamada, Zhou, & Barman, 2009; Scheiffele, Rietveld, Wilk, & Simons, 1999; Zhang, Pekosz, & Lamb, 2000). HA accounts for about 80% of the total envelope protein, followed by NA at 17 percent and (PA), polymerase basic protein 1(PB1), polymerase basic protein 2 (PB2), and polymerase basic protein 1-F2 (PB1-F2) (Fields BN, 2007). Three of these proteins, HA, NA, and M2, are in the viral envelope, which is a lipid bilayer that the virus obtains as it buds from the host cell's plasma membrane (Nayak et al., 2009; Scheiffele et al., 1999; Zhang et al., 2000). HA accounts for about 80% of the total envelope protein, followed by NA at 17 percent and

M2 at only 3% (Zhang et al., 2000). Under the lipid bilayer membrane lays the matrix, formed by M1, which surrounds the viral ribonucleoproteins (vRNPs). These vRNPs consist of the genomic segments wrapped by NP and associated with small amounts of NEP and the polymerase proteins to form the core of the virus (Nayak et al., 2009; Nayak, Hui, & Barman, 2004).

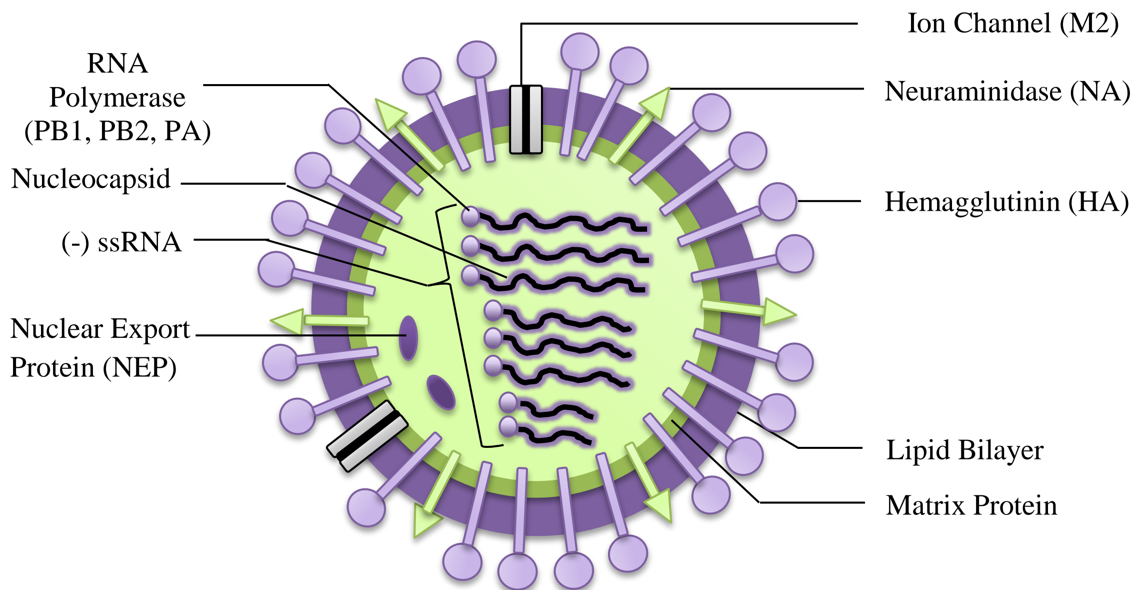


Figure 1. The influenza virus is roughly spherical in shape with a lipid bilayer envelope on its outer surface and matrix protein (M1) just inside of the lipid bilayer. The envelope has spikes of glycoproteins, neuraminidase (NA) and hemagglutinin (HA), as well as the ion channel (M2). Within the virus is nuclear export protein (NEP) and eight segments of negative sense RNA (-ssRNA) covered with nucleocapsid. On the RNA are several proteins (PB1, PB2, and PA).

The viral life cycle begins with entry into the host cell. This is facilitated by the HA homotrimer in the viral envelope that binds to sialic acid on the host cell's membrane surface (Figure 2). This binding induces receptor-mediated endocytosis and brings the virus into the cell's endosome (Skehel & Wiley, 2000). The endosome becomes acidified

to a pH of 5 to 6, which activates fusion of the viral and endosomal membranes and opens the M2 ion channel (Holsinger & Lamb, 1991; Skehel & Wiley, 2000). The viral core is acidified when the M2 ion channels are opened, releasing the vRNPs from M1 and allowing it to enter the host cell's cytoplasm (Pinto & Lamb, 2006). Next, the vRNPs enter the nucleus for transcription and translation. As the virus hijacks the cell's machinery for its own use, transcription begins by converting the negative sense viral RNA into positive sense mRNAs, which are then translated (Plotch, Tomasz, & Krug, 1978). Following translation, HA, NA, and M2 proteins are translated and inserted into the apical plasma membrane where the virus assembles and buds from its host (8). Replication follows and negative sense vRNP products are exported from the nucleus to the assembly site (Shapiro, Gurney, & Krug, 1987). Through scission, the virus buds from the infected cellular membrane and the virus particle is released from the host cell (Nayak et al., 2009). In order for the virus particle to leave the plasma membrane, the sialic acid residues from glycoproteins and glycolipids must be cleaved by NA. Without this, the virus particles would attach to sialic acid residues on plasma membrane proteins and could re-infect the original host cell rather than releasing and infecting neighboring cells. (Palese, Tobita, Ueda, & Compans, 1974).

The influenza A virus has several animal hosts including humans, birds, horses, whales, seals, mink, and swine. Most hosts are infected by only a small number of antigenic subtypes. However, waterfowl are the main reservoir of the virus and are susceptible to many antigenic subtypes (Vahlenkamp & Harder, 2006). In contrast to the site of replication in humans, influenza A replicates primarily in the cells that line the gastro-intestinal tract of waterfowl (Webster, Yakhno, Hinshaw, Bean, & Murti, 1978).

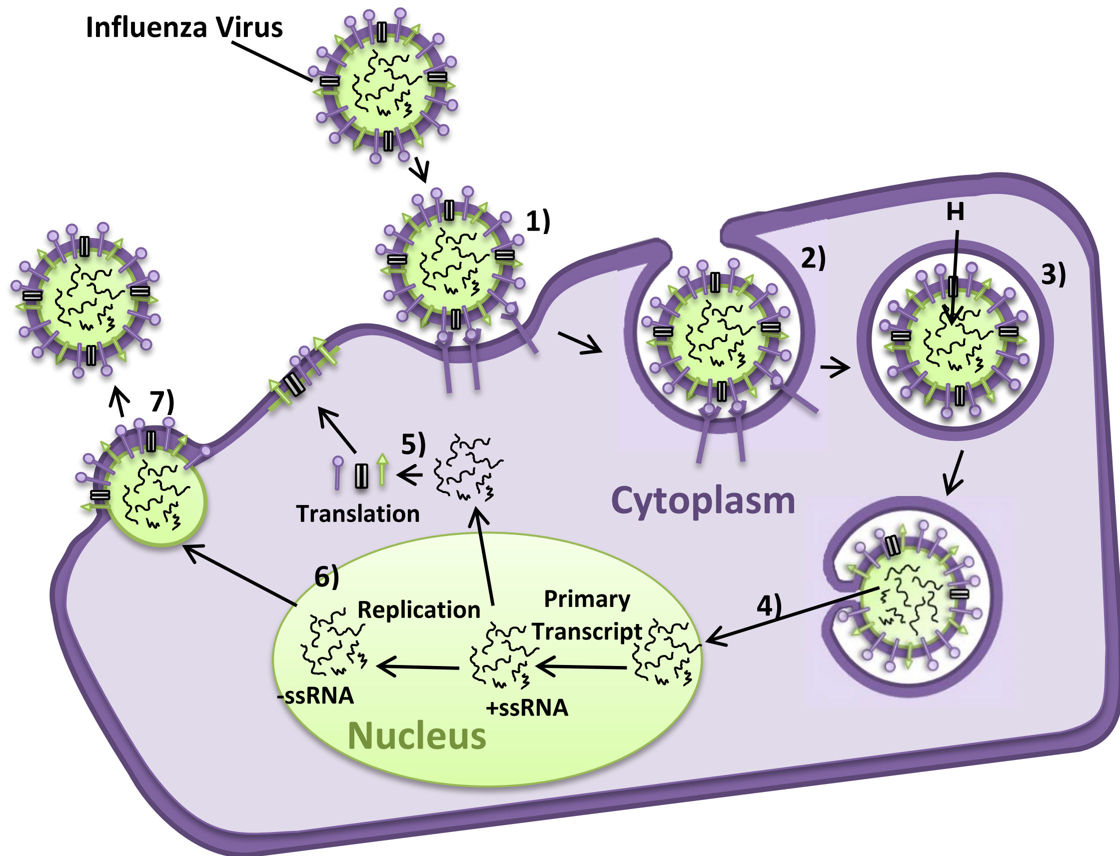


Figure 2. 1) The influenza virus attaches to epithelial cells of the respiratory tract by binding to sialic acid on the surface of the cell with viral hemagglutinin on the outer surface. 2) The virus is endocytosed and brought into the cell endosome. 3) Once inside, the virus uses an M2 ion channel to acidify the inside and aid in release of the vRNP from M1. 4) The viral RNA is brought to the cell nucleus where it undergoes primary transcription. 5) The mRNAs are then either translated into proteins that are localized to the cell membrane, 6) or used to replicate the genetic material that is then exported to the cytoplasm and packaged within each newly synthesized viral particle. 7) The progeny virus assembles at the cell membrane and, with fusion of the opposing viral and cellular membranes and scission of the virus buds from the infected cellular membrane, the virus particle can now infect a new cell.

Type A influenza viruses are divided into numbered subtypes corresponding to antigenic specificities of their HA and NA proteins (Simonsen, 1999). Currently 16 HA subtypes, numbered 1 through 16, and nine NA subtypes, numbered 1 through 9, are recognized. These subtypes are used to name each strain, along with the host of origin,

location of the first isolate, and year it was first isolated (Rohm, Zhou, Suss, Mackenzie, & Webster, 1996; Webster, Bean, Gorman, Chambers, & Kawaoka, 1992). While all subtypes of HA and NA can be found in waterfowl, most commonly in ducks, only HA subtypes 1, 2, and 3 and NA subtypes 1 and 2 have been found with stable lineages in the human population (Ito et al., 1998; Okazaki et al., 2000).

Although only a few subtypes of influenza A virus are found in humans, two processes called antigenic drift and antigenic shift help the virus to continuously evade the immune system and periodically cause large pandemics. Antigenic drift takes place when the virally encoded polymerase creates point mutations in the genes for the HA and NA glycoproteins. As the HA protein is a major antigenic determinant, the host's immune system selects against variants that are neutralized by antibodies while non-neutralized drift variants proliferate. Because of this, virus variants are able to reemerge annually and cause disease even in populations with large amounts of previous virus exposure (Webby & Webster, 2001).

Antigenic shift happens less frequently but is of considerable concern. It occurs during a process called reassortment when a single host cell is infected by two different strains of virus, resulting in progeny virus with a mixture of RNA segments from both parental viruses (Trifonov, Khiabanian, & Rabadan, 2009). Most avian influenza viruses are unable to replicate in human hosts, primarily because of receptor specificity limitations. Though all influenza viruses attach to cell-surface oligosaccharides with terminal sialic acid, avian and human viruses recognize different forms of these molecules. In aquatic birds, influenza viruses bind to NeuAα2,3Gal-terminated receptors whereas human influenza viruses bind to NeuAα2,6Gal (Couceiro, Paulson, & Baum,

1993; Ito et al., 1998; B. R. Murphy et al., 1982). Because swine have both NeuAα2,3Gal and NeuAα2,6Gal-terminated receptors on cells of their respiratory tract, they have been suggested as the "mixing vessel" capable of being infected by both avian and human influenza viruses, providing an opportunity for virus reassortment (Ito et al., 1998).

Studies have also shown that, through antigenic shift, avian strains that preferred NeuAα2,3Gal-terminated receptors could change their preference to NeuAα2,6Gal after several passages, thus providing the means for the virus to change its preferential receptor from avian to human sialic acid residues (Suarez et al., 1998). This antigenic shift could change the antigenic identity of the virus and cause wide and rapid spread of the new virus through the host population (Webby & Webster, 2001).

Immune Response to Influenza Virus

The first step in disease control and eventual clearance of the virus from the body is mediated by the innate immune response. Clearance is mounted by production of type 1 interferons (IFN), natural killer cells (NK), macrophages, and dendritic cells (DC). IFN α and β are released when viral RNA, which is produced during the viral lifecycle, is recognized by toll-like receptors in the host cell. IFN α and β then produce an antiviral response within neighboring host cells, inducing a change in gene transcription and putting them in an antiviral state. This can lead to viral RNA degradation and/or termination of protein synthesis. Further, IFN α and β can bind to NK cells, inducing lytic activity, allowing them to kill virus infected cells by releasing granules to induce apoptosis of the infected cell (K. Murphy, 2012). NK cells also produce IFNγ, TNFα, and MIP-1α, which may help to up-regulate T helper 1 (T_h1) cells, promote endothelial

activation, and attract phagocytic cells to the site of infection (McGill, Heusel, & Legge, 2009).

Macrophages and DCs are the two primary phagocytic cell types commonly found in the lungs. When in a steady state, macrophages produce low levels of cytokines and are less phagocytic (Holt, 1978). Upon viral infection however, they become activated and play an important role in early control of viral spread, prior to the adaptive response, by phagocytosing at heightened levels and producing inflammatory cytokines (McGill et al., 2009). These cytokines include IL-6 and TNFα, responsible for T and B cell growth and differentiation while also promoting endothelial activation and inflammation (Becker, Quay, & Soukup, 1991; K. Murphy, 2012).

Dendritic cells aid both the innate and adaptive immune response. In their naïve steady state, they can be found constantly surveying for pathogens and other foreign material in the airway epithelium, lung parenchyma, and the alveolar lung space (McGill et al., 2009). Once the DC has picked up viral antigen, through the process of phagocytosis or direct infection, the antigen is processed and peptides are displayed by the major histocompatibility complex (MHC) class I and II on the surface of the DC (K. Murphy, 2012). The DC then migrates to the draining lymph node where it presents the peptide on its MHC class I molecule to naïve $CD8^+$ T cells, causing activation of the cell, proliferation, and differentiation into cytotoxic T lymphocytes (CTL) (Belshe et al., 2007). These CTLs may leave the lymph node after 5-7 days to return to the lung and fight against the infection or they may become less active memory CTLs that survive long term for stimulation upon reinfection (Ridge, Di Rosa, & Matzinger, 1998). $CD4^+$ T helper (T_H) cells recognize peptides bound to the MHC class II molecules on the DC and

also on the surface of B cells that have endocytosed viral proteins (K. Murphy, 2012). Upon recognition, the T_H produces several cytokines, including IL-2, which stimulates T cell activation, and INFγ and TNFα, which induce resistance to viruses, inhibit cell proliferation, and promote endothelial activation and inflammation (Becker et al., 1991; Carding, Allan, McMickle, & Doherty, 1993; K. Murphy, 2012).

The interaction between T_H and B cells drives B cell proliferation and differentiation into memory B cells and plasma cells (Doherty, Brown, Kelso, & Thomas, 2009). Plasma cells most commonly produce antibody specific for HA and NA proteins, and to a lesser extent M2 proteins (Epstein et al., 1993). Initially, immunoglobulin M (IgM) antibody, with a low affinity for the antigen, is produced after stimulation. Over time, B cells undergo class switching and somatic hypermutation to produce IgG with higher affinity for antigen (K. Murphy, 2012).

These antibodies hinder the virus in several ways. Antibodies reduce further viral spread of the virus particle by binding to the HA protein, which interferes with viral binding to sialic acid residues on the surface of the host cell, and produces steric inhibition of attachment. If the virus achieves endocytosis, the antibody bound to HA could block fusion of the endosome and viral membranes, preventing release of the viral RNP. Antibody can also bind to NA protein and block separation of the virus from the host plasma membrane. This prevents release of the virions and infection of surrounding cells. Additionally, both antibodies and complement can agglutinate viral particles and function as opsonization agents to facilitate phagocytosis of the viral particles (Han & Marasco, 2011).

Measuring the Immune Response

Several methods are used to measure the antibody response to influenza vaccination. These include the hemagglutination inhibition (HI) assay, microneutralization (MN) assay, neuraminidase inhibition (NAI) assay, and enzyme-linked immunosorbent assay (ELISA). Each assay provides different information about the functional effect of a patient's antibody response.

In the HI assay, serum is mixed with virus then red blood cells (RBC) to determine the HA-specific antibody titer. Free RBCs fall to the bottom of the well and form a visible pellet. However, if the virus is able to bind via it's HA protein to the RBCs, it creates a lattice formation prevents pellet formation. If antibody from the patient serum binds to the virus HA it prevents RBC lattice formation, allowing pellet development. The reciprocal of the highest dilution of serum that is able to prevent lattice formation is the HI titer of the serum. The CDC has stated that an HI titer of 40 provides at least a 50% reduction in risk of infection (Potter & Oxford, 1979).

The MN assay is a two-step assay that is used to measure the neutralizing antibody titer. This assay involves mixing virus and dilutions of patient serum together to allow an influenza-specific antibody present to bind and neutralize the virus. The mixture is added to Madin Darby canine kidney (MDCK) cells, allowing the virus to attempt infection of the cells. The final step is an ELISA that detects virus infected cells. Lack of cell infection is indicative of neutralizing antibody presence (World, 2011). The CDC suggests that a MN titer ≥160 in adults corresponds to a HI titer of 40 ("Serum cross-reactive antibody response to a novel influenza A (H1N1) virus after vaccination with seasonal influenza vaccine," 2009).

The NAI assay measures the amount of antibody in serum able to bind viral NA protein and prevent it from carrying out its enzymatic activity. The viral NA enzyme is capable of releasing free sialic acid when it interacts with fetuin. The sialic acid is converted to a chromophore and extracted for spectrophotometric analysis. The more chromophore present, the higher the NA activity. When serum is added, antibodies bind to viral NA and block sialic acid binding and cleavage, and therefore chromophore production. The lack of chromophore signifies the presence of anti-NA antibody. Currently, the CDC has not established a protective NAI serum titer (World, 2011).

An ELISA can be used to measure all antibodies (Ab) that are produced in response to influenza infection or vaccination, not just those that are known to bind HA, NA or neutralize the virus. Additionally, ELISAs are useful for determining the subclass of antibody produced in an immune response. Most commonly, an indirect ELISA is used. To determine Ig subclass, secondary antibodies specific for IgE, IgA, IgM, and IgG can be used to learn more about the type of antibody response that was produced. (Kindt, 2007).

Vaccination

To combat influenza infection, vaccination is used to prime the immune system for rapid viral clearance. The CDC recommends that everyone six months of age and older be vaccinated, placing special emphasis on those who are pregnant, children under age five, anyone who is 50 years of age and older, those with chronic medical conditions, individuals who live in nursing homes, health care workers, and anyone who is in contact with those who are at a high risk for flu complications (Centers, 2011).

The influenza vaccine is available in two different forms in the United States: inactivated and live attenuated. The inactivated form is a trivalent, or quadravalent, mix of inactivated influenza viruses, which are chosen annually to reflect the viral strains most likely to be prominent in the coming season. Each year, isolates are collected from patient throats and sent to WHO surveillance laboratories to determine the strains that are most prominent. In March and April, the WHO releases the four strains that they believe will be in high circulation. The three or four strains used for the vaccine are re-considered because of continuous antigenic change of the virus, promoted by antigenic drift and shift. Because of this, annual re-vaccination is encouraged (World, 2011). Inactivated vaccine may be administered either intramuscularly (IM) for individuals that are 6 months or older or intradermally (ID) for individuals ages 18 to 64 years of age. A high dose of inactivated vaccine is also available for those over the age of 65 (Centers, 2011).

The second form of vaccine is the live attenuated vaccine. It is also a trivalent vaccine, comprised of the viruses recommended for the season. The key difference between the inactivated and attenuated vaccine is that the attenuated virus is still capable of limited replication. However, it is cold adapted, meaning that it replicates best at 25° C, so it has very little replication activity in the human body (World, 2011). The attenuated vaccine is administered intranasally and is approved for healthy individuals, age 2 through 49 years old, who are not pregnant (Centers, 2011).

Vaccines are administered in either a whole form, live attenuated, or in subunit form. Subunit vaccines contain only parts of the virus and may be comprised of split virus or one or more viral proteins. Split vaccines are produced by exposing the virus to

ether or detergent to break apart the virus so that it is no longer capable of infecting the host. Individual viral proteins provoke fewer side effects in the recipient. However, they also provide a weakened stimulus for the immune response (Hilleman, 2002).

Influenza vaccine may be administered intramuscularly, intranasally, or intradermally. IM vaccination requires delivering inactivated virus by needle into the muscle and relies on migrating antigen presenting cells or antigen drainage to a lymph node for an immune response. Intranasal administration requires inhalation of the live attenuated vaccine and provides an increased chance for production of secretory IgA as well as an alternative vaccine option for patients with an aversion to needles. However, live influenza vaccination is not recommended for children under age 2, those over the age of 49, or individuals with a weakened immune system (Organization, 2009; Tamura, 2010). The final method of vaccine administration is intradermally.

Avenues Suggested for Vaccine Sparing

Because of the annual vaccine shortage and the threat of a possible global pandemic, several avenues for increasing or sparing of precious vaccine have been proposed. First, methods are being developed to overcome our dependence on chicken eggs as the primary source of virus production. One method to achieve this is by adapting influenza strains to rapidly grow in mammalian cells. This culturing system would allow for mass production when scaled up in a bioreactor. There have also been efforts to adapt virus strains to grow in non-adherent cells, which would significantly increase the amount of virus that could be grown in a bioreactor (Genzel et al., 2014).

Many groups are working toward a universal vaccine that would provide protection against all strains of type A and B influenza viruses as another method of vaccine

sparing. Several methods of vaccine development have been suggested to achieve this. One suggestion is to produce a vaccine that induces production of broadly neutralizing antibodies against the conserved stem of the hemagglutinin (HA) protein on the surface of the virus (explained in detail later) by administering a "headless" HA protein (Ekiert et al., 2012). Using this method of immunization, boosters would be required every few years rather than annually.

Others have suggested that memory B cells must be properly stimulated to produce antibodies against the HA stem. To do this, individuals would be immunized with a rare strain of influenza virus so that only memory B cells that recognize the conserved regions of the HA stem are activated rather than B cells that recognize ever-changing regions in the HA protein head. Booster shots would consist of additional rare strains that would continue to up-regulate B memory cells that recognize the HA stem (Thomson et al., 2012).

Yet a third method of universal vaccine development has been suggested that focuses on activating cytotoxic T lymphocytes (CTL), which kill virally infected cells. While B cells generally react to whole surface proteins, CTLs react to processed internal proteins presented by infected cells. In a study done by Lillie, et al., two conserved internal proteins were attached to an inactivated vaccinia virus. A vaccine that boosts the CTL response would likely be paired with proteins that stimulate broad neutralizing antibodies.

Although a universal vaccine is an appealing solution, currently no universal vaccine is licensed for use. Because of this, ID vaccination has been suggested as a reliable alternative.

Targeting the Dermis through ID Vaccination

For some time, ID vaccination has been considered as a way to administer a smaller dose of vaccine and still achieve a protective antibody response. ID vaccination requires delivery of inactivated virus into the dermal layer of the skin. This method takes advantage of the rich supply of resident dendritic cells in the dermis, in contrast to muscle which has very few DCs. These resident DC (called Langerhans cells) search the dermis for antigens like the influenza virus. Upon infection of the DC or by endocytosis, the DC processes influenza proteins and displays them on MHC class I molecules. This is recognized by cytotoxic T cells and activates them (Bender, Bui, Feldman, Larsson, & Bhardwaj, 1995). In addition, DCs are able to stimulate the innate and adaptive immune response by processing viral antigen and presenting it on MHC class II. They then migrate to the draining lymph node and promote T cell activation. T cells stimulate B cells which produce antibody against the virus and form memory cells.

Research to support ID vaccination started as early as 1948 when Weller, et al. found that ID administration of antigens from the influenza virus induced localized redness and swelling in approximately 90% of his subjects. Additionally, a four-fold increase in HA-specific antibody was produced in most of those vaccinated (Weller, Cheever, & Enders, 1948). Since then, multiple studies have been conducted that proclaim the benefits of ID vaccination, including a review done by Young, et al. in 2011. In this review, over 200 low-dose ID studies were considered and 13 were compared that looked at the immune response to influenza vaccination by administration of 15, 9, and 3 μg of HA protein. Young, et al. concluded that there was comparable efficacy between ID and IM administration of influenza vaccine in all dose groups for the

18 to 60-year old population and comparable or superior efficacy in the >60-year old population (Young & Marra, 2011).

One of the largest known drawbacks of intradermal vaccination is that the target layer of skin is very thin and the technique for administration requires specially trained personnel. To combat this, several microneedle devices are under investigation to help deliver the appropriate amount of antigen at the correct skin depth (Prausnitz, Mikszta, Cormier, & Andrianov, 2009). Currently, there is one intradermal trivalent influenza vaccine on the United States market called Fluzone® Intradermal, which contains 9 μg of each antigen in a 0.1 ml dose (Centers, 2013b). While this is already lower than the standard intramuscular dose of 15 μg of each antigen, previous studies suggest that there may be a potential for a similar immune response with even less antigen administered per dose. However, one potential disadvantage of low-dose ID vaccination is that it may provoke an increase of IgE-mediated responses if a small amount of antigen is administered through annual influenza vaccination (Young & Marra, 2011).

Potential for Allergic Response to Low-Dose Vaccination

An IgE-mediated (also known as a type I) hypersensitivity reaction begins with a humoral antibody response fostered by T helper 2 (T_H2) cells. A type I hypersensitivity reaction is mounted in response to certain antigens, called allergens, and produces IgE antibody rather than IgM and IgG antibodies. IgE is most often produced in response to parasitic infections. However, some individuals have a hereditary genetic predisposition called atopy, which causes them to develop an immediate hypersensitivity reaction to antigens that are common in the environment (Wisniewski, Agrawal, & Woodfolk, 2013).

In addition to atopy, other factors regulate whether an allergic response will take place, including the mode of antigen presentation and the antigen dose (Parish, 1972). For example, if a very high or low dosage of antigen is given repeatedly (approximately >10 μM or <0.05 μM ovalbumin in mice), it may favor T_H2 activation, producing IgE rather than IgG, and supporting an allergic response (Hosken, Shibuya, Heath, Murphy, & O'Garra, 1995). Additionally, tissue culture studies have shown that priming doses of less than 50 ng/ml can induce T_H2 cell proliferation and lead to a type 1 response (Constant, Pfeiffer, Woodard, Pasqualini, & Bottomly, 1995).

Allergens that enter the body by the respiratory or digestive tracts or by the skin are taken up by macrophages or B cells. Once the antigen is inside of these cells, it is fragmented and displayed on the cell surface (Kidd, 2003). When allergen-specific T_H2 cells come into contact with the presented antigen, they secrete cytokines such as IL-4 and IL-13, which enhance B cell class switching to IgE and regulate clonal expansion of B cells (Corry & Kheradmand, 1999). T_H2 cells may also produce IL-4 and IL-9, which up-regulates mast cell production. Additionally, T_H2 cells may produce IL-5 and IL-9 which enhances eosinophil activity and accumulation. Mast cells also produce IL-4 and promote IgE antibody production from plasma cells (Lafaille, 1998). After the initial response, memory cells remain that will be reactivated upon later encounter with the antigen remain. IgE produced by plasma cells, either during the initial response or upon later exposure, binds to the FcεRI receptor on mast cells and eosinophils. Upon secondary exposure to the antigen, IgE bound to FcεRI receptors may be cross-linked (Figure 3). Cross-linking stimulates degranulation of the mast cells and eosinophils,

affecting the surrounding tissues by causing vasodilation, increased vascular permeability, and other inflammatory events (Kindt, 2007).

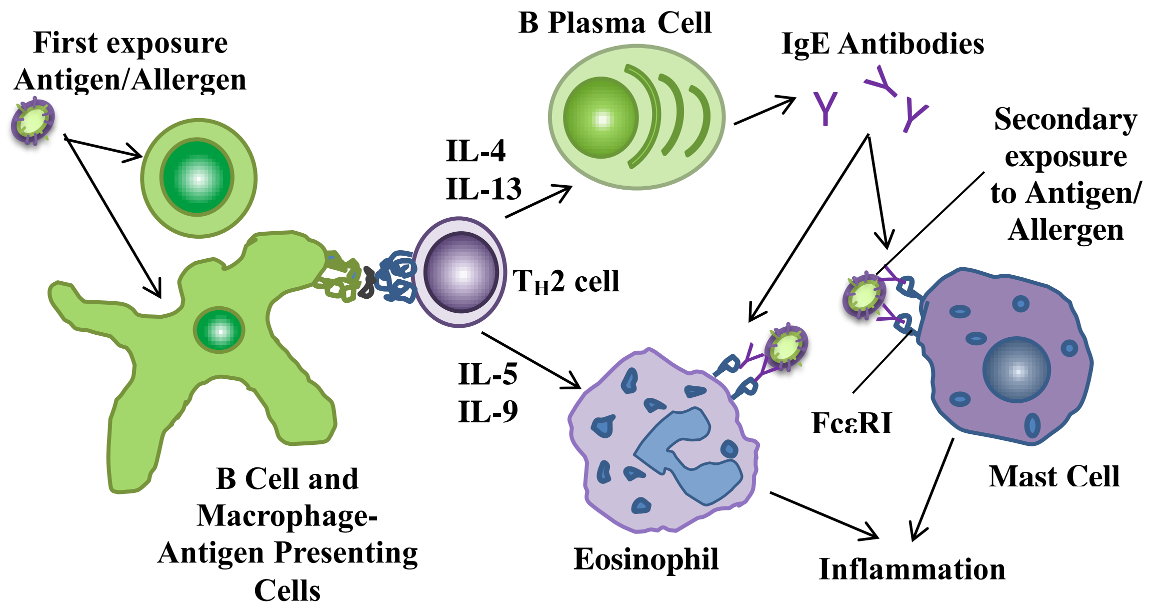


Figure 3. Type 1 Hypersensitivity reaction. Antigen (influenza virus shown) is picked up by antigen presenting cells (B cells and macrophages), processed, and displayed on the cell surface where it is recognized by T_H2 cells. This causes T_H2 cells to secrete IL-4 and IL-13 which promotes plasma cells to switch from producing IgG to IgE. T_H2 cells also produce IL-5 and IL-9 which up-regulate mast cell production and recruit eosinophils. IgE produced by plasma cells binds by its tail to FcεRI receptors on mast cells and eosinophils and can be cross-linked upon secondary exposure to the antigen. This causes inflammation in surrounding tissues.

Allergic reactions to influenza vaccines are a serious issue for individuals that mount an allergic response to egg proteins because many influenza vaccines currently available are produced by growing the virus in chicken egg amniotic fluid. Consequently, the vaccine contains egg proteins that may pose a threat to those with an egg allergy (Settipane, Siri, & Bellanti, 2009). In 2008, the CDC released a data brief stating that approximately 3 million children under the age of 18 reported a food or

digestive allergy in the year 2007. This exposed an 18% increase of reported food allergies between 1997 and 2007. They continued by explaining that children with food allergies are two to four times more likely to have other related allergies, with milk, eggs, peanuts, tree nuts, fish, shellfish, and soy accounting for over 90% of allergic reactions in affected individuals (Branum & Lukacs, 2008). Because of this, there is a concern that vaccination with a low dose of influenza vaccine annually may lead to a type 1 hypersensitivity response rather than a protective immune response.

HA and NA Data from Low Dose ID Vaccine Study at UW-LaCrosse

In 2004, a study was conducted by Taylor et al. in our lab that compared the standard IM dose of vaccination to 1/5 and 1/25 ID doses of Fluzone® 2004-2005 vaccine. Seroprotection rates (determined by hemagglutinin inhibition assays) showed similar rates of protection between the three vaccination groups for H1N1 (P=0.192, Figure 4). Additionally, serum samples were subjected to an indirect ELISA to examine the antibody response formed against whole influenza virus. There was no correlation found between the HA-specific antibody response measured by HIA titer and the whole influenza virus response measured by ELISA titer (Figure 5).

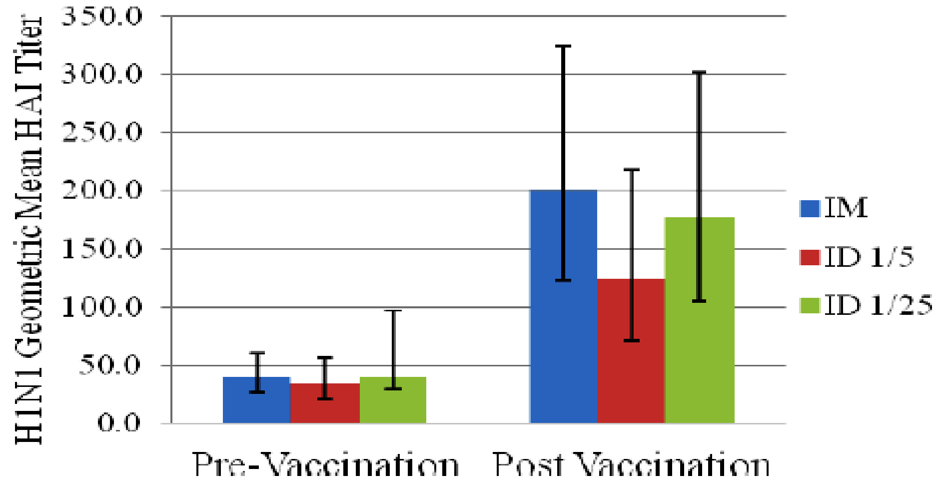


Figure 4. Geometric mean HAI antibody titers pre- and post-vaccination for A/New Caledonia (H1N1). Error bars represent 95% confidence intervals.

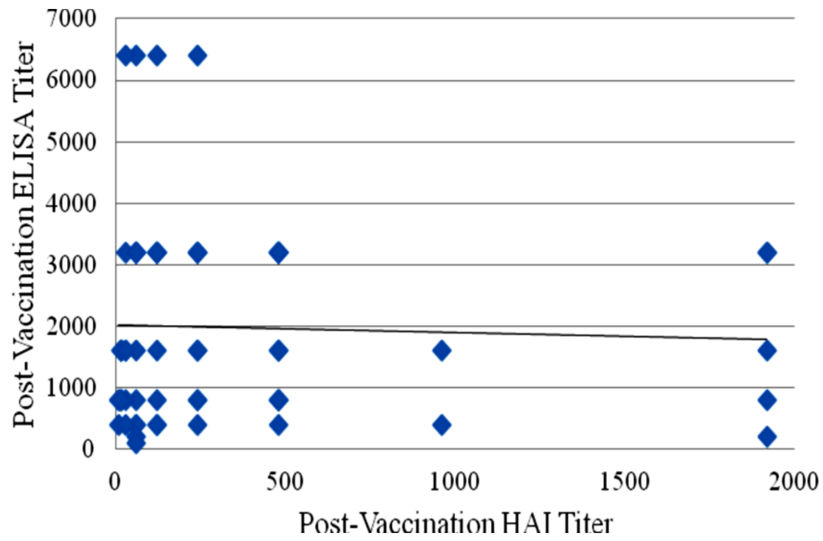


Figure 5. HAI antibody titers versus whole-virus ELISA antibody titers after 1/5 dose intradermal and standard dose intramuscular vaccination.

Since some subjects with a low HA-specific antibody titer had a high whole virus-specific antibody titer, NAI assays were done to determine if an antibody response to NA was formed in addition to the antibody response made to HA. There was no significant difference between the three vaccination groups either pre- or post-vaccination when NAI titers were compared (P=0.189, Figure 6). Additionally, a significant increase in NAI titer was observed post-vaccination (P<0.0005, Figure 6).

When compared to the NA antibody response, the antibody response to HA was higher after vaccination in all three vaccine groups. This is shown by the larger fold increase (P< 0.001) in HAI titer compared to NAI titer (Figure 7). Additionally, there was a weakly positive correlation between the HAI and NAI titers pre- and post-vaccination (Figure 8).

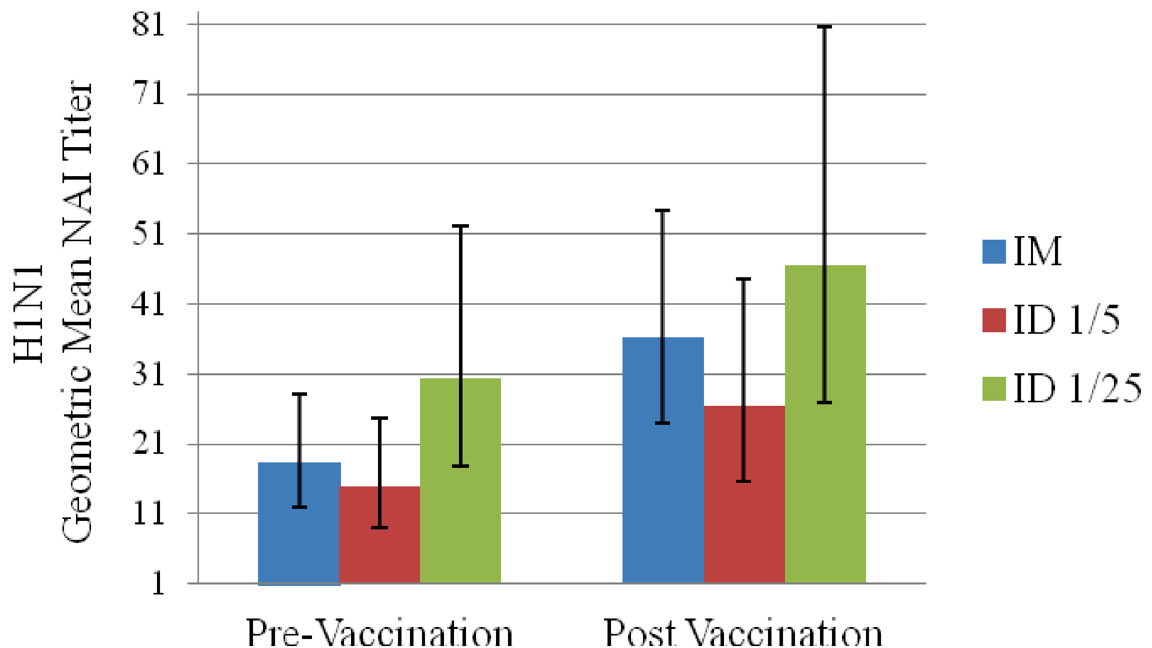


Figure 6. Geometric mean NAI antibody titers pre- and post-vaccination for A/New Caledonia (H1N1). Error bars represent 95% confidence intervals.

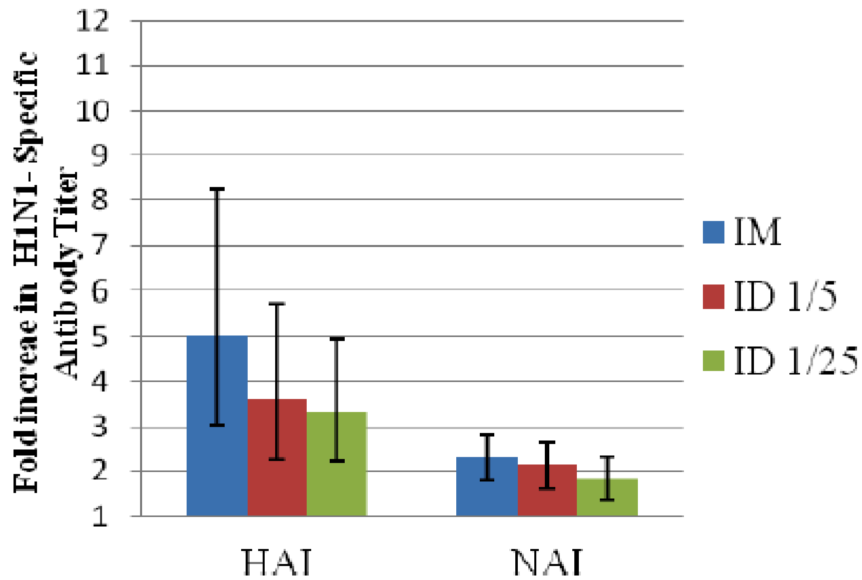


Figure 7. Fold increase in HAI post-vaccination with A/New Caledonia (H1N1). Error bars represent 95% confidence intervals.

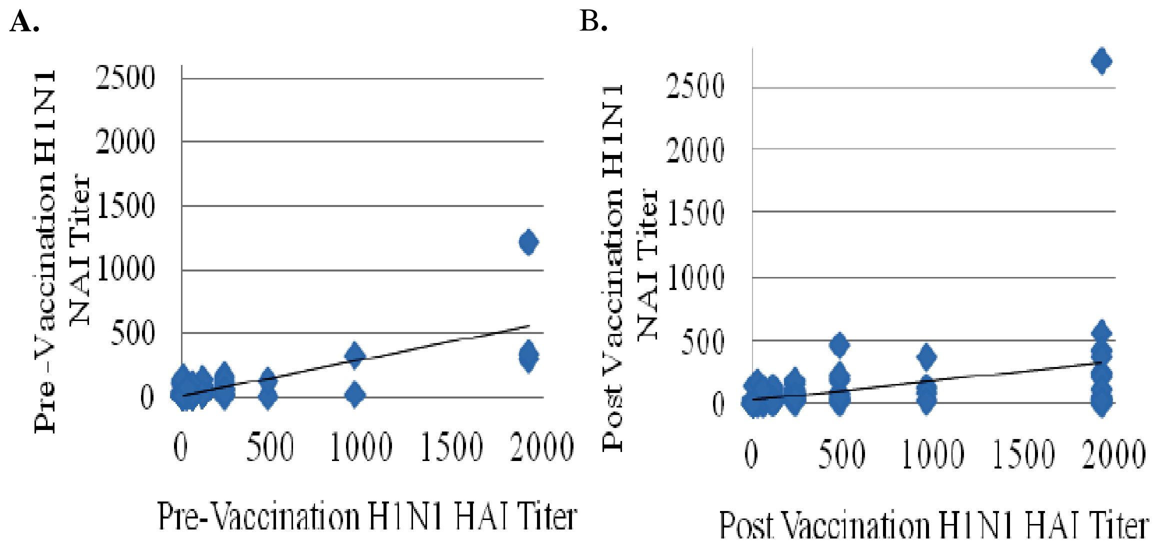


Figure 8. Virus specific HAI titer versus NAI titers. (A.) Pre-vaccination A/New Caledonia (H1N1)-virus specific HAI titers versus NAI titers (r2=0.556) (B.) Post-vaccination A/New Caledonia (H1N1)-virus specific HAI titers versus NAI titers (r2=0.132)

This previous work supports the hypothesis that low-dose ID vaccination produces a response similar to the standard IM dose, even at only 1/25 (0.6 µg) of the standard dose (15 µg). Although Th2 inducing studies have never been performed in human subjects, at least two studies have shown that reducing the amount of antigen necessary for a protective immune response by 1/10 significantly increased the amount of IL-4 produced, inducing a Th2 immune response (Constant et al., 1995; Ruedl, Bachmann, & Kopf, 2000). This creates a heightened concern that giving a low dose of vaccine (especially at 1/25 the standard dose) each year may eventually lead to an increase of patients developing an allergic response.

Low-dose ID influenza vaccination has not been administered repeatedly to date, so serum samples from repeated low-dose vaccine exposure are not available for IgE testing. However, we decided to examine the potential for IgE response increases by using an ELISA to compare IgE responses in first-time low dose ID vaccination versus standard dose IM vaccination. Additionally, the influenza-specific IgG response was tested via ELISA. This served as a positive control because we know, from previous work, that there was an antibody response mounted to the vaccine, though we are not sure if IgE was produced. The IgG data also adds to previous low dose influenza vaccination work which measured the HA and NA antibody titers.

Experimental Goals and Objectives

The main goal of this research was to use an indirect ELISA to compare influenza virus-specific total IgG and IgE levels in humans administered a low-dose ID influenza vaccine versus humans given a standard dose IM vaccination. This was done to assess the potential for low-dose vaccination to induce a higher allergic response, associated with

increased IgE production, rather than a protective IgG response. Alternatively, if a lower dose still evoked a high IgG response, this would provide support for lowering the standard dose given in the event of a serious influenza vaccine shortage. To do this, several objectives were established:

Objective 1: Purify influenza virus at a high titer in order to coat ELISA plates.

Objective 2: Optimize an influenza-specific ELISA for IgG and IgE to measure the antibody response. Subject serum samples from the standard IM and 1/5 and 1/25 ID dose groups to optimized ELISA.

Objective 3: Do statistical analysis on the resulting IgG and IgE Ab titers to compare titers among vaccination groups. Compare IgG titers with previously determined HA and NA titers.

METHODS

Human Subjects

Between September 22, 2004 and October 8, 2004, 117 human subjects were enrolled in this study, which was approved for use of human subjects by the Institutional Review Board of the University of Wisconsin-La Crosse (UWL). Subjects ranged in age from 18-52 and included students, faculty, and staff at the university that were not known to be immunocompromised or allergic to egg or influenza vaccine. All had given written informed consent. Six individuals were excluded because either sufficient blood samples or data were not collected, leading to a total of 111 sample subjects. Subjects were assigned to experimental groups by using a random numerical code produced in Microsoft Excel for each subject. The male to female ratio in each experimental group was matched as close as possible. Experimental groups had 34 individuals in the IM group, 40 in the 1/5 dose ID group, and 37 in the 1/25 dose ID group.

Vaccination

Subjects were vaccinated between October 4 and October 8, 2004 with a trivalent split subvirion vaccine called Fluzone® (2004-2005) in 5 ml multidose vials (Aventis Pasteur, Swiftwater, PA). The vaccine contained A/New Caledonia/20/99 (H1N1), A/Wyoming/03/2003 (H3N2) and B/Jiangsu/10/2003 (a B/Shanghai/361/2002-like strain) virus strains and was from lot #U1425AA. The vaccine virus was produced in embryonated chicken eggs, inactivated with formaldehyde, purified in a linear sucrose

density gradient, and disrupted with Triton X-100. The split virus was suspended in sodium phosphate-buffered isotonic sodium chloride solution with 15 µg HA of each virus strain per 0.5 ml dose. Vaccine was stored at 4°C and was shaken well before use. The IM group received 0.5 ml of vaccine in the upper arm deltoid muscle. The 1/5 dose ID group received 0.1 ml of vaccine into the inner forearm dermis and the 1/25 dose ID group received 0.1 ml of vaccine diluted 1:5 in sterile saline also into the inner forearm dermis. The IM group received a total of 15 µg HA of each virus strain while the 1/5 dose ID group received 3 µg HA/virus strain and the 1/25 dose ID group received 0.6 µg HA/virus strain. Vaccination and sample collection were performed at the UWL Student Health Clinic. The Student Health Clinic research nurse administered all IM vaccinations with a 3cc syringe and a 23g needle. An Allergy Associates of La Crosse nurse experienced in ID inoculation completed all ID vaccinations with a tuberculin syringe and a 26 g needle. For 30 minutes post vaccination, subjects were monitored for unfavorable reactions.

Sample Collection

Two 5 ml blood samples were collected from each subject into vacutainer tubes without additive. The first was collected prior to vaccination and the second was four weeks post-vaccination, from November 1-5, 2004. Each sample was processed within 4 hours after collection, separating serum from cells, and two aliquots of serum per subject were frozen at -20°C for future analysis.

Purified Influenza Virus Production

Before the ELISAs could be performed, purified A/New Caledonia/20/99 (H1N1) influenza virus at a high titer was produced. This was done by growing MDCK cells in

three CELLine (Wilson and Wolf, AD 1000) flasks, each contains a synthetic scaffold that supports very high densities of adherent cell growth and is enclosed by a membrane that allows for media exchange. The cells in these flasks were then infected with influenza virus. For each CELLine flask, ten 75 cm^2 flasks of MDCK cells were grown to 100% confluence in minimal essential medium (MEM) and 10% fetal bovine serum (FBS) (Appendix A) at 37°C with 5% CO_2. When at the desired confluence, all media was removed from the flasks and each was washed three times with 10 ml of phosphate buffered saline (PBS) at room temperature. Once all PBS was removed, each flask received 1 ml of typsin-EDTA and was incubated for 20 minutes at 37°C with 5% CO_2 to allow the cells to become free-floating. Cells and trypsin-EDTA from all flasks were pooled and 2 ml of MEM complete with additional glucose and L-glutamine (total of 5.4 g/L glucose and 4 mM L-glutamine) (Appendix A) and 3 ml of FBS were added. To wet the CELLine flask membrane and avoid damage to it, 50 ml of MEM complete with additional glucose and L-glutamine were added to the media compartment and allowed to sit for several minutes. Cells, media, and FBS were slowly added to the cell compartment and the additional 950 ml of MEM complete with additional glucose and L-glutamine were added to the media compartment. The CELLine flasks were incubated at 37°C with 5% CO_2 for eight days. Air trapped under the membrane was removed.

On day eight, the media was removed from the cell compartment and the cell compartment was rinsed three times with 10 ml PBS. One milliliter of MEM complete with extra glucose and L-glutamine and tosyl phenylalanyl chloromethyl ketone (TPCK) trypsin (2 μl/ml) was mixed with 1 ml of A/New Caledonia/20/99 (H1N1) influenza virus (HA titer: 64) and added to the cell compartment. The flask was incubated for 40

minutes at 37°C with 5% CO_2 to allow the virus to attach to the cells. After 40 minutes, an additional 15 ml of MEM complete with extra glucose and L-glutamine and TPCK trypsin (2 µl/ml) were added to the cell compartment and the flasks were incubated for four days at 37°C with 5% CO_2.

After four days, media was removed from the cell compartment and set aside. The cell compartment was rinsed three times with 10 ml of PBS and was pooled with the media. Pooled media and PBS were centrifuged at 1,500 revolutions per minute (RPM) for six minutes to remove all cellular debris.

The virus was concentrated using PEG- 6000 precipitation. To do this, 2.2 g of NaCl per 100 ml of culture fluid was dissolved by stirring constantly at 4°C. Next, 6 g of PEG- 6000 per 100 ml was added and the solution remained at 4°C with continuous stirring for at least 4 hours. The solution was centrifuged at 2,000xg for two hours to pellet the virus. After centrifugation, the supernatant was removed and the virus pellet was re-suspended in tris buffered saline (TBS) (Appendix B) at 1/50 of the original volume of culture fluid. The solution was sonicated three times at 25% and was placed in ice between each sonication to cool the liquid. This was done to break apart virus clumps that form when a large amount of virus is in a small amount of liquid.

A HA titer was done to determine the amount of virus by collecting type O blood and washing the red blood cells (RBCs) in a 1:10 ratio of blood to sterile PBS at room temperature. After adding PBS to the cells, they were centrifuged for 20-30 seconds to form a pellet and the supernatant was removed and discarded. After the third wash, sterile PBS was added to make a 1% solution of RBCs. Next, 50 µl of PBS was added to wells in a U-bottom 96 well plate. To make a two-fold serial dilution, 50 µl of virus was

added to the first well, mixed, and 50 µl was removed and transferred to the next well. This was continued until the desired number of dilutions was complete. Finally, 50 µl of the 1% RBCs was added to each well and it was allowed to sit for 60 minutes. After an hour, the titer was determined as the reciprocal of the last well in the dilution series in which RBCs did not form a pellet.

Measurement of Virus-specific IgE and IgG by ELISA

Indirect IgE and IgG ELISAs were performed on 32 serum samples. Eight samples were from the IM group, 12 samples were from the 1/5 ID group, and 11 samples were from the 1/ID group (pre and post vaccination).

First, 50 µl of a 1/29 dilution of A/New Caledonia/20/99 (H1N1) influenza virus (HA titer: 1024) in 1x TBS (for IgE and IgG ELISAs) was distributed into 94 of 96 wells of a microtitier plate. For the IgE ELISAs, the two positive control wells had 50 µl (approximately10 µg/ml) of purified human IgE added. The plate was incubated at 4°C overnight and rinsed three times with tris buffered saline and Tween 20 (TBST). All rinses were performed by a 96 well plate washer.

Next, 50 µL of a 1% milk in TBST solution was added to each well and incubated at 37°C for 30 min to block. After incubation, the plate was rinsed three times with TBST and, for the IgE sample wells, 50 µL of serum, producing a two-fold serial dilution starting at a 1/5 dilution and ending at 1/5120, was added in duplicate to patient wells. For the IgG sample wells, 50 µL of serum, producing a two-fold serial dilution starting at a 1/40 dilution and ending at 1/40960, was added in duplicate to appropriate wells. For the positive control wells, 50 µl of a 1/160 dilution of Dr. Bernadette Taylor’s serum was added and 50 µl of a BSA (0.35 mg/ml) was added to the negative control wells. The IgE

positive control wells received no serum or BSA. The plate was incubated at 37°C for 60 minutes to allow antibody in the serum to attach to virus on the plate.

After the incubation, the plate was washed three times with TBST. Next, for the IgE sample wells, 50 µl of a 1/2500 dilution (2.24 µg/ml) of goat anti-human IgE antibody conjugated to alkaline phosphatase in TBST was added to each well and a 1/2500 dilution (2.24 µg/ml) of whole molecule goat anti-human IgG antibody conjugated to alkaline phosphatase in TBST was added to each well of the IgG sample wells. The plate was incubated for 30 minutes at 37°C. The plate was again washed three times with TBST after incubation and 50 µl of p-nitrophenyl phosphate substrate was added. The plate was incubated at room temperature for 15 minutes to allow color development. The plate was analyzed by a microtiter plate reader measuring absorbance at 405 nm and generated data was saved electronically.

The IgE positive control wells received purified human IgE, block, goat anti-human IgE conjugated with alkaline phosphatase, and substrate. Two negative controls were used. One negative control was a no 1° Ab control in which wells were given virus, blocking agent, 0.05% BSA, goat anti-human IgE antibody with alkaline phosphatase conjugation and substrate and the second served as a "blank" with virus, blocking agent, goat anti-human IgE antibody with alkaline phosphatase conjugation and substrate.
For the IgG positive control sample wells, a 1/160 dilution of serum from Dr. Bernadette Taylor in TBST was used as a positive control because she had been vaccinated many times and had a high antibody titer against influenza virus. Two negative controls were used. One negative control consisted of virus, blocking agent, 0.05% BSA in TBST, whole molecule anti-IgG antibody with alkaline phosphatase conjugation and substrate

and the second served as a "blank" with virus, blocking agent, whole molecule goat anti-human IgG antibody with alkaline phosphatase conjugation and substrate. Antibody titers were determined by subtracting the blank well value from all wells and calculating the negative well value multiplied by two. Two times the negative control OD served as the cutoff for titer determination.

The titer for each sample was found by identifying the last OD reading that was above the cutoff value and determining the reciprocal of the dilution.

Statistical Analysis

Because antibody titers were right-skewed, as opposed to normally distributed, log 10-transformed values were used. An examination of histograms and boxplots for log10-transformed IgG antibody titers as well as Shapiro-Wilk normality tests for these within each treatment group showed that conditions were met for the use of parametric procedures on the log10-transformed IgG titers. Multivariate analysis of variance was used to determine if differences in mean log10-transformed IgG antibody titers exist among the three vaccination groups either pre-vaccination or post-vaccination. Repeated measures analysis of variance was used to compare the mean increases after vaccination in log10 (IgG titer) among and within the three vaccination groups. Pearson correlation coefficients and scatterplots were used to determine if log10-transformed IgG titers were linearly correlated with log10-transformed HA and NA titers, for pre-vaccination, post-vaccination, or in the fold increase, in all treatment groups combined. No adjustments were made for multivariate responses because Type II errors in this case could potentially be more serious than Type I errors.

A 5% significance level was used for all hypothesis testing. All statistical computations and graphs were made using SPSS Version 21 provided for the Center for Statistical Analysis at UWL.

RESULTS

Purity of Concentrated Influenza Virus for ELISAs

Since there is a very low amount of IgE present in serum (0.3 µg/ml) when compared to serum IgG levels (11 mg/ml), large amounts of purified virus were needed to develop an ELISA that would detect influenza-specific total IgG and IgE (Gonzalez-Quintela et al., 2008; Kindt, 2007). To do this, A (H1N1) New Caledonia influenza virus was grown in MDCK cells seeded in CelLine flasks and precipitated to produce purified virus. In order to ensure the purity of the concentrated A (H1N1) New Caledonia influenza virus, a 10% SDS PAGE gel was run and the gel was stained with a Color Silver Stain Kit (Pierce, Rockford, IL). The gel showed a clear band between 75 and 50 kilodaltons (Figure 9), consistent with the HA protein, which migrates around 65-75 kDa (Hoffman, Maguire, & Bennett, 1997). There is also a slightly lighter band just beneath, around 60 kDa, (Figure 9), which is consistent with the monomeric weight of NA. Additionally, there is a faint band around 30 kDa that would be consistent with the matrix protein (Shtyrya, Mochalova, & Bovin, 2009). Because the gel had such a clear staining pattern consistent with prior virus purification preformed in our laboratory, we were confident that there were few unwanted proteins in the virus sample and that the ELISA plates were coated with high amounts of purified influenza only.

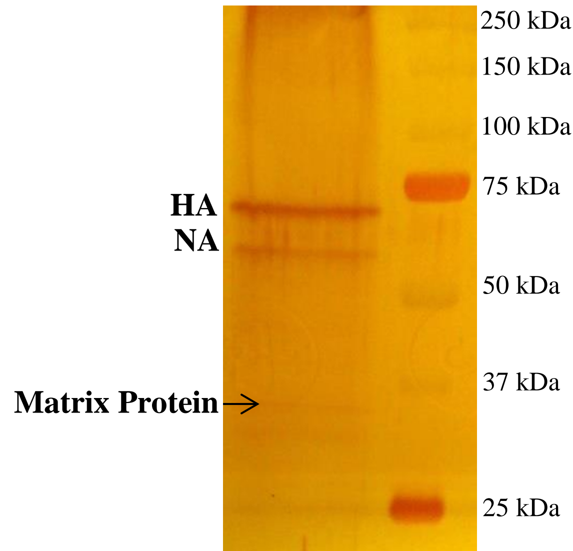


Figure 9. Left Lane: Purified influenza virus produced for coating ELISA plates was loaded on an SDS PAGE gel and stained with Color Silver Stain Kit (Pierce, Rockford, IL) to show purity of the sample. A band near 65-75 kDa was detected, consistent with the HA protein. A band near 60 kDa was visible, consistent with the NA protein, and a faint band was seen near 30 kDa, which may be the matrix protein. Right Lane: Molecular weight standards.

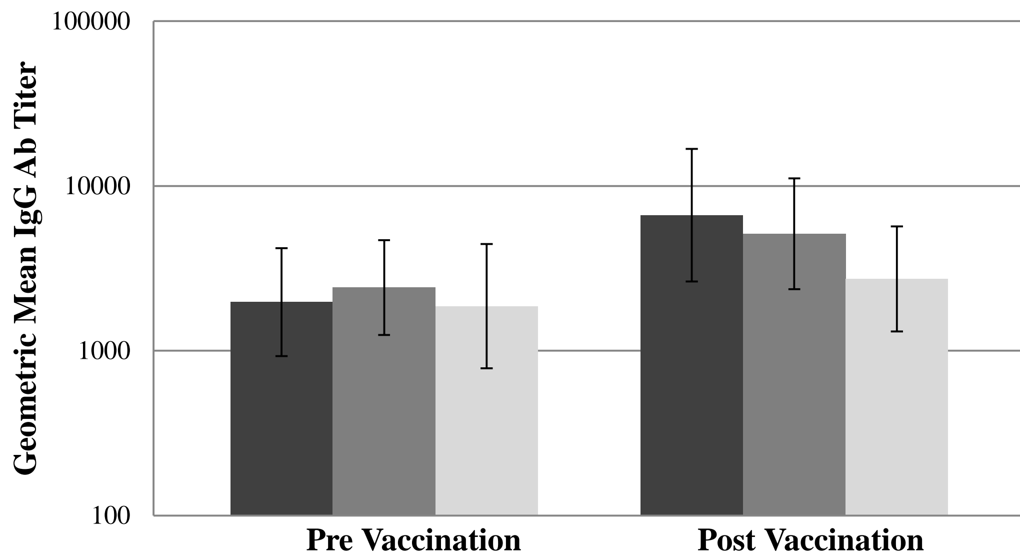
IgG Titers Specific for Influenza A (H1N1) New Caledonia Determined by ELISA

Total IgG measurements were done to give additional support to the HA and NA data found previously in our lab. Additionally, it also served as a positive control for the IgE ELISAs as there is a large amount of IgG in serum. If the IgG ELISAs did not produce positive results, there would be concern that the ELISA test was not working properly. Since the IgG ELISAs produced positive results, we could be confident that the ELISA was working properly. Geometric mean IgG influenza virus antibody titers were measured by IgG ELISA for IM, 1/5 dose ID, and 1/25 dose ID vaccination groups pre- and post-vaccination.

Of the serum samples tested with the influenza virus-specific IgG ELISA, eight samples were from the IM group, 12 samples were from the 1/5 ID group, and 11 samples were from the 1/25 ID group.

No statistical difference in mean log10-transformed IgG antibody titer was found among the three vaccination groups either pre-vaccination or post-vaccination ($P = 0.254$) (Figure 10A). While not statistically significant, there was an apparent dose dependent trend with lower antibody titers in lower dose vaccination groups (Figure 10A). Significant increases in mean IgG titer were observed after vaccination overall ($P < 0.0005$). No statistical differences were found in mean increase in log10(IgG titer) four weeks after vaccination among the three groups ($P = 0.109$), shown in Figure 10B. Again, while not statistically different, there was a clear trend toward lower titer increases with lower dose vaccination. Specifically, the IM dose group had a mean fold titer change of 5 and the 1/25 ID group had a mean fold titer change of less than 2 (Figure 10B).

A.



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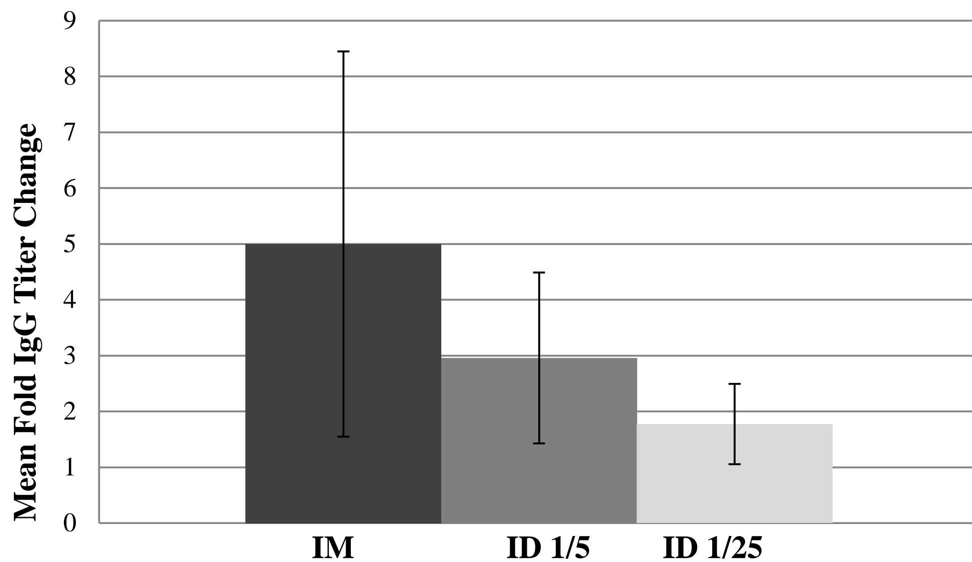

Figure 10. (A.) Geometric mean antibody titers for pre and post vaccination in IM, ID 1/5, and ID 1/25 groups. No statistically significant difference was found between the groups pre or post vaccination. Overall, significant increases in mean log10(IgG titer) were observed after vaccination ($P < 0.0005$). (B.) Influenza-specific mean titer increase following vaccination in IM, ID 1/5, and ID 1/25 vaccination groups. Error bars in both graphs represent 95% confidence intervals in each group.

Next, HA and NA titers that were previously found in our lab were compared to total IgG data. The pre-vaccination log10(IgG titer) was found to display a significant, positive linear correlation with log10-transformed titers for both HA ($r = 0.714$, $P < 0.0005$) and NA ($r = 0.524$, $P = 0.002$) (Figure 11A and 11C). This linear relationship was not as strong four weeks after vaccination for the HA titers with a Pearson correlation coefficient of ($r = 0.457$, $P= 0.011$) and was not found to be statistically significant for the NA titers ($r = 0.244$, $P = 0.187$) (Figure 11B and 11D). Additionally, the fold increase of HA and NA titers was compared to the fold increase of total virus-specific IgG. The fold increase in log10(IgG titer) was significantly positively correlated with the fold increases in the log10-transformed titers for both HA ($r = 0.491$, $P = 0.006$) and NA ($r = 0.504$, $P = 0.004$) (Figure 12). These correlations are only moderate in strength.

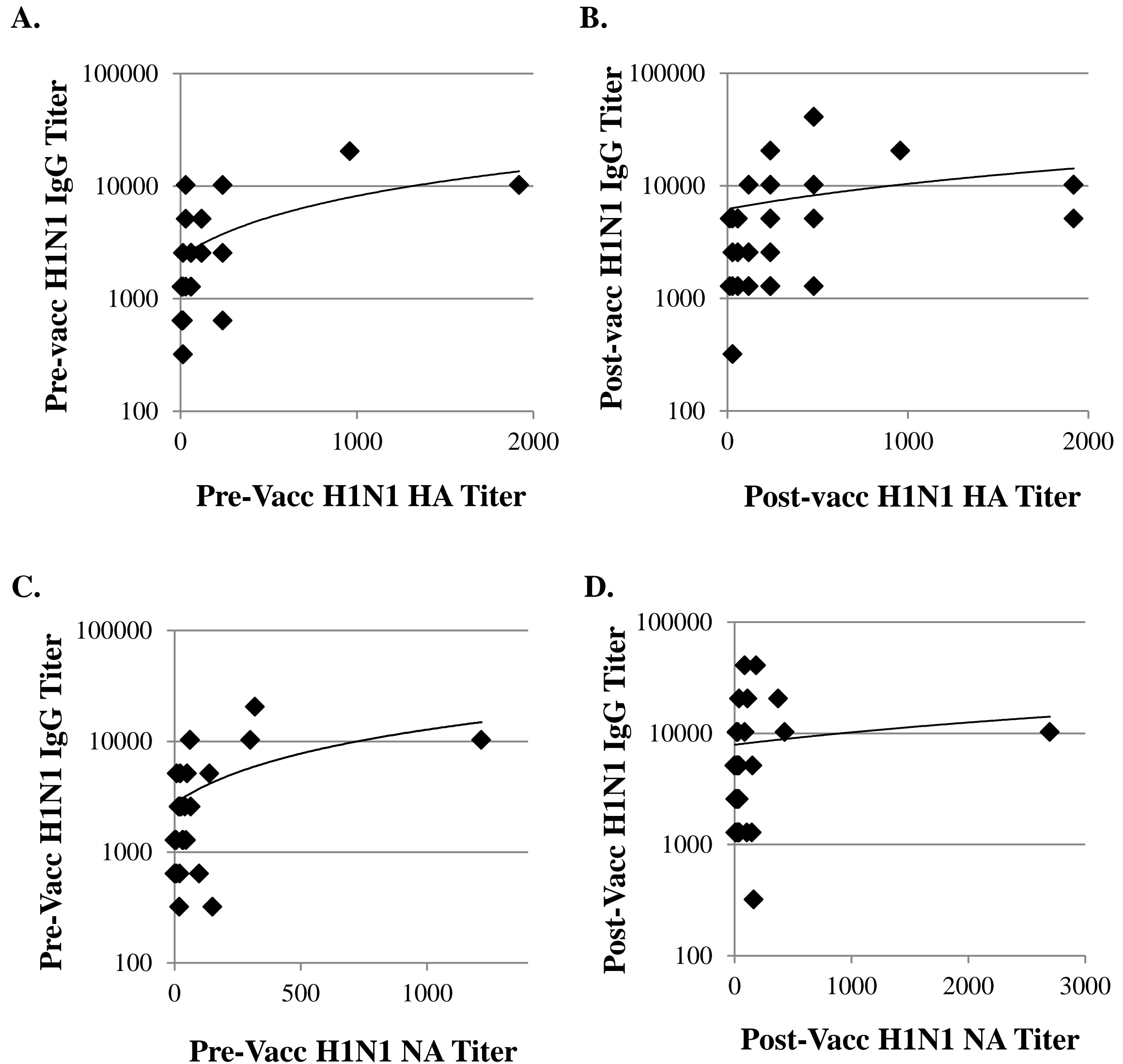

Figure 11. Virus-specific IgG titer versus HA and NA titers. (A.) Pre-vaccination A/New Caledonia (H1N1) virus-specific HA titers versus IgG titers ($r = 0.714$, $P < 0.0005$). (B.) Post-vaccination A/New Caledonia (H1N1) virus-specific HA titers versus IgG titers ($r = 0.457$, $P= 0.011$). (C.) Pre-vaccination A/New Caledonia (H1N1) virus-specific NA titers versus IgG titers ($r = 0.524$, $P = 0.002$). (C.) Post-vaccination A/New Caledonia (H1N1) virus-specific NA titers versus IgG titers ($r = 0.244$, $P = 0.187$).

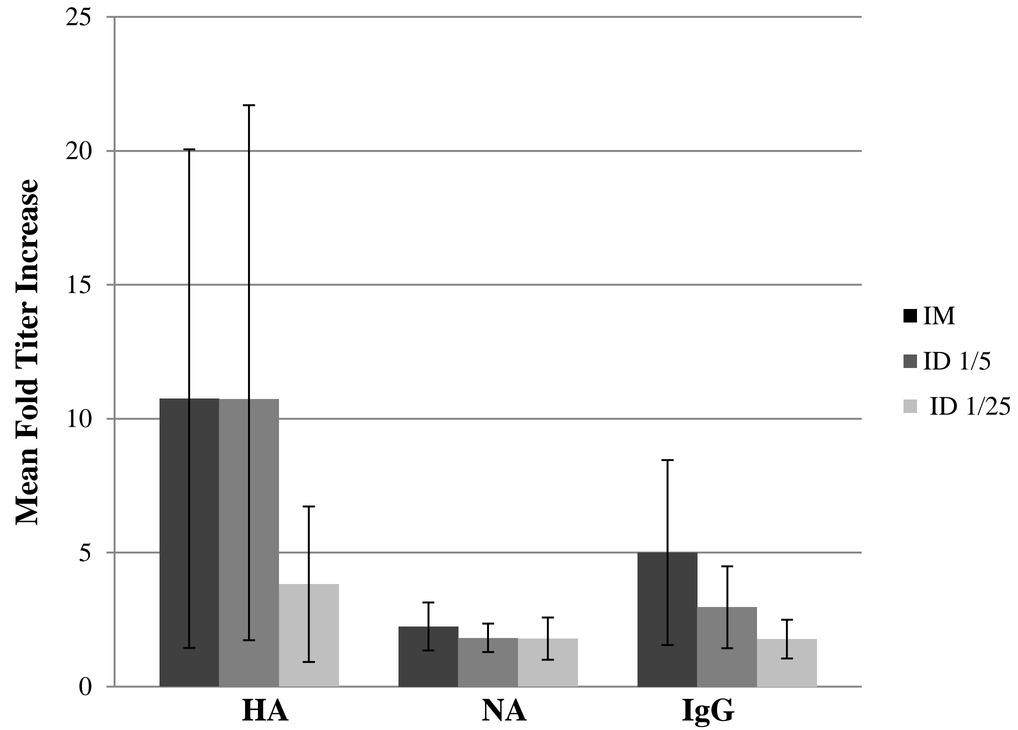

Figure 12. Mean fold increase in A/New Caledonia (H1N1) virus-specific HA ($r = 0.491$, $P = 0.006$), NA ($r = 0.504$, $P = 0.004$), and IgG titers four weeks post vaccination. Error bars represent 95% confidence intervals.

Subject Serum Influenza-specific IgE Titer Determined by ELISA

Serum samples that were tested for virus-specific total IgG were then subjected to an ELISA for virus-specific IgE. Since total IgG ELISAs were run in parallel with the IgE ELISAs, we could be confident that the ELISA was working properly.

None of the samples tested produced an OD reading above the cutoff value. Since each ELISA plate had a different negative control value (two times the negative control), the cutoff value was established for each plate individually and therefore varied between samples. Because of this, 1/5 post-vaccination serum sample OD readings from each vaccination group were plotted as a percentage of the cutoff value (Figure 13). Although none of the samples were above the cutoff value (displayed as 100%), there were a few samples in the 1/5 and 1/25 ID vaccination groups that were close to 80 percent. These samples could potentially have tested positive if undiluted serum was used or if serum samples had been collected sooner post-vaccination, since IgE responses peak and drop much more rapidly than IgG responses.

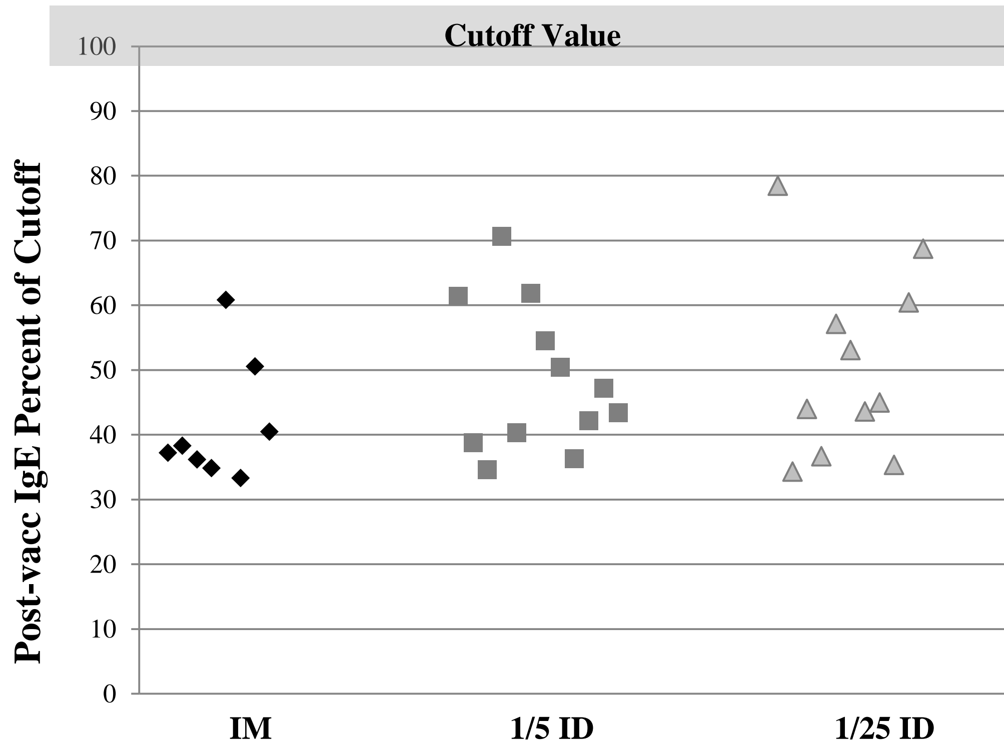

Figure 13. Post-vaccination IgE raw OD readings at the 1/5 serum dilution were all below the cutoff value (two times the negative) established for each sample. Each point represents a sample as a percentage of the total cutoff value, displayed here as 100 percent. Although a few samples in the 1/5 ID and 1/25 ID groups were close to 80 percent of the cutoff, no samples were above.

DISCUSSION

Previous low-dose ID influenza vaccination studies, which administered 1/5 and 1/25 doses, showed immune responses similar to those elicited from full-dose IM administration (Ansaldi, de Florentiis, Durando, & Icardi, 2012; Belshe et al., 2007; Beran et al., 2009; Jo et al., 2009; Ledgerwood et al., 2012; Van Damme et al., 2009). However, one foreseen drawback to low-dose vaccination may be the production of a T_H2 allergic response rather than a T_H1 response. Although Th2 inducing studies have never been performed in human subjects, at least two studies have shown that reducing the amount of antigen necessary for a protective immune response by 1/10 significantly increased the amount of IL-4 produced, inducing a Th2 immune response (Constant et al., 1995; Ruedl et al., 2000). This creates a heightened concern that giving a low dose of vaccine (especially at 1/25 the standard dose) each year may eventually cause more patients to develop an allergic response.

No Detection of Influenza Virus-specific IgE by ELISA in Patient Samples

None of the samples tested for influenza virus-specific IgE provided an OD reading above the cutoff (Figure 13). As the graph of 1/5 serum dilution post-vaccination samples shows, there are some samples in the 1/5 and 1/25 vaccination groups that were close to 80% of the cutoff value. There is a very low amount of IgE present in serum (0.3 μg/ml) when compared to serum IgG levels (11 mg/ml) (Gonzalez-Quintela et al., 2008; Kindt, 2007). Although undiluted sera may have produced measurable virus-specific IgE titers, limited serum volumes made testing undiluted samples impossible. In addition, the

serum samples were frozen and thawed several times to run multiple experiments, which may have caused denaturation of antibodies and may have depleted the small amount of IgE antibody present in the serum. A third explanation for low titers may be that patient serum was not collected when a maximum amount of IgE was in circulation. A study by Mitchell et al., using rats, showed that the biological activity of serum IgE peaked at day 10 post antigen exposure and declined rapidly (seven-fold) every day after (Mitchell, Moss, & Collins, 2002).

Intramuscular and Dose-sparing Intradermal Vaccinations Produce a Similar Total Virus-specific IgG Response

There were similar total IgG antibody responses mounted in response to the IM, ID 1/5, and ID 1/25 vaccine doses given, as shown in Figure 10a. There were no significant differences found between mean log10-transformed IgG titers among the three vaccination groups pre-vaccination or post-vaccination. Additionally, there were significant increases in mean log10 IgG titers overall after vaccination and no significant differences were found in the mean increase in log10 IgG titer four weeks post vaccination between the three vaccination groups. Figure 10b, which presents the mean change in IgG antibody titer, supports this by displaying a similar titer change across all three vaccination groups. Though it was not statistically significant, there was also a visible dose-dependent trend in in antibody production. Specifically, the lower the amount of HA given, the lower the antibody response (Figure 10).

Unfortunately, there are no established seroprotection or seroconversion standards for total influenza-specific IgG. The European Center for Disease Prevention and Control (ECDC) has established seroprotection and seroconversion standards that test the

ability of a vaccine to prevent clinical disease (vaccine efficacy) before licensure is granted by using HA antibody titer only. In adults 18-59 years of age, the mean fold increase in HA antibody titers following vaccination must exceed 2.5 (seroconversion factor). Additionally, at least 70% of vaccinated individuals must have a HA antibody titer of ≥40 (seroprotection rate) and at least a four-fold increase in HA antibody titers should be seen in 40% (seroconversion rate) of vaccinated individuals. For adults over 65 years of age, at least 60% of vaccinated individuals must have a HA antibody titer of ≥40 and at least a four-fold increase in HA antibody titers should be seen in 30% HAI (European, 1997).

Similarly, the United States Food and Drug Administration (FDA) uses the geometric mean titer GMT and rates of seroconversion as a recommendation for comparing vaccine efficacy. The seroconversion recommendation is defined as the percent of subjects with a minimum four-fold rise in HA titer. For example, a pre-vaccination HA titer < 1:10 and a post vaccination titer >1:40, or a pre-vaccination titer >1:10 and a minimum 4-fold rise HI titer (FDA/CBER, May 2007).

Although there are no established seroprotection or seroconversion standards for total influenza-specific IgG, there is a strong positive correlation between the level of antibody produced and the likelihood of clinical protection. Specifically, a relatively large amount of anti-influenza virus antibody leads to a positive clinical protection prospect. Therefore, mean total post-vaccination antibody titer is frequently used as a measurement of clinical protection (Nauta, Beyer, & Osterhaus, 2009).

Previously, influenza A/New Caledonia/20/99 (H1N1) HA and NA-specific antibody titers of pre and post vaccination serum samples from the same subjects were

measured by using hemagglutinin and neuraminidase inhibition assays. When titers from these two assays were compared to log10 IgG titers, the pre-vaccination log10 IgG titer was found to show a significant, positive linear correlation with titers for both HA and NA. However, at four weeks post vaccination this relationship was weak for the HA titers and not present for the NA titers (Figure 11). These data suggest that, in individual subjects, the total IgG antibody level is relatively reflective of HA and NA antibody levels before vaccination. Though the HA antibody increased somewhat parallel to the total IgG response after vaccination, NA antibody levels did not. Since the total amount of IgG is a mix of HA and NA inhibitory and non-inhibitory antibodies and antibodies against other viral proteins (like the matrix protein), it is not surprising that there is a much larger amount of total IgG than HA or NA alone.

Additionally, the fold increase in virus-specific IgG titer was significantly positively correlated with the fold increases in the antibody titers for both HA and NA (Figure 12). These correlations are only moderate in strength but suggest that there was a similar increase in total virus-specific IgG antibody production when compared to HA and NA antibody responses.

Together, the HA, NA, and total IgG data suggests that a similar antibody response was formed in all three vaccination groups. This adds evidence supportive of findings from others who have administered a 1/5 ID dose of vaccination and measured the antibody response by HIA (Young & Marra, 2011). However, no one has reported total IgG antibody responses after 1/5 ID influenza vaccination and no one has ever reported dose-sparing vaccination as low as 1/25 the standard dose. Since we began this study, one intradermal trivalent influenza vaccine has been approved for the United

States market called Fluzone® Intradermal. This new vaccine contains 9 µg of each antigen in a 0.1 ml dose, which provides 3/5 of the standard dose (Centers, 2013b). While this is lower than the standard intramuscular dose of 15 µg of each antigen, our data suggests that this dose could be further reduced to allow for a greater amount of vaccine coverage globally. Unfortunately, no data has yet been released on the total IgG response produced by Fluzone® Intradermal.

Summary

An influenza virus-specific IgG and IgE ELISAs were developed and used to measure the amount of corresponding serum antibody produced by patients vaccinated with a standard IM, 1/5 ID, or 1/25 ID vaccine dose. IgG levels increased significantly in all dose groups, suggesting a similar vaccine efficacy for all three groups. This supports use of a lower dose of vaccine for dose sparing benefits. Additionally, there was very little IgE detected in the serum samples. This may be because neat serum could not be used, serum samples were subject to multiple freeze-thaw cycles, or serum may not have been collected during peak IgE serum levels. IgE responses to low dose ID vaccination will require further study with time course data collected to elucidate any induced IgE antibodies and to more fully answer concerns about low dose vaccination.

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APPENDIX A

REAGENTS FOR H1N1 INFLUENZA PROPAGATION

Appendix A. Reagents for H1N1 Influenza Propagation

a.) MDCK growth medium for 75 cm^2 flasks

Minimum essential medium containing Earle's salts, L-glutamine, and essential amino acids (MEM) supplemented with (per 500 ml):
0.75 g sodium bicarbonate ($NaHCO_3$)
5 ml sodium pyruvate
5 ml pen-strep stock (100 U/ml penicillin G and 100 µg/ml streptomycin)
Fetal bovine serum (10% total)
Filter sterilize

b.) MDCK growth media for media compartment of CELLine flask

MEM supplemented with (per 500 ml):
0.75 g sodium bicarbonate ($NaHCO_3$)
5 ml sodium pyruvate
5 ml pen-strep stock (100 U/ml penicillin G and 100 µg/ml streptomycin)
1.75 g glucose
2.5 ml of 400 mM L-glutamine
Filter sterilize

c.) MDCK growth media for cell compartment of CELLine flask

MEM (per 500 mls):
0.75 g sodium bicarbonate ($NaHCO_3$)
5 ml sodium pyruvate
5 ml pen-strep stock (100 U/ml penicillin G and 100 µg/ml streptomycin)
1.75 g glucose
2.5 ml of 400 mM L-glutamine
Fetal bovine serum (20% total)
Filter sterilize

d.) Influenza growth media for 75 cm^2 flask

MEM was supplemented with (500 mls):
0.75 g sodium bicarbonate ($NaHCO_3$)
5 ml sodium pyruvate
5 ml pen-strep stock (100 U/ml penicillin G and 100 µg/ml streptomycin)
t-l-Tosylaminde-2-phenylethyl chloromethyl ketone-treated trypsin (2 µg/ml)
Filter sterilize

e.) Influenza growth media for 75 cm^2 flask

MEM was supplemented with (500 ml):
0.75 g sodium bicarbonate ($NaHCO_3$)
5 ml sodium pyruvate
5 ml pen-strep stock (100 U/ml penicillin G and 100 μg/ml streptomycin)
1.75 g glucose
2.5 ml of 400 mM L-glutamine
t-l-Tosylaminde-2-phenylethyl chloromethyl ketone-treated trypsin (2 μg/ml)
Filter sterilize

f.) Phosphate buffered saline (PBS)

900 ml ddH_2O
0.2 g KCL
0.2 g KH_2PO_4
8 g NaCl
1.15 g Na_2HPO_4
pH 7.2-7.4
1L ddH_2O
Filter sterilize