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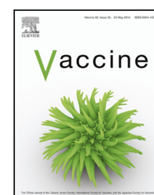

Induction of systemic and mucosal immunity and maintenance of its memory against influenza A virus by nasal vaccination using a new mucosal adjuvant SF-10 derived from pulmonary surfactant in young cynomolgus monkeys

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ABSTRACT

Induction of systemic and mucosal immunity and maintenance of its memory was investigated in 12 young male cynomolgus monkeys after intranasal instillation of flu vaccine using a new mucosal adjuvant SF-10 derived from pulmonary surfactant constituents. Split-product of influenza virus A/California/7/2009(H1N1)pdm hemagglutinin vaccine (HAv) at 15 μg with or without SF-10 and the adjuvant alone were instilled intranasally three times every 2 weeks. SF-10-adjuvanted HAv (SF-10-HAv) elicited significantly higher HAv-specific IgG and hemagglutinin inhibition (HI) titers in serum and HAv-specific secretory IgA and its neutralizing activities in nasal washes compared with HAv antigen and SF-10 alone. Significant cross-neutralizing activities of nasal washes after the third vaccination to several other H1N1 and H3N2 strains were observed. HI titers in serum and neutralizing activities in nasal washes reached peak levels at 6 weeks after initial vaccination, then gradually decreased after 10 weeks and returned to the baseline levels at 36 weeks. A single intranasal revaccination of SF-10-HAv at 36 weeks rapidly and significantly increased both immunity in serum and nasal washes compared with naïve monkeys. Revaccination by one or two doses achieved almost maximal immunity at 2 or 4 weeks after instillation. Statistically significant adverse effects (*e.g.*, body weight loss, elevated body temperature, nasal discharge, change in peripheral blood leukocyte and platelet counts) were not observed for 2 weeks after vaccination of SF-10-HAv, HAv or SF-10 and also during the experimental period. These results in young monkey model suggest the potential of clinical use SF-10 for intranasal flu vaccine.

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1. Introduction

Influenza virus is one of the most common respiratory pathogens that infects approximately 10–20% of the world's population each year, contributing to an excess in morbidity and mortality [1]. Severe influenza complications are most common in children younger than 2 years and elderly as well as individuals with compromised cardiac, pulmonary, or immune systems. The recent global spread of swine-origin H1N1 influenza A virus (IAV) highlighted the need for the development of effective vaccines for the prevention of viral infection and transmission. The currently available influenza vaccines administered intramuscularly or subcutaneously induce a predominantly IgG-mediated protection in the systemic immune compartment and significantly reduce hospitalization and deaths when they match antigenically the circulating viral strains [2,3]. However, this systemic vaccination neither results in adequate induction of antiviral secretory IgA (S-IgA), which provides a wide cross-protection, nor efficient prevention of infection at the airway mucosa, the site of viral entry and propagation [4,5], or cell-mediated responses in the respiratory tract [6].

In a recent report of a test-negative case–control design study based on influenza rapid diagnostic test, influenza vaccine administrated subcutaneously at one or two doses was ineffective against

Abbreviations: APC, antigen presenting cell; CVP, carboxyvinyl polymer; DPPC, 1,2-dipalmitoyl-phosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; GMP, good manufacturing practices; HI, hemagglutination inhibition; IAV, influenza A virus; OD, optical density; PA, palmitate; PFU, plaque-forming unit; PG, phosphatidylglycerol; S-IgA, secretory IgA; SSF, synthetic surfactant; SP, surfactant protein; TTS, 50 mM Tris–HCl (pH 8.0) containing 0.14 M NaCl and 0.05% Tween 20.

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either IAV or influenza B virus in 6–11-month-old infants [7]. Other reports also documented the ineffectiveness of trivalent inactivated influenza vaccine in reducing IAV infection attack rate in 6–24-month-old children [8]. Vaccine efficacy in children, especially in school-age children, is important with regard to achieving indirect protection of the elderly, because schools are the most efficient amplifiers of influenza epidemics in the community [7]. Therefore, the development of effective and safe vaccines for infants and children is desired.

It is well known that the first line of host defense and prevention is mucosal immunity, particularly nasopharyngeal immunity, which constitutes a major component of the immunological humoral and cell-mediated responses in the upper and lower respiratory tracts [4]. For this reason, mucosal vaccines and their adjuvants have been studied for over 40 years [9,10]. However, many such preparations were found to be ineffective or had safety problems [11]. At present, only nasal spray of live attenuated influenza vaccine [12] is available in the USA and Europe. However, it is only approved at present for individuals aged 2–49 years, and is not used in children aged 2 through 4 years with asthma or those with history of wheezing within the past 12 months [12–14]. In this regard, safer and more efficient influenza vaccines administrated into the nasal cavity, instead of influenza vaccines administered intramuscularly or subcutaneously or live attenuated influenza vaccine, for all persons aged >6 months, are strongly desired.

A key issue with regard to nasal vaccination is the poor efficiency of antigen uptake across the nasal mucosa due to rapid mucociliary clearance, the primary innate defense mechanism, which protects against invasion of bacteria and various antigens in air environment. To overcome this barrier and achieve effective immunity, immunostimulatory adjuvants that evoke activation of antigen presenting cells (APCs) and mucosal vaccine delivery vehicles have been introduced [15]. Many of the immunostimulatory adjuvants, however, do not maintain adequate APC stimulation and induce unexpected local inflammation and/or uncontrolled over-stimulation of APCs.

We have recently found that the bovine counterpart of human pulmonary surfactant, Surfacten®, has both safe and efficacious mucosal adjuvanticities by promoting IAV antigen delivery to APCs in mice and mini-pigs [16–18]. Surfacten® is free of pulmonary surfactant protein (SP)-A and -D and widely used in the treatment of respiratory distress syndrome in premature babies for more than 27 years worldwide without significant adverse effects, even at a relatively high clinical dosage of 120 mg/kg body weight [19]. The lung surfactant is effectively uptaken into alveolar cells and APCs of macrophages and dendritic cells. It has safety characteristics of rapid degradation *in vivo* with a short half-life of 5–11 h in the rat and rabbit lungs [20] and thus does not over-stimulate APCs, and has a short half clearance time of 43–116 min for Surfacten®-influenza virus hemagglutinin (HAv) complex in the mouse nasal cavity [17]. We have also recently developed a synthetic mucosal adjuvant SF-10, consisting of the three major human pulmonary surfactant lipids, a surfactant protein (SP)-C related peptide K6L16, and a carboxyvinyl polymer (CVP) as viscosity improver, and confirmed that induces significantly higher levels of anti-HAv-specific nasal wash S-IgA and serum IgG, compared to those induced by poly(I:C), a reported potent immunostimulatory adjuvant [21].

To evaluate the effects of the synthetic SF-10 adjuvant on systemic and local immunity and the efficacy of revaccination for maintenance of its memory, SF-10-HAv was intranasally administrated to young cynomolgus monkeys. The monkey model [22,23] has the advantages of a primate model that allows continuous sampling of sera and nasal washes over a long period of time and for revaccination, which allows monitoring of both local and systemic immune responses. The results of the present study demonstrated the efficacy of nasal instillation of SF-10-HAv in systemic antigen-specific IgG induction with hemagglutination inhibition (HI) activities in sera, local S-IgA induction with neutralizing activities in nasal washes and lack of statistically significant side effects. The results also showed that effectiveness of revaccination using SF-10-HAv.

2. Materials and methods

2.1. Animals and virus

Twelve young male cynomolgus monkeys (*Macaca fascicularis*) aged 30–32 months years (2.4–2.8 kg body weight), clinically healthy and free of infectious disease, were purchased from Vanny Bio-research Co. (Kandal, Cambodia) and housed separately in pens under controlled conditions of humidity (40 ± 5%), temperature (25 ± 1 °C), and light (12 h light/12 h dark cycle, light on at 8:00 a.m.) in the animal facility of Eve Bioscience Co. (Wakayama, Japan). Each monkey was checked and confirmed to be influenza-seronegative in sera and nasal washes, as determined by enzyme-linked immunosorbent assay (ELISA) (see below). The monkeys were fed 100 g of pellets (CLEA Japan, Inc., Tokyo, Japan) once a day, along with half fresh apple twice weekly and allowed access to water *ad libitum*. Animals were anesthetized by intramuscular injection of 10 mg/kg ketamine and 80 μg/kg xylazine for sample collection, rectal temperature, body weight measurements and veterinary observations to minimize suffering. At day −2 before vaccination, all monkeys were again tested for anti-HAv antibodies in nasal washes and serum. All animals were treated according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1996) and the study was approved by Animal Experimentation Ethics Committees of the Eve Bioscience Co. and the Tokushima University. IAV/California/7/2009(H1N1)pdm and IAV/Uruguay/716/2007(H3N2) were supplied by T. Odagiri (National Institute of Infectious Diseases, Tokyo, Japan) and IAV/Puerto Rich/8/1934(H1N1), IAV/WSN/1933(H1N1) and IAV/Aichi/2/1968(H3N2) were by M. Ohuchi (Kawasaki Medical School, Kurashiki, Japan).

2.2. Reagents

1,2-Dipalmitoyl-phosphatidylcholine (DPPC), phosphatidylglycerol (PG) and palmitate (PA) for the preparation of synthetic surfactant (SSF) were obtained from Nippon Fine Chemical (Osaka, Japan). Good manufacturing practices (GMP) grade synthetic SP-C related peptide K6L16 (KKKKKKLLLLLLLLLLLLLLLL) (96.8% purity) was from GenScript Japan (Tokyo). Carboxy vinyl polymer (CVP) was purchased from Sigma–Aldrich (St. Louis, MO).

2.3. Preparation of antigen, SSF and SF-10-HAv

IAV/California/7/2009(H1N1)pdm ether split antigen (15 μg HA, corresponding to 30.8 μg of viral protein) (Denka Seiken) was used as a HAv antigen. SSF was prepared by mixing three major lipids of Surfacten®, DPPC, PG and PA, and SP-C related synthetic peptide K6L16 as described previously [21]. Briefly, DPPC, PG and PA in chloroform/methanol 2:1 (v/v) and K6L16 peptide in methanol were mixed at a weight ratio of 75:25:10:2. Mixture was evaporated under a nitrogen stream, suspended with 10% ethanol and then mixed well with the Voltex Mixer (MaxiMix™ I, Therno Fisher Scientific Inc.). The samples were adjusted to pH 7.0 by adding a small amount of sodium hydroxide and periodically incubated 3 times, each for 3 min, at 42 °C. The SSF sample concentration was adjusted at 4 mg phospholipids/mL with 10% ethanol, and then lyophilized for storage at −20 °C.

Before vaccination, lyophilized SSF (weight expressed as that of phospholipids) was suspended in distilled water, mixed with HAv

(weight expressed as protein) in saline at a ratio of 10:1 (wt/wt) at 42 °C for 10 min with gentle mixing three times for 10 s and the SSF-HAv complex formation was conducted by lyophilization. Lyophilized SSF-HAv was dissolved in saline by gentle mixing and CVP in saline (pH 7.0–7.5) was added at a final concentration of 0.5% for increasing viscosity. The mixture of SSF adjuvant and CVP thickening additive was renamed as SF-10. The final solution of SSF + HAv + CVP, SF-10-HAv was used for nasal vaccination.

2.4. Vaccination procedure

For intranasal vaccination, anesthetized animals lying in the lateral position were immunized by intranasal instillation of 50 μL of the prepared SF-10-HAv into each nostril, and the procedure was repeated on two more occasions after 2-week interval. Animals received 15 μg HAv antigen with or without SF-10 each time. All animals showed significant induction of systemic and mucosal immunity after SF-10-HAv vaccination followed by return of their immunity to the baseline level during the experimental period for 36 weeks. At that stage, the animals were revaccinated with one or two doses of SF-10-HAv at 36 and 38 weeks after the first vaccination.

2.5. Sample collection

Serum and nasal aspirations for the assessment of antibody responses were collected from each animal at day −3 of the first vaccination, at day −1 of the second, third, fourth and fifth vaccinations and at the time points indicated in the figure during the experimental period for 40 weeks. For collection of nasal washes in anesthetized animals, 0.25 mL of sterile saline was instilled into each nostril and then head was moved gently. Nasal fluids were collected by aspiration from both nostrils over a period of 30 s on each side through a silicon tube, and trapped in a centrifuging tube connected with an evacuator. Finally, the inside of the silicon tube was rinsed with 1.0 mL of saline for collection. The nasal aspirates were immediately placed on ice, centrifuged at 500 × *g* for 5 min and the supernatant was stored at −30 °C until use.

Blood was collected from the femoral vein and serum was prepared under the same sampling schedule of nasal aspiration. To monitor the adverse effects of vaccination, blood samples were also collected for blood count analyses at day −3 before vaccination and at days 1, 15 and 29 after vaccination. Blood count analyses were conducted with autoanalyzer by LSI Medience Co. (Tokyo).

2.6. Enzyme-linked immunosorbent assay

Anti-HAv-specific antibodies in sera and nasal washes were evaluated by ELISA as described previously [16]. Briefly, 96-well plates (Nunc, Naperville, IL) were coated with HAv and bovine serum albumin (BSA) (0.1 μg/well each) in PBS overnight at 4 °C, then blocked with 2% BSA in 50 mM Tris–HCl (pH 8.0) containing 0.14 M NaCl and 0.05% Tween 20 (TTS) for 1 h at room temperature. The serum and nasal washes diluted with TTS containing 1% BSA were added to each well and incubated for 3 h at room temperature. The plate was washed six times with TTS, incubated with goat anti-monkey IgA (KPL, Gaithersburg, MD) or anti-monkey IgG (Nordic Immunological Laboratories, Eindhoven, The Netherlands) antibodies conjugated with horseradish peroxidase (Bethyl Laboratories, Montgomery, TX) for 2 h at room temperature. Color was developed by the addition of TMB substrate (Bethyl Laboratories), according to the instructions provided by the manufacturer. The chromogen produced was measured at 450 nm using a SPECTRA max PLUS384 autoreader (Molecular Devices, Tokyo). Antibody titers are defined as the reciprocal of the highest dilution of sample for which the optical density (OD) was at least twice the OD of the negative control samples before vaccination.

2.7. Hemagglutination inhibition assay

HI activity was measured as described previously [18]. Serum samples were treated overnight with RDE (II) "SEIKEN" (Denka Seiken Co.) at 37 °C to eliminate non-specific HI factors, and the assay was conducted according to the protocol for HI testing established by the World Health Organization. Briefly, 4 HA units of the vaccine antigen and 1% of washed chicken red blood cells were added to each well of 96-well plates, in which the test serum had been diluted, and then incubated for 30 min at room temperature. HI endpoint titers were determined as the reciprocal of the highest serum dilution that produced complete inhibition of hemagglutinating activity.

2.8. Neutralizing activities of nasal aspirates

For measurement of neutralizing activities of nasal aspirates, 200 plaque-forming units (PFU) of IAV/California/7/2009(H1N1)pdm were incubated with 100 μL of serially diluted nasal washes on ice for 2 h and then the virus titers in the mixtures were measured by the plaque assay on Madin–Darby canine kidney cells, as described previously [18]. Neutralizing activities were evaluated as titers that were determined as the reciprocal of the highest sample dilution that reduced the PFU level to half of that for the sample not treated with nasal aspirate. For cross-neutralization assay, nasal aspirates were also incubated with various IAV subtypes of IAV/Puerto Rich/8/1934(H1N1), IAV/WSN/1933(H1N1), IAV/Aichi/2/1968(H3N2) and IAV/Uruguay/716/2007(H3N2) for 2 h and neutralizing activities were measured by plaque assay.

2.9. Statistical analysis

All data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the unpaired Mann–Whitney's *U* test. A *P* value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. Kinetics of HAv-specific antibody responses in sera and nasal washes

To evaluate the mucosal adjuvanticity of SF-10 in young cynomolgus monkeys, we analyzed the time courses of induction of anti-HAv S-IgA in nasal washes and IgG in sera after intranasal instillation of SF-10-HAv (Tables 1 and 2). A significant induction of anti-HAv S-IgA titer was observed at 4 weeks after the second vaccination of SF-10-HAv and further exponential increase was detected at 6 weeks after the third vaccination. No significant induction of anti-HAv S-IgA titer in nasal washes was observed after intranasal instillation of HAv or SF-10 alone (Table 1).

Table 2 shows the serial changes in serum anti-HAv IgG titers. Significant and exponential induction of anti-HAv IgG titer was observed at 4 weeks after the second vaccination of SF-10-HAv and the induced titers were nearly maximum. A delayed increase in the titers was noted in some animals, reaching peak levels at 6 weeks after the third vaccination. Animals with near-maximum titers after the second vaccination showed no further increase in the titers after the third vaccination and the induction profile exhibited a plateau during the period of 4–6 weeks post-vaccination. No significant induction of anti-HAv IgG titers in serum was observed after vaccination with HAv or SF-10 alone.

Table 1
Effects of SF-10 on HAv-specific S-IgA titers in nasal washes.

Group	Animal number	HAv-specific S-IgA titers in nasal washes (weeks after first vaccination)			
		0	2	4	6
SF-10-HAv	1	1	1	16	64
	2	1	1	8	128
	3	1	1	8	32
	4	1	2	8	16
	Mean ± SD titer	1.0 ± 0	1.3 ± 1.0	10.0 ± 4.0*	60.0 ± 50.0*
SF-10	5	1	1	1	1
	6	1	1	1	1
	7	1	1	1	1
	8	1	1	2	1
	Mean ± SD titer	1.0 ± 0	1.0 ± 0	1.3 ± 1.0	1.0 ± 0
HAv	9	1	1	1	1
	10	1	2	1	1
	11	1	2	1	1
	12	1	1	1	1
	Mean ± SD titer	1.0 ± 0	1.5 ± 1.0	1.0 ± 0	1.0 ± 0

* $P < 0.05$, compared with HAv.

3.2. Induction of HI titers in serum and neutralizing activities in nasal washes

We analyzed the serial changes in serum HI titers (Table 3) and neutralizing activities in nasal washes (Table 4). The mean HI titers in animals vaccinated intranasally with SF-10-HAv at 4 weeks after the second vaccination were increased to ≥40, a value reported to be associated with protection from influenza illness in up to 50% of subjects in human challenge studies [24]. The HI titers in some animals were further increased to the double at 6 weeks after the third vaccination. In contrast, HI titers in the young animals vaccinated intranasally with either HAv or SF-10 alone were ≤20 during the experimental period for up to 6 weeks.

Mucosal S-IgA is primarily involved in protection of mucosal surfaces. Neutralizing titers against pathogen infection in nasal washes from animals vaccinated intranasally with SF-10-HAv started to increase at 4 weeks after the second vaccination and further increased at 6 weeks after the third vaccination (Table 4). Similar to the changes in HI titers, no neutralizing activities were detected in nasal washes in animals vaccinated intranasally with either HAv or SF-10 alone during the experimental period of 6 weeks.

3.3. Complications

The rectal temperatures of all animals in the three vaccination groups (SF-10-HAv, HAv and SF-10) measured every day at 10:00 a.m. from −3 to 6 days of the vaccination were 39.0 ± 0.5 °C within normothermic temperature range of cynomolgus monkeys and no statistically significant differences were observed among these groups. Similar findings were observed after two revaccination periods. Food consumption (estimated) was recorded once daily and no leftovers was detected in all animals during the experimental period. No significant change in body weight (Fig. 1) were observed in the three vaccination groups.

During the two weeks after vaccination, clinical examinations of nose skin reactions, such as erythema and edema, nasal discharge and sneezing were tested and no visible lesions were recorded in the three vaccination groups.

Blood hematology analyses of the number of leucocytes, platelets, neutrophils, eosinophils, basophils and lymphocytes at day −3 before vaccination and at days 1, 15 and 29 after each vaccination in all animals vaccinated with either SF-10-HAv, HAv or SF-10 were shown in Fig. 2. No statistically significant changes were observed in these parameters tested, although the levels of

Table 2
Effects of SF-10 on HAv-specific IgG titers in sera.

Group	Animal number	HAv-specific IgG titers in sera (weeks after first vaccination)			
		0	2	4	6
SF-10-HAv	1	1	1	6400	6400
	2	1	100	6400	6400
	3	1	100	3200	6400
	4	1	100	800	3200
	Mean ± SD titer	1.0 ± 0	75.3 ± 50.0	4200 ± 2723*	5600 ± 1600*
SF-10	5	1	1	1	1
	6	1	100	1	1
	7	1	1	1	1
	8	1	1	1	1
	Mean ± SD titer	1 ± 0	26.8 ± 50.0	1.0 ± 0	1.0 ± 0
HAv	9	1	1	1	1
	10	1	1	1	1
	11	1	1	1	1
	12	1	1	1	1
	Mean ± SD titer	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0

* $P < 0.05$, compared with HAv.

Table 3
Induction of HI titers after intranasal vaccination.

Group	Animal number	HI titers (weeks after first vaccination)			
		0	2	4	6
SF-10-HAv	1	1	10	80	80
	2	10	20	80	160
	3	1	20	80	160
	4	10	10	40	80
	Mean ± SD titer	5.5 ± 5.0	15.0 ± 6.0	70.0 ± 20.0*	120 ± 46*
SF-10	5	10	20	10	10
	6	10	10	10	10
	7	1	10	1	1
	8	1	1	1	1
	Mean ± SD titer	5.5 ± 5.0	10.3 ± 8.0	5.5 ± 5.0	5.5 ± 5.0
HAv	9	10	20	10	10
	10	10	10	10	10
	11	1	10	1	1
	12	10	20	10	10
	Mean ± SD titer	7.8 ± 5.0	15.0 ± 6.0	7.8 ± 5.0	7.8 ± 5.0

* $P < 0.05$, compared with HAv.

basophils at day 1 post vaccination in all three groups tended to increase transiently (Fig. 2E).

3.4. *Effect of revaccination of SF-10-HAv*

HAv-specific S-IgA titers and neutralizing activities, and HAv-specific IgG titers and HI titers, reached peak levels after three doses vaccination. The peak of immunity in nasal washes observed at 6 weeks after vaccination gradually returned to the initial levels within 36 weeks (Fig. 3A and B). The immunity in serum at 6 weeks also returned until 36 weeks, but more slowly than that in nasal washes, to slightly over or almost the level induced by one-dose vaccination in naïve animals (Fig. 3C and D).

To evaluate the long-term effect of intranasal vaccination of SF-10-HAv on immune memory, we studied the effects of revaccination using nasal washes and sera. In these experiments, monkeys vaccinated previously with SF-10-HAv were revaccinated at 36 and 38 weeks after the return of local and systemic immunity to almost the baseline levels. After one and two doses of revaccination with SF-10-HAv at 36 weeks and 38 weeks, anti-HAv S-IgA titers in nasal washes and IgG titers in sera were more rapidly induced along with the induction of neutralizing and HI titers, respectively, than naïve animals. The induced immunity after two doses revaccination was higher than that induced by the initial three doses vaccinations in naïve animals. The efficacy of induction by one dose revaccination was almost equivalent to that of the initial two doses vaccination in naïve animals (Fig. 3).

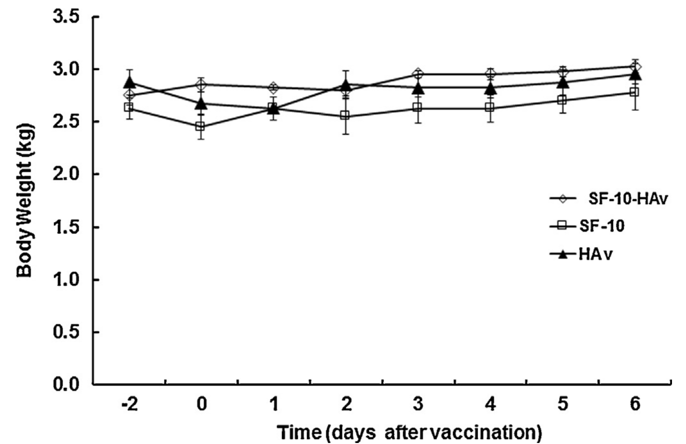

Fig. 1. Changes in body weight after intranasal vaccination. Monkeys received intranasal vaccination of 15 μg of HAv and/or SF-10 adjuvant (308 μg phospholipids). Data are expressed as mean ± SD.

Table 4
Induction of neutralizing activities in nasal washes after intranasal vaccination.

Group	Animal number	Neutralizing activities (weeks after first vaccination)			
		0	2	4	6
SF-10-HAv	1	1	1	16	32
	2	1	1	16	64
	3	1	1	4	32
	4	1	1	4	64
	Mean ± SD titer	1.0 ± 0	1.0 ± 0	10.0 ± 7.0	48.0 ± 18.0*
SF-10	5	1	1	1	1
	6	1	1	1	1
	7	1	1	1	1
	8	1	1	1	1
	Mean ± SD titer	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.0 ± 0
HAv	9	1	1	1	1
	10	1	2	1	1
	11	1	2	1	1
	12	1	1	1	1
	Mean ± SD titer	1.0 ± 0	1.5 ± 1.0	1.0 ± 0	1.0 ± 0

* $P < 0.05$, compared with HAv.

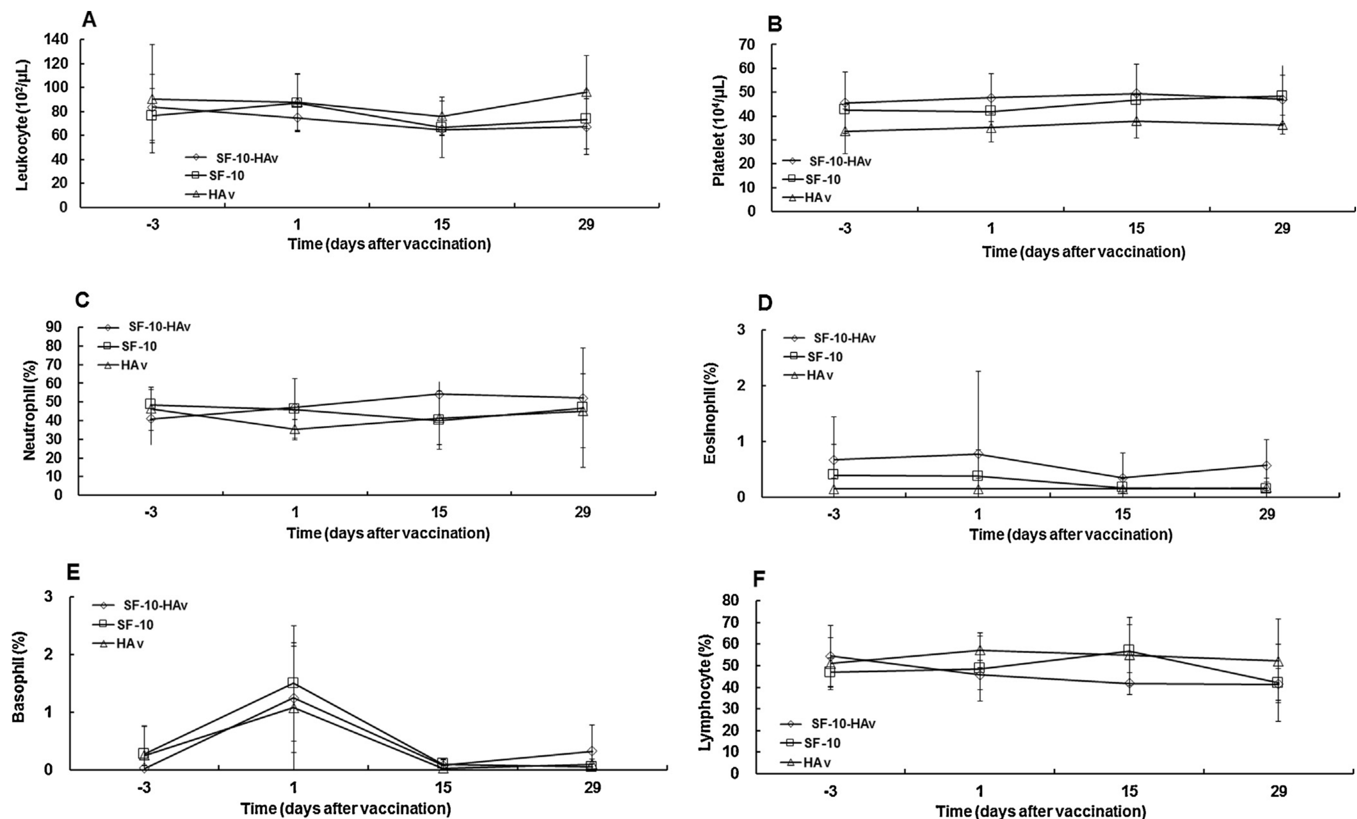

Fig. 2. Changes in leukocyte (A), platelet (B), neutrophil (C), eosinophil (D), basophil (E), and lymphocyte (F) counts in peripheral blood samples obtained after intranasal vaccination. Monkeys received intranasal vaccination of 15 μg of HAv and/or SF-10 adjuvant (308 μg phospholipids). Blood samples were collected from monkeys at day −3 of the first vaccination and at day 1 after each vaccination of the first, second and third. Data are mean ± SD.

3.5. Cross-neutralization of antibodies in nasal washes

Mucosal S-IgA is primarily involved in cross-protection against variant virus infection. To investigate cross-reactivity of induced antibodies in nasal washes after three doses vaccination, neutralizing activities against the original antigen virus IAV/California/7/2009(H1N1)pdm and various other subtypes, such as IAV/Puerto Rich/8/1934(H1N1), IAV/WSN/1933(H1N1), IAV/Aichi/2/1968(H3N2) and IAV/Uruguay/716/2007(H3N2), were measured (Table 5). Although the highest titer was detected to the original antigen virus by SF-10-HAv vaccination, significant cross-neutralizing activities were detected to IAV/WSN/1933(H1N1) with 37.5% and IAV/Aichi/2/1968(H3N2) with 11% of the original vaccine neutralizing activity. However nasal washes showed only less than 10% of the original vaccine neutralizing activity to IAV/Puerto Rich/8/1934(H1N1) and IAV/Uruguay/716/2007(H3N2) strains. No neutralization activities for all subtypes tested were observed in nasal washes from animals vaccinated with either HAv or SF-10 alone.

4. Discussion

Several potential mucosal adjuvants have been evaluated in the past, but no safe and/or efficacious mucosal adjuvant is available commercially at present. In many cases, improvement in antibody production by mucosal adjuvants is associated with risks of enhanced adverse reactions due to uncontrolled- and/or over-stimulation of APCs. To balance efficacy with safety, we used human pulmonary surfactant with a short half-life as constituents for mucosal adjuvant which is rapidly degraded *in vivo* and switching off APC stimulation. In addition, we improved the efficacy of synthetic mucosal adjuvant mimicking pulmonary surfactant by adding CVP to increase viscosity and used it as SF-10 adjuvant [21]. CVP at 0.5% in SF-10 increases the retention time of SSF-HAv complex in nasal cavity against mucociliary clearance and significantly increases the induction of anti-HAv S-IgA in nasal washes and IgG in serum [21].

We reported previously the results of intranasal instillation of HAv combined with the commercial product Surfacten® in 6–9-week-old minipigs just after the weanling period with immature immune system (as an animal model of the very young, the most high risk age, *i.e.*, younger than 2 years old) [18]. Intranasal instillation of Surfacten®-HAv elicited significantly higher serum HI titers than the antigen alone and also wide and significant cross-neutralizing activities against various IAV subtypes of H3N2 and H1N1 in nasal washes.

In the present study, we evaluated systemic and local immunity and the efficacy of revaccination of SF-10-adjuvant IAV/California/7/2009(H1N1)pdm HAv in young male cynomolgus macaques aged 30–32 months. Vaccination of school-age children is important to prevent the spread of the infectious diseases, because flu can spread rapidly in this age group to other students at the same school and family members at home. Induced immunity by SF-10 adjuvant was evaluated by the induction of HAv-specific IgG and HI activities in sera and HAv-specific S-IgA and its neutralizing activity in nasal washes. Intranasal vaccination with three doses, but not with one or two doses, of SF-10-HAv in monkeys induced maximal immunity of HAv-specific S-IgA in nasal washes and IgG in sera, probably because of the use of naïve monkeys. That three intranasal vaccinations were required to induce maximal immunity in naïve animals is consistent with similar results in specific pathogen-free mice reported previously [21]. However,

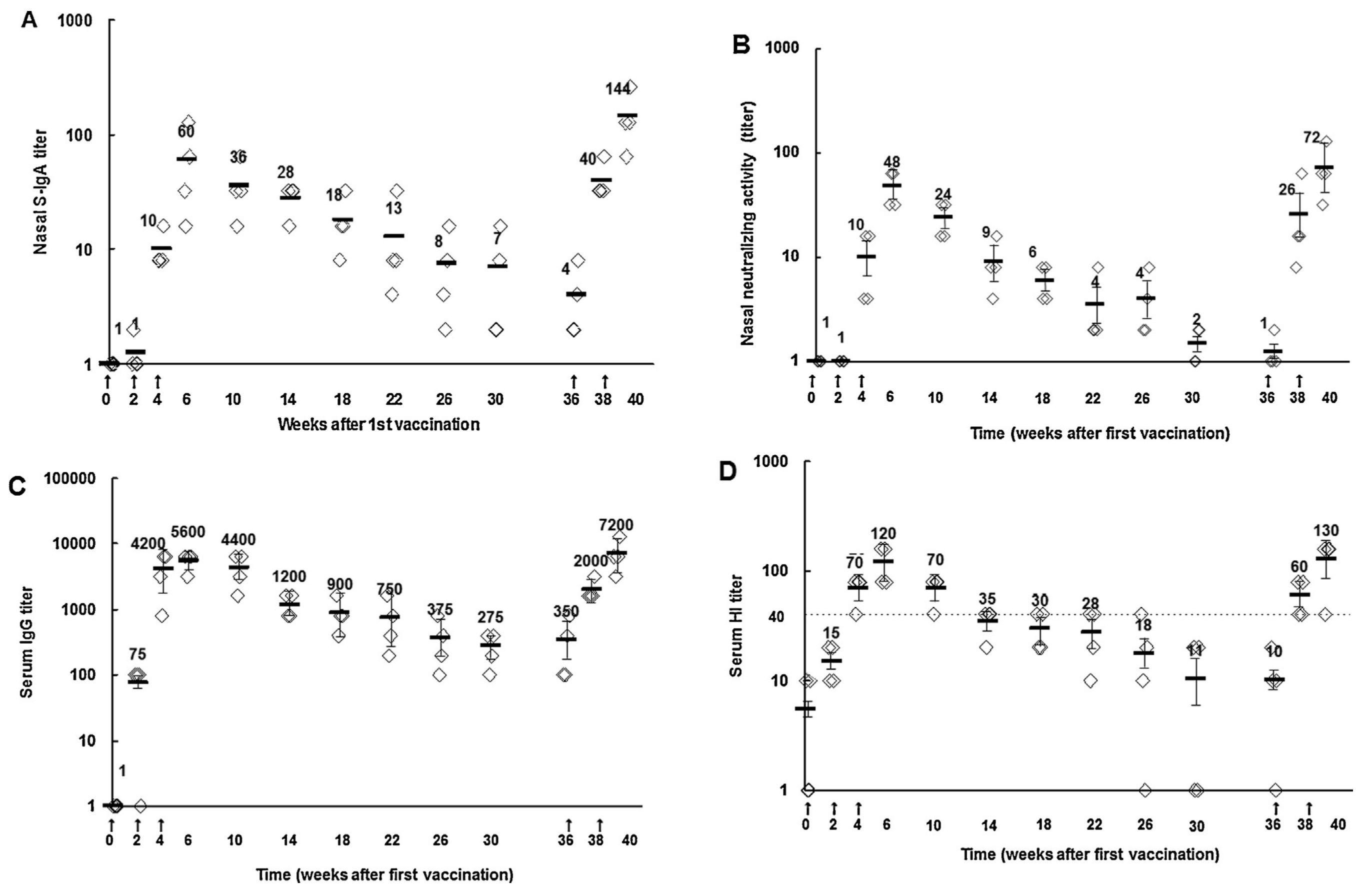

Fig. 3. Effects of revaccination on HAv-specific S-IgA titers (A), neutralization activity (B) in nasal washes, and HAv-specific IgG titers (C) and HI titers (D) in sera of monkeys vaccinated with SF-10-HAv. Monkeys were immunized by intranasal instillation with SF-10-HAv three times every 2-week interval, and then received additional fourth and fifth vaccinations as revaccination at 36 and 38 weeks after initial vaccination. Serum and nasal aspirations were collected from the monkeys at day −2 of the first vaccination, at day −1 of the second, third, fourth, and fifth vaccination and at the time points indicated in the figure during the experimental period. Anti-influenza HA-specific antibody titers in sera and nasal washes were evaluated by ELISA. Arrows in horizontal axis represent vaccinations. Data are mean titer ± SD of 4 animals per group.

revaccination with SF-10-HAv after the initial immunization for 36 weeks induced very rapid and potent production of their immunity in nasal washes and sera than in naïve monkeys. The induced levels were nearly equivalent to the levels of three doses vaccination in naïve animals. These results indicate that nasal vaccination using SF-10-HAv maintains immunological memory of HAv for at least 9 months in young monkeys. In addition, these results suggest that single-dose or two-dose (probably lesser possibility) vaccination with SF-10-HAv in children with immunological memory through previous infection or vaccination may be sufficient to achieve protective immunity, whereas naïve children require multiple doses vaccination. Furthermore, it is noticeable that significant

Table 5
Cross-neutralization activity of nasal washes against various IAV subtypes.

Group	Animal number	Neutralizing activities				
		A/California (H1N1)	A/PR8 (H1N1)	A/WSN (H1N1)	A/Aichi (H3N2)	A/Uruguay (H3N2)
SF-10-HAv	1	32	8	16	8	4
	2	64	8	32	8	4
	3	32	1	16	4	1
	4	64	1	8	1	1
	Mean ± SD titer	48.0 ± 18.0*	4.5 ± 4.0	18.0 ± 10.1*	5.3 ± 3.4*	2.5 ± 1.7
SF-10	5	1	1	1	1	1
	6	1	1	1	1	1
	7	1	1	1	2	2
	8	1	1	1	1	1
	Mean ± SD titer	1.0 ± 0	1.0 ± 0	1.0 ± 0	1.3 ± 0.5	1.3 ± 0.5
HAv	9	1	1	1	1	2
	10	1	1	1	1	1
	11	1	1	2	1	1
	12	1	1	1	1	1
	Mean ± SD titer	1.0 ± 0	1.0 ± 0	1.3 ± 0.5	1.0 ± 0	1.3 ± 0.5

* $P < 0.05$, compared with HAv.
A/California(H1N1): IAV/California/7/2009(H1N1)pdm; A/PR8(H1N1): IAV/Puerto Rich/8/1934(H1N1); A/WSN(H1N1): IAV/WSN/1933(H1N1); A/Aichi(H3N2): IAV/Aichi/2/1968(H3N2); A/Uruguay(H3N2): IAV/Uruguay/716/2007(H3N2).

cross-neutralizing activities against several IAV subtypes of H1N1 and H3N2 were observed in nasal washes of animals treated with SF-10-HAv.

We evaluated previously the safety of Surfacten® in minipigs [18], which is the original source of SF-10. Intranasal instillation of Surfacten®-HAv induced systemic and mucosal immunity in minipigs without any significant induction of inflammatory cytokines or migration of inflammatory cells at the site of immunization or in peripheral blood after vaccination [18]. In the present study, we also evaluated the safety of SF-10 in young male cynomolgus macaques. No significant adverse effects, such as change in body weight, body temperature, nasal discharge, failure of eating were observed during the experimental period. In addition, no statistically significant changes in the levels of leukocyte or platelet count, or changes in the percentages of neutrophils, eosinophils, basophils or lymphocytes in peripheral blood, were also observed during the 29-day period after vaccination of SF-10-HAv, HAv, and SF-10, although only basophils tended to increase transiently at day 1 post vaccination probably due to immune response to invasion by foreign substances (Fig. 2E).

In summary, mucosal adjuvant SF-10, a synthetic adjuvant derived from human pulmonary surfactant, showed safe mucosal adjuvanticity and elicited adequate serum and mucosal antibody responses and provided significant local and systemic immunity in young cynomolgus monkeys. Revaccination of SF-10-HAv after a decrease in antibody titer in sera and nasal washes to baseline levels, induced their immunity rapidly and more efficiently compared with naïve monkeys. The results suggest the potential clinical usefulness of SF-10 adjuvant for intranasal vaccination of HAv.

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Conflicts of interest: None.

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