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Intratracheal administration of influenza virus is superior to intranasal administration as a model of acute lung injury

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Abstract

Infection of mice with human or murine adapted influenza A viruses results in a severe pneumonia. However, the results of studies from different laboratories show surprising variability, even in genetically similar strains. Differences in inoculum size related to the route of viral delivery (intranasal vs. intratracheal) might explain some of this variability. To test this hypothesis, mice were infected intranasally or intratracheally with different doses of influenza A virus (A/WSN/33 [H1N1]). Daily weights, a requirement for euthanasia, viral load in the lungs and brains, inflammatory cytokines, wet-to-dry ratio, total protein and histopathology of the infected mice were examined. With all doses of influenza tested, intranasal delivery resulted in less severe lung injury, as well as smaller and more variable viral loads in the lungs when compared with intratracheal delivery. Virus was not detected in the brain following either method of delivery. It is concluded that compared to intranasal infection, intratracheal infection with influenza A virus is a more reliable method to deliver virus to the lungs.

Keywords

Influenza A infection; Inoculation; Mice; Lung injury; Intratracheal; Intranasal

The influenza A virus infection causes approximately 20,000–50,000 deaths in the United States every year (2010). New influenza strains generate recurring epidemics and pandemics with significant attributable morbidity, mortality and economic impact (Shrestha et al., 2011a). After the most recent H1N1 pandemic, the US Centers for Disease Control and Prevention estimated 61 million cases, 274,000 hospitalizations and 12,470 deaths occurred in the United States between April 2009 and April 2010 (Shrestha et al., 2011b). As a result of the significant mortality and morbidity associated with influenza A infection and the paucity of available therapies, investigators have turned to murine models in order to genetically dissect the molecular pathways responsible for the innate immune response to the infection.

The resulting literature shows a surprising variability in the severity and duration of the pneumonia resulting from influenza A infection and sometimes even different phenotypes between laboratories using genetically similar strains (Abdul-Careem et al., 2011; Hashimoto et al., 2007; Nhu et al., 2010; Shirey et al., 2013). Some of this variability might be attributed to the methods used to deliver the virus to mice. In one method, a viral aliquot is placed at the tip of the nose and aspirated during spontaneous breathing; in another, a tube is placed in the trachea under direct visualization and the virus is delivered through the tube directly into the lungs. In this study, these two methods were compared.

The protocol for the use of mice was approved by the Animal Care and Use Committee at Northwestern University. Murine adapted influenza A virus (A/WSN/33 [H1N1]) was kindly provided by Robert Lamb, Ph.D., Sc.D., Northwestern University, Evanston, IL. Madin–Darby Canine Kidney (MDCK) cells (American Type Culture Collection (ATCC), Manassas, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin G/streptomycin and 10% fetal bovine serum (37°C, 5% CO₂).

Eight-week-old (20–25 g) male C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice had free access to food and water at all times. For intratracheal administration, a 1 in., 18 gauge angiocath was cut diagonally to a length that ensured the tip was in the mid-trachea while the hub was in the mouth. A piece of tape was then placed on the needle of a Hamilton syringe toward the hub to mark the level of insertion required for the tip of the Hamilton syringe to extend just beyond the end of the angiocath. Isoflurane was administered to mice until adequate anesthesia was achieved. The animal was then placed on a plastic supportive table at an angle of 45° from the bench and the animal's upper teeth were secured under a loop of black braided silk attached to the end of the table closest to the operator. This position was maintained during the whole intubation procedure. A Novaflex light source was positioned several inches away from the mouth of the animal, in front of the operator, in order to illuminate the oropharynx. A set of blunted tweezers was used to gently pull the tongue out of the direct field of vision allowing visualization of the epiglottis and larynx. A metal spatula was employed as a laryngoscope blade for lifting the lower jaw. It was then advanced toward the larynx until the epiglottis was lifted, revealing the vocal cords. Keeping the laryngoscope in this position to maintain direct visualization of the vocal cords, a guide wire (obtained from a radial arterial line catheter) (Medline Inc., Mundelein, IL) was directed through the vocal cords over which the angiocath was placed using a Seldinger technique. To confirm placement, the catheter was briefly occluded by placing a gloved thumb over the orifice (3 breaths), which results in thoracoabdominal paradox if the tube is correctly placed. Different doses (350, 500, 750 or 1500 pfu/mouse) of influenza A virus (A/WSN/33 [H1N1]) in 50 µL of PBS were instilled by administering two 25 µL aliquots 10 s apart using a Hamilton syringe. The catheter was then removed. Operators were trained so that the entire procedure from anesthesia to removal of the tube could be performed in less than 2 min (Mutlu et al., 2007; Radigan et al., 2012). For intranasal administration, the neck of the anesthetized mouse was pulled slightly backwards with the body in a straight position to create a straight line between the nasopharynx and chest. Different doses (350, 500, 750 or 1500 pfu/mouse in 50 µL of PBS) of influenza A

virus (A/WSN/33 [H1N1]) were then administered at the nares in two 25 μ L aliquots and were aspirated into the lungs during spontaneous respiration.

Unanesthetized mice were weighed on a portable balance (Mettler-Toledo Inc., Columbus, OH) daily. The mice infected with influenza A virus (A/WSN/33 [H1N1]) were monitored for signs of distress (failure to respond to cage tapping, slowed respiration, failure of grooming and ruffled fur) every 8 h. Mice that developed these symptoms were euthanized and the death was recorded as mortality.

For the assessment of viral replication in the lung and brain, the mice were anesthetized after four days of inoculation with 500 pfu of the influenza A virus. The thorax was opened from the subxiphoid region to the neck, the inferior vena cava was clamped, and the right ventricle was perfused in situ with sterile PBS (>1 mL, until the lungs were clear) followed by removal of the heart and lungs en bloc. After removal of the lungs, the skull was opened and brain removed. The lungs and brain were placed on ice prior to and during homogenization (Tissue Tearor, 30 s) in a flow cytometry tube with 1 mL of PBS. An additional 2 mL of PBS was added to the resulting homogenate and subjected to homogenization using Dounce homogenizer (20 strokes). The lungs and the brain homogenates were then centrifuged at 4°C, and the pellet was discarded. MDCK cells (ATCC, Manassas, VA) were grown in 6-well plates to 100% confluency and incubated with serial 10-fold dilutions of influenza A-infected lung in DMEM and 1% bovine serum albumin (BSA) at 37°C. One hour later, the inoculums were aspirated (Jing et al., 2008). Replacement media (3 mL) [2.4% Avicel (FMC BioPolymer, Philadelphia, PA), 2 $\times$ DMEM, and N-acetyl trypsin (1.5 μ g)] was added to each well and the plates were incubated for 3 days (Matrosovich et al., 2006). The overlay was then removed and viral plaques were visualized using naphthalene black dye solution (0.1% naphthalene black, 6% glacial acetic acid, 1.36% anhydrous sodium acetate) (Jing et al., 2008; Matrosovich et al., 2006).

For the collection of BAL fluid, the trachea was exposed by careful blunt dissection using a forceps and a suture was placed behind it. A small hole in the trachea was made with a sharp tipped scissors into which a 20 gauge metal angiocath cut to a length of approximately 1 cm was placed. The suture was then tied tightly to create a seal around the angiocath (tracheostomy tube). After placement of the tracheostomy tube, 1.0 mL of PBS was slowly infused and aspirated three times, which was immediately placed on ice. A 200 μ L aliquot of BAL fluid was placed in a cytospin (1200 rpm for 5 min) and the slides were Wright stained for differential analysis. Cell count was measured using a hemocytometer (trypan blue 0.4%). The remaining BAL fluid (200 $\times$ g, 5 min) was centrifuged and BAL protein (Bradford) and cytokines (BD LSR II flow cytometer) were measured in the supernatant. FCAP Array™ Software Version 3.0 data were used for cytokine analysis. For histologic examination, the tracheostomy tube was placed and the right ventricle was perfused with approximately 1 mL of PBS (until the lungs cleared). The lungs and heart were then removed en bloc with the tracheostomy tube in place and inflated to exactly 15 cm of H₂O with 4% paraformaldehyde. We examined 5 μ m sections from paraffin-embedded lungs stained with hematoxylin–eosin using light microscopy. Lung water (edema) content was measured by opening the thorax from the subxiphoid region to the neck, removing both the left and right lungs and placing them into pre-weighed eppendorf tubes. Both lungs were

weighed before and after oven desiccation (>72 h) and the final wet-to-dry lung ratio was calculated.

Differences between groups were revealed using a t-test and Kaplan–Meier survival curve for mortality. Analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego CA, USA). Data are shown as means±standard error of the mean or standard deviation as indicated. We defined variability in the assay as the standard deviation of the assay divided by the mean. A *p* value <0.05 was considered statistically significant.

Mice were treated with influenza A WSN intratracheally and intranasally and followed for up to 22 days after infection and daily weight was recorded daily and mortality up to three times daily. There were significant differences in mortality between the intratracheal and intranasal infection for all doses of influenza administration (Fig. 1A–C). While the low dose (350 pfu/mouse) was associated with 50% mortality when administered intratracheally, none of the animals died when the virus was administered intranasally (Fig. 1A; **p* < 0.05). The intermediate dose (750 pfu/mouse) caused 100% mortality in the intratracheal administration group but only 30% mortality in the intranasal group (Fig. 1B; **p* < 0.0001). High dose (1500 pfu/mouse) caused 100% mortality in the intratracheal group but only 50% mortality in the intranasal group (Fig. 1C; intratracheal LD₅₀ 192 h and intranasal LD₅₀ 311 h; **p* < 0.0001). Compared with animals infected intratracheally, mice with intranasal inoculation lost less weight and the amount of weight lost at each day after infection was more variable in the intranasally compared with the intratracheally infected mice (Fig. 1D–F).

To determine the effect of different methods of administration of the influenza virus on viral replication in the lung, C57BL/6 mice were treated with 500 pfu of influenza A virus intratracheally and intranasally as described above and plaque assays were performed using lung homogenates on day 4 after influenza infection. Intranasal administration of the virus revealed more variability in viral replication compared to intratracheal inoculation (coefficient of variation intranasal 138.7% vs. intratracheal 61.08%) (Fig. 2).

To further examine the effects of intranasal inoculation on the brain, C57BL/6 mice were treated with influenza A virus (1500 pfu/mouse) intratracheally and intranasally as described above and plaque assays were performed using lung brain homogenates on day 2. No viral replication within the brain homogenates was observed following either intranasal or intratracheal delivery of the virus (data not shown).

Four days after inoculation with A/WSN/33 [H1N1] influenza A virus (500 pfu/mouse), mice infected intratracheally had more BAL fluid cellularity with significantly more neutrophils (Fig. 3A, B). Wet-to-dry ratio and total protein was also significantly higher in the intratracheally infected mice with less variability compared to mice infected intranasally (Fig. 3C, D). The inflammatory cytokines, including TNF-α and IL-6, were significantly higher (Fig. 3E, F) and the severity of lung injury evident upon careful examination of hematoxylin and eosin-stained lung sections was more severe in the mice infected intratracheally (Fig. 3G).

Mice represent an important model system in which genetic strategies can be employed in mammals to better understand the molecular pathogenesis of the innate immune response to influenza A infection. Furthermore, mice provide a preclinical model in which the molecular target for putative therapies targeting the virus or the host immune response can be genetically confirmed. These data suggest that the use of intranasal instillation as a method to administer viruses to mice delivers less and more variable inoculums of the virus to the lung when compared with intratracheal instillation via an endotracheal tube.

As murine respiratory tissues do not express the α (2–6) sialic acid residues that serve as a receptor for the influenza A virus in the human and ferret lung epithelium, mice are relatively resistant to influenza A infection. To overcome this limitation, investigators passaged human isolates of influenza A viruses in murine tissues (most commonly brain) and generated murine adapted viral strains. The most commonly used strains are the Influenza A/Puerto Rico/8/34 (PR/8) and Influenza A/WSN/33. Infection of mice with these viral strains results in a severe pneumonia and, with sufficiently large viral inoculums, in mortality of the animal (Van Reeth, 2000). There is evidence that the severity of the pneumonia and the associated mortality are directly related to the viral load in the lung—larger inoculums result in more severe pneumonia, more rapid weight loss and earlier mortality (Blazejewska et al., 2011; Nhu et al., 2010). Consistent with these findings, weight loss was more rapid and the requirement for euthanasia (mortality) occurred earlier in mice treated with the same inoculum of virus via the intratracheal compared with the intranasal route. Quantitative measurements of live virus using plaque assays performed 4 days after infection showed fewer and more variable numbers of live viral particles with intranasal administration compared with intratracheal administration of the virus, confirming that intranasal administration reduced the viral inoculum that reached the distal lung. Furthermore, at almost all doses, the variability in both weight loss and viral load was larger following intranasal administration of the virus, suggesting that intranasal administration reduces both the accuracy and precision of viral delivery to the distal lung.

In a study in influenza-infected ferrets, Hashimoto and colleagues found that CNS lesions were common when ferrets were infected intranasally and suggested this might alter the course of the disease (Bodewes et al., 2011). In this study, virus could not be recovered from the brains of mice infected using either the intratracheal or intranasal route, suggesting the differences in outcomes observed between the intratracheal and intranasal routes did not result from differential inoculation of the brain.

Our results are likely to be important for the delivery of other substances to the distal lung to induce pneumonia (e.g. the administration of live bacteria or LPS) and for the delivery of therapeutic substances to the lung. With relatively little training, intratracheal intubation can be performed in mice quickly and accurately with only brief anesthesia induced by the administration of isoflurane. Widespread adoption of intratracheal intubation as a method to deliver substances to the distal lung in murine models of influenza A pneumonia can both improve the accuracy of studies comparing lung injury severity with different genetic viral or murine strains, as well as improve their precision, thereby reducing the number of animals required to detect a statistically significant difference. Furthermore, standardization of the method of delivery across laboratories will facilitate the discovery of important

differences between the large numbers of genetically modified murine strains examined in these models worldwide.

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Abbreviation

pfu plaque forming unit

References

- Estimates of deaths associated with seasonal influenza—United States, 1976–2007. *MMWR Morb Mortal Wkly Rep.* 2010; 59:1057–1062. [PubMed: 20798667]
- Abdul-Careem MF, Firoz Mian M, Gillgrass AE, Chenoweth MJ, Barra NG, Chan T, Al-Garawi AA, Chew MV, Yue G, van Roojen N, Xing Z, Ashkar AA. FimH, a TLR4 ligand, induces innate antiviral responses in the lung leading to protection against lethal influenza infection in mice. *Antivir. Res.* 2011; 92:346–355. [PubMed: 21945041]
- Blaziejewska P, Koscinski L, Viegas N, Anhlán D, Ludwig S, Schughart K. Pathogenicity of different PR8 influenza A virus variants in mice is determined by both viral and host factors. *Virology.* 2011; 412:36–45. [PubMed: 21256531]
- Bodewes R, Kreijtz JH, van Amerongen G, Fouchier RA, Osterhaus AD, Rimmelzwaan GF, Kuiken T. Pathogenesis of Influenza A/H5N1 virus infection in ferrets differs between intranasal and intratracheal routes of inoculation. *Am J Pathol.* 2011; 179:30–36. [PubMed: 21640972]
- Hashimoto Y, Moki T, Takizawa T, Shiratsuchi A, Nakanishi Y. Evidence for phagocytosis of influenza virus-infected, apoptotic cells by neutrophils and macrophages in mice. *J. Immunol.* 2007; 178:2448–2457. [PubMed: 17277152]
- Jing X, Ma C, Ohgashi Y, Oliveira FA, Jardetzky TS, Pinto LH, Lamb RA. Functional studies indicate amantadine binds to the pore of the influenza A virus M2 proton-selective ion channel. *Proc. Natl. Acad. Sci. U. S. A.* 2008; 105:10967–10972. [PubMed: 18669647]
- Matrosovich M, Matrosovich T, Garten W, Klenk HD. New low-viscosity overlay medium for viral plaque assays. *Viol. J.* 2006; 3:63. [PubMed: 16945126]
- Mutlu GM, Green D, Bellmeyer A, Baker CM, Burgess Z, Rajamannan N, Christ-man JW, Foiles N, Kamp DW, Ghio AJ, Chandel NS, Dean DA, Sznajder JI, Budinger GR. Ambient particulate matter accelerates coagulation via an IL-6-dependent pathway. *J. Clin. Invest.* 2007; 117:2952–2961. [PubMed: 17885684]
- Nhu QM, Shirey K, Teijaro JR, Farber DL, Netzel-Arnett S, Antalis TM, Fasano A, Vogel SN. Novel signaling interactions between proteinase-activated receptor 2 and Toll-like receptors in vitro and in vivo. *Mucosal Immunol.* 2010; 3:29–39. [PubMed: 19865078]
- Radigan KA, Urich D, Misharin AV, Chiarella SE, Soberanes S, Gonzalez A, Perlman H, Wunderink RG, Budinger GR, Mutlu GM. The effect of rosuvastatin in a murine model of influenza a infection. *PLoS ONE.* 2012; 7:e35788. [PubMed: 22536437]
- Shirey KA, Lai WD, Scott AJ, Lipsky M, Mistry P, Pletneva LM, Karp CL, McAlees J, Gioannini TL, Weiss J, Chen WH, Ernst RK, Rossignol DP, Gusovsky F, Blanco JCG, Vogel SN. The TLR4 antagonist Eritoran protects mice from lethal influenza infection. *Nature.* 2013; 497:498–502. [PubMed: 23636320]
- Shrestha SS, Swerdlow DL, Borse RH, Prabhu VS, Finelli L, Atkins CY, Owusu-Edusei K, Bell B, Mead PS, Biggerstaff M, Brammer L, Davidson H, Jernigan D, Jhung MA, Kamimoto LA, Merlin TL, Nowell M, Redd SC, Reed C, Schuchat A, Meltzer MI. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009–April 2010). *Clin. Infect. Dis.* 2011a; 52:S75–S82. [PubMed: 21342903]

- Shrestha SS, Sverdlow DL, Borse RH, Prabhu VS, Finelli L, Atkins CY, Owusu-Edusei K, Bell B, Mead PS, Biggerstaff M, Brammer L, Davidson H, Jernigan D, Jhung MA, Kamimoto LA, Merlin TL, Nowell M, Redd SC, Reed C, Schuchat A, Meltzer MI. Estimating the burden of 2009 pandemic influenza A (H1N1) in the United States (April 2009–April 2010). *Clin. Infect. Dis.* 2011b; 52(Suppl. 1):S75–S82. [PubMed: 21342903]
- Van Reeth K. Cytokines in the pathogenesis of influenza. *Vet. Microbiol.* 2000; 74:109–116. [PubMed: 10799783]

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Fig. 1.

Mortality is higher and weight loss less variable when identical doses of influenza A are administered intratracheally compared with intranasally. (A, D) C57BL/6 mice were inoculated intratracheally and intranasally with influenza A virus 350 pfu/mouse, (B, E) 750 pfu/mouse, and (C, F) 1500 pfu/mouse and the need for euthanasia (mortality) was measured at least daily in the same mice. For all groups the initial number of mice for each treatment condition is between 8 and 10 animals, however, in D–F the number of mice in each group falls over the duration of the experiment by a number corresponding to the rate of mortality. Error bars indicate standard errors of the mean. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$.

Fig. 2.

Viral replication is higher and less variable when identical doses of influenza A are administered intratracheally compared with intranasally. C57BL/6 mice were treated with influenza A virus (500 pfu/mouse) intratracheally and intranasally. Four days after influenza A infection, the lungs were harvested and viral titers were measured using viral plaque assays. $n = 10$ for intranasal infection, $n = 9$ for intratracheal infection. $*p < 0.05$. Error bars indicate standard deviations, the standard deviation is 134% of the mean for intranasal infection and 61% of the mean for intratracheal infection.

Fig. 3.

Measures of lung injury and lung inflammation are higher and less variable when identical doses of influenza A are administered intratracheally compared with intranasally. C57BL/6 mice were inoculated intratracheally and intranasally with influenza A virus (500 pfu/mouse) and harvested 4 days later for assessment of lung inflammation and the severity of lung injury. (A) Total leukocyte counts and (B) neutrophil counts in BAL fluid, (C) wet-to-dry ratio of both lungs, a measure of total lung water, (D) total protein levels in the BAL fluid, (E, F) inflammatory cytokine levels, tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in BAL fluid were measured. (G) Lung injury was assessed histologically by examination of hematoxylin and eosin stained lung sections obtained using a TissueGnostics imaging system (100 $\times$). The left panels show a representative montage image from the intranasally (top) or intratracheally (bottom) infected mice. For all experiments, there were five mice in each group. * $p < 0.05$. Error bars indicate standard deviations.