H9N2 Influenza Whole Inactivated Virus Combined with Polyethyleneimine Strongly Enhances Mucosal and Systemic Immunity after Intranasal Immunization in Mice

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Influenza whole inactivated virus (WIV) is more immunogenic and induces protective antibody responses compared with other formulations, like split virus or subunit vaccines, after intranasal mucosal delivery. Polyethyleneimine (PEI), an organic polycation, is widely used as a reagent for gene transfection and DNA vaccine delivery. Although PEI recently has demonstrated potent mucosal adjuvant activity for viral subunit glycoprotein antigens, its immune activity with H9N2 WIV is not well demonstrated. Here, mice were immunized intranasally with H9N2 WIV combined with PEI, and the levels of local respiratory tract and systemic immune responses were measured. Compared to H9N2 WIV alone, antigen-specific IgA levels in the local nasal cavity, trachea, and lung, as well as levels of IgG and its subtypes (IgG1 and IgG2a) in the serum, were strongly enhanced with the combination. Similarly, the activation and proliferation of splenocytes were markedly increased. In addition, PEI is superior as an H9N2 WIV delivery system due to its ability to greatly increase the viral adhesion to mucosal epithelial cells and to enhance the cellular uptake and endosomal escape of antigens in dendritic cells (DCs) and further significantly activate DCs to mature.

Taken together, these results provided more insights that PEI has potential as an adjuvant for H9N2 particle antigen intranasal vaccination.

The rise and spread of the low-pathogenic avian H9N2 influenza virus have seriously increased the risk of a new influenza pandemic. H9N2 viruses have prevailed in chickens in China in recent years and have constantly undergone reassortment, and novel genotypes have continued to emerge (1–3). A novel H7N9 reassortant subtype was recently found to cause severe human respiratory infections in China (4). Bioinformatic analyses for the H7N9 virus revealed that its six internal genes were from H9N2 avian influenza viruses of chickens (5). Thus, the elimination of low-pathogenic avian H9N2 influenza virus in poultry becomes even more important in influenza prevention.

The nasal cavity of the respiratory tract is the primary entry site of the H9N2 influenza virus, and the viral infection could be discontinued if intranasal immunity is well established (6). Compared with live attenuated influenza vaccines or subunit influenza vaccines (such as purified viral hemagglutinin [HA]) or neuraminidase [NA] proteins), whole inactivated H9N2 influenza vaccines have more advantages, including an improved safety profile, higher immunogenicity, more effective ability of establishing cross-protection at the pathogen’s entry site, and stronger cross-presentation of antigens by dendritic cells (DCs) for a CD8+ T cell response against viruses (7–9). However, mucosal immunization by intranasal delivery with inactivated virus alone is often poorly effective. Unlike systemic immunization, nasal antigens must cross various barriers (compact epithelium, mucociliary clearance, and mucus) before they contact with submucosal immune cells (10). Many researchers used various immunopotentiators, such as CpG DNA and cholera toxin (CT), to target the downstream immune system or used mucadhesive particulate carrier systems, such as thermally sensitive hydrogel (8), to prolong the nasal residence time when combined with influenza whole inactivated virus (WIV) via intranasal immunization.

Polyethyleneimine (PEI), a cationic polymer, exhibits a high positive charge density when protonated in aqueous solutions and is considered a promising candidate for transfection or delivery of DNA and oligonucleotides (11). PEI has also been used to increase the immune effect of DNA vaccines, probably because of its cellular targeting and uptake (12). A recent study showed that PEI has potent mucosal adjuvant activity for viral subunit soluble glycoprotein antigens, including gp140 derived from HIV-1 and hemagglutinin protein from the influenza virus. It is possible that PEI could coat H9N2 WIV (larger granular antigens) and improve the mucosal and systemic immunity after intranasal immunization.

In this study, H9N2 WIV combined with PEI was used to immunize mice through the nasal cavity. Following immunization, the local and systemic immune responses were measured. Furthermore, mouse bone marrow-derived dendritic cells, as the most powerful antigen-presenting cells, were used to evaluate antigen uptake, cross-presentation efficiency, and DC maturation.
Heat-inactivated viruses were prepared at 56°C for 0.5 h. Duck/NanJing/01/1999 [H9N2] were generously supplied by the Jiangsu Academy of Agricultural Sciences (Nanjing, China) and purified by using discontinuous sucrose density gradient centrifugation, as previously described (16). Antibodies PE-CD40 (1C10), FITC-major histocompatibility complex class II (MHC-II) (M5/114.15.2), PerCP-Cy5.5-CD69 (H1.2F3), APC-CD3 (17A2), FITC-CD4 (GK1.5), PE-CD8 (GK1.5), or respective isotype controls were obtained from eBioscience (San Diego, CA, USA). Other antibodies included hors eradish peroxidase (HRP)-conjugated anti-mouse IgG, IgG1, IgG2a (Santa Cruz, CA, USA), and IgA (Southern Biotech, Birmingham, AL, USA). Cholera toxin B subunit (CTB) was from Absin (Shanghai, China). Branched PEI (25 kDa) was from Sigma (St. Louis, MO, USA), and it was used as surrogate nasal epithelium because of its similar biophysical properties, such as forming a tight monolayer, cilia, secreting mucus (13–15).

Animals. C57BL/6 and BALB/c mice (6 weeks old, specific-pathogen-free [SPF]) were from the Animal Research Center of Yangzhou University (Yangzhou, China). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Agricultural University and followed National Institutes of Health guidelines for the performance of animal experiments.

Preparation of H9N2 WIV-PEI complexes. Influenza viruses (A/Duck/Nanjing/01/1999 [H9N2]) were generously supplied by the Jiangsu Academy of Agricultural Sciences (Nanjing, China) and purified by using a discontinuous sucrose density gradient centrifugation, as previously described (16). Heat-inactivated viruses were prepared at 36°C for 0.5 h. Inactivation of the virus was confirmed by inoculation into 10-day-old SPF embryonated eggs for three passages. The quantity of purified whole inactivated viruses was measured with a Micro BCA protein assay kit (Thermo Fisher, Waltham, MA, USA). H9N2 WIV-PEI complexes were made according to the method that was used to prepare a polymer-DNA complex, with modifications (17). H9N2 WIV was dissolved in phosphate-buffered saline (PBS) (0.01 M) to adjust the concentration to 5 mg/ml. PEI was dissolved in triple-distilled water to yield different concentrations. H9N2 WIV-PEI complexes were prepared by adding the H9N2 WIV solution to the PEI solution at an equal volume and vortexing for 0.5 min at room temperature. The H9N2 WIV-PEI complexes were analyzed using native polyacrylamide gel electrophoresis (PAGE). The gel formula contains 6.7 ml 30% acrylamide–0.8% methylene bisacrylamide, 10 ml 4× phosphate buffer (pH 5.8 to 8.0), 23.08 ml Milli-Q water, 0.2 ml 10% ammonium persulfate, 0.02 ml N,N,N′,N′-tetramethylbenzenedi-amine (TEMED). After electrophoresis (15 mA) and 6 h on ice, gels were stained with Coomassie blue (Invitrogen). For viral labeling, viruses were incubated for 1.5 h at 37°C. After being washed 3 times with PBST, the plates were washed 3 times with PBST. Then, 2-fold serial dilutions of serum samples or lavage fluid from mice were applied to the plates and incubated for 1.5 h at 37°C. After being washed 3 times, plates were incubated with HRP-conjugated anti-mouse IgG (total IgG, IgG1, and IgG2a) in serum and secretory IgA (sIgA) in mucosal wash (nasal, tracheal, and lung wash) was measured by enzyme-linked immunosorbent assay (ELISA), as described previously (18). Briefly, ELISA plates were coated overnight at 4°C with 2 µg H9N2 WIV/well and then blocked by incubation with 1% (wt/vol) bovine serum albumin (BSA) in PBS containing 0.05% Tween (PBST) for 1 h at 37°C. Thereafter, the plates were washed 3 times with PBST. Then, 2-fold serial dilutions of serum samples or lavage fluid from mice were applied to the plates and incubated for 1.5 h at 37°C. After being washed 3 times, plates were incubated with HRP-conjugated anti-mouse IgG (total IgG, IgG1, and IgG2a) or IgA antibodies for 1 h. The plates were washed 5 times and were incubated with 3,3′,5,5′-tetramethylbenzidine (TMB). After 20 min, the reaction was stopped with sulfuric acid, and the optical density (OD) was measured using a Bio-Rad microplate reader (Model 550). Results are shown as the means ± SD. *, P < 0.05; **, P < 0.01; *** P < 0.001.
Antigen adhesion and uptake. In order to explore the adhesion of H9N2 WIV on the surface of mucosal epithelial cells in vitro, Calu-3 cells (5 × 10⁵ cells) were incubated with DyLight 405/633-H9N2 WIV (H9N2 WIV concentration, 14.3 μg/ml) or DyLight-H9N2 WIV-PEI complexes for 72 h, and CD69 activation was performed using FACS in vitro; meanwhile, the proliferative response was detected using WST-8 assays according to the manufacturer’s instruction.

Phenotype of DCs. In vitro, the DCs were incubated with H9N2 WIV or H9N2 WIV-PEI complexes (PEI/H9N2 WIV weight ratios of 0.04, 0.08, and 0.12) for 24 h. Next, the DCs were harvested and incubated with FITC-MHC-II, PE-CD40, or the respective isotypes at 4°C for 30 min, per the manufacturer’s guidelines. The colocalization analysis was performed by the colocalization module of the ZEN 2012 software. The colocalization rate (CLR) was calculated according to the following equation (21): CLR = colocalization area/antigen channel area.

Statistical analysis. The results were expressed as the means ± the standard deviations (SD). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett’s t test to evaluate variations between groups; a P value of <0.05 was considered statistically significant.

RESULTS Characterization of H9N2 WIV-PEI complexes. Based on electrostatic interactions between the positive amino groups on PEI and the negative surface charges on H9N2 WIV, we mixed PEI and H9N2 WIV in different weight ratios to prepare the H9N2 WIV-PEI complexes. Native PAGE analysis showed that H9N2 WIV measured at 450 nm (OD₄₅₀). The criteria were judged as follows: the OD₄₅₀ of the positive control should be more than 1.0, and the OD₄₅₀ of the negative controls should be lower than 0.2. Results were expressed in the ratio of OD₄₅₀ produced by the serum or mucosal wash samples compared to the negative-control serum or mucosal wash (P/N). Samples with a ratio higher than 2.1 were considered positive. The titer was expressed as the highest dilution of antibody producing P/N ratio values of ≥2.1.

Hemagglutination inhibition assay. Hemagglutination inhibition (HI) titers in serum were determined according to the procedure described previously (19). Briefly, samples were serially 2-fold diluted in 96-well round-bottom plates. Then, four hemagglutination units of H9N2 antigen were added to each well, and the plates were incubated for 30 min at room temperature. Finally, 1% chicken erythrocyte suspension was added to each well, and plates were incubated statically for at least 30 min at room temperature. The highest dilution capable of preventing hemagglutination was scored as the HI titer.

T-cell restimulation and proliferation. Furthermore, splenic lymphocytes were isolated from the immunized mice and were assessed for the percentages of CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T cells by fluorescence-activated cell sorting (FACS). Another part of the isolated splenic lymphocytes (5 × 10⁶ cells) were restimulated with H9N2 WIV for 72 h, and CD69 activation was performed using FACS in vitro; meanwhile, the proliferative response was detected using WST-8 assays according to the manufacturer’s instruction.

Generation of DCs. DCs were isolated and cultured using our advanced method (20). Briefly, bone marrow cells were obtained from femurs and tibias of wild-type male C57BL/6 mice and cultured in medium (RPMI 1640; Invitrogen) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL-4) (PeproTech, Rocky Hill, NJ). After 60 h of culture, the medium was gently discarded, and fresh medium was added. On day 6, nonadherent and loosely adherent cells were harvested and subcultured overnight in complete medium. On day 7, only cultures with >90% cells expressing CD11c by FACS were used.

Antigen adhesion and uptake. In order to explore the adhesion of H9N2 WIV on the surface of mucosal epithelial cells in vitro, Calu-3 cells

FIG 3 PEI assists H9N2 WIV in enhancing the level of serum antibody. Antigen-specific serum IgG titers (A), IgG1 titers (B), IgG2a titers (C), and HI titers (D) of immunized mice (n = 12) 2 weeks after the last vaccination. Data are shown as the means ± SD. *, P < 0.05; **, P < 0.01.
alone, as negative charges, was not seen in the gel. Interestingly, when H9N2 WIV combined with PEI, with an increase of the mixing ratio, more viruses quickly migrated toward the cathode. These data suggested that H9N2 WIV could effectively combine with PEI through strong electrostatic interactions (Fig. 1).

**PEI assists H9N2 WIV in enhancing the local immune responses.** To verify whether PEI could be an effective adjuvant in response to H9N2 WIV, the mice were intranasally immunized three times with H9N2 WIV alone, H9N2 WIV-PEI complexes, or H9N2 WIV in combination with the known mucosal adjuvant CTB. Specific IgA levels were determined by ELISA (Fig. 2). The results showed that the mucosal IgA levels in nasal (Fig. 2A), tracheal (Fig. 2B), and lung (Fig. 2C) washes induced by PEI/CTB plus H9N2 WIV were substantially greater than by the antigen-alone treatment (*P* < 0.01).

**PEI facilitates H9N2 WIV to trigger the systemic immune responses.** To analyze whether PEI could assist H9N2 WIV in enhancing the systemic immune responses, specific serum antibody levels were determined by ELISA 28 days after the primary immunization (Fig. 3A through C). The results showed that PEI/CTB plus H9N2 WIV induced much higher serum antigen-specific IgG (Fig. 3A), IgG1 (Fig. 3B), and IgG2a (Fig. 3C) antibody titers than H9N2 WIV alone (*P* < 0.01). The hemagglutination inhibition (HI) titer in the serum was also analyzed, and similar changes in the HI titer (Fig. 3D) were observed. Furthermore, splenocytes were isolated from the immunized mice and were re-stimulated with H9N2 WIV in vitro. The percentages of CD3⁺CD4⁺ (Fig. 4A and C) and CD3⁺CD8⁺ (Fig. 4B and D) T cells, the CD69 expression (Fig. 5A and B), and the proliferative index (Fig. 5C) were markedly increased in the group of PEI/CTB plus H9N2 WIV compared with those of the antigen alone. Altogether, these results indicated that H9N2 WIV plus PEI effectively induced systemic immune responses after intranasal immunization.

**PEI improves the viral adhesion to Calu-3 cells and the cellular uptake of H9N2 WIV by DCs.** Adhesion of the antigen on the surface of mucosal epithelial cells is crucial for the subsequent uptake by submucosal DCs. Thus, we explored whether PEI could assist H9N2 WIV in attaching to Calu-3 cells. Compared to H9N2 WIV alone, H9N2 WIV combined with PEI strongly promoted viral adhesion on the surface of Calu-3 cells, which were analyzed

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**FIG 4** PEI assists H9N2 WIV in enhancing the percentages of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells. At 28 days after the primary immunization, the percentages of CD3⁺CD4⁺ (A and C) and CD3⁺CD8⁺ (B and D) spleen T cells from the immunized mice were analyzed by FACS. Data are shown as the means ± SD. *, *P* < 0.05; **, *P* < 0.01.
by CLSM and FACS (Fig. 6A through C). Next, we investigated whether PEI could also assist H9N2 WIV in uptake by DCs. Our FACS data showed that PEI efficiently enhanced viral uptake by DCs (Fig. 6D and E). Of note, the PEI-H9N2 WIV complex group that was prepared at a weight ratio of 0.08 had the best capability of uptake.

**PEI enhances the cellular endosomal escape of H9N2 WIV in DCs.**

Except for with cellular uptake, efficient endosomal escape of the exogenous antigen plays a key role in inducing cross-presentation (22). Thus, we studied the subcellular distribution of the antigen after internalization by DCs. CLSM was used to observe the position of labeled lysosomes and antigens. As shown in Fig. 7A and B, most of H9N2 WIV colocalized with lysosomes in the antigen-alone group (CLR/H11005 89.1%). In the H9N2 WIV-PEI complex group, part of H9N2 WIV escaped from lysosomes (CLR/H11005 72.1%), suggesting an effective antigen escape from lysosomes and release to the cytoplasm.

**PEI assists H9N2 WIV in enhancing the phenotypic maturation of DCs.**

Maturation of DCs is crucial for the initiation of downstream immune responses. To investigate whether PEI modulated the DC maturation, the expressions of major histocompatibility complex class II (MHC-II) and CD40 on the DCs were analyzed by FACS after exposure to H9N2 WIV-PEI complexes (PEI/H9N2 WIV weight ratios of 0.04 and 0.08) for 24 h. The expression of MHC-II (Fig. 8A and C) was upregulated compared with that of the antigen alone. Meanwhile, the CD40 expression profile (Fig. 8B and D) on the DCs exhibited a similar change in the group of H9N2 WIV-PEI complexes (PEI/H9N2 WIV weight ratio of 0.08). PEI alone did not induce the expressions of MHC-II and CD40 compared with those in the control group. These findings suggested that PEI has the capability of assisting H9N2 WIV in enhancing the phenotypic maturation of DCs.

**DISCUSSION**

Here, we demonstrated that PEI as a potent mucosal adjuvant also effectively combined with H9N2 WIV and elicited robust local and systemic immunity compared with CTB. These data confirmed that the adjuvant activity is not limited to several soluble glycoprotein antigens (23). Although H9N2 WIV particles are larger granular antigens, they could easily interact with the positively charged PEI because of their negative charges (24). In addition, we also chose a branched (25 kDa) but not linear PEI that probably provided a larger contact area. Similarly, branched PEI is the best research form in terms of gene delivery (25). Recently, it was reported that branched PEI drove a mixed Th1/Th2-type adaptive immune response after systemic delivery (26).

Generally, the nasal mucosa is composed of a pseudostratified columnar ciliated epithelium, which forms tight barriers for clearance of foreign various microbial pathogens. In the human nasal cavity, the half-time of nonmucoadhesive formulation clearance is about 20 min (27). It may not allow sufficient retention for antigen to be absorbed through the nasal mucosa (28). Furthermore, because nasal mucosa is negatively charged, the negatively charged H9N2 WIV is more difficult to adhere to the surface of the mucosa. Another concern is the size of the antigens; our previous studies suggested that OVA (a soluble model antigen) can be easily transported through the nasal mucosal epithelium, whereas fluorescent latex particles (FLP; macromolecule particles, 30 nm to ~100 nm) had weak abilities to cross the mucosal barriers (10). Thus, finding ways to improve the adhesion of granular antigens to mucosa is a critical issue for H9N2 WIV intranasal immunization. Our current data demonstrated that PEI, using an electric adsorption principle, assisted more H9N2 WIV to attach to the Calu-3 cells. Similarly, chitosan is a mucoadhesive polymer that has been studied for mucosal vaccine delivery (29).
However, the unfavorable water solubility of chitosan restricted its development (30).

Dendritic cells (DCs), as immune sentinels, form a tight cellular network underneath the nasal mucosa. They not only timely capture submucosal pathogenic microbes but also have a special ability to sample luminal antigens by using their transepithelial dendrites (31,32). In addition, continually stimulated epithelial cells can secrete a large amount of C-C ligand chemokines (such as CCL20) that recruit distant DCs (33). Thus, the longer antigens stay on the nasal epithelial cells, the more opportunities for submucosal DCs to take up luminal antigens. In an in vitro assay, our data showed that PEI also provided assistance to H9N2 WIV in enhancing uptake by DCs, which may use their electricity adsorption ability. DC maturation plays a vital role in innate immune responses and subsequent adaptive immunity (34). Our results and those of others (23) demonstrated that PEI alone did not induce DC maturation by detecting the expression of phenotypic markers. However, when incubation of H9N2 WIV was combined with PEI on DCs, the expressions of MHC-II and CD40 were significantly improved compared with H9N2 WIV alone. One possibility is that PEI provides more assistance for H9N2 WIV to enter the DCs. Once H9N2 WIV entered the DCs, their viral genomic single-stranded RNA (ssRNA) was able to activate their ligand toll-like receptor 7 (TLR7) and then initiate an innate immune response of DCs (35).

Cross-presentation also plays an important role in influenza
vaccines. Regulating the antigen-processing process inside DCs and presenting them via the major histocompatibility complex class I (MHC-I) pathway is the critical step to activate CD8⁺ T cells and elicit robust cytotoxic T lymphocytes (CTL) (22, 36). Efficient antigen escape from the lysosome can be presented easily through the MHC-I pathway (37). Our results suggested that PEI can efficiently improve the endosomal escape of H9N2 WIV and could possibly enhance antigen cross-presentation. This was in

FIG 7 PEI enhances the cellular endosomal escape of H9N2 WIV in DCs. (A) Intracellular trafficking of internalized H9N2 WIV or H9N2 WIV-PEI complexes (PEI/H9N2 WIV weight ratio of 0.08) was observed by CLSM. Lysosomes (red), H9N2 WIV (green). Bars, 5 μm. (B) Overlay analysis shows the detailed colocalization information: empty lysosome (1), escaped antigen (2), and colocalized antigen (3). Data are representative of more than three independent experiments.

FIG 8 PEI assists H9N2 WIV in enhancing the phenotypic maturation of DCs. DCs were stimulated with medium, PEI, H9N2 WIV (H9N2 WIV concentration, 14.3 μg/ml), or H9N2 WIV-PEI complexes (PEI/H9N2 WIV weight ratios of 0.04, 0.08, and 0.12) for 24 h. The expressions of MHC-II (A and C) and CD40 (B and D) on DCs were analyzed by FACS. All of the data are shown as the means ± SD of three replicates and are representative of three independent experiments. *, P < 0.05; **, P < 0.01.
line with the result that H9N2 WIV combined with PEI highly increased the number of CD8+ T cells compared with antigen alone in vivo. One explanation for endosomal escape was a proton sponge effect of cationic polymers in the acidic endosome. In DCs, the pH at a mildly acidic level allows some antigens to escape from endosomes for cross-presentation (38). PEI, using its powerful sponge effect, effectively buffered the influent protons in the endosome and prevented further rapid acidification, which resulted in a greater promotion of the endosomal escape of H9N2 WIV.

In conclusion, H9N2 WIV, as a granular antigen, combined with PEI, remarkably enhanced the antigen uptake and cross-presentation, improved the DC maturation, and further elicited robust local mucosal and systemic immune responses after intranasal immunization. These features are beneficial to prevent an H9N2 influenza pandemic.

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