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Aerosol Vaccination Induces Robust Protective Immunity to Homologous and Heterologous Influenza Infection in Mice

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Abstract

Live-attenuated influenza vaccine (LAIV) delivered by large droplet intranasal spray is efficacious against infection. However, many of the large droplets are trapped in the external nares and do not reach the target nasal airway tissues. Smaller droplets might provide better distribution yielding similar protection with lower doses. We evaluated 20 and 30 micron aerosol delivery of influenza virus in mice. A 15 second aerosol exposure optimally protected against homologous and heterologous influenza infection and induced a robust immune response. These results demonstrate the feasibility of nasal vaccination using aerosolized particles, providing a strategy to improve vaccine efficacy and delivery.

Keywords

influenza; vaccination; aerosol

1. Introduction

Seasonal influenza virus infections cause over 200,000 hospitalizations and an estimated 36,000 deaths in the United States annually [1,2]. Most clinical cases occur in young children where the infection rate is generally twice that of adults [3,4]; however, the elderly are very susceptible to influenza complications and related deaths [5–7]. Annual immunization against influenza is the best prophylaxis against infection. The Advisory Committee on Immunization Practices (ACIP) currently recommends influenza vaccination for about 85% of the population of the United States [8]. Because the infection rate is so high in children, targeting children between 6 months and 16 years of age for influenza

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Dr. Papania is co-inventor of the aerosol device used in this study and thus has a financial interest in the product.

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vaccination programs could reduce the incidence of influenza infection by 65–97% [9]. In 2007 the ACIP recommended annual influenza vaccination for all school-age children [10]. To achieve high annual coverage, safe and effective vaccine delivery methods combining high levels of acceptance in children and adolescents with ease of administration will be essential. Delivery methods which provide dose-sparing to expand the vaccine supply would also be extremely useful.

In the U.S. two types of licensed vaccines are approved for use for the prevention of influenza; a trivalent inactivated vaccine (TIV; multiple vaccine manufacturers) delivered by intramuscular injection, and a live-attenuated influenza vaccine (LAIV; FluMist™) delivered as a large particle intranasal spray. Both vaccines reduce the risk and severity of infection with influenza viruses homologous with the vaccine strains, however, LAIV generally provides better cross protection against drifted strains. The mechanisms of the enhanced heterologous protection are not clear but may include improved mucosal immunity generated by the mucosal route of vaccination and improved cellular immunity generated by the LAIV infection.

As a vaccine delivery method, intranasal LAIV vaccination offers several important advantages over intramuscular injection, in addition to enhanced cross protection against drifted influenza strains. In the developing world, reuse of contaminated needles is a major concern, there is a common aversion to the pain of injection which may decrease acceptance of vaccination, as well as the risk to healthcare workers of accidental injury with a contaminated needle [11]. Intranasal LAIV vaccine (FluMist™) is needle-free and avoids the major problems associated with vaccination by injection; however, there is a need to improve the efficiency and acceptability of intranasal vaccine delivery methods.

LAIV (FluMist™) is administered using a Becton-Dickenson AccuSpray™ device which generates large vaccine particles [mass median aerosol diameter (MMAD), >70 microns], and is a high speed spray. Particle size and speed are key factors that determine where an aerosol will deposit in the airway. Because large fast-moving particles cannot navigate narrow airway passages, the largest particles tend to be trapped in the external nares and do not reach the internal nasal airways which are the target of nasal vaccination. In addition, droplets deposited in the nose tend to drip out, reducing the acceptability of nasal sprays. The large droplets which do enter the nasal airways tend to roll back toward the pharynx causing unpleasant sensation; a common complaint in people receiving nasal sprays. These droplets also have limited surface contact with the nasal airway tissues and limited residence time in the nasal airway which may reduce their immunologic impact.

In contrast, very small particles (< 5 microns) can navigate the entire airway and deposit in the lungs. For LAIV, pulmonary deposition is unnecessary and it is undesirable as it increases the risk of adverse events. Thus, for ideal nasal deposition of a vaccine aerosol, the droplets should be small enough to get into the internal nasal airway but also have a MMAD greater than 10 microns to minimize lung deposition [12,13]. Several studies beyond those for influenza virus suggest that delivery of appropriate vaccine particle sizes can lead to effective immunity. For example, field trials have shown that measles vaccine administered by small particle aerosol can boost or induce virus-specific antibody responses in vaccinated or previously seronegative subjects [14–17]. In addition, measles virus aerosol vaccination evokes a stronger and longer lasting antibody response compared to measles vaccine administered by injection as measured by serum antibody response [18], and vaccination by the aerosol route is less susceptible to interference by preexisting virus neutralizing antibodies [19,20]. In this study, we evaluated the parameters required for effective aerosol delivery of influenza virus in mice using controlled 20 and 30 micron MMAD particle aerosols as proof of concept for LAIV aerosol delivery. Mice exposed to aerosols containing

live influenza virus were protected against homologous influenza virus challenge as well as lethal heterologous challenge. The results suggest that influenza can be effectively delivered by aerosol exposure to generate potent immunity against influenza virus challenge, and controlled vaccine particle aerosolization may offer a solution for improving current influenza vaccination strategies.

2. Materials and methods

2.1. Viruses

Influenza viruses A/Hong Kong/1/68 × A/Puerto Rico/8/34 (X31; H3N2) and A/PR/8/34 (PR8; H1N1) were grown in the allantoic cavity of 10-day old embryonated chicken eggs for 48 h at 37°C. Stock viruses were aliquoted and stored at −80°C until use. The 50% tissue culture infectious dose (TCID₅₀) of the stock viruses were determined by the Reed and Meunch method [21].

2.2. Aerosol nebulizer

The nebulizer used for aerosol delivery of influenza viruses was manufactured by Creare Inc. (Hanover, NH). The nebulizer delivers aerosols with specific mass median aerosol diameter (MMAD) particle sizes utilizing an ultrasonic vibrating mesh technology, and includes a disposable aerosolizing element (DAE).

2.3. Mouse studies

Specific-pathogen free female BALB/c mice (Harlan, Indianapolis, IN) aged 6–8 weeks old were housed in microisolator cages, and food and water were provided *ad libitum*. In all studies, no less than 5 mice per treatment were examined in at least two independent assays. All animal studies were reviewed and approved by the University of Georgia Investigational Animal Care and Use Committee. Mice were anesthetized with Avertin (2, 2, 2 tribromoethanol) by intraperitoneal injection prior to inoculation.

The aerosol was administered to mice using a hydrophobic plastic conical mask that covered the nares and mouth of the mice. There was no detectable loss of influenza virus viability post-nebulization as determined by infectious virus plaque assay (data not shown). Parameters tested included aerosol exposure time, volume of aerosol emitted, aerosol flow rate, and titer of virus in the aerosol suspension. The volume of aerosol emitted and aerosol exposure time were evaluated simultaneously using either 20 or 30 micron MMAD aerosols, a low titer of X31 (10² TCID₅₀/ml), varying amounts of exposure time (15, 30, or 45 sec) at a flow rate of 1 cc/min. The 20 and 30 micron MMAD particle size was chosen to limit deposition of virus to the upper airways while facilitating broader distribution of the particles in the nasal passages. The MMAD particle size of 30 microns generates very few respirable particles as assessed by the device manufacturer, i.e. < 1% by volume are in the respirable range (1 to 5 microns) while 20% of the particles are in 5 to 20 microns range. For studies evaluating titer of virus in the aerosol suspension, log-fold increasing concentrations of X31 virus ranging from 10¹ to 10⁵ TCID₅₀/ml were delivered to mice using 30 micron aerosol particles for 15 sec/mouse at a flow rate of 1 cc/min. Studies evaluating the nebulizer flow rate required for effective delivery of virus examined flow rates of 2 cc/min., 1 cc/min., 0.5 cc/min., and 0.1 cc/min. using 30 micron particles, a standard concentration of virus (10³ TCID₅₀/ml), and a 15 sec exposure time. Due to the limitations of the model system large droplet delivery was emulated by topical intranasal inoculation. For intranasal (i.n.) inoculation, mice received 0.05 ml/nare for a total inoculation of 0.1 ml X31 virus using a pipet. To evaluate the protective efficacy associated with different delivery routes, mice were inoculated intramuscularly (i.m.) with 10⁷ TCID₅₀/0.1 ml X31 virus, i.n. with 10⁵ TCID₅₀/0.1 ml X31 virus, or by aerosol (30 micron particles, 15 sec, 1 cc/min.) with

104 TCID₅₀/ml X31 virus (H3N2) and 14 days post-inoculation i.n. challenged with X31 (105 TCID₅₀/0.1 ml), or with a lethal dose (103 TCID₅₀/0.1 ml) of antigenically distinct PR8 influenza virus (H1N1). Five mice from each group were euthanized 3 days post challenge to collect nasal washes and lungs for virus titer determination.

2.4. Collection of nasal wash and lung samples

Lungs and nasal washes were collected from euthanized mice at day 3 post inoculation (p.i.) and frozen at -80°C until all specimens could be assayed together to minimize biological variation. Nasal washes of mice were performed using 0.5 ml of phosphate buffered saline (PBS) containing 0.5% bovine serum albumin, penicillin (4000 U/ml) (Calbiochem, Gibbstown, NJ), streptomycin (800 µg/ml) (Sigma, St. Louis, MO), polymyxin B (400 U/ml) (MP Biochemicals, LLC, Solon, OH), and gentamicin (100 µg/ml) (Gibco, Carlsbad, CA). Nasal washes were thawed in a 37°C water bath and vortexed. To prepare lung tissue, 1 ml of PBS containing antibiotics and 0.5% bovine serum albumin was added to each sample. Samples were homogenized using the TissueLyser (Qiagen) then centrifuged at 10,000 rpm for 5 minutes. The TCID₅₀ was determined for each sample as previously described [22]. Briefly, 10-fold serial dilutions of samples from 10⁻¹ to 10⁻⁶ were made in Modified eagles medium (MEM) with TPCK [L-(tosylamido-2-pheyl) ethyl chloromethyl ketone]-treated trypsin (Worthington Biochemical Corporation, Lakewood, NJ) (1 µg/ml). Dilutions of each sample were added to Madin-Darby canine kidney (MDCK; ATCC) cells (4 wells/dilution; 200 µl/well) and the cells were incubated for 48 h at 37°C. The contents of each well were tested for hemagglutination and the TCID₅₀ was calculated by the Reed and Meunch method [21].

2.5. ELISA

The levels of IgA and IgG antibodies against influenza were determined from lung homogenates as previously described [23]. Briefly, each well of a 96 well EIA plate (Costar, Cambridge, MA) was coated with 100 µl of sucrose gradient purified X31 (4 µg/ml, Charles River Laboratories) in 0.05M carbonate/bicarbonate buffered saline and incubated at 4°C overnight. Plates were blocked with PBS containing 10% normal horse serum at room temperature for 1 h. After three washes with PBS containing 0.05% Tween 20, serial dilutions of lung homogenates were added to the wells and incubated for 2 h at room temperature. After three washes, the wells were treated with goat anti-mouse IgA, IgG1, or IgG2a-horseradish peroxidase conjugates (Bethyl Laboratories, Montgomery, TX) for 1 h at room temperature. Unbound conjugates were removed and 3, 3', 5, 5'-tetramethylbenzidine (TMB) containing H₂O₂ (Vector Laboratories) was used to develop color. Color development was stopped after 25 minutes using 1M H₂SO₄ and the optical density was read at 450 nm.

2.6 Hemagglutination inhibition assay

Serum samples were collected from each mouse prior to inoculation with influenza virus, and 14 days post challenge. All sera was treated with receptor-destroying enzyme (RDE, Denka Seiken Co., LTD. Toyko, Japan) and tested in a hemagglutination inhibition assay (HAI) with 0.5% chicken red blood cells (CRBCs) as previously described [24]. Viruses were diluted to contain four agglutinating units in PBS.

2.6. Statistical analysis

Comparison of virus titers in nasal wash and lung homogenates was performed using one-way ANOVA and Tukey's Multiple Comparison post-hoc test. GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA) and Microsoft Excel were used for statistical analysis. Differences with p<0.05 were considered to be statistically significant.

3. Results

3.1. Evaluation of Aerosol Parameters

To determine how aerosol distribution and particle size affected delivery and levels of virus deposition, mice were inoculated with live X31 influenza virus by mask aerosol delivery or i.n. droplet administration (Figure 1). Because the volume of aerosol emitted is related to aerosol exposure time, these two parameters were evaluated simultaneously (Figure 1A). The results showed no statistical difference in the level of infectious virus isolated from nasal washes or lungs of mice administered X31 using 20 or 30 micron median aerosol diameter particles, or between the routes of administration. The findings suggest the level of virus detected in the nasal wash and lungs were independent of the delivery time or volume delivered; however, there was a trend for higher mean virus titers in the lungs compared to nasal washes (Figure 1A). The range of mean virus titers isolated from nasal washes of mice administered 30 micron aerosol particles was $10^{3.5}$ to $10^{4.0}$ TCID₅₀/ml, and the range in the lungs was $10^{5.5}$ to $10^{6.1}$ TCID₅₀/ml across the exposure times. Similarly, mice administered 20 micron particles had mean virus titers in nasal washes ranging from $10^{3.7}$ to $10^{4.9}$ TCID₅₀/ml, and mean virus titers in lungs ranging from $10^{4.3}$ to $10^{5.6}$ TCID₅₀/ml across the different exposure times. Given the similar results for aerosol administration of 20 or 30 micron median particle sizes of X31, and because the 30 micron DAE cartridges did not clog during administration, the 30 median aerosol diameter particle size was used for the remaining studies.

It has been suggested the local concentration and deposition pattern of vaccine within the airways may influence the effectiveness of the local and systemic immune responses. To determine whether the titer of virus in the aerosol suspension affected deposition, mice were administered varying amounts of virus from 10^1 – 10^5 TCID₅₀/ml X31 as 30 micron median aerosol particles or i.n. administered the virus (Figure 1B). For mice exposed by aerosol, virus titers in nasal washes and lungs decreased with decreasing virus dose titers and were below the limit of detection in mice administered the lowest dose of virus (10^1 TCID₅₀/ml). This dose-dependent effect was statistically significant for the lung titers evaluated (Figure 1B).

The results for dose-dependent aerosol delivery using X31 virus were compared to intranasal (i.n.) delivery of log-fold decreasing concentrations of X31 virus (10^5 to 10^1 TCID₅₀/ml). There were no significant differences ($p > 0.05$) between the amount of infectious virus isolated from either nasal washes or lungs of mice inoculated with varying doses of X31 (Figure 1B). The mean virus titers isolated from nasal washes of i.n. inoculated mice ranged from $10^{3.9}$ to $10^{5.4}$ TCID₅₀/ml, and the mean virus titers isolated from lungs ranged from $10^{6.0}$ to $10^{6.3}$ TCID₅₀/ml showing that even at very low doses (10^1 and 10^2 TCID₅₀/ml); infectious virus is readily delivered to both the upper and lower airways by i.n. delivery. However, delivery of infectious virus to the lower airway is not a preferred vaccination strategy, as this that may contribute to enhanced pathogenesis [25]. In contrast, lower airway delivery of infectious virus was significantly ($p < 0.05$) lower at these lower doses (10^1 and 10^2 TCID₅₀/ml) using aerosol exposure, and peak virus titers were similar following aerosol or i.n. delivery at higher virus concentrations, i.e. 10^4 to 10^5 TCID₅₀/ml.

As the liquid flow rate can affect the amount of aerosol the animal is exposed to, the nebulizer flow rate required for effective delivery of live virus was determined (Figure 1C). There was no significant difference in the level of infectious virus recovered from nasal washes at any flow rate tested, i.e. 2 cc/min (2), 1 cc/min. (1), 0.5 cc/min (0.5), and 0.1 cc/min (0.1), although there was evidence of a dose-dependent response. The mean virus titers isolated from nasal washes ranged from $10^{4.0}$ to $10^{5.0}$ TCID₅₀/ml and higher virus titers (10^5

TCID₅₀/ml) were recovered from nasal washes of mice exposed to a higher flow rate. Similarly, there was no significant difference in the level of virus recovered from the lungs at the flow rates examined; however, no infectious virus was detected in the lungs using a 0.1 cc/min. flow rate. The mean virus titers isolated from the lungs ranged from $<10^1$ to $10^{6.3}$ TCID₅₀/ml. There was a trend toward higher mean virus titers in the lungs, but no statistical difference compared to nasal wash virus titers, a finding consistent with a similar trend for mice administered 10^3 TCID₅₀/ml in the dose-response study (Figure 1B).

3.2. Serum Antibody Responses

A hemagglutination inhibition (HAI) antibody titer of 1:40 is considered to be the minimum serum antibody titer required for protection against influenza infection [26]. To determine if aerosol administration induces adequate HAI antibody titers, serum HAI antibody responses were determined at day 14 post-aerosol administration in mice (Table 1). The mean HAI serum antibody titer in mice was directly related to the titer of X31 administered where the lowest virus titer (10^2 TCID₅₀/ml) administered induced the lowest mean serum antibody response (mean HAI titer 28) and the highest virus titer (10^6 TCID₅₀/ml) administered induced the highest mean serum antibody response (mean HAI titer 213). Based on a 1:40 HAI antibody titer indicative of protection [26], 100% of mice administered a 10^4 TCID₅₀/ml aerosol dose or higher had protective levels of serum antibodies.

3.3 Aerosol Delivery Protects Against Challenge

To evaluate the protective efficacy associated with different vaccination routes, mice were inoculated i.m., i.n., or by aerosol with X31 (H3N2) and 14 days post-inoculation challenged with X31, or with a lethal dose of antigenically distinct (H1N1) PR8 (Table 2). Intramuscular inoculation was compared against aerosol delivery as this is the most common method for immunization in humans. In order to induce a similar serum antibody response the dose of virus used for intramuscular vaccination was two logs higher than the dose of virus used for either intranasal or aerosol inoculation. The mean virus titers in the nasal wash and lungs of naïve mice challenged with X31 were $10^{4.2}$ TCID₅₀/ml and 105.0 TCID₅₀/ml, respectively. In contrast, naïve mice challenged with PR8 succumbed having mean PR8 titers in nasal wash of $10^{1.8}$ TCID₅₀/ml and in the lungs of $10^{4.9}$ TCID₅₀/ml. As anticipated, mice i.m. inoculated and challenged with X31 had no detectable levels of infectious virus in nasal washes or lungs, and all mice survived challenge. In contrast, mice i.m. inoculated with X31 and challenged with PR8 succumbed to the heterologous challenge, and had mean PR8 titers in nasal washes of $10^{1.4}$ TCID₅₀/ml and 104.9 TCID₅₀/ml in the lungs. Interestingly, both survival and the mean PR8 titers in nasal washes and in the lungs were similar between naïve and i.m. inoculated mice suggesting that i.m. inoculation does not provide sufficient heterologous protection. However, both i.n. and aerosol inoculation provided heterologous protection from PR8 challenge in which all mice survived. There was no detectable levels of infectious PR8 in nasal washes but similar mean virus titers (range $10^{2.2}$ to $10^{3.0}$ TCID₅₀/ml) in the lungs that was significantly different ($p<0.05$) from the amount of virus isolated from the lungs of i.m. PR8 challenged mice. These results show that the route of vaccination is important for inducing protective immunity.

3.4 Aerosol Exposure Induces Both Systemic and Mucosal Immunity

To evaluate a measure of vaccine efficacy, the levels of serum antibody to HA were determined in mice after different inoculation routes (Table 3). All mice were seronegative (<10) for PR8 as measured by HAI 13 dpv (data not shown). The mean HAI serum antibody titers were not tested for naïve mice; however, naïve mice challenged with X31 had a mean HI titer of 192. Mice receiving i.m. X31 inoculation had a mean HAI titer of 124 that was boosted to 480 after X31 challenge, and because these mice succumbed after PR8 challenge,

HAI titers were not tested. In contrast, i.n. inoculated mice had a mean HAI titer of 224 that was boosted to 736 after X31 challenge, but had a lower mean HAI titer of 70 after PR8 challenge. A similar but substantially more robust secondary response was observed in aerosol exposed mice where following vaccination the mice had a mean HAI titer of 156 which was boosted to 685 after X31 challenge, and following PR8 challenge, had a mean HAI titer of 140 which was 2-fold higher compared to i.n. inoculated mice. Taken together, the results from Tables 2 and 3 indicate that aerosol exposure induces superior antibody responses compared to other inoculation routes, induces sterilizing immunity against homologous challenge, and provides a level of protection against heterologous lethal challenge.

Mucosal immunity has a role in protection against influenza infection as mucosal IgA antibodies have the ability to neutralize influenza at the point of viral entry [27]. The mucosal antibody responses were determined in the lungs of mice inoculated by different routes to determine the levels of IgA, IgG1, and IgG2a (Figure 2). Lung IgA levels were higher in i.n. inoculated mice compared to aerosol or i.m. inoculated mice (Figure 2A). Interestingly, lung IgA titers were highest for i.m. inoculated mice compared to aerosol or i.n. inoculated mice after challenge (Figure 2B). The mucosal antibody findings for IgA are consistent with the report that in absence of pre-immune antibody re-infection accelerates the production of IgA [28]. Both i.n. and aerosol inoculated mice had similar levels of IgG1 at day 14 post-inoculation; however, IgG1 levels were lower for i.m. inoculated mice (Figure 2C). Following challenge, there was no substantial increase in IgG1 levels for any group, and all groups of mice had similar levels of IgG1 (Figure 2D). IgG2a levels were similar for all groups of mice at day 14 post-inoculation (Figure 2E), and all groups had higher but similar levels of IgG2a after challenge (Figure 2F).

4. Discussion

Traditional methods of vaccination using intramuscular or subcutaneous routes have been successful but have limitations and safety issues. Additionally, these routes of vaccination often do not generate adequate immunity at the sites of infection particularly for mucosally transmitted viruses [29]. Aerosol vaccination provides a method to overcome these obstacles, and offers advantages that include noninvasive delivery, reduced risk of transfer of blood-borne pathogens, elimination of needles, reduced medical waste, and potential dose-sparing [30]. The need to improve methods of vaccine delivery has been recognized by the World Health organization (WHO), particularly for measles virus (MV) vaccination [20], and recent studies evaluating aerosol administration of MV vaccine has shown aerosol vaccination to be a promising non-invasive alternative to subcutaneous (s.c.) injection [20,31,32].

In this study, we examined aerosol delivery of influenza virus by means of a nebulizer that uses ultrasonic vibrating mesh to generate aerosol particles. We tested aerosol parameters that included median particle size, dose volume, and virus titer in the aerosol suspension, delivery time, and flow rate for optimal deposition of influenza virus in the airways. Assessing these parameters in mice can be difficult because the nares and airways are small, and the level of anesthesia can affect the outcome. These difficulties are well-documented for i.n. instillation in mice where the relative distribution of the instillation agent between the upper and lower respiratory tract and lungs is heavily influenced by delivery volume and the level of anesthesia [33]. For example it has been shown that as the volume of fluid is increased during nasal instillation in anesthetized mice, there is a concomitant increase in relative dosing to the lungs [33]. Moreover, any volume beyond 0.05 ml that is i.n. instilled results in a level lower respiratory tract deposition [33]. Thus, it is not surprising that the

distribution of aerosolized particles in the airways is dependent in part on volume delivered, particle size and flow rate.

In this study, we show no statistical difference in the level of infectious virus isolated from nasal washes or lungs of mice administered X31 using aerosol delivery of 20 or 30 micron median diameter particles, and as expected, the viral titers in nasal washes and lungs decreased with decreasing virus dose titers. The aerosolization rate required for effective delivery of live virus was evaluated at ranges of 0.1 cc/min. to 2.0 cc/min.; however, there was no significant difference in the level of infectious virus recovered from nasal washes at any flow rate tested. Interestingly, no infectious virus was detected in the lungs using a 0.1 cc/min suggesting this flow rate preferentially delivers virus to the upper airways. Of the three delivery routes examined in this study, i.n. and aerosol delivery were the most effective for eliciting high serum antibody titers against HA and reduced virus replication in the respiratory tract of mice challenged with homologous or heterologous virus, and aerosol delivery of X31 evoked robust immune responses even at very low doses (10^2 TCID₅₀/ml).

Several important findings regarding the induction of immune responses following vaccination or challenge are reported here. One is that i.m. inoculation with X31 induced lower mean serum anti-HA antibody titers and no detectable cross-protection to PR8 challenge, while both i.n. and aerosol delivery protected against homologous and heterologous challenge. These findings are consistent with a previous study that showed nasal instillation or small particle aerosol vaccination of mice with an attenuated, temperature-sensitive recombinant influenza A virus induced similar levels of virus neutralizing antibodies and provided similar levels of protection as measured by recovery from a sub-lethal challenge with a virulent virus [34]. Another finding is that lung IgA levels were higher for i.n. immunized mice compared to aerosol or i.m. inoculated mice prior to challenge, but at day 7 post-challenge, lung IgA titers were considerably higher for i.m. vaccinated mice compared to the other inoculation routes. It has previously been reported in mice that maximal secondary IgA responses in the respiratory tract are achieved by a combination of intranasal priming and boosting with vaccine [35]. However, our finding that i.m. inoculation boosts for higher mucosal IgA levels following challenge has also been observed in a different influenza virus study that examined parenteral DNA vaccination and boosting with antigen-matched recombinant adenovirus which induced strong IgA responses and better protection against morbidity following H1N1 and H5N1 challenge [36]. In the challenge study shown in this report, i.n. or aerosol inoculation and challenge also induced substantial increases in lung IgG1 and IgG2b. While induction of IgG1 has a role in protection against influenza, the major serum isotype present in mice that survive lethal virus challenge is IgG2a even in the presence of low levels of IgG1 [37–39]. Studies have shown that generation of IgG2a antibodies is associated with increased influenza vaccine efficacy as this isotype is more efficient at viral clearance [40,41]. In the studies reported here, all inoculation routes induced similar levels of IgG1 both after vaccination and challenge; however, the aerosol exposed group had a higher increase in IgG2a levels after challenge compared to the i.n. inoculation group. These results further suggest aerosol vaccination may be a strategy to improve vaccine efficacy for influenza.

Existing licensed influenza vaccines include inactivated and live attenuated influenza virus formulations. Aerosol delivery is being developed for vaccinating against influenza virus infection using live attenuated influenza A virus. We evaluated aerosol delivery of influenza in mice, the most common model for preliminary vaccine efficacy studies. In these studies a mouse-adapted influenza A virus was used because live attenuated human influenza viruses do not replicate in mice; in general human influenza A viruses do not replicate in mice without prior adaptation [42]. The ferret is considered to be the most suitable animal model for preclinical evaluation of human influenza vaccines. Influenza infection in ferrets closely

mimics that in humans with respect to clinical signs, pathogenesis, and immunity and ferrets are naturally susceptible to infection with human influenza A viruses [43]. Ferrets also share marked similarities to humans in terms of lung physiology, airway morphology and cell types present in the respiratory tract, including the distribution of α -2,6-linked sialic acids, the receptor for human influenza viruses [44,45]. The further development of aerosol immunization using live attenuated influenza virus will use ferrets because it is the more relevant animal model for influenza vaccine studies.

With the burgeoning need for the development safe and effective vaccination strategies for influenza, this study shows that aerosol delivery induces robust and protective immunity. Further, the results from aspects of the study suggest that reducing aerosol flow rate may allow for upper airway targeted vaccine delivery, and that exceedingly low titers of virus can be used to vaccinate for robust antibody responses to HA. Clearly, aerosol vaccination strategies that induce robust and protective immunity and obviate the issues associated with parenteral vaccination offer an advantage, but perhaps the greatest is that aerosol vaccination offers a natural route of infection leading to immunity at the site of natural infection.

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Figure 1.

Evaluation of aerosol parameters in a mouse model of influenza virus infection. The results represent the mean virus titers in nasal washes and lungs 3 days post exposure of five BALB/c mice per group. (A) Evaluation of particle size, exposure time, and volume of aerosol emitted using 10^2 TCID₅₀/ml of X31 and a flow rate of 1 cc/min. (B) Evaluation of virus titer in the aerosol suspension using varying titers of virus in 30 micron particles at 1 cc/min. and 15 sec exposure time. (C) The effects of varying flow rates on delivery of 30 micron particles using 10^3 TCID₅₀/ml of X31 and a 15 sec exposure time. For all studies, mice received 100 μ l of virus for intranasal (i.n.) inoculation. Graphs depict representative results from one representative infection experiment. *Indicates statistically significant differences as compared to aerosol mean lung titer at 10^1 TCID₅₀/ml and † aerosol mean lung titer at 10^5 TCID₅₀/ml ($p < 0.05$, one-way ANOVA and Tukey's Multiple Comparison post-hoc test).

Figure 2.

Virus-specific mucosal antibody responses in mice after various vaccination routes. IgA, IgG1, and IgG2a levels in lungs of mice were determined 14 days post vaccination (dpv) (A, C, and E respectively) and 7 days post challenge (dpc) (B, D, and F respectively). Antibody levels were measured using five mice per group except the aerosol 7 day post challenge group (n=4) and naïve group (n=3).

Table 1

Serum HAI Antibody Response in Mice 14 Days After Aerosol Administration.

Titer of X31 in Aerosol (TCID ₅₀ /ml)	% with titer ≥40	Mean HAI Antibody Titer	HAI Antibody Range
10 ²	20	28	<10–160
10 ³	80	70	20–160
10 ⁴	100	120	40–320
10 ⁵	100	156	80–160
10 ⁶	100	213	160–320

n=10 for all groups except 10⁶ (n=6)

Table 2

Virus Replication in the Respiratory Tract of Vaccinated Mice After Challenge.

Vaccine Group	Survival (%)		Mean Virus Titers 3 dpc [TCID ₅₀ (log ₁₀ /ml)] (no. of mice shedding virus/total no. of mice challenged)					
			X31			PR8		
	X31	PR8	Nasal washes	Lungs		Nasal washes	Lungs	
i.m.	100	0	<1 ^a (0/5)	<1 (0/5)		1.4± 0.38 (2/5)	4.9 ±0.25 (5/5)	
i.n.	100	100	<1 (0/5)	<1 (0/5)		<1 (0/5)	3.0 ±1.36* (2/5)	
Aerosol	100	100	<1 (0/5)	<1 (0/5)		<1 (0/5)	2.2 ±0.86* (2/5)	
Naïve	100	0	4.2 ±0.22 (5/5)	5.0 ±0.19 (5/5)		1.8 ±0.63 (3/5)	4.9 ±0.19 (5/5)	

^a limit of detection

* indicates statistically significant differences as compared to i.m. vaccine group (p<0.05, repeated measures ANOVA).

Table 3

Serum Hemagglutination Inhibition Antibody Titers After Vaccination and Challenge.

Vaccine Group	Mean Serum Antibody Titer 13 dpv ^a (range)	Mean Serum Antibody Titer 14 dpc ^b (range)	
		X31 Challenge	PR8 Challenge
	X31	X31	PR8
i.m.	124(40–320)	480(320–640)	NT ^c
i.n.	224 (160–320)	736(320–1280)	70 (40–80)
Aerosol	156(80–320)	685 (320–1280)	140 (80–320)
Naive	NT	192 (160–320)	NT

^a days post vaccination; n=10^b days post-challenge; n=5^c Not tested