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Graduate Studies

ANTIBODY RESPONSE TOWARD INFLUENZA VIRUS NEURAMINIDASE PROTEIN AFTER LOW DOSE INTRADERMAL VERSUS INTRAMUSCULAR FLUZONE® VACCINATION IN HUMANS

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College of Science and Health
Clinical Microbiology Concentration

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ANTIBODY RESPONSE TOWARD INFLUENZA VIRUS NEURAMINIDASE PROTEIN AFTER LOW DOSE VERSUS INTRAMUSCULAR FLUZONE® VACCINATION IN HUMANS

By Katie M. Bauer

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ABSTRACT

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Influenza is a contagious respiratory illness which results in hospitalization of more than 200,000 people and the death of around 36,000 people in the United States every year. The best way to reduce the impact of influenza is through immunization. Current seasonal vaccination is designed to induce a neutralizing antibody response against the viral envelope protein hemagglutinin (HA). An antibody response toward influenza viral envelope protein, neuraminidase (NA), has also been shown to contribute toward protection from influenza disease. Low-dose intradermal vaccination has been proposed as a vaccine-stretching measure. Intradermal influenza vaccination may elicit a strong anti-NA antibody response, despite the vaccine containing less NA than HA, because of the abundant antigen presenting cells found in the skin. This research compares the magnitude of antibody response to influenza virus protein NA elicited by the influenza vaccine (Fluzone®, 2004-2005) administered intramuscularly (standard dose) and intradermally (1/5 dose and 1/25 dose). A neuraminidase inhibition assay was used to determine the NA inhibition antibody titer of vaccinated subjects against A/New Caledonia/20/99 (H1N1) and A/Wyoming/03/03 (H3N2). It was also used to detect cross-reactive NA-specific antibodies against a novel avian influenza strain A/Turkey/Ontario/6625-1/98 (H6N1). Results showed that virus-specific NA inhibition antibody titers were similar among vaccination groups and that the seasonal influenza vaccine produces cross-reactive antibodies to influenza virus NA protein.

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INTRODUCTION

Basics of influenza infection and vaccination. Influenza (the flu) is a contagious respiratory illness caused by influenza virus. The flu can cause mild to severe illness with symptoms of high fever, headache, dry cough, sore throat, and myalgia. On average, each year more than 200,000 people are hospitalized and about 36,000 people die from influenza virus infection in the United States (11).

The best way to reduce the impact of influenza virus infection and subsequent morbidity and mortality in the human population is through immunization. Current methods of seasonal vaccination are directed toward mounting a neutralizing antibody response against the virus. This is accomplished through a vaccine that is primarily composed of the influenza virus envelope protein, hemagglutinin (HA), to which an antibody response is known to protect against subsequent infection by the same strain of the virus (20, 21, 32, 34, 54, 62). An antibody response toward the other influenza envelope protein, neuraminidase (NA), has also been shown to contribute toward protection from influenza disease (23, 47-49, 69, 72, 73). Neuraminidase is present in the vaccine, although the amount per dose has not been standardized like that of HA. Low abundance of NA in the vaccine may lead to a less than optimal protective antibody-mediated response toward the protein. This is particularly unfortunate for individuals who have a poor antibody-mediated response toward HA and therefore may not be protected by the vaccine. Also, antibodies toward NA have been shown to be cross-

reactive among strains within a subtype (8, 31, 55, 66, 74, 75, 84) which may help dampen the effect of small antigenic changes of the virus known as antigenic drift.

Influenza classification. Influenza virus is a member of the *Orthomyxoviridae*, a family of single-stranded negative sense RNA viruses with segmented genomes. Influenza viruses are divided into three genera, *Influenzavirus A, Influenzavirus B,* and *Influenzavirus C,* based on the glycoprotein composition of their viral envelope. Influenza A and B virus envelopes contain two different glycoproteins, hemagglutinin (HA) and neuraminadase (NA) while influenza C virus envelopes have a single external glycoprotein, hemagglutinin esterase-fusion protein. The surface glycoproteins (HA and NA) of influenza A viruses have much greater amino acid sequence variability than those of influenza B viruses. Because of this variability, influenza A viruses are subdivided according to which antigenic determinants of HA (16 known types) and NA (9 known types) they possess (45).

Influenza virus structure. Influenza A virus is made of a viral envelope containing glycoproteins, wrapped around a central core. The central core contains the viral RNA genome and viral proteins needed to protect the RNA of the virus, replicate the virus, and assemble new virus progeny. The influenza A virus genome has eight negative sense RNA segments from which ten polypeptides are encoded (Fig. 1). Three of these polypeptides are inserted into the lipid envelope: hemagglutinin (HA), involved in cell entry; neuraminidase (NA), involved in cell exit; and matrix protein 2 (M2), involved in uncoating of the virus. Under the viral lipid envelope lies matrix protein 1 (M1) which aids in viral assembly in the host cell cytoplasm Inside M1 are the viral ribonucleoproteins (vRNP) which are composed of the genomic RNA segments,

nucleocapsid/nucleoprotein (NP), which coat the eight RNA segments, and RNA polymerase complex proteins (PB1, PB2, and PA). Non-structural proteins (NS1 and NS2) are also found in small quantities within the virus particle (46).

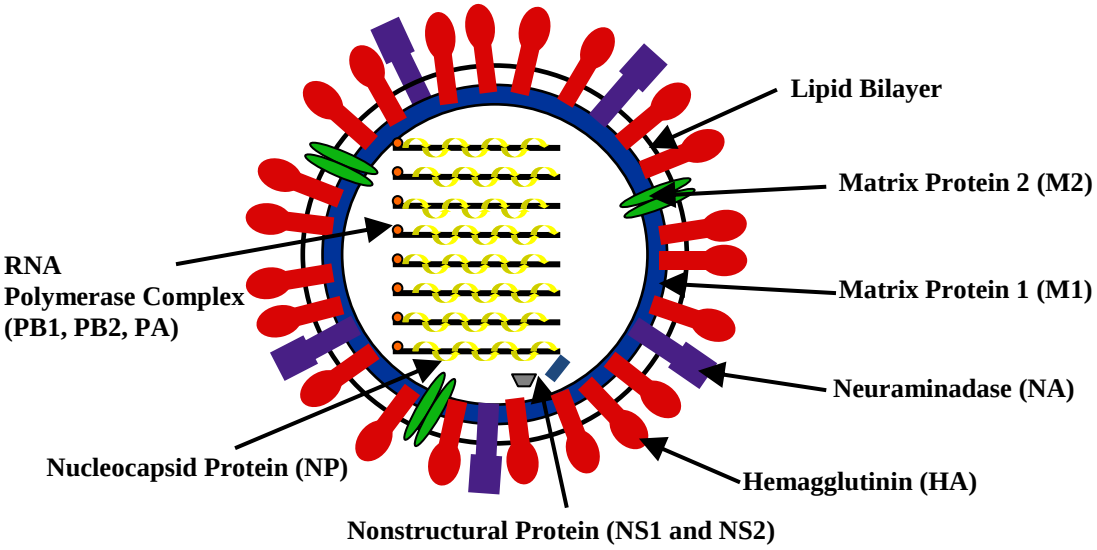


FIG. 1. Influenza virus structure.

Influenza virus life cycle. Influenza virus glycoprotein HA initiates infection of a eukaryotic host cell by binding to 5-N-acetyl neuraminic acid (sialic acid) residues on the surface of the cell, which triggers endocytosis of the virus (Fig. 2, steps 1-2). The viral envelope then fuses with the endosomal membrane, an event triggered by a conformational change in HA under low pH conditions. This allows the vRNPs to enter the cytoplasm (Fig. 2, steps 3-4) (81). Influenza vRNPs enter the nucleus through translocation across the nuclear pore complex (Fig. 2, step 5). Viral genomic RNAs are transcribed into mRNA in the nucleus and steal the host cap from the host cell's mRNA, which allows viral mRNAs to be translated into viral proteins by host cell ribosomes in the cytoplasm (Fig. 2, step 6). The translation of M1, NP, NS1, PA, PB1, and PB2

proteins occurs on free ribosomes (Fig. 2, step 7), whereas translation of HA, NA and M2 proteins occurs on membrane-bound ribosomes of the rough endoplasmic reticulum (RER) (Fig. 2, step 8). After translation, viral proteins HA, NA, M1, NS1, and M1 are delivered to the plasma membrane where they are incorporated into new virus particles (Fig. 2, step 9) (45). Viral RNA pol proteins and NP are transported back to the nucleus to aid in viral genome transcription and replication (Fig. 2, step 10) Inside the nucleus, the negative sense viral genomic RNA (gRNA) is replicated through the synthesis of a complementary RNA (cRNA) intermediate (Fig. 2, steps 11-12). New vRNPs leave the nucleus and go to the host cell surface where they are packaged in a budding viral particle (Fig. 2, steps 13-14). The matrix protein, M1 is an important mediator of viral assembly because it binds vRNPs, the cytoplasmic tails of the glycoproteins, and other M1 proteins to form a shell beneath the plasma membrane (Fig. 2, step 15). Once all the viral proteins and vRNPs are assembled at the host plasma membrane, the virus buds from the host cell. Final release of the virus relies on NA to cleave sialic acid residues from the surface of the host cell so that the virus does not rebind to the receptors, which would prevent the virus from being released into the extracellular space (Fig. 2, step 16) (81).

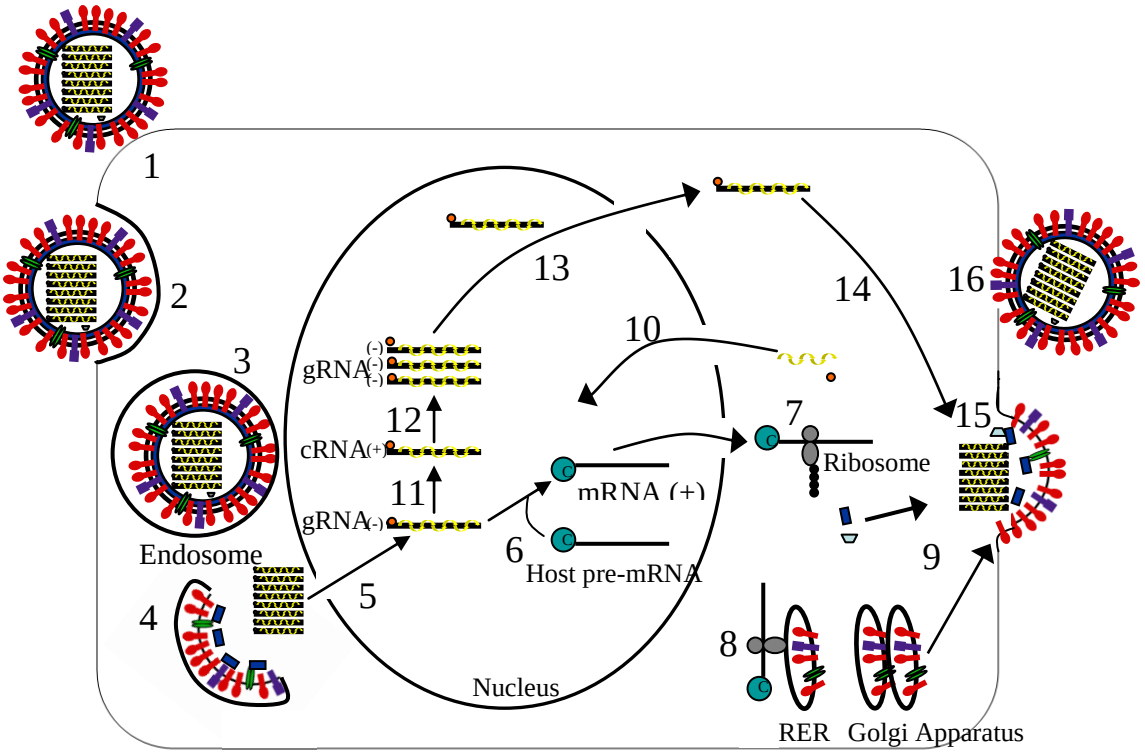


FIG. 2. Life cycle of influenza virus

Structure and function of influenza HA and NA viral proteins. The influenza virus envelope contains about 500 HA spike glycoproteins (M_w 375,000 Da). Each HA is a homotrimer with a large ectodomain which forms rod-like structures that project radially from the viral surface. The distal domain of each trimer contains 3 binding sites that are specific for sialic acid residues on the host cell surface. The HA is synthesized as a precursor polypeptide HA_0 (M_w 77,000 Da) which requires proteolytic cleavage into HA1 (M_w 50,000 Da) and HA2 (M_w 27,000 Da) before it becomes functional and the virus particle can infect host cells. The cleavage results in the generation of a terminal hydrophobic peptide on HA2 that is needed for the virus to fuse with the host endosomal membrane (45).

The influenza virus envelope contains about 100 NA glycoproteins (M_w 220,000 Da). Each NA is a homotetramer made up of a mushroom-shaped head with four

domains which is enzymatically active and a stalk region that is attached to the membrane. The active sites cleave terminal sialic acid residues, therefore allowing efficient viral release and preventing further viral infection of same cell (45).

Adaptive immune response to influenza virus. The immune response to influenza viral proteins is dictated by how the viral proteins are processed by the immune system. All influenza viral proteins are translated during viral replication and can serve antigens inside the infected host cell. These endogenous viral antigens undergo cytoplasmic degradation, which provokes cell-mediated immunity. Newly translated virus proteins are broken down by proteasomes in the infected host cell cytoplasm. The resultant peptides are loaded onto MHC I molecules and expressed on the cell surface (Fig. 3 A). The peptide-MHC I complex is recognized by T cell receptors of CD8+ T cells (Tc cells) which destroy the infected cells. Membrane bound antigens, HA, NA and M2 can also stimulate a dendritic cell-mediated response resulting in presentation with MHC I by dendritic cells through a process called cross-presentation (Fig. 3 B). In this process, phagocytosed proteins are transported out of the phagosome and processed by cytoplasmic proteosomes just as endogenous proteins are. Alternatively, membrane bound antigens can remain in the endocytic pathway of antigen presenting cells and be degraded by the phagolysosome. The peptides of these antigens are loaded onto MHC II molecules and expressed on the surface of the antigen presenting cell (Fig. 3 C). The peptide-MHC II complex is recognized by CD4+ T helper cells (Th cells). Th cell activation results in the production of cytokines (Fig. 3 D) which trigger B lymphocytes specific for the antigen to differentiate into plasma cells, and produce antigen specific antibodies (Fig. 3 E). (30, 75).

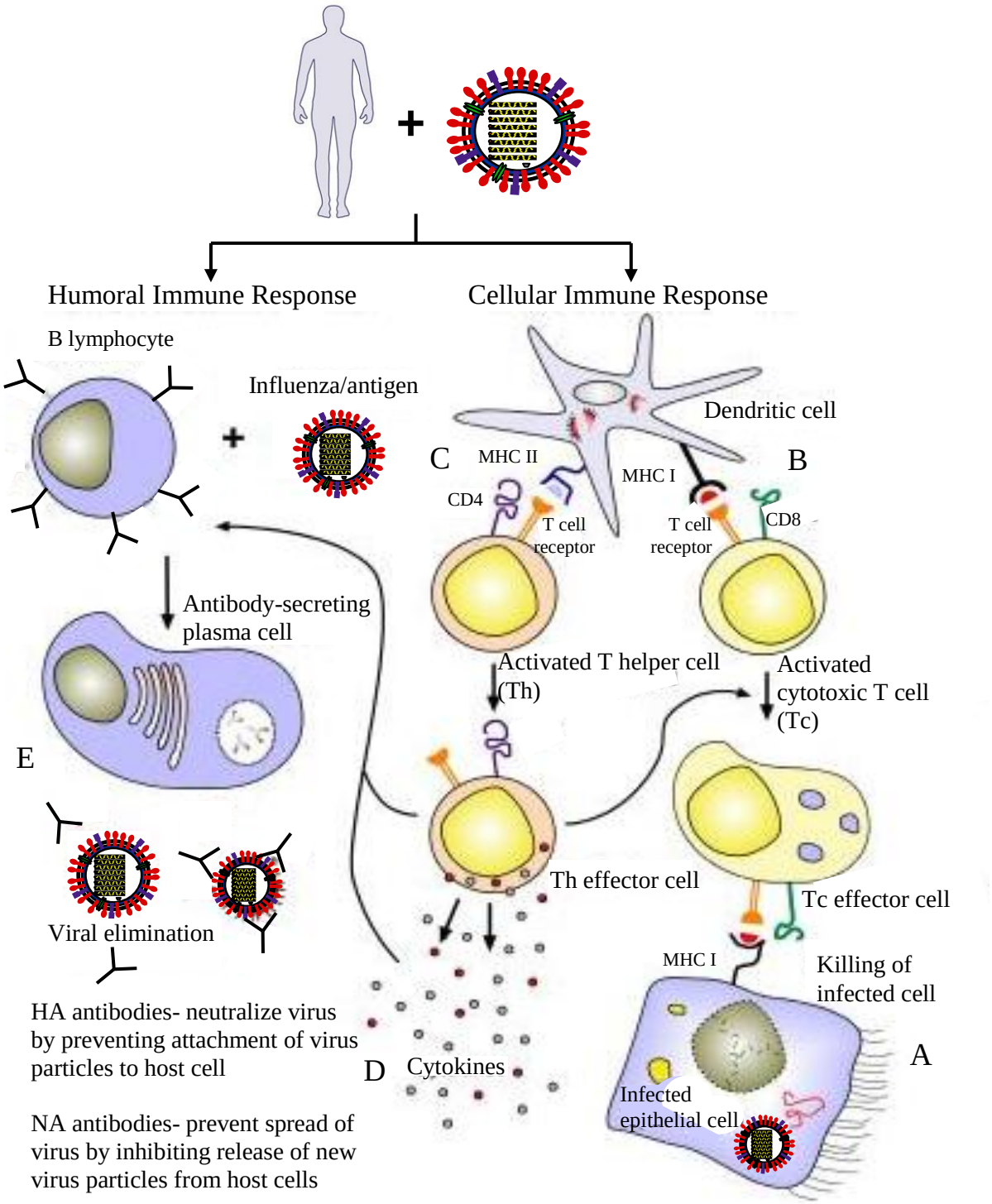


FIG. 3. Immune response mediated toward influenza virus. Infection with influenza virus results in a cellular immune response which kills virus infected cells as well as a humoral immune response which results in the production of antibodies that neutralize or inhibit release of influenza virus.
Modified diagram from Influenza Report

Antibodies to influenza viral proteins. The goal of current vaccination is to produce neutralizing antibodies against HA. These antibodies block viral attachment to the host target cell by binding to HA epitopes close to the ligand binding site (30). Response to influenza virus vaccines is measured by quantifying serum HA-specific antibody titers using the hemagglutination inhibition (HI) assay. The HI assay measures the ability of antibodies to block HA binding to sialic acid- containing receptors on red blood cells (58). Measurement of vaccination efficacy is currently based primarily on HA antibody titer.

Other antibodies, besides HA antibodies that block viral attachment to the host cell, play an important role in interfering with influenza virus replication. For instance, antibodies made against the HA stalk cross-link monomers of HA and in doing so prevent membrane fusion (30, 50). Antibodies to influenza virus NA prevent neuraminidase activity, which is needed for the release of newly formed virus from the host cell (30). Non-neutralizing antibodies binding directly to any accessible epitope can lead to Fc-receptor-mediated binding of macrophages, phagocytosis, and inactivation of the virus (53). Antibodies toward M2 have been demonstrated to lead to antibody dependent cellular cytotoxicity by NK cells as well as to prevent assembly of virus particles at the cell surface and alter distribution and surface expression of M2 (36, 37). Even though non-neutralizing anti-influenza antibodies cannot prevent infection with influenza virus; they can promote viral clearance, reduce virus spread, and thereby reduce the duration, morbidity, and mortality of influenza disease (10, 38).

Antigenic drift and shift. Antigenic drift is a continual, gradual process through which new strains of influenza virus emerge. It occurs because an error-prone RNA polymerase creates point mutations within the HA, NA, and other viral protein gene segments, resulting in minor changes in their respective amino acid sequences (71). These subtle changes in HA and NA result in new antigenic sites and natural selection drives the emergence of virus strains that are less effectively recognized by previously made antibodies. Reformulation of influenza vaccines each year is an attempt to protect against new human influenza strains that have emerged due to antigenic drift.

Influenza A also undergoes antigenic shift, which is the process by which two or more different strains of influenza infect an individual cell and recombine to form a new subtype with a mix of genes from the original strains. Reassortment of HA and/or NA RNA segments between influenza strains can lead to a sudden change in surface antigen expression (45). Because pigs are susceptible to avian, human and swine influenza viruses, they can be infected with influenza viruses from different species (e.g., chickens and humans) at the same time (70, 85). If this happens, it is possible for the genes of these viruses to reassort and create a new virus. For example, if a pig was infected with a human influenza virus and an avian influenza virus at the same time a new virus that had most of the genes from the human virus, but a HA or NA from the avian virus could be produced. The resulting new virus would likely be able to infect humans and spread from person to person, but it would have surface proteins (HA or NA) not previously seen in influenza viruses that infect humans, and therefore to which most people have little or no immune protection (70, 85). A recent example of antigenic shift is a swine H1N1 strain that emerged in Mexico in February 2009. This virus contains PB2 and PA genes of

North American avian virus origin, a PB1 gene of human H3N2 virus origin, HA (H1), NP, and NS genes of classical swine virus origin, and NA (N1) and M genes of Eurasian avian-like swine virus origin (56). Antigenic drift (and shift) is unpredictable and therefore scientists are unable to prepare vaccines in advance that are effective against them. Consequently, the emergence of a new subtype of the virus that transmits easily among humans can cause a global pandemic in a very short time.

Fluzone® production and composition. Influenza vaccination strategies try to include new strains of influenza virus circulating in the human population. The viruses in the vaccine change each year and depend upon international surveillance and scientists' estimations about which types and strains of viruses will circulate in the coming year (11). Fluzone®, is split-virion, inactivated vaccine which is typically administered intramuscularly. Each vaccine contains three influenza viruses; one A(H3N2) virus, one A(H1N1) virus, and one B virus. According to the manufacturer, Fluzone® is prepared from influenza virus propagated in embryonated chicken eggs. The virus-containing fluids are harvested and inactivated with formaldehyde. The influenza virus is concentrated and purified in a linear sucrose density gradient solution and then disrupted with Triton X-100, producing a split virus. The lipo-philic fraction is further purified by chemical means and suspended in sodium phosphate-buffered isotonic sodium chloride solution. Each 0.5 ml dose is formulated to contain a total of 15 µg HA per virus strain (68).

Although, the vaccine is produced to contain large amounts of influenza HA glycoprotein, it also contains NA and internal virus proteins M1 and NP (5, 14, 64). It has been estimated NA makes up 6.9-8.5% of an influenza viral particle’s total protein

while NA contributes 9.6%-12.7% of the total protein found in split-virion vaccines (76). Therefore, the NA level in each split-virion vaccine is 1.4-1.6-fold higher than in the corresponding viral particle (76). Analysis of eleven batches of trivalent, split-virion vaccines from 1997-1999 showed that the NA content of influenza B viruses range from 5.1-6.9 µg/ml (3). If this were also true of the influenza A viruses contained in the vaccine and all trivalent, split-virion vaccines contain roughly the same NA content per strain, each 0.5 ml dose of vaccine would contain 2.5-3.5 µg NA per virus strain.

Correlation between HA inhibition titer and influenza vaccine efficacy. Before a new vaccine is licensed it is tested and proven to be protective (33). Vaccine efficacy is a measure of direct protection against disease in subjects who have been vaccinated compared with subjects who have not been vaccinated. Vaccine effectiveness is often confused with vaccine efficacy although they are distinctly different, yet related concepts. Essentially, vaccine effectiveness is how well a vaccine reduces disease in a population in the real world. Vaccine efficacy is usually measured by randomized, double-blind, controlled clinical trials looking for decrease in attack rates while vaccine effectiveness is often measured by retrospective case control studies (79).

Influenza vaccine efficacy has been determined through immune response studies, challenge studies, field trials, and case control studies. In challenge studies healthy volunteers were vaccinated and then challenged with wild type or live attenuated influenza virus. These studies showed that there is a positive linear correlation between pre-challenge HA antibody titers and the percentage of people that are protected from influenza disease upon challenge (20, 32). In a study performed during a natural influenza A epidemic in 1951 that included 2852 men, the probability of clinical infection

closely correlated with pre-epidemic homologous HI antibody titer. Individuals with a titer of 16 had an attack rate of 1.5% while those without detectable antibodies toward HA had an attack rate of 18% (54). The study also showed no cases of influenza in individuals with HI antibody titers of 32 or greater (54). Many other studies have shown a clear correlation between resistance to infection and antibody levels to HA before exposure to influenza virus, specifically that HA antibody titers in the range of 30-40 confer 50% protection of the population against infection (21, 34, 62).

Efficacy studies clearly indicate that clinical protection from influenza vaccines closely correlates with their antigenicity. Therefore, it is generally accepted that vaccine-induced HA antibody titers specific for disease causing strains in the community are a good surrogate marker for clinical efficacy. An HA antibody titer $\geq$40 represents the protection threshold beyond which serious injury is unlikely to occur (13). In Europe, influenza vaccines are now tested annually for immunogenicity as part of a marketing approval procedure. Approval requires that the mean fold increase in HA antibody titers following vaccination in individuals aged 18-60 years should exceed 2.5 (seroconversion factor), with at least 70% of those vaccinated having a HA antibody titers of $\geq$40 (seroprotection rate) and at least a four-fold increase in HA antibody titers seen in at least 40% (seroconversion rate) (17). The Food and Drug administration approves licensure of the influenza vaccine in the United States, using seroconversion rate and seroprotection rate as criteria for licensure (25).

Problems with current influenza vaccination. There is a need for an improved influenza virus vaccine or a vaccine strategy that will protect a larger percentage of the human population from multiple strains of circulating influenza virus. Despite the use of a consistent formulation, dose administration, and route of administration the protective immune response to seasonal influenza vaccination is quite heterogeneous among humans (19, 27). Failure to mount protective neutralizing antibody responses against influenza A virus HA is part of the problem (28, 51). Efficacy of the vaccine is solely measured by ability of the vaccine to induce antibody production toward HA. When the match between the vaccine and circulating strains is good, efficacy rates of healthy adults range from 70-90% but are much lower in healthy elderly 65 years of age or greater (26-77%) and immunocompromised individuals (18, 24, 57, 61). New analysis of flu studies from 1967-2011 determined that clinical effectiveness of the seasonal influenza vaccine for individuals 18-65 is 59%, not 85-95% effective as previously thought (59). In addition to efficacy problems of the current influenza vaccine against targeted influenza viruses, the current vaccination strategy is solely to mount protection against influenza strains that are in that vaccine. The 2009-2010 pandemic caused by a new influenza A (H1N1) virus of swine origin and the pandemic threat caused by the highly pathogenic avian influenza A viruses (H5N1 and H2N2) have increased the interest in developing vaccines that can induce broad protective immunity.

Improved influenza vaccination through the intradermal route. Current inactivated vaccines given intradermally may induce strong antibody responses toward HA and other viral proteins, even at lower doses because of the abundant antigen presenting dendritic cells, found in the skin. Antibody production to NA for example

could lead to better protective response toward influenza virus resulting in a reduction of the incidence and/or severity of infections (22). If intradermal vaccination produces antibody production toward NA, intradermal vaccination may offer protection to those who respond poorly to HA. Additionally, antibodies toward NA have been shown to be more cross-protective than antibodies toward HA due to the more highly conserved nature of the NA protein and may therefore offer some protection against strains of influenza not in the vaccine (8, 31, 55, 66, 74, 75, 84).

Studies have shown that there is a decreased immunogenicity toward NA antigen when it is delivered in conjunction with HA in conventional vaccine strategies, a phenomenon known as antigenic competition (39, 40, 42). A previous study showed that supplementation of conventional influenza vaccine with NA induces a balanced immune response without antigenic competition (41). However, no studies were found that looked at the effect of inoculation sites or low-dose vaccination on antigenic competition of influenza vaccination.

The skin as an immune system organ. The skin is a critical barrier between the host and the environment. It is subject to a variety of potentially damaging agents including toxins, radiation, and microorganisms. In order to defend against microbial organisms, the skin serves as an important immune system organ. The skin is has a dense population of resident dendritic cells which have the capacity to traffic to and from the skin. Muscle, on the other hand, is almost devoid of these professional antigen-presenting cells. Dendritic cells are specialized immune system cells that have the ability to take up and process antigens from the periphery of the skin then migrate and transport the antigens to secondary lymphoid organs, where they stimulate naïve T cells (52).

Inactivated influenza virus proteins, when presented by dendritic cells, have been shown to stimulate both CD4+ T helper cells as well as CD8+ T cytotoxic cells (Fig. 3), (6). This process begins when extracellular viral proteins are endocytosed by the dendritic cell and degraded by lysosomal enzymes. After migration to secondary lymphoid organs, dendritic cells present pieces of the proteins (T-cell epitopes) loaded onto MHC-II which activates CD4+ T helper cells (52). In dendritic cells only, inactivated influenza virus can also enter a pathway known as cross-presentation where the extracellular proteins are internalized by dendritic cells and access the endogenous pathway for antigen processing and are presented with MHC-I which activates CD8+ T cytotoxic cells (6). Activated T helper cells promote humoral immunity by stimulating B cells to make antibody to the virus. Activated T cytotoxic cells promote cellular immunity by killing virus-infected cells.

Support for low dose intradermal influenza vaccination. Because of large dendritic cell populations in the skin, the skin may be an effective route to administer vaccines, including influenza, even at lower doses. To date, there have been no clinical efficacy reports on low-dose intradermal vaccination followed by live influenza challenge (26). However, as early as 1948, Weller, et al., observed that intradermal administration of influenza antigens induced localized redness and swelling in 90% of subjects and a four-fold increase in HA-specific antibody response in the majority of subjects (80). A study by Beran showed that intradermal (ID) vaccination of 2/5 dose and 1/5 dose did not differ significantly in post vaccination protective HA antibody titers (≥40) compared to standard dose intramuscular (IM) vaccination, (ID=88.2.3% and 88.5% respectively, IM=96.9%) (7). A similar study by Belshe et al. showed that intradermal vaccination of

3/5 dose, 2/5 dose, and 1/5 dose did not differ significantly in post vaccination protective HA antibody titers (≥32) compared to standard dose intramuscular vaccination, (ID=89.3%, 89.7%, and 82.8% respectively, IM=93.3%) (5). More than 15 studies have shown similar results, demonstrating that low dose intradermal vaccination, as low as 1/5 dose, elicits an antibody response that is not significantly different than standard dose intramuscular vaccination (2, 4, 5, 43, 60).

A vaccine shortage in 2004 led some doctors to give low-dose intradermal vaccinations in order to stretch the supply. Some of these doctors tracked patients in order to check for the efficacy of this method of immunization. For example, Dr. Kathryn Kirkland immunized 1602 healthy employees and volunteers intradermally using one-fifth the standard intramuscular dose and found that it decreased incidence of influenza disease by 52% compared to non-vaccinated individuals (78).

Intradermal influenza vaccination has shown superior immunogenicity in elderly adults and immunocompromized individuals compared to intramuscular administration. A study in which elderly adults were given a trivalent intradermal influenza vaccination at the standard intramuscular dose (15 μg of HA per strain) showed post vaccination geometric mean titers in the intradermal group were 48%-70% higher than those in an intramuscular vaccination control group (35). A study by Gelinck et al. compared standard dose intramuscular vaccination and 1/5 dose intradermal influenza vaccination in cancer patients being treated with anti-tumor necrosis factor (TNF)-alpha (n=50), human immunodeficiency virus (HIV)- infected patients (n=80) hematologic stem cell transplantation (HSCT) patients (n=26), and healthy controls (41). The protection rates were similar after intramuscular or low-dose intradermal vaccination in the four study

groups (29). The researchers looked further looked at a subgroup of 21 subjects with the most severe immunodeficiencies, including 10 HIV-positive patients with very low CD4 T cell counts and 11 HSCT patients. They found that those who received intradermal influenza vaccination had exceptionally high protection rates for immunodeficient patients (67-83%) compared to the protection rates of the patients who received intramuscular influenza vaccination (13-27%) (29). Another study that compared intramuscular vaccination and 1/5 dose intradermal vaccination in HIV-positive patients found a significantly higher percentage of responders, as measured by an increase in HA antibody titer, in the intradermal group (42%) compared to the intramuscular group (35%) (44).

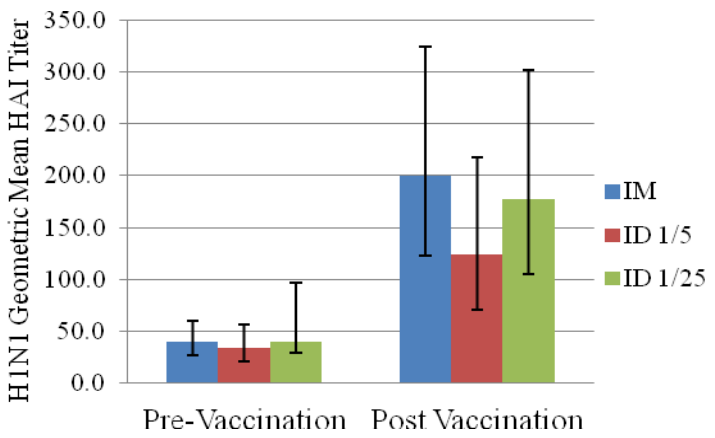
A low-dose intradermal trivalent influenza vaccination, Intanza®/IDflu®, was approved for use in seasonal influenza vaccination in Europe and Australia in February 2009. The vaccine comes in two different dosage formulations, with the dosage of 9 µg of HA per strain approved for individuals 18-59 and standard dosage of 15 µg of HA per strain approved for individuals 60 years of age or older (1). A similar vaccine composition made by Sanfoni-Pasteur, intradermal Fluzone®, was approved by the FDA and is available to health-care providers in the U.S. for the 2011-2012 influenza season (67).

UW-La Crosse low dose intradermal influenza vaccination study. A study done at UW-La Crosse by Taylor et al. compared standard dose intramuscular vaccination to both 1/5 and 1/25 dose intradermal vaccination of Fluzone® 2004-2005. Hemagglutinin inhibition assays showed that seroprotection rates were similar among

vaccination groups for H1N1 (post-vaccination, P=0.192) and H3N2 (post-vaccination, P=0.620) (Fig. 4) (77).

To further investigate the antibody response toward low-dose intradermal Fluzone® vaccination, UW-La Crosse students performed indirect ELISAs to examine the antibody response to whole influenza virus. No correlation was found between the HA-specific antibody titer (HIA titer) and whole influenza virus-specific antibody titer (ELISA titer) of each subject (Fig. 5). The finding that some subjects with low HA-specific antibody titers have high whole-virus specific antibody titers leads to the question of which other influenza viral protein/s the subjects are making antibodies against and whether these antibodies contribute toward protection from subsequent influenza virus infection. Of particular interest is if individuals make antibodies to NA, which are known to contribute toward protection from influenza disease.

A



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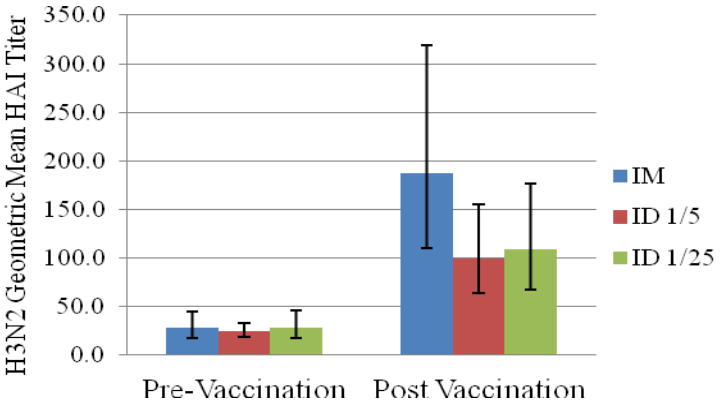


FIG. 4. Geometric mean HAI antibody titers pre and post vaccination for A/New Caledonia (H1N1) (A) and A/Wyoming (H3N2) (B). Error bars represent 95% confidence intervals.

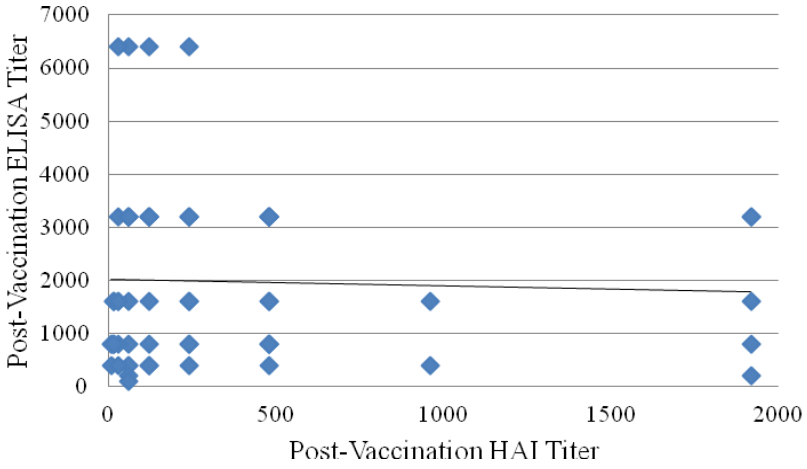


FIG. 5. HAI antibody titers versus whole-virus ELISA antibody titers after 1/5 dose intradermal and standard dose intramuscular vaccination against A/Wyoming (H3N2).

Protective antibody response to neuraminidase As previously mentioned, antibodies that bind to the active site of influenza virus NA prevent neuraminidase activity, which is needed for the release of newly formed virus from the host cell (30). Antibodies to NA thus directly affect the yield of virus produced within an infected individual because they prevent infection of uninfected host cells. Although antibodies to NA cannot prevent infection of host cells, they may suppress subsequent virus replication and reduce morbidity and mortality (30). Neuraminidase specific antibodies have been shown to contribute to protection from clinical presentation of disease in mice. Kilbourne et al. showed that vaccination of mice with a recombinant NA2 protein reduced the replication of virus in mice as measured by median infective dose of virus infection and suppressed disease as measured by total body weight loss (49). Johansson et al. showed similar results vaccinating mice with purified influenza neuraminidase (38).

The appearance of the first influenza H3N2 strain in 1968, A/Hong Kong/1968 (H3N2), allowed researchers to investigate the role of NA antibodies in the development and clinical presentation of disease. Prior to 1968, H2N2 strains of influenza A virus were circulating in the general population, thus people produced antibodies against NA2. However, with the emergence of the new HA3 subtype any protective immunity toward H3N2 infection was mediated by antibodies to the NA and/or M2 protein(s) only. Volunteers were tested for preexisting serum antibodies to NA2 by neuraminidase inhibition assay (NIA) to establish their NA antibody levels prior to challenge. Subjects were inoculated intranasally with an infectious dose of A/Hong Kong/68 (H3N2). Volunteers who developed influenza disease with fever possessed low levels of anti-NA antibodies prior to challenge. Those who had illness without fever had intermediate

levels of anti-NA and those who displayed no apparent infection had significantly higher levels of anti-NA prior to challenge. The duration of illness and amount of virus shed were also inversely related to pre-challenge NA antibody titer. These findings provide direct evidence that NA antibodies reduce the severity and presentation of influenza disease (55).

Efficacy of influenza vaccines is currently measured solely based upon HA antibody titers even though NA antibodies contribute toward immunity to influenza. Therefore, the ability of current vaccines to induce protective levels of NA-specific antibodies is unknown. This aspect is particularly relevant to consideration of those individuals who fail to mount a strong HA-specific antibody response and thus are said to be unprotected according to current vaccination licensure criteria. Studies have shown that 9-15% of individuals who receive standard seasonal influenza vaccination do not mount protective HA titers to any given strain of influenza virus (12, 61, 65). Differences in antibody response to HA protein results from genetic variability of MHC between individuals (61, 84). Variation in MHC alleles from person to person determine which antigens people will present and thus respond to. It has been shown that mice that mount a poor anti-HA response because of deficiencies in MHC repertoire are still able to mount a strong anti-NA response. This implies that in humans, a vaccine which elicits a protective antibody response toward NA will improve protective efficacy for those that respond poorly to HA (84).

The NA-specific antibody mediated immune response also provides significant cross-protection against influenza virus strains within the same subtype (16). These are viruses in which the neuraminidase protein has “drifted” in its amino acid sequence. The

degree of relatedness between the NA used in an immunization and the NA of viral challenge correlates with degree of cross-protection. This was observed in the previously mentioned study by Murphy et al. where preexisting antibodies to NA2 of a previously circulating H2N2 strain provided protection against A/Hong Kong/1968 (H3N2). The NA proteins of these influenza viruses must have been similar enough that antibodies made toward H2N2 provided cross-protection toward H3N2. It has been suggested that NA-specific antibodies to circulating, or vaccine acquired A/New Caledonia/20/99 (H1N1) may be protective against avian H5N1 viruses (31, 63). A study conducted by Sandbulte et al. investigated this hypothesis, testing cross protection by three different methods: NA1 DNA (from A/New Caledonia/20/99 (H1N1)) vaccination of mice, passive transfer of anti-NA serum in mice, and detection of cross-reactive H5N1 antibodies in human volunteers (66). Significantly, 50% of NA1 DNA-immunized mice survived infection with 10 times the LD_{50} of an H5N1 virus recovered from a human victim in 2004 (A/Vietnam/1203/04), demonstrating that immunity to NA protects mice. Mice that received whole serum from NA1 DNA-immunized mice (from previous experiment) survived infection of 10 times LD_{50} of the H5N1 strain, which demonstrates that humoral immunity is at least partially protective. Finally, about 20% of human volunteers (n=38) tested had low inhibitory activity against an avian H5N1 influenza strain based on neuraminidase inhibition assay (66).

In summary, antibodies to influenza glycoprotein NA provide protection through inhibiting viral progeny spread to new host cells therefore reducing the likelihood, duration, and degree of influenza illness (23, 47-49, 55, 69, 72, 73). Inducing antibody production toward NA through vaccination may improve the efficacy of influenza

vaccines, particularly for individuals who are not able to mount a protective HA antibody response (84), and offer some cross-protection from challenge with closely related influenza viruses (8, 31, 55, 66, 74, 75, 84).

Research goals and experimental design. The goal of this research was to compare the antibody response toward influenza virus neuraminidase protein after low-dose intradermal versus standard dose intramuscular influenza vaccination in humans. To accomplish this, subject serum was tested by the neuraminidase inhibition assay for the presence of functionally relevant NA-specific antibodies produced through vaccination against three influenza virus strains; two strains which were found in the vaccine and one avian influenza strain not contained in the vaccine.

This research addressed three specific questions:

1. Did subjects in all vaccination groups mount equally strong antibody responses to neuraminidase after vaccination?
2. Did subjects make neuraminidase antibodies that were cross-reactive against a different influenza strain within the same NA subtype (H6N1 vs H1N1)?
3. Did subjects who did not make protective levels of antibodies toward HA have NA titers which may help to protect them from influenza disease?

MATERIALS AND METHODS

Vaccination of human subjects and sample collection. The influenza vaccine used in the UW-L low-dose intradermal vs standard dose vaccination study was Fluzone® (2004-2005), a trivalent subvirion vaccine (Aventis Pasteur Swiftwater, PA). The virus strains contained in the vaccine were A/New Caledonia/20/99 (H1N1), A/Wyoming/03/2003 (H3N2) and B/Jiangsu/10/2000.

A total of 111 subjects aged 18-50 were included in the low-dose intradermal influenza study. An intramuscular vaccination group (n=34) received an injection of 0.5 ml of vaccine, containing 15 µg HA from each of the three strains into the deltoid muscle of the upper arm. A 1/5 dose intradermal vaccination group (n=40) received an injection of 0.1 ml of vaccine containing 3 µg of HA from each virus strain into the dermis of the inner forearm. A 1/25 dose intradermal vaccination group (n=37) received an injection of 0.1 ml of 1/5 diluted vaccine containing 0.6 µg HA from each virus strain into the dermis of the inner forearm. Blood samples were collected from subjects immediately before vaccination and four weeks post-vaccination. Serum was separated from blood cells and stored at -20ºC. This part of study was performed by Dr. Mary Morris, Dr. Bernadette Taylor, and former graduate students.

Hemagglutination inhibition assay. Hemagglutination inhibition (HA-I) titers were determined according to World Health Organization protocols (82). Briefly, subject serum was diluted in a two-fold dilution series from 1/15-1/1920. The serum dilutions

were reacted with each of the three influenza virus strains in the 2004-2005 vaccine and turkey red blood cells. The reciprocal of the last serum dilution that inhibited hemagglutination of the red blood cells by influenza virus was reported as the HA-I titer. This part of the study was performed by Christy Kelly, a former graduate student and Dr. Bernadette Taylor.

Human influenza virus propagation. Human influenza viruses A/NewCaledonia/20/99 (H1N1) and A/Wyoming/03/03 (H3N2) were obtained from the CDC and propagated in Madin-Darby canine kidney (MDCK) cells [ATCC# CCL-34]. These cells were grown to 90% confluence in 75 cm^2 flasks containing 20 ml MDCK growth media (Appendix A) at 37°C with 5% CO_2. Upon confluence, MDCK growth media was removed from MDCK monolayer and the monolayer was washed two times with room temperature PBS (Appendix A). Virus stocks were removed from an -80°C freezer, thawed in cool water, and kept at 4°C to maintain viability. The viruses were diluted 1/100 in 1 ml of influenza growth media. (Appendix A). The flasks were inoculated with 1 ml of virus and rotated to cover monolayer with inoculum. The flasks were incubated for 30 min-1hr at 37°C with 5% CO_2 for virus adsorption. Additional influenza growth media was added to inoculated flasks. Flasks were incubated at 37°C with 5% CO_2 for 3 days. Cytopathic effect was observed as rounding up of cells and detachment from culture flask. Supernatant was centrifuged for 5 min at 300 x g, 4°C to pellet cellular debris. Each human virus was passaged multiple times in MDCK cells in order for the viruses to become fully adapt to the cell line. A/New Caledonia/20/99 (H1N1) was passaged 7 times which resulted in a plateaued max virus-specific HA titer of 64. Neuraminidase activity of the A/New Caledonia/20/99 (H1N1) supernatant was

sufficient for NAI assays after these passages. A/Wyoming/03/03 (H3N2) was passaged 12 times which resulted in a plateaued max virus-specific HA titer of 64. Nearaminidase activity of the A/Wyoming/03/03 (H3N2) supernatant was not sufficient for NAI assays after these passages; therefore this virus was concentrated by Polyethylene glycol precipitation before the virus was used for NAI assays.

Avian influenza virus propagation. Avian influenza virus A/Turkey/Ontario/6625-1/98 (H6N1) obtained from National Veterinary Services Laboratories (NVSL), U.S. Department of Agriculture was propagated in embryonated chicken eggs. Briefly, specific pathogen-free chicken eggs from Sunnyside Hatchery in Beaver Dam, WI were received at 4 days post fertilization and placed in a 37°C egg incubator with rotating shelves. At 10 days post fertilization, virus (stock concentration unknown) was diluted 1/100 in 10 T antibiotic media (Appendix B), as recommended by NVSL, and 0.1 ml of virus dilution was inoculated into the allantoic cavity of each egg. Eggs returned to the 37°C egg incubator, then candled on day 1 and day 2 post inoculation. No dead eggs were observed. On day three post-virus inoculation, eggs were removed from the incubator and placed at 4°C overnight to kill the embryos. The virus was harvested by collecting allantoic fluid. Pooled allantoic fluid was centrifuged at 4°C for 5 min at 500 x g to pellet any blood cells and tissue fragments. Aliquots of virus-containing allantoic fluid were placed at -80°C for storage.

Neuraminidase assay. The proper virus dilution to be used in the neuraminidase inhibition assay was established by determining the viral dilution that had one unit of NA activity. Influenza viruses were diluted in a 2-fold dilution series (1, 1/2, 1/4, 1/8). Each virus dilution (50 µl) was added to 50 µl PBS (Appendix C) and 100 µl fetuin (Appendix

C) in 13 X 100 mm borosilicate glass tubes (Fig. 6, step 1). A fetuin control tube was made by adding 100 µl PBS and 100 µl fetuin to a 13 X 100 mm borosilicate glass tube. Each tube was covered and incubated in a 37°C water bath for 18 hours (A/NewCaledonia/20/99 (H1N1) and A/Wyoming/03/02 (H3N2)) or 1 hour (A/Turkey/Ontario/6625-1/98 (H6N1)) (Fig. 6, step 2), which resulted in the release of free N-acetyl neuraminic acid. Tubes were removed from the water bath and allowed to cool for 2 min at room temp. Periodate reagent (Appendix C) (0.1 ml) was added to each tube, shaken well, and left at room temp for 20 min (Fig. 6, step 3), which converted N-acetyl neuraminic acid to beta-formyl pyruvic acid. Arsenite reagent (Appendix C) (1 ml) was added to each tube and tubes were shaken vigorously until the brown color disappeared (Fig. 6, step 4). Thiobarbituric acid reagent (Appendix C) (2.5 ml) was added to each tube (Fig. 6, step 5). The tubes were shaken vigorously and placed in a boiling water bath for 15 min (Fig. 6, step 6), which resulted in the production of a pink chromophore. The tubes were removed from the boiling water bath and placed in an ice bath for at least 5 min. The tubes were removed from the ice bath and 3 ml of Warenoff reagent was added to each tube (Fig. 6, step 7). The tubes were covered separately with parafilm and vortexed, then centrifuged in swinging buckets at 10 x g (Fig. 6, step 8). The upper (butanol) phase of each sample was transferred to a cuvette and the optical density was measured at a wavelength of 549 nm using a spectrophotometer (Fig. 6, step 9). The fetuin control sample was used as a blank. The proper virus dilution to be used in the neuraminidase inhibition assay was determined by constructing an NA activity curve for each influenza virus sample tested. The curves were constructed by plotting

NA activity (OD of each virus sample at 549 nm) versus virus dilution. One unit of NA activity was defined as the dilution of virus that had an OD of 0.5.

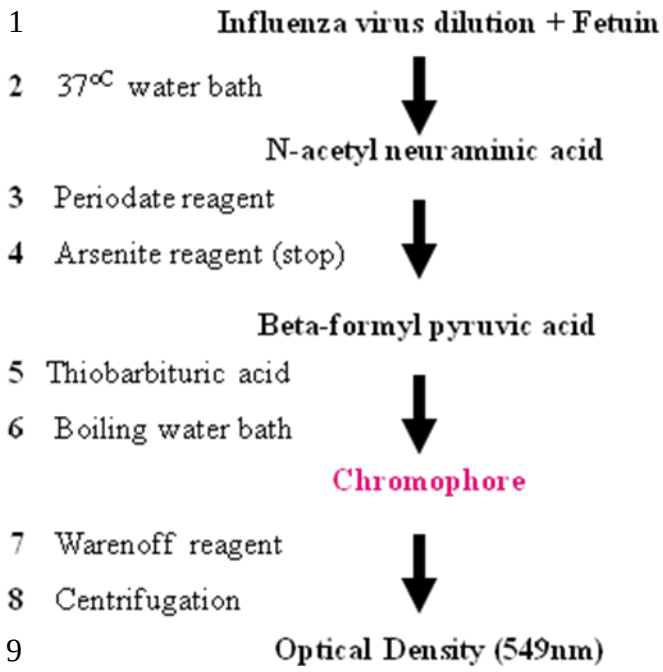


FIG. 6. Neuraminidase assay procedure

Neuraminidase inhibition assay. The neuraminidase inhibition assay tests for the presence of functionally relevant NA-specific antibodies. Influenza NA is an enzyme that cleaves sialic acid from the host cell that enables the release of virus from the infected cell. The neuraminidase inhibition assay is based on antibodies preventing neuraminidase activity by binding to NA and preventing release of free sialic acid.

The neuraminidase inhibition assay was used to determine the NA inhibition antibody titer of each subject's serum pre and post-vaccination against A/New Caledonia/20/99 (H1N1) and A/Wyoming/03/03 (H3N2). It was also used to detect cross-reactive NA-specific antibodies against a novel avian influenza strain

A/Turkey/Ontario/6625-1/98 (H6N1). The neuraminidase inhibition assay tests for the presence of functionally relevant NA-specific antibodies Influenza NA is an enzyme that cleaves sialic acid from the host cell that enables the release of virus from the infected cell.

Pre and post-vaccination subject serum was diluted in 0.5 log 10 steps. Subject serum was diluted $10^{-0.5}$, 10^{-1}, $10^{-1.5}$ and 10^{-2} when testing for NA inhibition titers against human influenza viruses and 10^{0}, $10^{-0.5}$, 10^{-1} and $10^{-1.5}$ when testing for NA inhibition titers against the avian influenza virus. Each serum dilution (50 µl) was added to a 50 µl virus dilution and 100 µl fetuin in 13 X 100 mm borosilicate glass tubes. Four virus control tubes were made by adding 50 µl PBS, 50 µl virus, and 100 µl fetuin to borosilicate glass tubes. A fetuin control tube was made by adding 100 µl PBS and 100 µl fetuin in a 13 X 100 mm borosilicate glass tube. The remaining protocol is identical to NA assay above.

Optical density data were converted to NA inhibition titers. The fetuin control sample was used as a blank and the NA activity of virus used for each run was determined by averaging the OD of the virus control tubes. The OD of the virus control represented 0% inhibition of NA activity (OD 0% inhibition). The percent inhibition of NA activity of each serum plus virus sample was determined by subtracting the OD of each serum plus virus sample from the OD 0% inhibition and dividing the product by OD 0% inhibition. The NA inhibition titers are defined as the serum dilution that inhibits virus NA activity by 50%. The NA inhibition titers of each subject serum sample were determined by plotting the dilution of subject serum vs. % NA activity inhibition of each serum sample and calculating the serum dilution that inhibited virus NA activity by 50%.

Duplicate runs were performed on each pre- and post-vaccination subject serum. Materials and methods were the same for duplicate runs except subject serum dilutions were altered to best fit NA inhibition titer determined by the first run. For instance, if a subject's serum had a first run NA inhibition titer of $10^{-1.75}$ against A/New Caledonia/20/99 (H1N1), serum dilutions for the second run were 10^{-1}, $10^{-1.5}$, 10^{-2}, $10^{-2.5}$ instead of $10^{-0.5}$, 10^{-1}, $10^{-1.5}$ and 10^{-2}. The NA inhibition titers of subject serum for duplicate runs had to be within a two- fold non- logarithmic dilution of each other in order to be considered good duplicate values. For example, if duplicate runs gave titers: $10^{-1.75}$ and $10^{-1.85}$, the non logarithmic reciprocal dilution for each would be 56.2 ($1/10^{-1.75}$) and 70.8 ($1/10^{-1.85}$). The reciprocal dilutions 56.2 and 70.8 are within a two-fold value of each other, and are thus good duplicate values. Subject serum samples that did not have good duplicate NA inhibition titers were analyzed a third time by NA inhibition assay.

For data analysis, all duplicate NA inhibition titers were averaged and converted to non logarithmic titers, for example $1/((10^{-1.75} + 10^{-1.85})/2) = 62.67$.

Statistical analysis. Histograms, boxplots, and Shapiro-Wilk normality tests were conducted on $\log_{10}$-transformed antibody titers to assess normality of the data for suitability with parametric inference procedures. The titers were highly skewed to the right and all of the samples rejected the hypothesis of normality at $\alpha=0.05$ both before and after vaccination for each virus and vaccination group. Although the $\log_{10}$-transformed titers were not normal, parametric analysis techniques were determined to be adequate for the use on $\log_{10}$- transformed titers. Nonparametric techniques would be reported if they reached a different statistical conclusion than the parametric procedure.

Oneway analysis of variance (ANOVA) was used to determine if differences existed between the three vaccination groups when comparing the geometric mean titers. A paired samples T test was used to determine if differences existed between fold increases in titers after vaccination. Pearson Chi-Square tests were used to analyze the difference in NAI seroconversion rates between vaccination groups as well as for comparing the percentage of subjects with cross-reactive antibodies toward NA. Pearson correlation was used to investigate a correlation between H1N1 and H6N1 antibody titers. ANOVA was used to show the distribution of antibodies to H6N1 across categories of H1N1. Spearman correlation was used to investigate a correlation between HAI and NAI titers. Paired T tests were used to compare mean fold increase differences between HAI and NAI antibody titers. A 5% significance level was used for all hypothesis testing. All statistical computations and graphs were made using SPSS Version 19.

RESULTS

Human subject serum analyzed by NAI Assay. Of 111 subject sera that were analyzed by HAI assay, 111 were analyzed by NAI against A/New Caledonia/20/99 (H1N1), 107 against A/Wyoming/03/03 (H3N2), and 107 against A/Turkey/Ontario/6625-1 (H6N1). The breakdown of subject sera analyzed by NAI according to vaccination group is summarized in Table 1. Some subjects were not analyzed by NAI assay for H3N2 and H6N1 because of insufficient serum.

TABLE 1. Subjects analyzed by NAI assay

	Number of subjects in each vaccination group			
Influenza Virus Strain	IM	ID 1/5	ID 1/25	Total
A/New Caledonia (H1N1)	34	40	37	111
A/Wyoming (H3N2)	34	38	35	107
A/Turkey/Ontario (H6N1)	34	37	36	107

Virus-specific NAI antibody titers do not differ among vaccination groups. The geometric mean NA-specific antibody titers for the IM, ID 1/5 dose, and ID 1/25 dose vaccination groups were measured by NAI assays. Pre and post-vaccination NAI titers against A/New Caledonia/20/99 (H1N1), A/Wyoming/03/03 (H3N2), and A/Turkey/Ontario/6625-1/99 (H6N1) are shown in shown in Table 2 and Figure 7. No significant differences were found in the pre-vaccination titers between the three vaccination groups for H1N1 (P=0.189), H3N2 (P=0.211), or H6N1 (P=0.730).

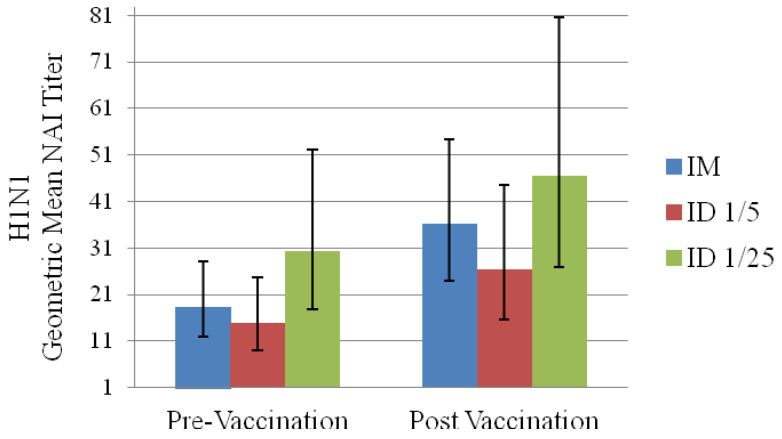
No significant differences were found in the post-vaccination titers between the three vaccination groups for H1N1 (P=0.393), H3N2 (P=0.129), or H6N1 (P=0.370). Significant increases in antibody titers (log_{10}) were observed post-vaccination in all groups (P<0.0005 for H1N1, P<0.0005 for H3N2, P=0.035 for H6N1, Fig. 8). NAI seroconversion rates (percentage of subjects that demonstrated an NA-specific antibody titer >2.5) between the three vaccination groups were not significantly different for H1N1 (P=0.129) or H6N1 (P=0.145). However, seroconversion rate for H3N2 was significantly higher for the ID 1/5 dose group than the IM and ID 1/25 dose groups (P=0.024, Table 3).

TABLE 2. Geometric mean virus-specific NAI antibody titers pre- and post-vaccination

Influenza Virus Strain	Geometric mean antibody titer[a]					
	IM		ID 1/5		ID 1/25	
	Pre	Post	Pre	Post	Pre	Post
A/New Caledonia (H1N1)	18.3 (12.0-28.0)	36.1 (24.0-54.3)	15.0 (9.1-24.7)	26.5 (15.7-44.6)	30.4 (17.7-52.2)	46.5 (26.8-80.5)
A/Wyoming (H3N2)	3.6 (2.4-5.6)	6.3 (4.3-9.3)	2.3 (1.6-3.3)	4.4 (2.7-6.9)	2.6 (1.8-3.9)	3.5 (2.4-5.3)
A/Turkey/Ontario (H6N1)	1.31 (1.07-1.62)	1.33 (1.04-1.71)	1.19 (1.02-1.39)	1.30 (1.08-1.56)	1.26 (1.08-1.48)	1.32 (1.12-1.57)

[a]Value (95% confidence interval)

A



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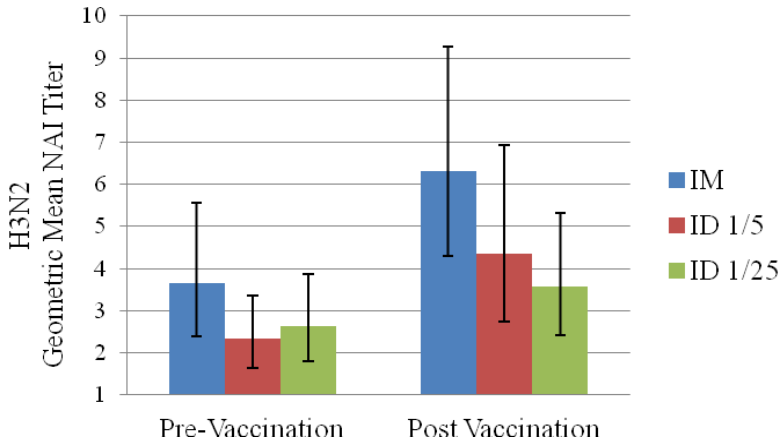

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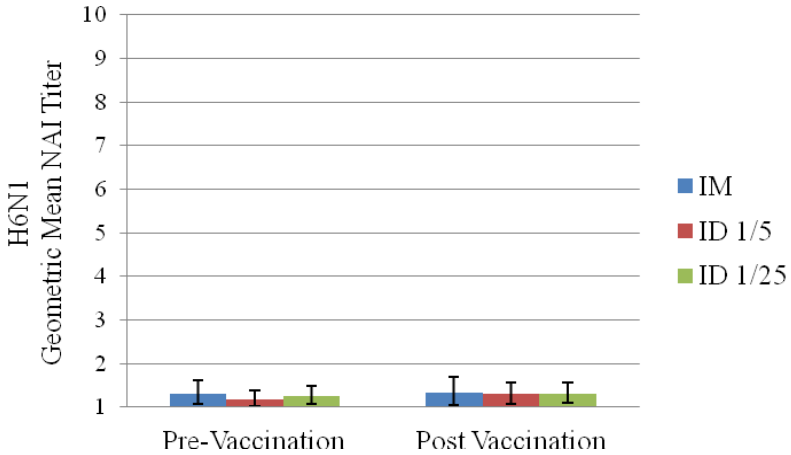

FIG. 7. Geometric mean NAI antibody titers pre- and post-vaccination for A/New Caledonia (H1N1) (A), A/Wyoming (H3N2) (B), and A/Turkey/Ontario (H6N1) (C). Note the Y axis scale is larger for H1N1 than H3N2 and H6N1. Error bars represent 95% confidence intervals.

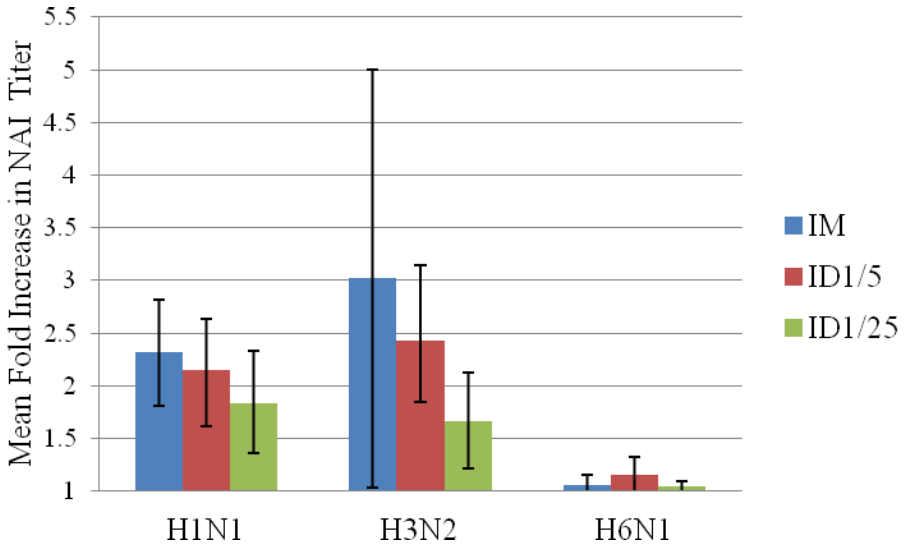

FIG. 8. Fold increases in virus specific NAI titers four weeks post-vaccination toward A/New Caledonia (H1N1), A/Wyoming (H3N2), and A/Turkey/Ontario (H6N1). Error bars represent 95% confidence intervals.

TABLE 3. NAI seroconversion rates (percent of subjects with increase in NA-specific titer >2.5)

	IM		ID 1/5		ID 1/25	
Influenza virus strain	# subjects/total	rate	# subjects/total	rate	# subjects/total	rate
A/New Caledonia (H1N1)	11/34	32.4	12/40	30.0	5/37	13.5
A/Wyoming (H3N2)	6/34	17.6	15/38	39.5	5/35	14.3
A/Turkey/Ontario (H6N1)	0/34	0.0	2/38	5.3	0/36	0.0

Vaccination produces cross-reactive antibodies to influenza virus NA protein in a small percentage of subjects. Some subjects had detectable H6N1 NA-specific antibodies pre-vaccination (16-27% in all vaccination groups) and post-vaccination (21-31% in all vaccination groups). These subjects had cross-reactive antibodies to NA, as measured by A/Turkey/Ontario (H6N1)-specific NAI titers greater than 1 (Fig. 9). Vaccination route did not have an impact on the subjects ability to make cross reactive antibodies post-vaccination (P=0.685, Fig. 9).

Pearson correlation showed a weak positive relationship between H1N1 and H6N1 NAI titers both pre-vaccination (r=0.323, *P*=0.001) and post-vaccination (r=0.285, *P*=0.003). To test for this correlation, subjects were categorized based upon pre-vaccination H1N1 NAI titers and analyzed against pre-vaccination H6N1 NAI titers of subjects within each group (Fig. 10). Likewise, subjects were categorized based on post-vaccination H1N1 NAI titers and analyzed against post-vaccination H6N1 NAI titers of subjects within each group (Fig. 10). Note: These categories were chosen based upon the post-vaccination geometric mean H1N1-specific titer for all vaccination groups (35.1) representing a medium response toward NA. This categorization also allowed for similar subject numbers within each category being analyzed. The distribution of H6N1 NAI titers was shown to increase across categories of increasing titer against H1N1 both pre-vaccination (*P*=0.008) and post-vaccination (*P*=0.014) indicating that subjects with high NA titers against H1N1 have more cross-reactive NA-specific antibodies against H6N1.

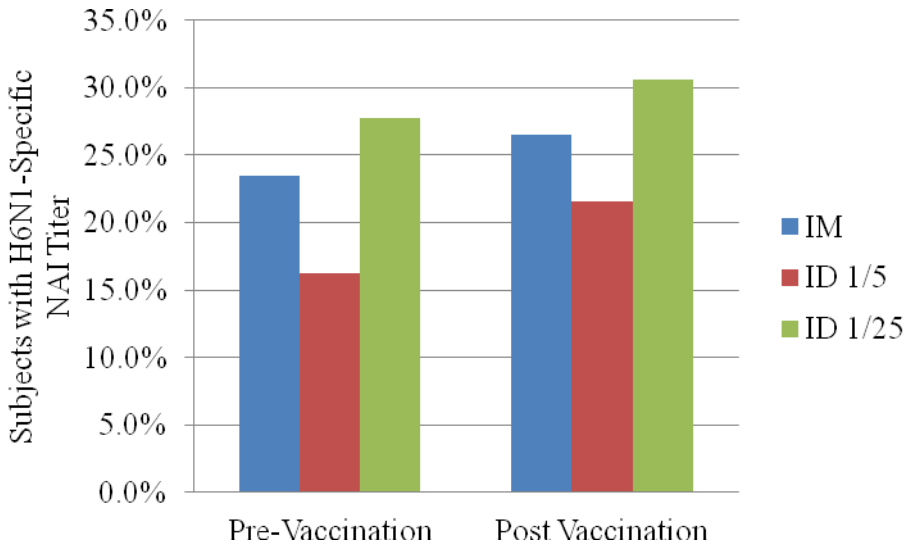

FIG. 9. Percentage of subjects with A/Turkey/Ontario (H6N1)- specific NAI titers greater than 1.

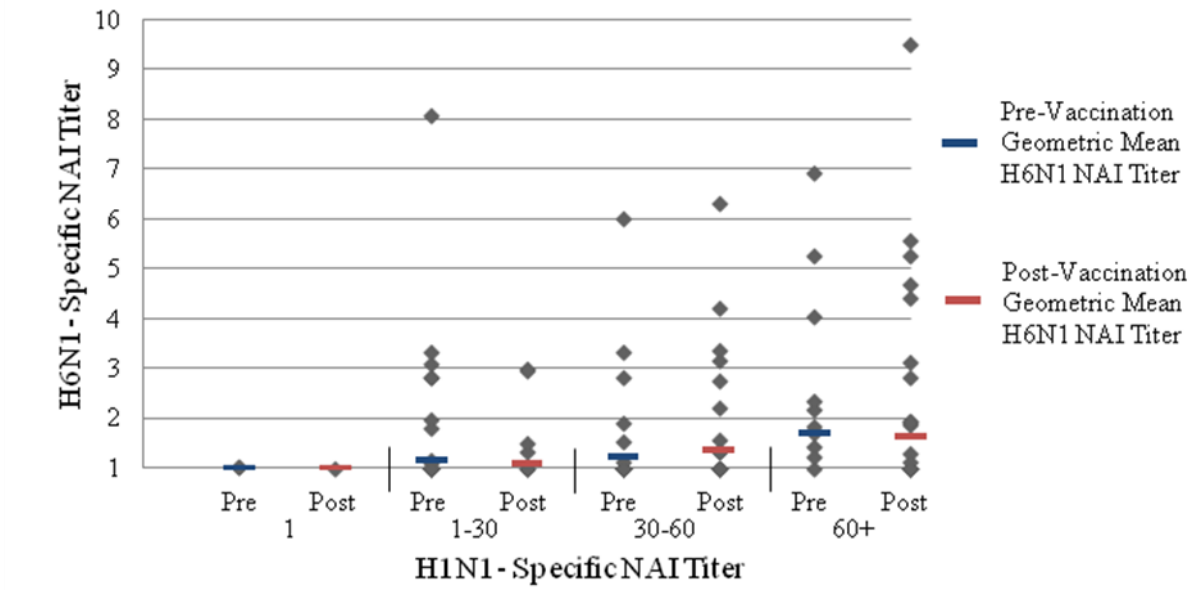

FIG. 10. A/Turkey/Ontario (H6N1)-specific NAI titers of subjects with low (titer=1), medium low (titer=1-30), medium high (titer=30-60), and high (titer=60+) H1N1-specific NAI titers pre- and post- vaccination.

Antibodies toward influenza virus NA may protect some individuals who do not mount a protective antibody response to HA. There was a weak positive correlation between virus specific HAI titers and NAI titers both pre-vaccination and post-vaccination (Fig. 11). Although this correlation is present when considering all the subjects in the study, this correlation is highly variable when looking at an individual subject's antibody responses toward HA and NA, as evident by the low r^2 value of each of these comparisons (Fig. 11).

The antibody response to HA was stronger than the antibody response toward NA after vaccination as the fold increase in HAI titers was significantly higher than fold increase in NAI titers for all vaccination groups against H1N1 ($P<0.001$) and H3N2 ($P<0.001$, Fig. 12). However, some individuals had low HAI titers but high levels of NAI antibodies after vaccination.

Twenty three out of 111 (20.7%) subjects failed to make a seroprotective HAI titer (HA-specific antibody titer ≥40) against A/New Caledonia (H1N1) post-vaccination while 25 out of 111 (22.5%) subjects failed to make a seroprotective HAI titer against A/Wyoming (H3N2) post-vaccination. The subjects that were considered unprotected from influenza disease based on low HAI titers were grouped according to virus-specific post-vaccination NAI titers (Fig. 13). Most poor responders to HA also had low post-vaccination NAI titers. Four out of 23 (17.4%) poor HA responders had high post-vaccination NAI titers (>50) against A/New Caledonia (H1N1) while three out of 25 (12%) poor HA responders had high post-vaccination NAI titers (NAI>10) against

A/Wyoming/03/03 (H3N2). These individuals already had high NAI titers prior to vaccination, but their titers increased post-vaccination for H1N1 (fold increase 1.1-1.7) and H3N2 (fold increase 1.6-2.1).

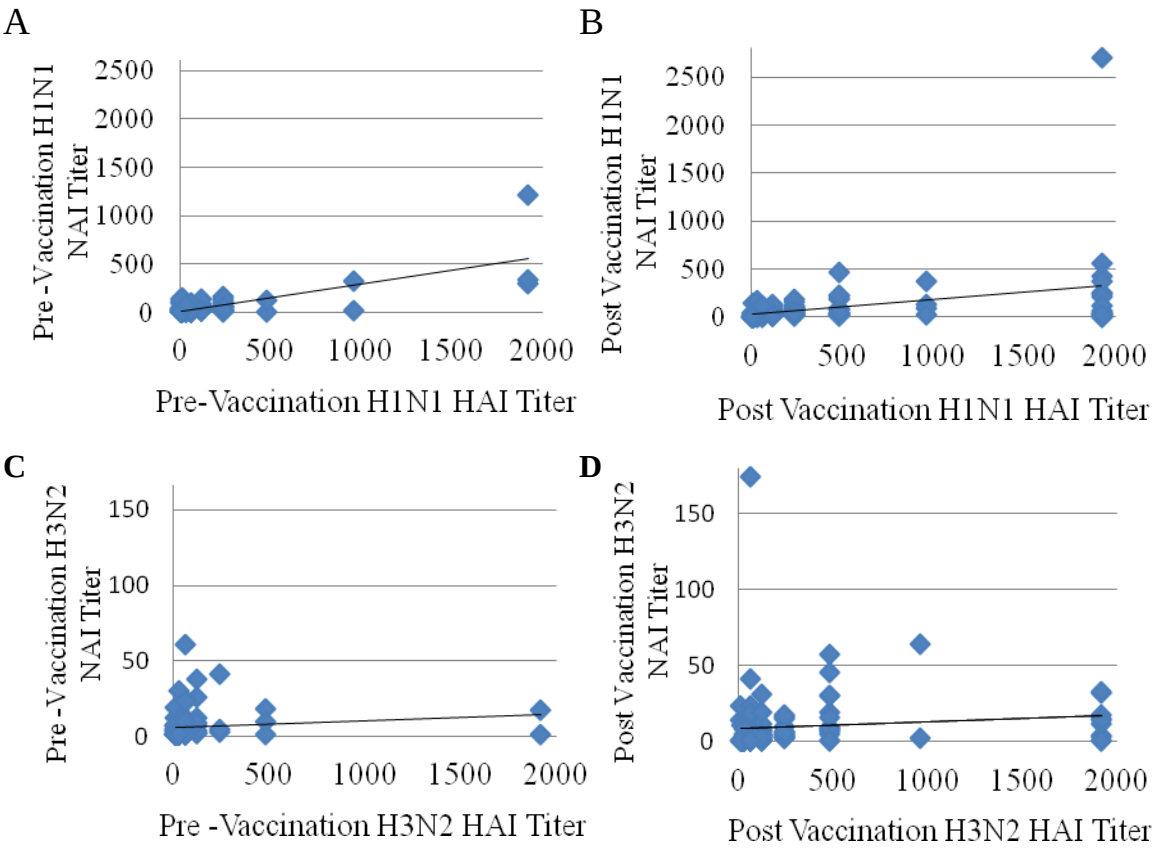

FIG. 11. Virus specific HAI titer versus NAI titers. Pre-vaccination A/New Caledonia (H1N1)-virus specific HAI titers versus NAI titers (r^2=0.556) (A). Post-vaccination A/New Caledonia (H1N1)-virus specific HAI titers versus NAI titers (r^2=0.132) (B). Pre-vaccination A/Wyoming (H3N2)- virus specific HAI titers versus NAI titers (r^2=0.015) (C). Post-vaccination A/Wyoming (H3N2)- virus specific HAI titers versus NAI titers (r^2=0.133) (D).

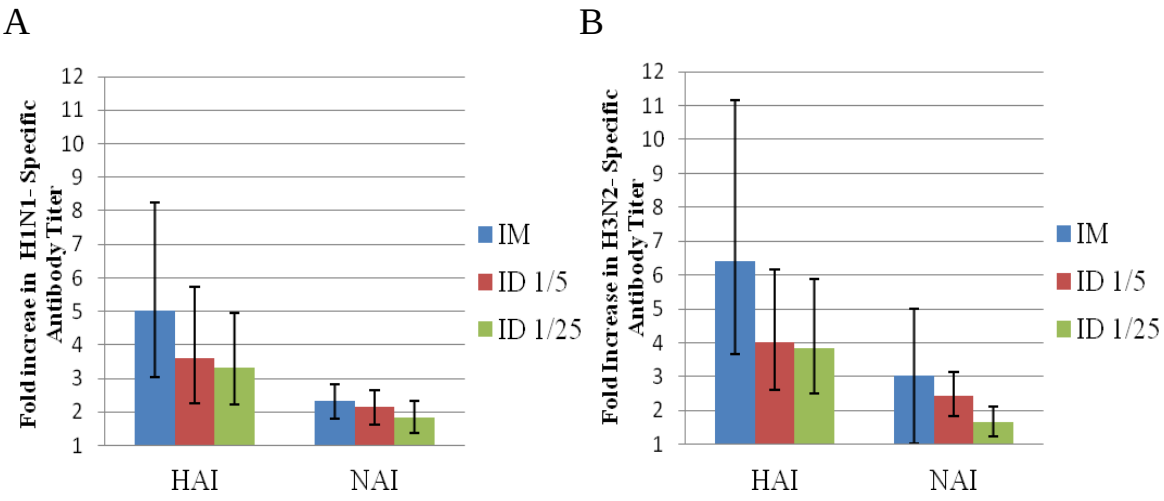

FIG. 12. Fold increase in HAI and NAI titers post-vaccination with A/New Caledonia (H1N1) (A) and A/Wyoming (H3N2) (B). Error bars represent 95% confidence intervals.

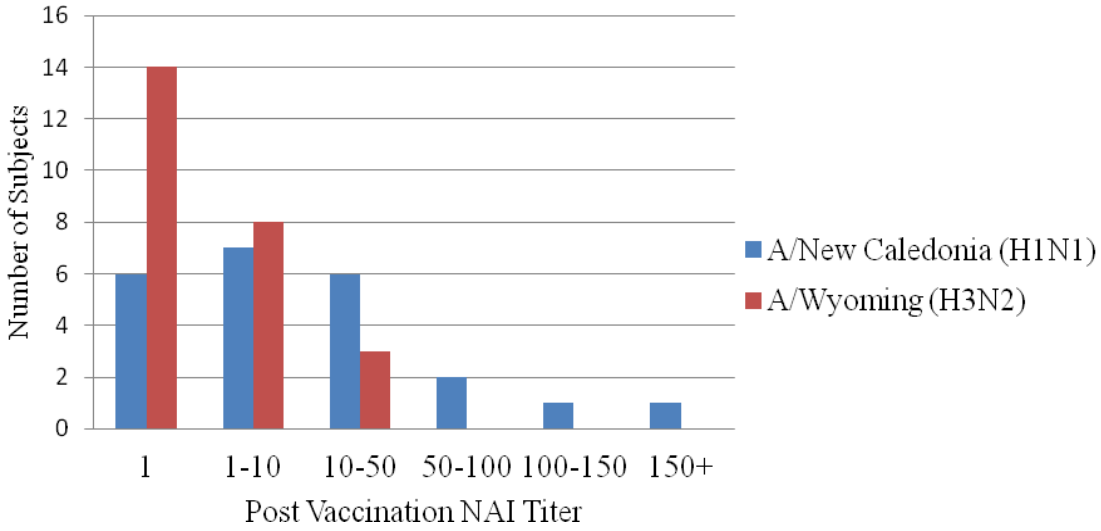

FIG. 13. Distribution of post-vaccination NAI titers among subjects with unprotective post-vaccination HAI titers (HAI titer <40).

DISCUSSION

Intradermal influenza vaccination is a promising alternative to conventional intramuscular vaccination. Previous studies have shown that intradermal administration of 1/5 and 1/25 dose of influenza vaccine has immunogenicity comparable to intramuscular vaccination based solely on induction of HA-specific antibodies (2, 4, 5, 7, 43, 60, 77). The results of this study which measured antibodies against the NA protein after low dose intradermal vaccination supports the previous findings, that immunogenicity of the vaccine is not significantly reduced even at 1/25 dose, as the IM and ID vaccination groups had comparable post-vaccination NAI antibody titers against each of the viruses tested. Combined functional HA and NA antibody titers did not correlate with whole virus antibody titers (as measured by ELISA, Fig. 14 versus Fig. 15). This illustrates that virus antigens induce variable antibody responses in different individuals and that there are yet other viral antigen-specific antibodies that must be contributing to the overall immune response in the subjects studied.

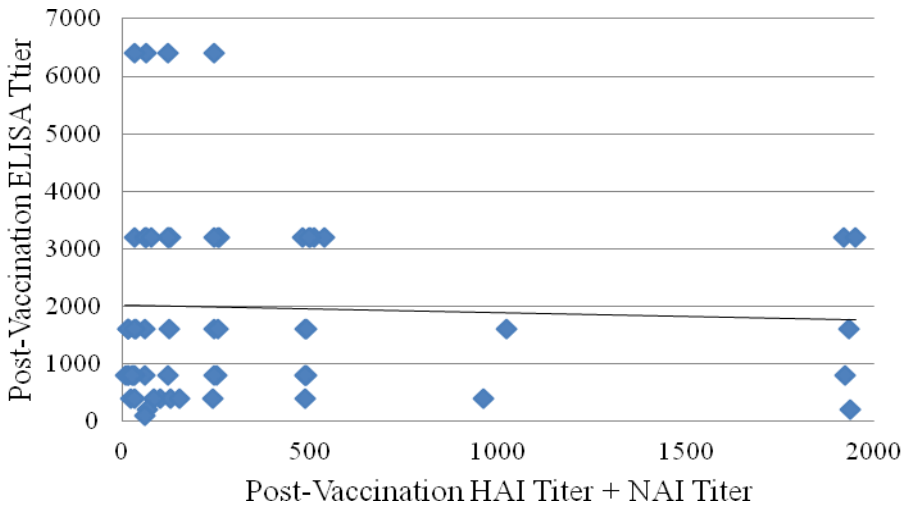

FIG. 14. Sum of HAI titers and NAI titers versus whole virus ELISA antibody titer after low-dose intradermal versus standard dose intramuscular influenza vaccination.

Seasonal influenza vaccination induced production of NA-specific antibodies in all vaccination groups. The pre and post-vaccination NAI titers of subjects varied greatly depending on the influenza virus strain tested. However, the post-vaccination NAI titers increased relative to the pre-vaccination NAI titers for each strain. The pre-vaccination NAI titer for all vaccination groups against A/New Caledonia H1N1 was 20.2 (15.2-26.8), which was much higher than against A/Wyoming H3N2, 2.8 (2.3-3.5), and A/Turkey/Ontario/6625-1/99, 1.3 (1.1-1.4). It is not surprising that the pre-vaccination A/Turkey/Ontario/6625-1/99 NAI titers were lower than that of the human influenza strains because the human subjects have not been previously exposed to a turkey influenza virus strain. Therefore, any turkey strain NA-specific antibodies must be cross-reactive antibodies produced by exposure to a human influenza virus with similar NA epitopes. One reason for the difference in pre-vaccination NAI titers against the two human strains is that the A/New Caledonia/20/99 (H1N1) strain had been circulating in the human population much longer than the A/Wyoming/03/03(H3N2)

strain. Previous exposures to this virus probably allowed individuals to build NA-specific antibody responses against A/New Caledonia/20/99 (H1N1). A/New Caledonia/20/99 (H1N1) was first isolated from a human in 1999 and was a prevalent strain of influenza virus circulating in the human population for several years prior to the UW-La Crosse low dose intradermal influenza vaccination study in 2004. A/New Caledonia/20/99 (H1N1) was included in the seasonal influenza vaccines during the years of 2000, 2001, 2002, 2003, in addition to the 2004 seasonal influenza vaccine investigated in the study (83). In comparison, A/Wyoming/03/03 (H1N1) was first isolated from a human in 2003 and was only contained in the seasonal influenza vaccine for the year 2004-2005, which was investigated in this study (83).

There was an increase in NAI titers post-vaccination for all influenza virus strains tested. Vaccination route did not have an impact on the increase in NAI titer for any of the influenza strains. Subjects in all vaccination groups had comparable fold increases in NAI vaccination titer in A/New Caledonia/20/99 (H1N1) and A/Wyoming/03/03 (H3N2). This suggests that subjects had similar NA-specific antibody responses to the human influenza strains. The fold increase in NAI titer for A/Turkey/Ontario/6625-1/99 (H6N1) was significantly lower than the fold increases in NAI titers against the human influenza strains. This is not unexpected since the subjects were not vaccinated against the turkey influenza strain. The small rise in NAI titer showed that low levels of cross-reactive antibodies were produced, likely as a result of vaccination with human influenza virus A/New Caledonia/20/99 (H1N1).

If there were an increased focus on induction of antibody toward NA through vaccination, similar standards to the CPMP criteria for influenza vaccine licensure could

be established to evaluate the NA-specific antibody response of influenza vaccination. The vaccination group with the highest NAI seroconversion rate against A/New Caledonia (H1N1) was the IM group at 32.4% while the highest seroconversion rate against A/Wyoming (H3N2) was the ID 1/5 dose group at 39.5%. Based on a minimum NAI seroconversion rate of 40% (minimum for HAI seroconversion rate) none of the vaccine groups would meet this criteria for either human influenza strains tested.

Seasonal influenza vaccination induced production of NA-specific cross-reactive antibodies. Vaccination with Fluzone® induced cross-reactive antibodies to A/Turkey/Ontario/03/03 (H6N1) in all vaccination groups, as indicated by fold increases in NAI titers greater than 1 and a 3.8% increase in number of subjects with detectable A/Turkey/Ontario/03/03 (H6N1) specific NAI titers post vaccination. This data supports the concept that immunization with seasonal influenza vaccines can induce cross-reactive NA-specific antibodies against distantly related influenza virus strains. A positive correlation was shown between H1N1-specific NAI titers and H6N1-specific NAI titers. This suggests that individuals who have a high NAI titer to a particular influenza strain that they have been vaccinated against or exposed to naturally will also have more cross-reactive NA-specific antibodies to influenza viruses containing the same NA type. It is unknown if cross-reactive NA-specific antibodies would confer protection against infection although such antibodies may reduce the severity of disease symptoms.

Seasonal influenza vaccination induced NA-specific antibodies which may protect some individuals from influenza disease. Subjects in all vaccination groups had generally stronger antibody responses toward HA vs NA following vaccination as shown by a greater fold increase in HAI titers than NAI titers. Other studies have demonstrated

a decreased magnitude of antibody response toward NA antigen when it is mixed with HA indicating antigenic competition between the HA and NA proteins in conventional influenza vaccines (39, 40, 42). We did not investigate if NAI titers would be higher post-vaccination if HA were removed from the vaccine, therefore we cannot speculate on the magnitude of antigenic competition in this study. However we can say that if antigenic competition between HA and NA exists, low-dose intradermal influenza vaccination did not reduce this phenomenon in comparison to standard dose intramuscular vaccination

Variation in MHC alleles and antibody and T cell receptor genes from person to person determine which antigens people will make strong antibody responses to. Our results showed that the magnitude of antibody response to HA and NA was variable from individual to individual. However we did see a weak positive correlation between HAI titers and NAI titers when considering all of the subjects in the study for both A/New Caledonia/20/99 (H1N1) and A/Wyoming/03/03 (H3N2). Since some individuals with low HAI titers also have low NAI titers while others have high HAI and NAI titers it appears that there is some relationship between antibody responses toward the two proteins overall.

Antibody response toward NA may improve protective efficacy of influenza vaccination particularly for individuals who do not make seroprotective HAI titers. Unfortunately, a seroprotective NAI titer has not been established and efficacy studies need to be done to resolve what NAI titer correlates with immunity to influenza disease (9). Previous studies that have determined protective NAI titer have reported varied titer findings (9). In one study that used plasmid DNAs expressing NA from a H5N1 strain, a

mean NAI titer of 210 resulted in survival of mice that were challenged with the wild type H5N1 virus (15). In a different mouse study using a reassortment NA vaccine, a mean NAI titer of 120 was required to protect against illness (69). It is hard to compare these mouse studies head to head since the studies are measuring different outcomes. Additionally, the relevance of mouse protective NAI titers in human influenza infection and disease is unknown. Controlled human studies need to be done to determine protective NAI titers. Therefore we cannot say to what degree NA antibody titers found in this study may contribute to protection from disease in individuals who do not make seroprotective HAI titers.

In this study, most poor-HA responders were found to have low post-vaccination NAI titers as well. Four individuals who did not make seroprotective HAI titers did have high NAI titers (>50) against A/New Caledonia/20/99 (H1N1). Three individuals who did not make seroprotective HAI titers did have high NAI titers (>10) against A/Wyoming/03/03 (H3N2). All of these subjects had high pre-vaccination NAI titers. These individuals represent a percentage of the general population that do not make seroprotective HA titers but do have antibodies toward NA than may offer them protection from disease.

Individuals who have low HAI titers in addition to antibodies toward NA may be protected from influenza disease due to contribution of antibodies against both of these proteins. Sixteen out of twenty three individuals who did not have seroprotective HAI titers possessed at least some antibodies against HA (HAI titer $\geq$15) for A/New Caledonia/20/99 (H1N1). Of these, 13 also had antibodies toward NA (NAI titer>1) for A/New Caledonia/20/99 (H1N1). There were 21 out of 25 individuals who did not have

seroprotective HAI titers that did have some antibodies against HA (HAI titer ≥15) for A/Wyoming/03/03 (H3N2). Of these, 9 also had antibodies toward NA (NAI titer>1) for A/Wyoming/03/03 (H3N2). The contribution of protection from disease by NA antibodies in these individuals is impossible to say at this point. However these individuals represent a percentage of the population that might be protected from influenza disease because, event though they have low-moderate HAI antibody titers, they also have antibodies against NA protein.

Summary. In summary, the neuraminidase inhibition assay was used to determine the anti-NA titers of subjects vaccinated intradermally (1/5 and 1/25 dose) versus intramuscularly against A/New Caledonia/20/99 (H1N1) and A/Wyoming/03/03 (H3N2). Cross-reactive NA-specific antibody titers against a novel avian influenza strain A/Turkey/Ontario/6625-1/98 (H6N1) were also measured. Results showed an increase in NAI titers post-vaccination for all influenza virus strains tested and that vaccination route did not have an impact on the increase in NAI titer for any of the influenza strains. Results also showed that the seasonal influenza vaccine produces a small cross-reactive antibody response to a novel influenza virus NA protein. NA-specific antibodies may contribute to protective efficacy of low-dose vaccination as well as traditional intramuscular vaccination. Finally, these findings further support low-dose intradermal administration of seasonal influenza vaccination as being an effective alternative to conventional standard dose intramuscular vaccination.

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APPENDIX A

REAGENTS FOR HUMAN INFLUENZA VIRUS PROPAGATION

Appendix A. Reagents for Human Influenza Virus Propagation

a.) MDCK growth media

Supplement 500 ml minimum essential medium containing Earle's salts, L-glutamine, and essential amino acids (MEM) with:

0.75 g sodium bicarbonate ($NaHCO_3$)

5 ml sodium pyruvate

5 ml penicillin-streptomycin stock (100 U/ml penicillin G and 100 µg/ml streptomycin)

Add fetal bovine serum (FBS) to 10% before use

b.) Influenza growth media

Supplement 500 ml MEM with:

0.75 g sodium bicarbonate ($NaHCO_3$)

5 ml sodium pyruvate

5 ml penicillin-streptomycin stock (100 U/ml penicillin G and 100 µg/ml streptomycin)

Add ι-1-Tosylamide-2-phenylethyl chloromethyl ketone- treated trypsin to a final concentration of 2 µg/ml before use

c.) 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

Add ddH_2O to 1L

Filter sterilize

APPENDIX B

REAGENTS FOR AVIAN INFLUENZA VIRUS PROPAGATION

Appendix B. Reagents for Avian Influenza Virus Propagation

a.) 10 T Antibiotic medium

950 ml Basal media

1.21 g trizma base
26 g tryptose broth
1 L ddH_2O
Autoclave for 20 min on slow exhaust

50 ml Antibiotics for 10 T

15 ml sterile PBS (Appendix A)
6.3 g (1,586 U/mg) penicillin
2.68 g (747 U/mg) streptomycin
13 ml kanamycin sulfate (50 mg/ml)
20 ml gentocin (50 mg/ml)
0.04 ml myostatin (5,000,000)
pH to 6.6 with 1N NaOH
Add sterile PBS to 50 ml final volume

APPENDIX C

REAGENTS FOR NEURAMINIDASE AND NEURAMINIDASE INHBITION ASSAY

Appendix C. Reagents for neuraminidase and neuraminidase inhibition assay

a.) Fetuin

20 ml phosphate buffer, pH 5.9

81 ml 0.4M sodium dihydrogen orthophosphate (NaH_2PO_4)

19 ml 0.4M disodium hydrogen orthophosphate (Na_2HPO_4)

pH to 5.9, Adjust pH if necessary with appropriate phosphate component

20 ml ddH_2O

0.5 g fetuin

Store in aliquots at -20°C

b.) Periodate reagent

4.28 g sodium meta-periodate

38 ml ddH_20

Dissolve by heating. Cool to room temperature and add:

62 ml 85% ortho-phosphoric acid

Mix well and store at room temperature in a dark bottle away from light

c.) Arsenite reagent

10 g sodium arsenite (meta)

7.1 g anhydrous sodium sulfate

100 ml ddH_2O

Dissolve by heating. Cool to room temperature and add:

0.3 ml concentrated sulfuric acid

Store at room temperature

d.) Thiobarbituric acid reagent

35.5 g anhydrous sodium sulfate

3 g thiobarbituric acid

500 ml ddH_2O

Dissolve by heating in a boiling water bath. Store at room temperature

Reagent may precipitate after about 10 days depending on the quality of thiobarbituric acid, whereupon fresh reagent should be prepared.

e.) Warenoff reagent

475 ml 1-Butanol

25 ml concentrated hydrochloric acid

Store in dark bottle at room temp in a location suitable for flammable reagents

f.) Phosphate-buffered saline (0.01M), pH 7.3 (PBS)

40 g sodium chloride (Na Cl)

1 g potassium chloride (KCl)

5.75 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

1 g potassium phosphate, dibasic, anhydrous (KH_2PO_4)

5 L ddH_2O