ORIGINAL ARTICLE

Intranasal immunization with live attenuated influenza vaccine plus chitosan as an adjuvant protects mice against homologous and heterologous virus challenge

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Abstract Our previous studies have proven the adjuvanticity of chitosan in mice when administered with inactivated and subunit influenza vaccine. In this study, we investigated the adjuvant effect of chitosan on the immunogenicity and protective efficacy of a live attenuated influenza vaccine. Mice were inoculated intranasally with live attenuated influenza vaccine plus chitosan and then challenged with a high, lethal dose of homologous or heterologous virus. Antibody responses, secretion of IFN-γ by spleen cells, body weight loss, survival rates, and residual lung virus titers were tested. The results demonstrated that live attenuated influenza vaccine with chitosan adjuvant not only protected mice completely against challenge with the homologous virus but also provided good cross-protection against a heterologous virus. In addition, chitosan as adjuvant could significantly increase the levels of antigen-specific antibodies and the population of IFN-γ-secreting T cells. These results reveal the potential of chitosan as a candidate adjuvant for use in a live attenuated influenza vaccine.

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Introduction

Influenza is a highly contagious and acute respiratory disease that causes high morbidity and mortality in humans every year. Vaccination is the most effective strategy to prevent and control influenza infections [1]. The currently available licensed influenza vaccines include inactivated influenza vaccines and live attenuated influenza vaccines. A live attenuated influenza vaccine has several advantages over an inactivated influenza vaccine. A live attenuated influenza vaccine can be administered conveniently through the nasal mucosal pathway, it elicits robust mucosal immunity and cellular responses, and it can provide a longer-lasting immune response [2, 3]. However, live attenuated influenza vaccines are only applicable to the population between 2 and 49 years old, and the immune response induced by live attenuated vaccines in elderly people is relatively weak compared to the inactivated influenza vaccine [4]. Thus, a high dosage of live attenuated vaccine is required to induce a sufficient immune response [5]. In an attempt to improve the immunogenicity of live attenuated vaccines and reduce the dosage required, the use of adjuvant would be useful. Some studies have shown that adjuvants can greatly improve the immunogenicity of live attenuated influenza vaccines and minimize the amount of antigen required, which can greatly alleviate the pressure on the limited global influenza vaccine manufacturing capacity [6, 7].

A number of mucosal adjuvants, such as cholera toxin and *Escherichia coli* heat-labile toxin, have been investigated for their ability to improve the vaccine-specific immune response [8]. Chitosan is a natural deacetylated polysaccharide from chitin in crustaceans (e.g., shrimp, crab), insects, and other invertebrates. It has several distinctive biological activities, including non-toxicity, non-irritant, non-antigenicity, fine bioadhesivity, biocompatibility, and biodegradation, which has been widely applied in the pharmaceutical and food industries [9–11]. Recently, Rauw et al. demonstrated that chitosan had potential as an adjuvant in poultry given by the ocular-nasal inoculation route with live Newcastle disease vaccine. The results showed that chitosan enhanced the cellular immune response of live Newcastle disease vaccine and promoted its protective effect. This was the first report that chitosan can be used as an adjuvant for a live virus vaccine in poultry [12, 13]. Several other studies also demonstrated that chitosan was an effective and safe adjuvant for an inactivated influenza vaccine [14–19]. Bacon et al. [14] verified that intranasal immunization with an influenza subunit vaccine plus chitosan stimulated broader immune responses in a mouse model. In addition, Ghendon et al. and our group confirmed that chitosan could significantly enhance the immunogenicity and the protective effect of an inactivated H5N1 influenza vaccine [15–17]. However, the properties of chitosan as an adjuvant for live attenuated influenza vaccines have never been tested. Therefore, the aim of this study was to investigate the effect of chitosan as a mucosal adjuvant for a live attenuated influenza vaccine.

In this study, a temperature-sensitive live-attenuated vaccine strain was successfully rescued using reverse genetics techniques and administrated intranasally to mice with chitosan as an adjuvant. Our studies first demonstrated that, in a mouse model, chitosan significantly enhanced the immunogenicity of a live attenuated influenza vaccine, and the live attenuated influenza vaccine plus chitosan protected mice effectively against challenge with homologous influenza virus as well as heterologous influenza virus.

Materials and methods

Cells and viruses

293T and MDCK cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10 % fetal bovine serum (FBS). Influenza viruses used in this study included a mouse-adapted A/PR/8/34(PR8) (H1N1) and A/Chicken/Jiangsu/11/2002 (H9N2). The H9N2 influenza viruses were isolated in Jiangsu Province, China. A mouse-adapted strain of the A/Chicken/Jiangsu/11/2002 (H9N2) virus was

then obtained by 10 lung-to-lung serial passages in BALB/c mice as described previously [20]. The mouse-adapted A/Chicken/Jiangsu/11/2002 (H9N2) virus, now fatal for infected mice, was used to challenge immunized mice. These viruses were stored at −70 °C.

Cloning and generation of viruses by reverse genetics

Strain A/PR/8/34 was inoculated into 10-day-old chicken embryos, allantoic fluid was collected 72 h afterwards, and viral RNA was extracted using TRIzol Reagent (Invitrogen). The RNA was reverse-transcribed into single-stranded cDNA according to the instructions provided with the cDNA synthesis kit (Promega). Specific primers were synthesized by a method described previously [21] and used for the amplification of various gene segments of virus by PCR. Amplified gene segments were then sequenced by the dideoxy method using an ABI 3130 Genetic Analyzer (Applied Biosystems).

PCR products were digested with *BsmB*I and *Bsa*I and cloned between the *BsmB*I sites of the bidirectional transcription/expression vector pHW2000 [22]. Recombinant plasmids carrying the different gene segments of the A/PR/8/34(PR8) (H1N1) virus were named pHW-PB2, -PB1, -PA, -HA, -NP, -NA, -M and -NS, respectively. Mutations causing temperature sensitivity (*ts*) and attenuation (*att*) in PB1 (K391E, E581G, and A661T) and PB2 (N265S) were introduced by site-directed mutagenesis using overlap extension PCR as described previously [23]. The mutated plasmids were named pHW-mPB1 and pHW-mPB2.

Recombinant virus was rescued as described previously [22, 24]. Briefly, 1 µg of each plasmid (pHW-mPB2, -mPB1, -PA, -HA, -NP, -NA, -M and -NS) was combined with 18 µl of the transfection reagent Lipofectamine 2000 (2 µl per µg DNA, Invitrogen), incubated at room temperature for 30 min, and then transferred to monolayers of 10^6 293 T cells in 6-well plates. Six hours later, the mixture was removed from the cells and replaced with Opti-MEM (Gibco-BRL) containing 0.3 % BSA and 0.01 % FCS. Seventy-two hours after transfection, the culture medium was collected and used to inoculate 10-day-old SPF chicken embryos for virus propagation.

Adjuvant, immunization and challenge

Chitosan was purchased from Sigma (USA). A 0.4 % (w/v) solution of chitosan was prepared in a 0.2 M sodium glutamate solution (pH 4.5). The rescued virus suspension was mixed with 0.4 % chitosan or PBS in a 1:1 (v/v) ratio. Anesthetized BALB/c mice aged 6–8 weeks were inoculated intranasally (i.n.) with the mixed solution in a volume of 40 µl (20 µl per nostril) and challenged i.n. 21 days later with $100 \times LD_{50}$ of PR8 virus or mouse-adapted H9N2

virus. The survival rate, clinical symptoms and bodyweight of the mice were monitored for at least 2 weeks.

Collection of samples

Blood samples were collected three days after virus challenge. Mice were anesthetized with chloroform and then bled from the heart with a syringe. The sera were separated and stored at -20 °C for determination of PR8 virus-specific antibodies. After bleeding, the mice were incised ventrally along the median line from the xiphoid process to the point of the chin. The trachea and lungs were taken out and washed three times with a total volume of 2 ml of PBS (containing 0.1 % BSA). The bronchoalveolar washes were collected for virus titration [20].

Detection of virus-specific antibody

Antibody titers were determined by ELISA as described in our previous studies [20, 25]. ELISA was performed sequentially in a 96-well polystyrene microtiter plate containing (1) inactivated PR8 virus, (2) serial two-fold dilutions of sera from mice, (3) goat anti-mouse IgG, IgG1, and IgG2a Ab (Southern Biotechnology Associates, Inc. USA) conjugated with biotin, (4) streptavidin conjugated with alkaline phosphatase (Southern Biotechnology Associates, Inc. USA), and (5) p-nitrophenyl-phosphate. The amount of chromogen produced was measured based on absorbance at 414 and 405 nm in a Labsystems Multiskan Ascent Autoreader (model 354, Finland). The Ab-positive cutoff values were set as mean $+ 2 \times$ SD of control sera. The ELISA Ab titer was expressed as the highest serum dilution giving a positive reaction.

Gamma interferon (IFN-γ) ELISPOT assay

Spleen cells were isolated from mice for IFN-γ ELISPOT assay three weeks after the immunization. The assay was performed according to the instruction manual (U-CyTech, The Netherlands). Briefly, 96-well plates of the ELISPOT Multiscreen Assay System (Millipore) were coated with anti-mouse IFN-γ capture Abs and incubated for 24 h at 4 °C. On the following day, the plates were washed and blocked with 200 µl of blocking solution R for 1 h. Next, 1×10^5 splenocytes from the immunized mice were added to each well and stimulated overnight at 37 °C in 5 % CO_2 in the presence of RPMI 1640 (negative control), Con A (positive control), or 10 µg of inactivated PR8 virus per ml. After 24 h of stimulation, the cells were washed and incubated at 37 °C for 1 h with biotinylated detector antibodies (UCyTech). The plates were washed, and streptavidin-HRP conjugate (U-CyTech) was added to each well and incubated at 37 °C for 1 h. The plates were washed,

and reagent from the AEC coloring system (U-CyTech) was added to each well. The plates were then washed with distilled water and dried at room temperature. Spots were counted by an automated ELISPOT Bioreader 4000 (Bio-Sys Limited Germany). The results were expressed as the number of spot-forming cells (SFC) per 10^6 splenocyte cells in the ELISPOT experiment.

Virus titration

A bronchoalveolar wash was serially diluted tenfold starting from a dilution of 1:10, inoculated on to Madin-Darby canine kidney (MDCK) cells, and examined for cytopathic effect two days later. The virus titer of each specimen, expressed as the 50 % tissue culture infectious dose ($TCID_{50}$), was calculated by the Reed-Muench method. The virus titer in each experimental group was expressed as the mean $\pm$ SD of the virus titer per ml of specimens from five mice in each group.

Statistical analysis

The results of test groups were evaluated by Student's t-test; if the P-value was less than 0.05, the given significance level, the difference was considered statistically significant. The survival rates of the mice in the test and control groups were compared using Fisher's exact test.

Results

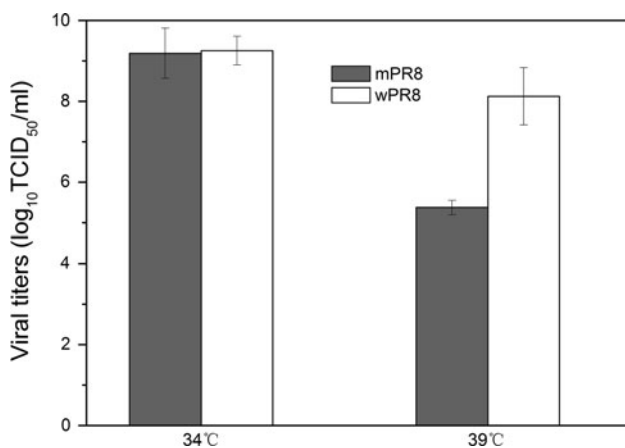### Generation of a live attenuated influenza vaccine

A live attenuated influenza vaccine with mutations in PB1 (K391E, E581G, and A661T) and PB2 (N265S) was constructed as described in Materials and methods. As expected, the rescued viruses exhibited more than a 2-log difference in growth at 34 °C and 39 °C (Fig. 1), which is characteristic of the *ts* phenotype [23]. The *att* phenotype was confirmed by evaluating the replication of mPR8 influenza virus and the survival rate of mice (Tables 1 and 2) [26].

Chitosan co-administered with live attenuated influenza virus vaccine reduces homologous influenza-induced mortality and enhances viral clearance

One hundred five female BALB/c mice aged 6-8 weeks were randomly divided into seven groups, with 15 mice in each group. Mice were vaccinated intranasally with live attenuated vaccine (10, 100, or 1000 $TCID_{50}$) with or without chitosan in the adjuvanted and non-adjuvant group, respectively. The rest were immunized with PBS alone as

the control group. Three weeks after the immunization, all of the mice were challenged intranasally with $100 \times LD_{50}$ ($10^{5.6}$ $TCID_{50}$) of homologous PR8 virus. Three days after the lethal challenge, bronchoalveolar washes were collected from five mice of each group for lung virus titration, as described in "Materials and methods". To evaluate the vaccination effect against homologous A/PR/8/34 (H1N1) virus, the remaining 10 mice in each group were observed daily for 14 days after challenge to monitor changes in body weight loss and to record mortality.

The results showed that the protection provided by live attenuated influenza vaccine against the homologous virus depended on the administered dosage of vaccine and adjuvant. As shown in Table 3 and Fig. 2, the survival rates of the mice immunized with live attenuated influenza vaccine alone at a dose of 10 $TCID_{50}$, 100 $TCID_{50}$ or 1000 $TCID_{50}$ at day 14 after the virus challenge were 0 % (0/10), 0 % (0/10) and 100 % (10/10), respectively, and the survival rates with the same dosage of live attenuated influenza vaccine in combination with chitosan adjuvant were 20 % (2/10), 100 % (10/10) and 100 % (10/10), respectively. The survival data demonstrated that a better protective effect on mice was achieved when using a higher immunizing dose. Meanwhile, the survival rate was significantly higher in the adjuvanted moderate (100 $TCID_{50}$) vaccine group than in the corresponding non-adjuvanted formulation group ($p<0.05$), suggesting that chitosan significantly enhanced the protective effect induced by live attenuated influenza vaccine.

Bodyweight changes in the mice were observed daily for 14 days after challenge (Fig. 2). All of the mice in the 10 $TCID_{50}$ non-adjuvanted group, the 100 $TCID_{50}$ non-adjuvanted group, and the control group significantly lost weight (Fig. 2B and D) and died within seven days (Fig. 2A and C) after challenge. However, the mice in the 10 $TCID_{50}$ vaccinate-adjuvanted group, with a 20 % survival rate, recovered within two weeks after challenge (Fig. 2A and B). Excellent protection was achieved in mice immunized with 100 $TCID_{50}$ with chitosan. These mice

Fig. 1 Mutant PR8 virus (mPR8) is temperature sensitive. MDCK cells in a 96-well plate were infected with the mutant virus and incubated for three days at 34 °C and 39 °C. The temperature-sensitive phenotype was determined by measuring the virus titer

Table 2 Survival rate of mice inoculated intranasally with different doses of wPR8 and mPR8 influenza virus

Virus used for inoculation[a]	$TCID_{50}$ of virus inoculated per mouse	No. of survivors/ no. tested
wPR8	10^7	0/6
	10^6	0/6
	10^5	0/6
	10^4	1/6
	10^3	5/6
	10^2	6/6
mPR8	10^7	0/6
	10^6	1/6
	10^5	5/6
	10^4	6/6
	10^3	6/6
	10^2	6/6

[a] Six mice in each group received different doses of mutated PR8 virus (mPR8) and wild PR8 virus (wPR8) i.n., respectively

Table 1 The mPR8 mutated viruses are restricted in replication in the lower respiratory tract of mice

Virus[a]	Virus replication in trachea-lungs[b]					
	Day 2		Day 3		Day 4	
	Titer	Virus detected/total	Titer	Virus detected/total	Titer	Virus detected/total
wPR8	5.3 ± 0.3	5/5	5.3 ± 0.3	5/5	5.4 ± 0.3	5/5
mPR8	ND[c,d]	0/5	1.8[d]	1/5	1.8[d]	1/5

[a] Groups of 6- to 8-week-old BALB/c mice received 10^5 $TCID_{50}$ of mutated PR8 virus (mPR8) and wild PR8 virus (wPR8) i.n., respectively

[b] Trachea-lungs from groups of five mice were harvested on days 2, 3, 4 postinfection. Virus titers are expressed as mean $\pm$ SD

[c] ND, not detected

[d] Significant difference compared with the corresponding value for the wPR8-inoculated group ($p<0.05$)

Table 3 Protection of mice against lethal homologous PR8 virus challenge by intranasal administration of live attenuated vaccine plus chitosan

Immunogen	Dose of LAIV ($TCID_{50}$)	Protection against challenge with various viruses	
		Lung virus titer ($log_{10}TCID_{50}/ml$)	No. of survivors/no. tested
LAIV + chitosan	1000	ND[a,b]	10/10[b]
LAIV	1000	ND[a,b]	10/10[b]
LAIV + chitosan	100	ND[a,b,c]	10/10[b,c]
LAIV	100	5.1 ± 0.9	0/10
LAIV + chitosan	10	4.5 ± 2.6	2/10
LAIV	10	6.4 ± 0.5	0/10
PBS	–	6.4 ± 0.3	0/10

Fifteen mice in each group were immunized with PBS or different doses of live attenuated vaccine (LAIV) with or without chitosan. Three weeks post-vaccination, mice were challenged with a lethal dose ($100 \times LD_{50}$) of homologous PR8 virus. Bronchoalveolar washes were collected 3 days postinfection for titration of lung virus. The survival rates of mice 14 days postinfection were determined. Results are expressed as means $\pm$ SD of five tested mice in each group

[a] ND, not detected

[b] Significant difference compared to the mice in the PBS control groups ($p < 0.05$)

[c] Significant difference compared with the corresponding value for the group inoculated with live attenuated influenza vaccine alone ($p < 0.05$)

displayed no obvious weight loss, and no deaths occurred in this group (Fig. 2C and D). A similar situation was observed in the group immunized with 1000 $TCID_{50}$ of the live attenuated influenza vaccine with or without chitosan after challenge (Fig. 2E), indicating that a high dose of vaccine alone can induce an efficient immune response to protect mice against viral infection.

On day 3 post-challenge, five mice from each group were sacrificed, and virus titers in the lungs were measured (Table 3). The data showed that the lung viral titers in immunized mice declined as the dose of live attenuated influenza vaccine increased. Except for the 10-$TCID_{50}$-immunized groups, the residual lung virus titers in the immunized groups were significantly lower than those of the control group ($p<0.05$). Moreover, virus titers of the mice immunized with 10 $TCID_{50}$ and 100 $TCID_{50}$ of live attenuated influenza vaccine plus chitosan were lower than those of mice immunized with the same dose of live attenuated influenza vaccine alone. This indicated that the chitosan could accelerate the clearance of virus in the lung after challenge of mice vaccinated with 10 $TCID_{50}$ and 100 $TCID_{50}$ of live attenuated influenza vaccine. In addition, we did not detect virus in organs of mice inoculated with 1000 $TCID_{50}$ of live attenuated influenza vaccine alone, indicating that vaccination with the highest amount of live attenuated influenza vaccine provided complete protection.

Co-administration of live attenuated influenza vaccine with chitosan increases the specific antibody response

Mice were immunized according to procedures described above. Three weeks after the immunization, five mice in each group were sacrificed to obtain sera and washes for detection of specific IgG and IgA antibody, respectively, by ELISA. The results are shown in Table 4. Compared to the PBS control group, the immunized groups produced considerably higher amounts of virus-specific IgG antibodies. Meanwhile, a high dosage of the live attenuated influenza vaccine induced a relatively high titer of IgG antibodies. The IgG titers of the mice immunized with live attenuated influenza vaccine plus chitosan were higher than those of the mice immunized with live attenuated influenza vaccine only. The results indicated that chitosan adjuvant could enhance IgG Ab titers to the live attenuated influenza vaccine. Furthermore, a more interesting result was that the IgG titer produced in the 100 $TCID_{50}$ vaccine-adjuvanted group was even higher than that in the 1000 $TCID_{50}$ non-adjuvanted group, indicating that chitosan adjuvant may result in at least a 10-fold dose-sparing effect in mice. To assess the levels of Abs in the mucosal compartment, we detected the production levels of IgA Abs in nasal washes of mice of all groups. Interestingly, co-administration with 100 $TCID_{50}$ of live attenuated influenza vaccine with chitosan resulted in significantly higher levels of IgA titers than in the corresponding non-adjuvanted group (Table 4).

Additionally, in order to evaluate the Th bias of the Ag-specific humoral immune response induced by chitosan, we determined the IgG isotypes in serum. Generally, IgG1 antibodies are associated with a Th2 response, while IgG2a antibodies are associated with a Th1 response [34]. Although low dosage of live attenuated vaccine with chitosan failed to induce detectable antibody, IgG1 and IgG2a antibody titers were obviously higher in the moderate- and high-dosage vaccine-adjuvanted groups than in
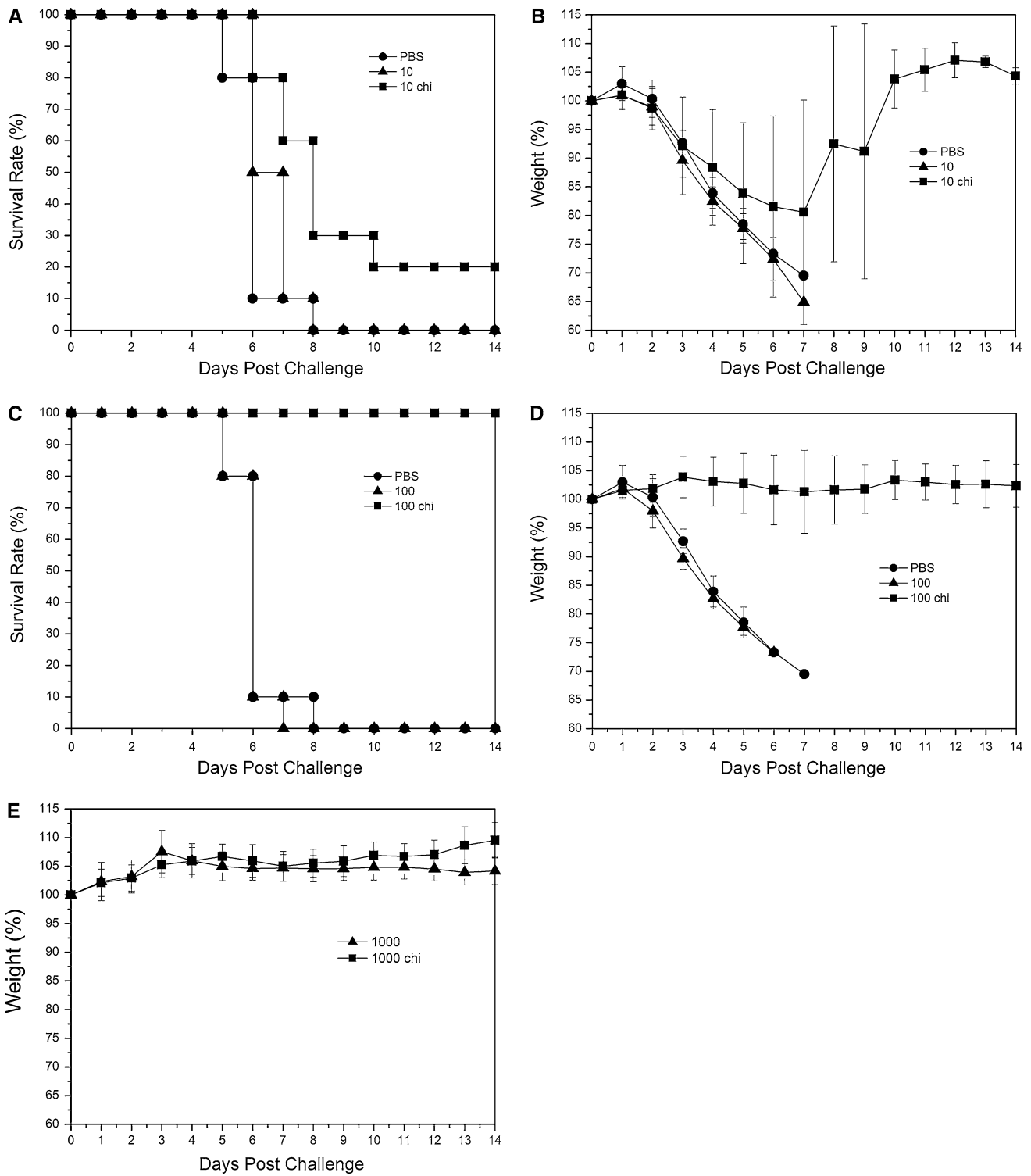
Fig. 2 Protection of mice against lethal challenge with homologous virus. Ten mice in each group were immunized intranasally with various doses of vaccine, either alone or in combination with chitosan. The PBS group served as a negative control. Three weeks after the immunization, mice were challenged with a lethal dose ($100 \times LD_{50}$) of influenza PR8 virus. Survival (**A** and **C**) and weight loss (**B**, **D** and **E**) were monitored for 14 days

Table 4 Antibody responses in mice induced by intranasal administration of live attenuated vaccine with or without chitosan

Immunogen	Dose of LAIV (TCID$_{50}$)	Ab responses (ELISA, 2^n)			
		Serum IgG	Serum IgG2a	Serum IgG1	Nasal wash IgA
LAIV + chitosan	1000	$14.4 \pm 0.5^{a,b}$	$16.0 \pm 1.0^{a,b}$	$12.6 \pm 0.9^{a,b}$	2.8 ± 0.5^{b}
LAIV	1000	11.6 ± 1.1^{b}	13.2 ± 1.3^{b}	7.8 ± 1.5^{b}	2.0 ± 0.0^{b}
LAIV + chitosan	100	$13.2 \pm 1.6^{a,b}$	$15.8 \pm 1.9^{a,b}$	$12.5 \pm 1.3^{a,b}$	$2.5 \pm 0.6^{a,b}$
LAIV	100	8.0 ± 1.0	<3	<3	ND^c
LAIV + chitosan	10	9.0 ± 1.0^{a}	<3	<3	ND^c
LAIV	10	6.0 ± 0.0	<3	<3	ND^c
PBS	–	–	–	–	–

Five mice in each group were immunized with PBS or different doses of live attenuated vaccine (LAIV) with or without chitosan. Three weeks post-vaccination, the serum samples were examined by ELISA for specific IgG, IgG2a and IgG1 Abs, respectively. Results are expressed as means $\pm$ SD of five tested mice in each group

[a] Significant difference compared with the corresponding value for the group inoculated with live attenuated influenza vaccine alone ($p < 0.05$)

[b] Significant difference compared to the mice in the PBS control groups ($p < 0.05$)

[c] ND, not detected

the corresponding non-adjuvanted groups ($p<0.05$) (Table 4). Collectively, these results indicated that chitosan could broadly stimulate the adaptive immune response.

Chitosan enhances antigen-specific T-cell responses

Cellular immune responses to live attenuated influenza vaccine were assessed by measuring IFN-γ secretion in mouse splenocytes. BALB/c mice were immunized once with 100 TCID$_{50}$ and 1000 TCID$_{50}$ of live attenuated influenza vaccine with or without chitosan. Mice immunized with PBS only were used as a blank control. Splenocytes were harvested for IFN-γ ELISPOT assay three weeks after the immunization. The average number of spots in triplicate wells of each sample was used to calculate the number of splenocytes secreting IFN-γ.

The spot data are shown in Fig. 3. Only a few non-specific spots were detected for the control group (≤ 5 spots/10^6 cells), whereas the number of positive non-specific spots (concanavalin-stimulated) was up to 1,500/10^6 cells (data not shown). Compared with the PBS control group, a significant number of PR8-specific IFN-γ-secreting splenocytes were detected in all of the immunized groups, and the number of IFN-γ-secreting splenocytes in the immunized groups increased with increasing immunization dosage. Co-administration of vaccine with chitosan induced substantially more specific IFN-γ-secreting lymphocytes at the same dosage level ($p<0.05$). These results indicate that chitosan could significantly increase the number of antigen-specific T cells. In conclusion, chitosan enhanced both the humoral and cellular immune response to live attenuated influenza vaccine.
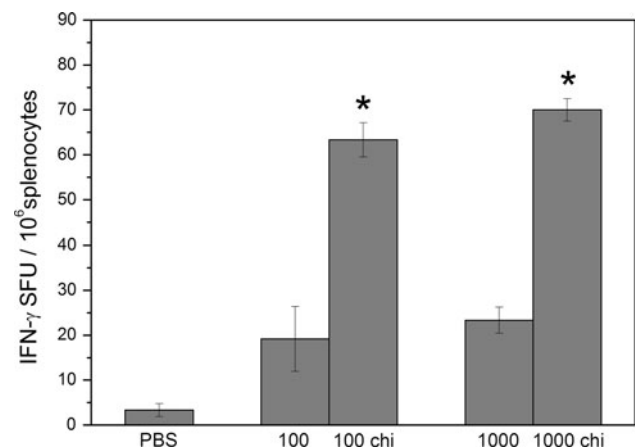
Fig. 3 Detection of IFN-γ secreted from splenocytes by ELISPOT assays. Splenocytes harvested from mice 21 days after vaccination were stimulated with 10 µg/ml inactivated PR8 vaccine for 30 h and scored in ELISPOT assays for IFN-γ-producing cells. The values represent the average of quadruplicate wells containing cells from mice and are expressed as mean $\pm$ SD. The results are expressed as the number of SFC per 10^6 input cells. *Significant difference ($p < 0.05$)

Co-administration of chitosan with live attenuated influenza vaccine confers protection against heterologous virus challenge

To explore if the live attenuated influenza vaccine plus chitosan could provide protection against heterologous influenza viruses, one challenge experiment using the A/Chicken/Jiangsu/7/2002 (H9N2) avian influenza strain was carried out. Based on the above results, 1000 TCID$_{50}$ of live attenuated influenza vaccine with or without adjuvant could elicit an efficient immune response to protect mice against viral infection, whereas 10 TCID$_{50}$ of live attenuated influenza vaccine could not conferred efficient

protection against viral infection, even in combination with chitosan. Thus, we chose 100 $TCID_{50}$ of live attenuated influenza vaccine for the heterologous challenge experiment. Forty-five female BALB/c mice aged 6-8 weeks old were randomly divided into three groups, with 15 mice in each group. Two groups of mice were separately immunized once with 100 $TCID_{50}$ of live attenuated influenza vaccine with or without chitosan. The remaining group, immunized with PBS only, was used as control. Three weeks after the immunization, all of the mice were challenged intranasally with $100 \times LD_{50}$ of the H9N2 avian influenza virus. Lung virus titers, body weight changes and survival rates were measured to evaluate the ability of the vaccine to protect mice against the heterologous influenza virus.

As shown in Table 5 and Fig. 4A, all of the mice in the PBS control group, when challenged with H9N2 virus, died within 7 days. The survival rates of the mice immunized with live attenuated influenza vaccine with and without chitosan against H9N2 virus challenge were 100 % (10/10), and 0 % (0/10), respectively. Three days after the challenge, the residual lung virus titers in the adjuvanted vaccine group was significantly lower than those in the non-adjuvanted vaccine group ($p<0.05$), indicating that chitosan could accelerate the clearance of virus in lung (Table 5). Weight loss was observed for 28 days after challenge, as shown in Fig. 4B. Although mice in the control group and the non-adjuvanted vaccine group lost body weight quickly and died within 7 days, all the mice in the adjuvanted vaccine group regained weight and made a complete recovery within four weeks after challenge. These results indicate that immunization with 100 $TCID_{50}$ of live attenuated influenza vaccine with chitosan adjuvant not only provided protection against homologous virus but also provided protection against heterologous virus.

Discussion

Influenza A viruses cause a highly contagious, acute respiratory disease responsible for human suffering and an economic burden every year. Although current influenza vaccines have had some success at reducing morbidity and mortality, development of novel vaccines that are easy to administer and can generate cross-protective immunity will be needed for wide clinical application. In recent years, a few studies have reported that chitosan can be used as a mucosal adjuvant [14–19]. Although vaccination with inactivated influenza, pertussis, diphtheria, and tetanus vaccines containing chitosan has been demonstrated to enhance the antigen-specific systemic and local antibody responses significantly in animal models [14, 27–29], the adjuvant properties of chitosan associated with live attenuated influenza vaccines have never been reported. Thus, in the present study we investigated chitosan as an adjuvant for live attenuated influenza vaccine in a mouse model.

In this study, the results demonstrated that chitosan used as a mucosal adjuvant of a live attenuated influenza vaccine could significantly increased not only systemic immunity but also mucosal immunity, producing a high level of mucosal IgA. The mortality of mice in adjuvanted vaccine groups was effectively reduced against a high lethal challenge dose of homologous H1N1 virus, indicating that using chitosan can significantly lower the dose of vaccine antigen required to reach the same level of immunity. The protective immunity afforded by the adjuvanted vaccine might correlate with the increased PR8-specific IgG and IgA levels, since protective immunity was more evident when a sufficient level of PR8-specific Abs was induced (Table 4). These results indicate that the Abs played a major role in the immune protection provided by the live attenuated vaccine, whereas cellular immunity assisted in the protection.

Table 5 Protection of mice against lethal heterologous H9N2 virus challenge by intranasal administration of live attenuated vaccine plus chitosan

Immunogen	Dose of LAIV ($TCID_{50}$)	Protection against challenge with various viruses	
		Lung virus titer ($\log_{10}TCID_{50}$/ml)	No. of survivors/no. tested
LAIV + chitosan	100	6.6 ± 0.3[a,b]	10/10[a,b]
LAIV	100	7.4 ± 0.4	0/10
PBS	–	7.6 ± 0.3	0/10

Fifteen mice in each group were immunized with PBS or 100 $TCID_{50}$ of the live attenuated vaccine (LAIV) with or without chitosan. Three weeks post-vaccination, mice were challenged with a lethal dose ($100 \times LD_{50}$) of A/Chicken/Jiangsu/11/2002(H9N2). Bronchoalveolar washes were collected 3 days postinfection for titration of lung virus. The survival rate of mice 28 days postinfection was determined. Results are expressed as mean $\pm$ SD of five tested mice in each group

[a] Significant difference compared with the corresponding value for the group inoculated with live attenuated influenza vaccine alone ($p < 0.05$)

[b] Significant difference compared to the mice in the PBS control groups ($p < 0.05$)
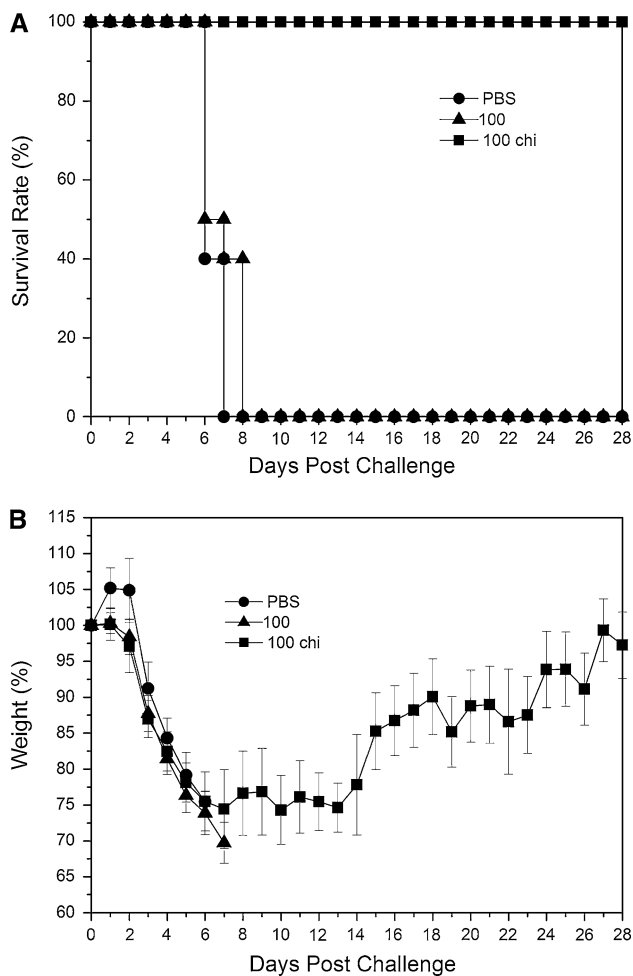
Fig. 4 Protection of mice against lethal challenge with heterologous virus. **A** Ten mice in each group were immunized intranasally with PBS or 100 $TCID_{50}$ of the mutated virus with chitosan. Three weeks post-vaccination, mice were challenged intranasally with $100 \times LD_{50}$ wild H9N2 virus. Survival of the mice is plotted on the graph for each group. **B** The mice in A were weighed daily after challenge. The average weight of the mice as a percent of the starting weight is graphed $\pm$ S.D.

In general, induction of influenza-virus-specific antibodies by immunization closely correlates with protection against influenza virus infection [2], but the subtype of the antibodies mediating protection is less clear. The currently available licensed influenza vaccines include inactivated influenza vaccines and live attenuated influenza vaccines, and inactivated influenza vaccines are composed of two possible forms of antigen preparation: inactivated whole virus vaccines and split vaccines (including subunit vaccines). BALB/c mice typically respond to natural infection with influenza virus and inactivated whole-virus vaccines with a Th1-type immune response [30, 31], which is associated with the production of IgG2a antibodies that can accelerate the clearance of viruses and increase protection

against lethal influenza challenge. However, the major antibody isotype present in the sera of mice vaccinated with split vaccines is IgG1 [32], which is stimulated during Th2-type immune responses and has the ability to neutralize viral particles. In this study, we evaluated IgG1 and IgG2a antibody responses as well as IFN-γ-producing T cells to analyze the contribution of Th2- and Th1-related immune mechanisms. The results revealed that vaccination with live attenuated influenza vaccine, with or without adjuvant, induced mainly IgG2a antibody. On the other hand, our data showed that addition of the chitosan adjuvant to the live attenuated influenza vaccine significantly enhanced both the IgG2a and the IgG1 antibody response when compared to immunization with non-adjuvanted vaccine. A similar result was obtained in a previous study, showing that chitosan-adjuvanted ovalbumin induced significantly increased antigen-specific Th1 and Th2 immune responses in mice [33]. Th1-type immune responses and mixed Th1/Th2 humoral immune responses have been showed to be preferred over Th2-type responses in a mouse influenza virus challenge model [31, 34]. Our results show that when mice were challenged with $100 \times LD_{50}$ of homologous or heterologous influenza virus, the addition of chitosan significantly increased the survival rates. Creation of significantly more IFN-γ-secreting T cells and higher IgG2a antibody titers was able to accelerate the clearance of heterosubtypic virus [35], which could give a reasonable explanation why chitosan-adjuvanted vaccines provided better protection against heterosubtypic virus challenge in our study.

Currently the commercially available *FluMist*®, a cold-adapted live attenuated influenza vaccine, requires immunization with high dosage to elicit an adequate immune response. In order to reduce the amount of vaccine required, addition of an appropriate adjuvant to vaccine formulations may be a possible solution. In this study, we evaluated chitosan as an adjuvant for a live attenuated influenza vaccine in a mouse model. As a result, 100 $TCID_{50}$ of chitosan-adjuvanted vaccine elicited higher IgG antibody titers than that elicited by 1000 $TCID_{50}$ of vaccine alone, suggesting that chitosan could effectively reduce the amount of vaccine required. Since the amount of the commercial *FluMist*® required for administration to humans is usually as high as 10^7 $TCID_{50}$, it is worth further study to determine if chitosan can be used as an adjuvant for live attenuated influenza vaccines in order to reduce the dosage required, limit side effects and save costs.

The use of adjuvant also can significantly increase the protective efficacy of vaccines. Several reports have been published about adjuvant effects of chitosan for influenza vaccines [14–19]. Ghendon et al. showed that chitosan as an adjuvant for H5 inactivated influenza vaccines significantly enhanced antibody titers and protective efficiency

not only against homologous influenza viruses but also against drift variants [17]. Our previous studies showed that immunization with subunit influenza vaccine M1 or M2 proteins plus chitosan adjuvant not only provided complete protection against homologous H9N2 virus but also provided protection against heterologous H1N1 and H5N1 virus to a certain extent [36, 37]. Consistent with previous studies, our data clearly indicated that the addition of chitosan to the live attenuated influenza vaccine significantly increased protective immunity. The mechanism of chitosan in enhancing the humoral and cellular immune response is still not quite clear. Some studies have suggested that chitosan may absorb more antigens across the nasal mucosa by slowing down mucociliary clearance, thus maintaining the contact of antigen with the mucosa for a longer time [38–40]. Other studies have shown that chitosan could activate components of the nonspecific immune system, such as macrophages and natural killer cells, and could induce immune responses to bacteria, fungi and tumors [41–44]. Therefore, chitosan may offer a danger signal and act as an adjuvant. The detailed mechanism of the adjuvant effect of chitosan will require further investigation.

Developing an efficient and safe adjuvant for influenza vaccines is the objective of our study. Cholera toxin (CT) and heat-labile toxin (LT) have already been successfully used as adjuvants for inactivated influenza vaccines [8]. However, co-administration of vaccine with CT or LT would redirect antigen into the CNS and provoke unnecessary inflammation [45, 46]. Therefore, the development of adjuvants must proceed cautiously and fully address these safety concerns. Recently, Ghendon et al. [17] showed that chitosan did not induce IgE antibodies or antibodies against chitosan itself, indicating that chitosan would be a safe adjuvant in a mouse model. Furthermore, chitosan appeared to be non-toxic and well tolerated by human subjects in preclinical trials [18, 19]. More importantly, chitosan was approved by the FDA as a constituent of many food products and pharmaceutical excipients [9–11]. Taken together, these facts supported that chitosan could be a promising adjuvant that is safe for vaccines.

In conclusion, this proof-of-concept study demonstrates that a single intranasal immunization of live attenuated influenza vaccine adjuvanted with chitosan could enhance the protective immunity of both humoral and cellular immune responses, which resulted in effective protection against homologous as well as heterologous influenza virus challenge. Our study demonstrates for the first time that chitosan, a derivative of the natural amino polysaccharide chitin, could be used as a potential adjuvant candidate for a live attenuated influenza vaccine and provides valuable information for further research.

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