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Potency of an inactivated influenza vaccine prepared from a non-pathogenic H5N1 virus against a challenge with antigenically drifted highly pathogenic avian influenza viruses in chickens

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ABSTRACT

Antigenic variants of H5N1 highly pathogenic avian influenza virus (HPAIV) have selected and are prevailing in poultry populations in Asia. In the present study, the potency of inactivated influenza vaccine prepared from a non-pathogenic H5N1 avian influenza virus, A/duck/Hokkaido/Vac-3/2007 (H5N1), was assessed by challenging with H5N1 HPAIV variants, A/muscovy duck/Vietnam/OIE-559/2011 (H5N1), A/whooper swan/Hokkaido/4/2011 (H5N1), and A/peregrine falcon/Hong Kong/810/2009 (H5N1) belonging to clades 1, 2.3.2.1, and 2.3.4, respectively. All chickens immunized with the Vac-3 vaccine survived without showing any clinical signs after intranasal challenge either with A/whooper swan/Hokkaido/4/2011 (H5N1) or A/muscovy duck/Vietnam/OIE-559/2011 (H5N1). After challenge with A/peregrine falcon/Hong Kong/810/2009 (H5N1), 10 out of 12 vaccinated chickens survived and the other 2 died on 4 or 7 post-challenge days. The Vac-3 vaccine of 2.4-fold antigen concentration conferred complete protective immunity in chickens against challenge with A/peregrine falcon/Hong Kong/810/2009 (H5N1).

1. Introduction

Avian influenza caused by H5N1 highly pathogenic avian influenza virus (HPAIV) has spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first outbreak occurred at a goose farm in Guangdong province in China (Smith et al., 2006; Xu et al., 1999). In recent intensive surveillance studies in Asia, especially in China, genetically different viruses of clades 2.3.2, 2.3.4, and 7 were characterized as dominant isolates from poultry and wild birds (Kou et al., 2009; Smith et al., 2009; Jiang et al., 2010). In the updated grouping of H5 HPAIVs, it was reported that the clades of H5N1 viruses were divided into one or more newly defined second-, third-, and/or fourth-order clades, e.g. recent H5N1 isolates that had been categorized into clade 2.3.2 were defined as

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clade 2.3.2.1 (WHO/OIE/FAO H5N1 Evolution Working Group, 2012). In Japan, H5N1 HPAIVs belonging to clade 2.3.2.1 were isolated from dead whooper swans in 2008 (Uchida et al., 2008; Okamatsu et al., 2010), fecal samples of ducks that flew from Siberia in October 2010 (Kajihara et al., 2011), and from wild birds and domestic poultry in 2011 (Sakoda et al., 2012). Antigenicity of H5N1 HPAIVs belonging to clades 2.3.2.1 and 2.3.4 was distinct from that of other HPAIVs and non-pathogenic avian influenza viruses (Okamatsu et al., 2010; Smith et al., 2009), suggesting that antigenic variants of H5N1 HPAIV have been selected during circulation in poultry populations.

A reassortant influenza virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04), was generated using two non-pathogenic avian influenza viruses, A/duck/Mongolia/54/2001 (H5N3) and A/duck/Mongolia/47/2001 (H7N1). Both viruses were isolated from fecal samples of migratory ducks (Soda et al., 2008b). Vac-1 vaccine prepared from Dk/Vac-1/04 conferred immunity to suppress the manifestation of clinical signs and the amount of virus shed in chickens after challenge with H5N1 HPAIVs belonging to clades 2.2 and 2.5 (Isoda et al., 2008). Vac-1 vaccine induced a high level of HI antibody response in chickens, lasting as long as 138 weeks after vaccination (Sasaki et al., 2009b). Vac-1 vaccine confers protective immunity against antigenically drifted H5N1 HPAIV, A/whooper swan/Hokkaido/1/2008 (H5N1) (Ws/Hok/08), belonging to clade 2.3.2.1 in chickens (Okamatsu et al., 2010).

In the present study, we prepared a vaccine from A/duck/Hokkaido/Vac-3/2007 (H5N1) (Dk/Vac-3/07), which is antigenically closely related with Dk/Vac-1/04, and growth potential in embryonated chicken eggs was higher than that of Dk/Vac-1/04 (Soda et al., 2008b). The potency of the Vac-3 vaccine was assessed by challenge with recently prevailing antigenic variant HPAIVs.

2. Materials and methods

2.1. Viruses

Dk/Vac-3/07 was generated in our laboratory as a reassortant influenza virus between A/duck/Hokkaido/101/2004 (H5N3) and A/duck/Hokkaido/262/2004 (H6N1), isolated from fecal samples of migratory ducks (Soda et al., 2008b). The following HPAIV isolates were used: A/muscovy duck/Vietnam/OIE-559/2011 (H5N1) (Mdk/VN/11), was isolated from an apparently healthy muscovy duck in Viet Nam in 2011, A/whooper swan/Hokkaido/4/2011 (H5N1) (Ws/Hok/11), isolated from a dead whooper swan found on the waterside of lake Ohnuma in Hokkaido, Japan (Sakoda et al., 2012), and A/peregrine falcon/Hong Kong/810/2009 (H5N1) (Pf/HK/09) was kindly provided by Dr. Luk S. M. Geraldine, Tai Lung Veterinary Laboratory (Hong Kong SAR, China). All viruses used in the present study were propagated in 10-day-old embryonated chicken eggs at 35 °C for 30–48 h and infectious allantoic fluids were stored at −80 °C until use.

The complete nucleotide sequence of Dk/Vac-1/04 and Dk/Vac-3/07 have been registered in GenBank/EMBL/DDBJ (Accession numbers: AB259709-AB259716 and AB355926-AB355933, respectively) (Soda et al., 2008b). It is also revealed that the all genes of Dk/Vac-1/04 were closely related to Dk/Vac-3/07 (98% similarity in HA gene, 97% similarity in NA gene, and more than 99% similarity in the other genes).

2.2. Generation of recombinant viruses

In addition to Dk/Vac-3/07, vaccine was prepared from attenuated Pf/HK/09. Viral RNA was extracted from the allantoic fluid of embryonated chicken eggs infected with Pf/HK/09 using a commercial kit (TRIzol LS Reagent, Invitrogen, Carlsbad, CA, USA) and reverse transcribed with the Uni12 primer (Desselberger et al., 1980) and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR-based amplification of the full-length HA and NA gene segments was performed using universal primer sets (Hoffmann et al., 2001). The PCR products were inserted into the vector pHW2000 (Hoffmann et al., 2000) using an In-Fusion HD Cloning Kit (Takara Bio Inc., Otsu, Shiga, Japan). To generate a mutant virus with polybasic amino acid residue RRRK deletions at the HA cleavage site, amin acid mutation residue T (codon ACA) were substituted into the HA cleavage site of the Pf/HK/09 strains using a Quick Change II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Attenuated Pf/HK/09, Pf/mut (H5N1), was generated by reverse genetics methods according to Hoffmann et al. (2000). Pf/mut (H5N1) possesses the gene encoding the mutant HA of Pf/HK/09, in which polybasic amino acid at the cleavage site was deleted, NA of Pf/HK/09, and the backbone of Dk/Vac-1/04 internal genes. To confirm attenuation of Pf/mut (H5N1), the IVPI test was carried out according to the OIE (World Organization for Animal Health) manual (OIE, 2008).

2.3. Vaccine preparation

Vac-3 vaccine and Pf/mut vaccine were prepared from Dk/Vac-3/07 or Pf/mut (H5N1), respectively. Dk/Vac-3/07 or Pf/mut (H5N1) was inoculated into the allantoic cavity of 10-day-old embryonated chicken eggs and the eggs were then incubated at 35 °C for 48 h. After the allantoic fluid was harvested, formalin was added to a final concentration of 0.2%, and the mixture was incubated at 4 °C for 7 days to inactivate the viruses. Virus inactivation was confirmed by inoculation of the formalin-treated samples into embryonated chicken eggs. The inactivated Dk/Vac-3/07 and Pf/mut (H5N1) virus suspensions were concentrated by ultrafiltration using the Vivaflow 200 (Sartorius AG, Goettingen, Germany), then diluted with phosphate-buffered saline (PBS, pH7.2) to give the required hemagglutinin titer concentration. The inactivated viruses, light liquid paraffin, sorbitan monooleate, and polysorbate 80 were mixed in a volume ratio of 9:36:4:1 and then agitated to obtain emulsion. The Vac-3 vaccine of 2.4-fold antigen concentration was also prepared and designated as Vac-3 conc. vaccine. Vac-3 vaccine contains inactivated virus of 756 HA per dose and was prepared from the dilution of infectious allantoic fluid 1:1 with PBS, 378 HA per dose of Pf/mut vaccine at 1:1, and 1843 HA per dose of Vac-3 conc. vaccine at 2.4:1, respectively.

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2.4. Antigenic analysis of the viruses

Polyclonal antisera were prepared from chickens immunized with inactivated Dk/Vac-3/07, A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04), A/whooper swan/Mongolia/3/2005 (H5N1) (Ws/Mon/05), Mdk/VN/11, Ws/Hok/11, or Pf/HK/09. Each virus inactivated with formalin was inoculated once or twice into the lower thigh muscle of chickens. Two weeks after the final immunization, serum was obtained from each vaccinated chicken and used for a hemagglutination-inhibition (HI) test to assess antigenic relationships among H5 influenza viruses. HI test was performed according to Isoda et al. (2008). The differences within 4-fold HI titers were determined as antigenically related, whereas over 4-fold determined as antigenically different.

The antigenic specificities of H5 viruses, Mdk/VN/11, Ws/Hok/11, and Pf/HK/09, were assessed by the fluorescent antibody method using monoclonal antibodies (MAb) to the HA according to the method of Soda et al. (2008a). MDCK cells infected with each of the H5 influenza virus were fixed with 100% acetone 8 h post-inoculation. Reactivity patterns of the H5 viruses with MAbs were investigated with FITC-conjugated goat anti-mouse IgG (ICN Biomedicals Inc., Irvine, CA, USA) using a fluorescent microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany).

2.5. Potency test of vaccines in chickens

One hundred and ten chickens (White leghorn) were hatched and raised in our laboratory. Half a milliliter of Vac-3 vaccine was injected into the lower thigh muscle of 54 four-week-old chickens. Three weeks later, 18 vaccinated and 4 non-vaccinated seven-week-old chickens in 3 groups were challenged intranasally with 100 50% chicken lethal dose (CLD_{50}) of Mdk/VN/11, Ws/Hok/11, or Pf/HK/09. One hundred times CLD_{50} of Mdk/VN/11, Ws/Hok/11, and Pf/HK/09 was $10^{6.3}$, $10^{5.7}$, and $10^{5.5}$ 50% egg infectious dose (EID_{50}), respectively. Twelve out of 18 vaccinated chickens in each group were monitored for their clinical signs for 2 weeks, and 6 chickens in each group were sacrificed 3 days post-challenge (d.p.c.).

Pf/mut and Vac-3 conc. vaccines were injected into the lower thigh muscle of 2 groups of 18 four-week-old chickens. Three weeks later, 18 vaccinated and 4 non-vaccinated chickens in the 2 groups were challenged intranasally with 100 CLD_{50} of Pf/HK/09. Twelve out of 18 vaccinated chickens in each group were monitored for their clinical signs for 2 weeks, and 6 chickens in each group were sacrificed 3 d.p.c.

When chickens died or were sacrificed, tracheal and cloacal swabs were collected and soaked in minimum essential medium (MEM), and their tissues (trachea, lungs, kidneys, and colon) were collected aseptically. To make 10% suspensions with MEM, the collected tissue samples were homogenized using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). Serial 10-fold dilutions of the suspentions with PBS were inoculated into 10-day-old embryonated chicken eggs and incubated at 35 °C for 48 h. EID_{50} of viruses was determined by the method of Reed and Muench (1938) and expressed as EID_{50} per milliliter of swab or gram of tissue, respectively. Sera were collected from all of the 90 vaccinated and 20 non-vaccinated chickens just before challenge and examined for the presence of antibodies against the vaccine strains and challenge virus strains by the HI tests. Challenge studies were carried out in self-contained isolator units (Tokiwa kagaku, Tokyo, Japan) at a BSL3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. Animal experiments were authorized by the Institutional Animal Care and Use Committee of Hokkaido University (approval numbers: 09-0119 and 10-0007) and all experiments were performed according to the guidelines of this committee.

3. Results

3.1. Antigenic analysis

H5N1 HPAIVs used as the vaccine strain and challenge virus in the present study were antigenically analyzed by the HI tests (Table 1). Dk/Vac-3/07 is antigenically closely related to Ck/Yamaguchi/04 and Ws/Mon/05, but is different from Ws/Hok/11 and Pf/HK/09. The recent H5N1 HPAIV isolates belonging to clades 1.1, 2.3.2.1, and 2.3.4 were antigenically different.

H5N1 HPAIVs used as the vaccine strain and challenge virus in the present study were antigenically analyzed using a panel of MAbs recognizing six different epitopes on the HA of A/duck/Pennsylvania/10218/84 (H5N2). Each of the MAbs bound to the low pathogenic avian influenza

Table 1
Antigenic analysis of H5N1 subtype avian influenza viruses using polyclonal antibodies.[a]

Virus	Clade[b]	Antiserum to					
		Vac-3	Yama/04	Mon/05	VN/11	Hok/11	HK/09
A/duck/Hokkaido/Vac-3/2007	–	128	16	32	4	8	<4
A/chicken/Yamaguchi/7/2004	2.5	128	128	128	32	32	<4
A/whooper swan/Mongolia/3/2005	2.2	128	256	512	128	128	64
A/muscovy duck/Vietnam/OIE-559/2011	1.1	256	64	16	256	16	16
A/whooper swan/Hokkaido/4/2011	2.3.2.1	32	16	64	16	128	<4
A/peregrine falcon/Hong Kong/810/2009	2.3.4	16	8	8	16	16	128

Vac-3, A/duck/Hokkaido/Vac-3/2007; Yama/04, A/chicken/Yamaguchi/7/2004; Mon/05, A/whooper swan/Mongolia/3/2005; VN/11, A/muscovy duck/OIE-559/2011; Hok/11, A/whooper swan/Hokkaido/4/2011; HK/09, A/peregrine falcon/Hong Kong/810/2009.

[a] Homologous titers are underlined.

[b] "–" indicate lineages not belonging to clade 0–9.

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Table 2
Antigenic analysis of H5 influenza viruses using monoclonal antibodies.

Virus[a]	Clade[b]	Monoclonal antibodies[c]						
		I (88)	II (145)	III (157)	IV (168)		V (169)	VI (205)
		D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2
LPAIV								
A/duck/Pennsilvania10218/1984 (H5N2)	–	+	+	+	+	+	+	+
A/duck/Hokkaido/Vac-3/2007 (H5N1)	–	+	+	+	+	+	+	+
HPAIV								
A/Vietnam/1194/2004 (H5N1)	1	+	+	+	+	+	–	+
A/chicken/Yamaguchi/7/2004 (H5N1)	2.5	–	+	+	+	+	–	+
A/whooper swan/Mongolia/3/2005 (H5N1)	2.2	+	+	+	+	+	–	+
A/muscovy duck/Vietnam/OIE-559/2011 (H5N1)	**1.1**	**+**	–	–	–	–	–	**+**
A/whooper swan/Hokkaido/4/2011 (H5N1)	**2.3.2.1**	**+**	–	–	–	–	–	–
A/peregrine falcon/Hong Kong/810/2009 (H5N1)	**2.3.4**	–	–	–	–	–	–	–

[a] Viruses indicated in bold were used in the challenge study.
[b] "–" indicate lineages not belonging to clade 0–9.
[c] Reactivity of monoclonal antibodies against the HA of A/duck/Pennsylvania/10218/1984 (H5N2) to the representative H5 viruses was compared using fluorescent antibody methods. Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies is indicated in parentheses.

viruses (LPAIVs) and HPAIVs isolated before 2005, and few MAbs bound to the antigens of Mdk/VN/11, Ws/Hok/11, and Pf/HK/09. It was demonstrated that the epitopes recognized by these MAbs were conserved in LPAIVs and HPAIVs isolated before 2005, but not in recently prevailing HPAIVs (Table 2).

3.2. Efficacy of the Vac-3 vaccine in chickens

Fifty-four vaccinated chickens and 12 non-vaccinated chickens were challenged intranasally with each of the HPAIVs, Mdk/VN/11, Ws/Hok/11, and Pf/HK/09. The serum HI titers of the vaccinated chickens varied with each of the challenge viruses. The survival rates of the chickens challenged with each of the three HPAIVs are shown in Fig. 1. All vaccinated chickens survived without showing any disease signs after challenge either with Mdk/VN/11 or Ws/Hok/11, whereas two vaccinated chickens died after challenge with Pf/HK/09. All non-vaccinated chickens challenged with any of the HPAIVs died within 2 to 4 d.p.c. (Fig. 1A–C).

To evaluate the potential of Vac-3 vaccine to induce immunity for the prevention of virus shedding, we tried to recover the virus from swabs and tissues of the vaccinated and non-vaccinated chickens after challenge with each of HPAIV, Mdk/VN/11, Ws/Hok/11, and Pf/HK/09 (Table 3). Infectivity titers of the recovered viruses from vaccinated chickens were lower than those of non-vaccinated chickens after challenge with Mdk/VN/11 or Pf/HK/09 3 d.p.c. Infectious viruses were recovered from tracheal swabs and the organs of vaccinated chickens 3 d.p.c. with Ws/Hok/11, although the titers of viruses recovered from these birds were lower than those from non-vaccinated chickens.

3.3. Efficacy of the Vac-3 conc. vaccine against Pf/HK/09 in chickens

In order to enhance the efficacy of Vac-3 vaccine, the antigen concentration of Vac-3 vaccine was increased ×2.4 and designated as Vac-3 conc. vaccine. The Vac-3 conc. vaccine was assessed for efficacy against a challenge with Pf/HK/09. Pf/mut vaccine prepared from Pf/mut (H5N1) (IVPI = 0.00) was also assessed for its potency as the homologous control. Thirty-six chickens immunized either with Vac-3 conc. or Pf/mut vaccine and 8 non-vaccinated chickens were challenged intranasally with Pf/HK/09.

Table 3
Virus recovery from chickens vaccinated with Vac-3 vaccine challenged with H5N1 HPAIVs.

Challenge virus	Vaccination	Sampling d.p.c.[a]	No. of chickens	HI titer[b]		Virus recovery					
						No. of chickens from which each virus was recovered [GM value of the virus titer (log 10)]					
				Dk/Vac-3/07	Challenge virus	Swab (log EID50/ml)		Tissue (log EID50/g)			
						Tracheal	Cloacal	Trachea	Lungs	Colon	Kidneys
Mdk/VN/11	Vaccinated	3	6	256	8–64	1 (≤0.7)	0	0	0	0	0
	Non-vaccinated	2†	4	<4	<4	4 (5.5)	4 (5.3)	4 (8.6)	4 (9.3)	4 (8.5)	4 (9.4)
Ws/Hok/11	Vaccinated	3	6	256–512	16–64	2 (≤1.0, ≤1.3)	0	3 (4.3)	4 (5.1)	5 (4.4)	4 (4.0)
	Non-vaccinated	3–4†	4	<4	<4	4 (4.2)	4 (3.2)	4 (6.4)	4 (8.2)	4 (6.9)	4 (7.9)
Pf/HK/09	Vaccinated	3	6	64–512	<4–16	2 (≤0.7, ≤1.3)	0	0	1 (3.5)	0	0
	Non-vaccinated	2†	4	<4	<4	4 (4.9)	4 (5.4)	4 (7.6)	4 (9.1)	4 (8.3)	4 (8.8)

[a] Swab and tissue samples were collected on the following days from sacrificed (no mark) or dead (†) chickens.
[b] The range of HI titers using either Dk/Vac-3/07 or each challenge virus before challenge is indicated.

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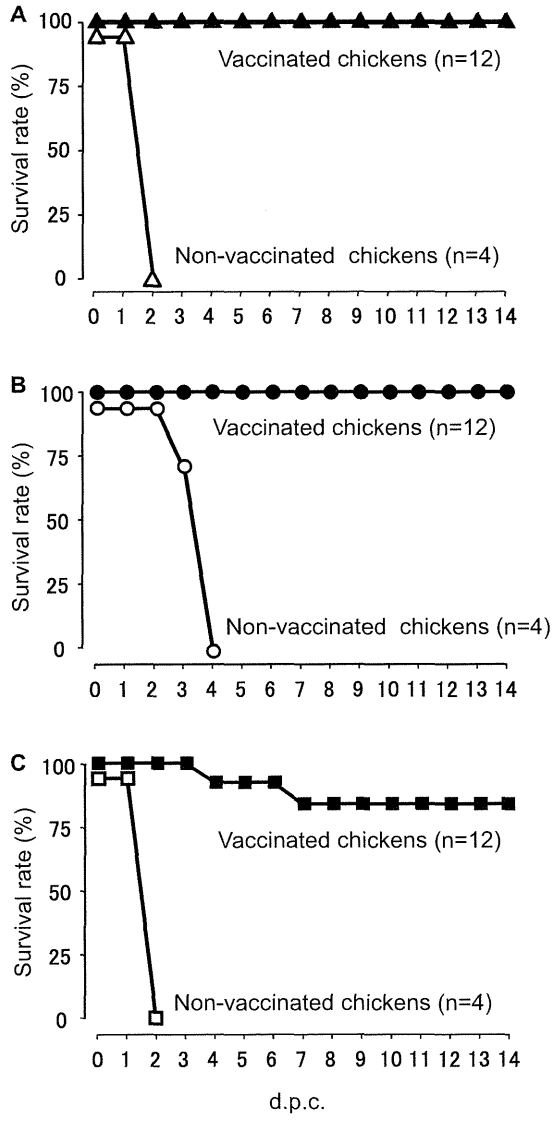

Fig. 1. Survival rates of chickens vaccinated with Vac-3 vaccine after challenge with H5N1 HPAIVs. Twelve four-week-old chickens from each group were immunized intramuscularly with 0.5 ml of Vac-3 vaccine. Three weeks after vaccination, the vaccinated chickens were challenged with 100 CLD_{50} of Mdk/VN/11(A), Ws/Hok/11(B), and Pf/HK/09 (C), respectively.

HI titer to Pf/HK/09 of the sera of the chickens immunized with Vac-3 conc. vaccine was 4-16 HI, which is similar to those of the chickens immunized with Vac-3 vaccine (Table 4). The survival rates of the chickens challenged with Pf/HK/09 are shown in Fig. 2. All vaccinated chickens survived without showing any disease signs after the challenge with Pf/HK/09 (Fig. 2A and B). All non-vaccinated chickens challenged with Pf/HK/09 died within 3 d.p.c. Viruses were not recovered from swabs or organs of any of the chickens immunized with Pf/mut vaccine after the challenge with Pf/HK/09 (Table 4). Virus was scarcely recovered from the tracheal swab of chicken immunized with Vac-3 conc. vaccine and the viral titer was lower than in non-vaccinated chickens after challenge with Pf/HK/09.

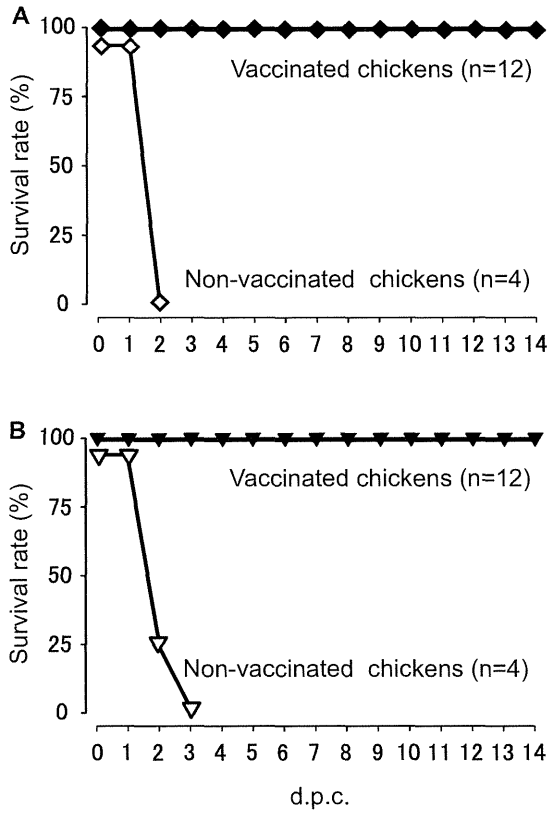

Fig. 2. Survival rates of chickens vaccinated with Pf/mut vaccine (A) and Vac-3 conc. vaccine (B) after challenge with Pf/HK/09. Twelve four-week-old chickens from each group were immunized intramuscularly with 0.5 ml of Pf/mut vaccine or Vac-3 conc. vaccine. Three weeks after vaccination, these vaccinated chickens were challenged with 100 CLD_{50} of Pf/HK/09.

4. Discussion

Antigenic variants of H5N1 HPAIVs have been selected in poultry under immunological selection pressure (Cattoli et al., 2011; Grund et al., 2011). In the present study, it was demonstrated that H5N1 HPAIVs prevailing recently in Asia were antigenically different from non-pathogenic avian influenza virus and H5N1 HPAIVs isolated before 2005 (Table 1). We previously demonstrated that an inactivated avian influenza vaccine prepared from Dk/Vac-1/04 conferred protective immunity and reduced the amount of virus shedding when chicken was challenged with Ck/Yamaguchi/04, Ws/Mon/05, and Ws/Hok/08 (Isoda et al., 2008; Okamatsu et al., 2010). In the present study, we prepared an inactivated influenza vaccine from Dk/Vac-3/07, which is a reassortant generated between non-pathogenic avian influenza viruses isolated from wild water birds. It is assumed that Vac-3 vaccine has similar potency with Vac-1 vaccine against recent H5N1 HPAIVs since Dk/Vac-3/07 is antigenically similar to Dk/Vac-1/04. However, the growth potential of Dk/Vac-3/07 is better than that of Dk/Vac-1/04. It is possible to generated concentrated Vac-3 vaccine using Dk/Vac-3/07. The

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Table 4
Virus recovery from chickens challenged with Pf/HK/09.

Vaccination	Sampling d.p.c.[a]	No. of chickens	HI titer[b]		Virus recovery					
					No. of chickens from which each virus was recovered [GM value of the virus titer (log 10)]					
			Dk/Vac-3/07	Pf/HK/09	Swab (log EID50/ml)		Tissue (log EID50/g)			
					Tracheal	Cloacal	Trachea	Lungs	Colon	Kidneys
Pf/mut	3	6	16–32	128–512	0	0	0	0	0	0
Vac-3 conc.	3	6	256–1024	4–16	1 (1.7)	0	0	0	0	0
Control	2–3†	8	<4	<4	8 (5.1)	8 (5.3)	8 (7.8)	8 (9.0)	8 (8.3)	8 (8.7)

[a] Swab and tissue samples were collected on the following days from sacrificed (no mark) or dead (†) chickens.
[b] The range of HI titers using either Dk/Vac-3/07 or Pf/HK/09 before challenge is indicated.

potency of Vac-3 vaccine was assessed by challenging with antigenically drifted H5N1 HPAIVs isolated in 2009 and 2011. Vac-3 vaccine conferred protective immunity to suppress the manifestation of clinical signs and virus shedding in chickens challenged with antigenically drifted H5N1 HPAIVs belonging to clades 1.1, 2.3.2.1, and 2.3.4. In order to clarify why the efficacy of Vac-3 vaccine was not sufficient to protect all vaccinated chickens from the challenge with Pf/HK/09, we prepared Pf/mut vaccine, which was antigenically homologous with Pf/HK/09 (data not shown). All chickens immunized with Pf/mut vaccine survived for 14 days without showing any clinical signs and viruses were not detected from the swabs and tissues of the chickens. These results correspond to the findings that Pf/HK/09 is antigenically different from Dk/Vac-3/07 compared with Mdk/VN/11 and Ws/Hok/11. To improve the efficacy of Vac-3 vaccine, antigen concentration was increased for Vac-3 conc. vaccine preparation. HI antibody responses of vaccinated chickens correlated with the antigen concentration in H5N1 (Sasaki et al., 2009a) or H7N7 (Maas et al., 2009) influenza virus vaccine. Inactivated whole particle vaccine confers protective immunity against a challenge with viruses antigenically drifted from the vaccine strain to chickens by increasing the antigen concentration (Hwang et al., 2011). Vac-3 conc. vaccine conferred protective immunity to all vaccinated chickens after the challenge with Pf/HK/09. The vaccine with increased antigen concentration induced sufficient immunity to protect from infection with variant H5N1 HPAIV in chickens.

In the present study, it was demonstrated that the vaccine prepared from non-pathogenic avian influenza virus conferred protective immunity against the challenge with antigenically drifted H5N1 HPAIVs, indicating that Vac-3 vaccine induces sufficient immunity in chickens. The results of the antigenic analysis indicate broad antigenic diversity among H5N1 HPAIVs prevailing recently in Asia (Table 1). The vaccine prepared from recent H5N1 HPAIVs may not be completely effective against HPAIVs belonging to different clades. Since the misuse of vaccines lead to the silent spread of antigenically drifted viruses, it is recommended that avian influenza vaccine should be applied very carefully in addition to the stamping-out policy. There is an urgent need to eradicate H5N1 HPAIV from Asia by stamping-out without misusing vaccines (Table 4).

5. Conclusion

All chickens immunized with the Vac-3 vaccine survived without showing any clinical signs after intranasal challenge either with A/whooper swan/Hokkaido/4/2011 (H5N1) or A/muscovy duck/Vietnam/OIE-559/2011 (H5N1). The Vac-3 conc. vaccine of 2.4-fold antigen concentration conferred complete protective immunity in chickens against challenge with A/peregrine falcon/Hong Kong/810/2009 (H5N1).

Conflict of interest statement

The authors declare that they have no conflict interests.

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Review

Mucosal IgA responses in influenza virus infections; thoughts for vaccine design

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ABSTRACT

The current challenge in influenza vaccine design is to induce long-lasting protection not only against the vaccine strain, but also against drifted (point mutations in the surface antigens HA or NA) and even shifted (exchange of genome segments) strains. Several immune mediators that can induce cross-protection have been described, such as CD4 T-cells, CD8 T-cells and antibodies, including IgA. However, most vaccines are now administered intramuscularly or subcutaneously and subsequently relatively little is known on the role of local, mucosal responses. Since local IgA responses have been shown to play an important role in responses to natural infection, and IgA responses in mice were shown to also be involved in cross-protection, the research on mucosal influenza vaccines is currently expanding. However, the functioning of the mucosal immune system, especially in the respiratory tract, is just beginning to be revealed. Here, the current knowledge on the induction of IgA, the role of influenza specific IgA producing B-cells in anti-influenza immunity as well as the role of humoral memory responses induced upon vaccination will be reviewed.

Contents

1. Introduction

Seasonal influenza A virus infections cause millions of cases each year with the highest risk of complications in very young and very old people as well as immunocompromised patients, all lacking a strong immune response. In addition, also more infectious or pathogenic strains can infect people, such as the 2009 pandemic influenza A virus (A(H1N1)pdm09), or highly pathogenic avian influenza A H5N1 virus, respectively. In contrast to seasonal influenza viruses, highly pathogenic strains can be more threatening for young, healthy people in whom tissue damage can be the result of overly powerful host inflammatory responses [1].

In the case of both seasonal and newly evolved strains, the most efficient way to fight the disease is preventing it by means of vaccination. However, current influenza vaccines are effective against a single type of influenza only, thus for the seasonal vaccine necessitating the presence of multiple strains, as well as a yearly renewal of the vaccine. In addition to the possibility of a mismatch of the vaccine with the actual circulating influenza strains, newly evolved strains, such as H5N1 and A(H1N1)pdm09, highlighted the need for improved cross-protection. Ideally, a universal influenza vaccine would be developed, that induces a strong and long lasting memory response which is cross-protective to drift variants as well as across subtypes of the influenza virus (Fig. 1). Many factors are

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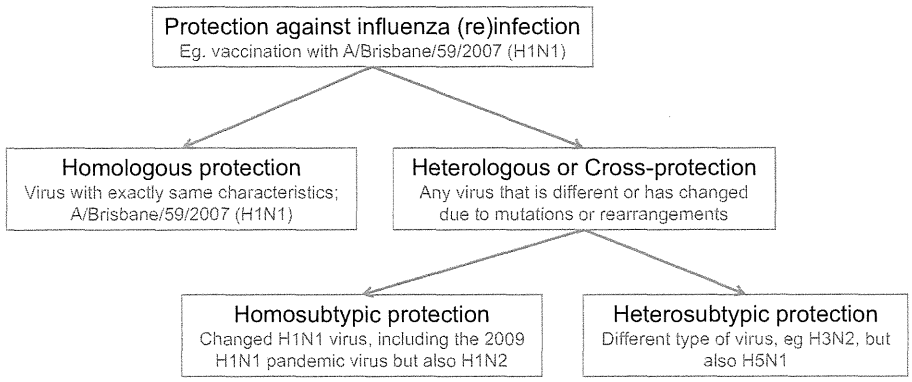

Fig. 1. Overview of different terms related to different types of protection. Since no official definition is available for these terms, this figure shows how these terms are used in this review. The definitions are based on the type of haemagglutinin antigen that the virus contains. For each term some virus strains that will fit the term are given in grey, based on an example of protection against A/Brisbane/59/2007 (H1N1).

known to play a role in cross-protection, but in this review we will focus on the current knowledge on the role IgA could play in realising universal protection. Importantly, rational design of IgA inducing vaccines has so far been hampered by a lack of knowledge: since local, tissue-specific, immune responses, including IgA, are often not measured [2], relatively little information is available. Consequently, the importance of the presence of IgA as well as the mechanisms via which IgA responses are induced and maintained are just beginning to be revealed [3].

2. The role of IgA in (cross-)protection from influenza infection

Pre-existing secretory IgA (S-IgA) antibodies can provide immediate immunity via their unique capability to eliminate a pathogen before it even passes the mucosal barrier and enters the human body [4], also termed immune exclusion [5]. Furthermore, IgA has also been shown to be very effective at disarming viruses in virus-infected secretory epithelial cells and in redirecting antigens to the lumen when they enter the lamina propria [3] (Fig. 2). These responses are all non-inflammatory, since IgA, unlike IgG, does not fix complement and thus does not activate the inflammatory complement pathway [6]. Therefore, a strong IgA response could be particularly important in case of highly pathogenic strains, where most complications are caused by uncontrolled pro-inflammatory responses.

Although the roles of S-IgA and serum antibodies are difficult to investigate independently, infection models in knockout mice showed that S-IgA normally does play an important role in protection against influenza [7,8]. Moreover, transfer of S-IgA from respiratory tract washings from immunized to naïve mice was shown to provide protection to challenge with a homologous or drifted strain [9] and several studies in mice showed induction of strong homosubtypic as well as modest heterosubtypic cross-protective IgA antibodies (Table 1).

Thus, IgA contributes to, but is not essential for the establishment of cross-protection to influenza. Interestingly, all mediators of cross-protection, such as CD8 T-cells [27], CD4 T-cells [28] or B-cells [29] seem to be partially redundant, since high degrees of protection were also observed in mice lacking CD4 T-cells, CD8 T-cells or B-cells [21,30,31].

3. IgA production

Antigen specific antibody producing B-cells can develop at two different types of locations, extrafollicular and in germinal centres (GC), and in a T-cell dependent or independent manner [32]. GC function as a specialized environment to support affinity maturation mediated by activation-induced deaminase (AID) induced somatic hypermutation [33]. In addition AID is involved in the production of the preferred antibody class, by influencing class switch recombination (CSR) of the heavy chain [33,34]. Most IgA memory B-cells (B_{Mem}) and long-lived IgA plasma cells develop in the GC of peripheral lymphoid organs and require T-cell help via

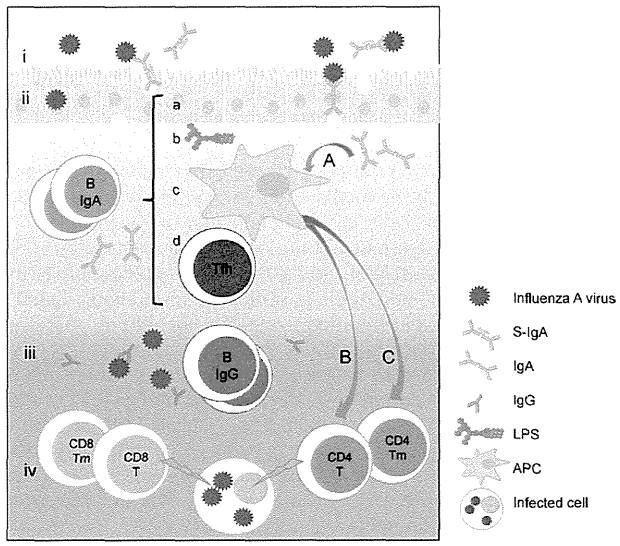

Fig. 2. The role of IgA in protection from influenza infection; function, induction and interaction. Function (i–iv): IgA can neutralize influenza viruses at the mucosal interface (i), even before they actually enter the host by crossing the mucosal barrier, thus existing IgA is the first line of defence upon reinfection or infection after vaccination. In addition, IgA can effectively clear the virus in infected epithelial cells (ii). Virus that could not be destroyed by IgA and successfully entered the host can be eliminated by IgG (iii), which is the main protection on which seasonal vaccination is based, and (iv) cytotoxic CD8 T-cells target host cells that were infected. *CD4 cells can also kill infected cells, but are mostly involved in helping B- and CD8* T-cells to eliminate the influenza virus. Induction (a–d): induction of IgA (by influencing for example class switching or proliferation of plasmablasts) is influenced by tissue signals, for example from epithelial cells (a), innate signals, like viral or commensal products such as LPS that activate innate receptors (b), by different types of APC (c) and by helper T-cells, mostly Tfh (d). Interaction (A–C): IgA antibodies were found to influence the function of antigen presenting cells (APC) (A) and so indirectly have a share in the activation of effector T-cells (B) as well as activation of memory responses (C). Tfh: follicular helper T-cell, Tm: memory T-cell, APC: antigen presenting cell, S-IgA: secretory IgA.

Table 1
IgA mediated cross-reactivity to influenza A viruses in a mouse model.

Vaccine type	Adjuvant	Vaccine strain(s)	Cross-responsive strain(s)		Ref.
			Homo-subtypic	Hetero subtypic	
Only homosubtypic					
HA	CTB	H1N1	H1N1		[7]
HA	CTB + 0.2% holotoxin	H1N1	H1N1		[10]
HA	LTB + LT	H1N1	H1N1		[11]
HA	CTB	H1N1, H3N2	H1N1, H3N2		[12]
HA	CTB	H3N2	H3N2		[13]
Homosubtypic and heterosubtypic					
HA	Surf clam microparticles	H1N1 H3N2	H1N1	H1N1	[14]
HA	PolyI:C	H1N1 H3N2	H1N1	H1N1	[15]
HA	Chitin microparticles	H1N1 H3N2	H1N1	H1N1	[16]
HA	Mutant CT	H1N1 H3N2	H1N1	H1N1	[17]
HA	CTB	H1N1 H3N2	H1N1 H3N2	H3N2 H1N1	[9]
HA	CTB	H1N1	H1N1	H3N2	[18]
HA	CTB	H1N1 H3N2	H1N1	H1N1	[19]
HA	CTB	H1N1 H3N2	H1N1	H1N1	[20]
WIV	CT	H1N1	H1N1	H3N2	[21]
Only heterosubtypic					
HA	PolyI:$C_{12}U$	H1N1, H3N2		H5N1	[22]
HA	PolyI:$C_{12}U$	H1N1, H3N2		H5N1	[23]
LAIV and IIV[a]	–	H2N2		H5N1	[24]
WIV (formalin)[b]	–	H1N1, H1N2, H2N2, H3N1, H3N2, H5N4, H9N2		H5N1	[25]
WIV (formalin)	+/−LT	H3N2		H5N1	[26]

Only articles are included in which cross-protective IgA antibodies were demonstrated to be present. Vaccines were administered intranasally.
[a] Inactivated influenza virus, different from WIV.
[b] Ether split vaccine did not induce heterosubtypic protection.

CD40L (CD154) and TGFβ1, although T-cell independent B-cell class switching in GC might be mediated by interaction with dendritic cells (DC) and stromal cells, including follicular DC [35].

Also at extrafollicular mucosal sites antibodies can develop both with and without the help of T-cells, the latter involving BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand) [36,37]. Although hypermutation, necessary for affinity maturation, was reported to be minimal at these sites [38], antigen-specific antibody producing B-cells that developed at this site were shown to play a role in prevention of reinfection [39], and to lead to the generation of an IgG and IgA producing B_{Mem} subset [40]. Recombinatorial, transcriptional and signalling events underlying IgA class switching were recently reviewed [32]. Below, we will highlight the immunological parameters that could be a target for the induction of IgA production upon influenza vaccination (Fig. 2).

3.1. Mucosal tissue

The inductive sites of the mucosal immune system include mucosa-associated lymphoid tissue (MALT) as well as local and regional draining lymph nodes. The mechanisms leading to IgA CSR have mostly been studied in the gut and were found to be influenced by the specific environmental factors at this site, mainly created by commensal bacteria and their products [41]. Much less is known about the respiratory tract, which is not populated with as many commensals as the intestinal tract. However, recently it was shown that commensals do play an important role in protection against influenza infection, since antibiotic treatment of mice reduced CD4 T-cell, CD8 T-cell as well as antibody responses, including IgA. Immunity to influenza viruses could be restored by nasal administration of LPS but also by rectal administration of TLR agonists (LPS, CpG, polyI:C) [42]. These findings indicate that signals from distal mucosal regions can support immune priming in the respiratory tract, although it needs further investigation to find whether support from distal regions is preferential over an intact commensal system in the respiratory tract.

3.2. Innate sensing

Influenza viruses interact with several types of pattern recognition receptors, including TLR family members, but also with members of the RIG-I like receptor (RLR) family and the Nod like receptor (NLR) family [43] as well as several members of the C-type lectin receptor family [44]. For vaccination purposes, also members of these receptor families that are not activated by a natural infection have been targeted. For example, flagellin, that activates TLR5, was also shown to promote IgA production and heterosubtypic protection when incorporated in the membrane of influenza VLPs [45]. Similarly, PolyI:PolyC12U, activating TLR3, was shown to induce heterosubtypic protection through IgA antibodies when administered intranasally [23]. Moreover, the effect of this TLR3 ligand was shown to act in a synergistic manner with the TLR-2 ligand zymosan [46].

Recently, several possible models were developed that implicate an essential role for TLR signalling in CSR. Classically, only two signals were described to be necessary to induce CSR in naïve B-cells: presentation of the antigenic peptides on MHC class II molecules after antigen binding to the B-cell receptor and secondly, activation of these B-cells via cytokines and CD40-CD40L interaction with antigen specific T-cells. Currently, TLR signalling

is thought to sometimes provide an important third signal [47] and it has been reported that MyD88 was necessary to induce protection in primary, but not secondary, influenza infection. IgA levels in MyD88$^{-/-}$TRIF$^{-/-}$ mice were reduced in saliva, however, in serum, BALF and nasal wash, levels were similar to those in WT mice and thus induced in a TLR independent manner [48]. In contrast, in another study it was shown that TLRs can play a role in both T-cell dependent and independent IgA responses at both mucosal and systemic levels [49]. This inconsistency might partially be explained by the finding that MyD88 is not only involved in TLR signalling, but it also interacts with TACI (Transmembrane Activator and CAML-Interactor) which is involved in both T-cell dependent as well as independent class switching. Thus, in mice lacking MyD88, both TLR, and TACI signalling will be affected [50].

3.3. APC bridging innate and adaptive immunity

Some antigen presenting cells have been associated with induction of IgA responses, including pDC (plasmacytoid DC), Tip-DC (TNF and Inducible nitric oxygen species (iNOS) Producing DC) and LAPC (late-activator APC).

pDC, highly appreciated in anti-influenza responses for the induction of type 1 interferon, Th1 and cytotoxic responses, were found to also enhance B-cell expansion and differentiation into CD27^{high} plasmablasts upon TLR7 stimulation [51]. Interestingly, pDC were found to be necessary for optimal mucosal IgA and serum IgG production in primary, but not booster influenza vaccination schedules, upon vaccination with live attenuated virus, inactivated whole virus or split virus. In contrast, pDC were not essential for raising a response to live virus [52].

Upon influenza infection, Tip-DC (TNF and inducible nitric oxygen species (iNOS) producing DC) were first known for their production of large amounts of both TNF and NO upon infection with highly virulent strains, thereby inducing tissue damage [53]. However it was recently found that NO – when present in controlled amounts – can induce TGF-βRII expression on B-cells, thereby enabling T-cell dependent IgA class switching. Also, MyD88 signalling downstream of TLR2, 4 and/or 9, needed to induce iNOS, was involved in T-cell independent IgA secretion, in a BAFF and APRIL-dependent manner [54].

Another type of APC that might play an important role was recently identified and designated LAPC, or late-activator APC. Whereas influenza-activated DC are most active around day 3 after infection and induce a response with many Th1 type characteristics, LAPC peak around day 8 after infection and induce a Th2-type polarization, resulting in IgA, IgG1 and IgG2 antibody production, and downregulation of anti-viral Th1-type responses [55].

3.4. Adaptive CD4 T-cell responses

CD4 T-cells are well known for supporting humoral and cellular responses and in addition they can activate innate immunity [28] and display cytolytic potential [56]. The role of CD4 helper T-cells in enhancing B- and CD8 T-cell, immune responses is dependent on the ability of the CD4 T-cells to present antigen on their surface in the context of MHCII molecules as well as the cytokine environment they create.

Importantly, vaccination with peptide variants of the desired epitopes that possess high affinity interactions with the MHC molecules in the host, will increase the amount of these antigens presented on CD4$^+$ T-cells, thereby promoting priming of T and B-cells that interact most efficiently with each other [57]. This could be used to expand the CD4 T-cell repertoire specific for the most genetically conserved regions of influenza HA and NA antigens, thereby enhancing cross-protective neutralizing antibody responses [57]. It was suggested that via priming in the upper respiratory tract, this way also the antigen specificity of IgA antibodies might be influenced by vaccination.

In addition to the MCH molecules, also the cytokine environment created by different T-cell subsets will influence immune responses. The predominant subset of CD4$^+$ T-cells responsible for the generation of high-affinity, class-switched antibodies are follicular helper T-cells (Tfh), that were defined in 2000 by Schaerli et al. as well as Breitfeld et al., based on their surface CXCR5 expression and their key role in antibody production in GC. Tfh are involved in the formation of GC and in the induction of CD40L, IL-2, IL-4, IL-10, TGFβ and IL-21, thereby promoting B-cell proliferation, CSR and somatic hypermutation, resulting in highly specific class switched plasma cells and long-lived memory cells [58,59]. TGFβ was also found to play an important role in IgA class-switching, since mice deficient in TGFβII receptor on B-cells, were hardly producing IgA [60] and S-IgA could not be detected after mucosal vaccination in these mice [61]. Moreover, in the GC, TGFβ and IL-21, produced by Tfh, were found to synergize to stimulate the generation of high numbers of IgA plasmablasts [58].

Recently, it was found that blood circulating cells expressing CXCR5 might be related to Tfh. Three subsets were found; Th1, Th2 and Th17, of which the latter most strongly induced IgA responses [62]. In addition, CXCR5 was also found on a subset of peripheral blood central memory cells and these were proposed to enable quick and efficient secondary antibody mediated immune responses [63].

4. Humoral memory

The essence of vaccination is priming of the immune system with an antigen to induce a quick and effective immune response upon a subsequent encounter of the pathogen bearing that antigen. Success for all current vaccines is based on long-lived antibody production with high affinity, with antibodies shown to be maintained for 75 years after smallpox vaccination [64]. Whether this can be achieved by influenza vaccination needs to be investigated, however, in the serum of people who were naturally infected during the 1918 influenza pandemic, B_{Mem} could be isolated from serum at least up to 70 years after the last encounter [65]. Moreover, these antibodies were also active against the mild A(H1N1)pdm09 [66]. Humoral immune memory is provided by recirculating B_{Mem} and long lived plasma cells, typically residing in the bone marrow [67]. However, much needs to be discovered about the role of B_{Mem} in protection to subsequent influenza infections, regarding for example isotype expression and localization.

In mice, proliferating T-cells were shown to obtain gut- and skin-homing properties during antigen priming in mesenteric and the peripheral lymph nodes, respectively [3]. The factors inducing this are largely unknown, although retinoic acid produced by DC was found to contribute to gut-homing, whereas vitamin D3 metabolites contribute to skin-homing.

It has been stated that probably similar mediators can induce homing to the respiratory tract, with Waldeyer's ring and/or cervical lymph nodes functioning as the antigen stimulation site for T and B-cells with respiratory tract homing properties [3]. Thus, antigens reaching lymph nodes in mucosa-associated lymphoid tissue (MALT) might stimulate mucosal immunity in the same region. Recently it was also found that TGFβ and IL-21, produced by Tfh, not only synergize to induce IgA class switching, but also to simultaneously downregulate CXCR5 and upregulate CCR10 on plasmablasts, enabling their exit from GC and migration towards local mucosa, verifying that Tfh cells play an important role in establishing high-affinity and long-term responses [58]. This was confirmed by a

study that found Tfh to be an important reservoir of memory cells in secondary responses to antigen [68].

B_{Mem} that are produced in GC in the acute phase of infection are known to circulate and to spread to secondary lymphoid tissues. In mice, 8–12 weeks after infection many influenza specific IgA and IgG B_{Mem} were present in these lymphoid tissues, but also in the lung. Upon vaccination, lymph node and lung analysis revealed a higher frequency of IgA B_{Mem} after intranasal compared to intramuscular vaccination, however even upon intranasal vaccination levels of B_{Mem} detected in the lung were very low compared to levels induced by infection [69].

When an intramuscular inactivated vaccine was compared to an intranasal, live attenuated vaccine in a human study, both memory IgG and IgA responses in the circulation were higher after intramuscular administration of the inactivated vaccine, but local memory responses were not determined in this study [70].

Further insight into which homing receptors are involved in B_{Mem} dispersion and how this relates to for example the pathogen, the type of immune activation and the site of induction would be very useful for rational vaccine design. In addition, different types of B_{Mem} might be programmed for specific functions at a specific location. It was recently found that at least two distinct types of IgA B_{Mem} exist: $CD27^+$ and $CD27^-$ IgA B_{Mem}. The former are produced in GC whereas the latter are GC independent and most likely produced locally. The $CD27^-$ B-cells were shown to be induced independent of T-cells, since similar levels of these cells were found in CD40L deficient patients as compared to healthy controls [40]. However, whether these differences are also reflected in their localization and functions, remains to be investigated.

Currently, long-term memory responses induced by influenza vaccines are not tested before use. However, recent studies in mice, ferrets and humans showed that the heterologous protective capacities of prior vaccination depends greatly on the type of vaccine used as well as the type of subsequent vaccination or infection and can be beneficial [71], but also detrimental [72–74]. The latter could have severe consequences in case of a pandemic and was therefore intensively studied using data based on A(H1N1)pdm09 infections. However, conflicting data have been reported and prior vaccination with a seasonal trivalent vaccine has been associated with either increased illness due to A(H1N1)pdm09 [75,76], no association [77–79] or an association with protection of A(H1N1)pdm09 related illness [80–83]. Importantly, all these studies represent association studies and many confounding factors might be unknown, as is stated by the authors as well. Thus no thorough data are available, but improved epidemical studies will pose a great challenge since it is extremely difficult to take the full history of exposure to influenza antigens in humans into account.

Using a different approach, research on human monoclonal antibodies derived from plasma cells or B_{Mem} suggested that vaccination for seasonal influenza would mainly lead to activation of B_{Mem} responsive against dominant epitopes. In contrast, exposure to an antigen representing a major antigenic shift would increase chances of activating and expanding rare heterosubtypic B_{Mem} recognizing highly conserved epitopes, due to absence of competition by pre-existing B_{Mem} recognizing the dominant epitopes [84,85]. Thus, subsequent vaccination with several highly diverse influenza variants might induce good cross-protection. Importantly, the induction of long-term responses should also be studied, since it was shown that only antibodies derived from B_{Mem}, but not from long living plasma cells, were able to strongly neutralize escape mutants from West Nile virus [86].

5. Implications for vaccine design

Seasonal influenza vaccines are currently prepared, based on the prediction of the strain that might cause the epidemic in the following season. These vaccines are mostly injected intramuscularly or subcutaneously and are designed to prevent the onset of the disease induced by the specific vaccine strains, but these vaccines neither induce cross-protection nor prevent infection, since they mostly induce neutralizing IgG antibody in the serum (Fig. 2).

In contrast, influenza vaccines that are currently being designed are mostly based on other mechanisms than induction of IgG, that induce a broader cross-protection, with the ultimate ambition a so called 'universal influenza vaccine'. Several characteristics of the influenza virus as well as the people that should receive the vaccination have to be taken into account. The diverse and more cross-responsive response upon infection compared to current vaccination would suggest that the induction of several, if not all, immunological effectors that can add to cross-protection would more likely be capable to optimally kill viruses with diverse characteristics and at different locations in the body (Fig. 2). Reasoning based on the vaccine recipients would lead to the same conclusion, since people of different ages, sexes as well as genetic and environmental backgrounds will respond differently to vaccination and might rely on different immune mediators for their protection [28,87]. This is in agreement with prospective findings on the key immunological responses induced by the successful yellow fever vaccine, which was identified as 'broad, polyfunctional and persistent, integrating all effector cells of the immune system' [88]. Importantly, different effector cells will not only work next to each other, but will also interact. For example, CD4 T-cells facilitate B-cell responses but B-cells were reported to in their turn also influence CD4 T-cells in several ways [89]. Also IgA was shown to influence T-cells, since $IgA^{-/-}$ mice showed reduced T-cell priming and memory responses upon influenza vaccination, due to impaired APC function that could be overcome with IL-12 [90] (Fig. 2). More knowledge on these interactions will further take vaccine design out of its traditional methodology based on trial-and-error, towards a more rational approach.

Importantly in this respect, natural influenza virus infection was shown to be superior to vaccination with inactivated virus in inducing cross-protection against infection by mutated viruses within a particular subtype of the A-type virus in humans [91–93]. Inactivated virus in its turn has been shown to be more immunogenic than split vaccines, which is in agreement with the general finding that effectiveness and safety of vaccines are usually inversely correlated.

Thus, both whole virus particles as well as split-product seasonal vaccines can induce strong protection against the homologous virus [94]. However, heterosubtypic immunity is not observed when vaccination is performed using an ether-split vaccine, whereas in the same study administration of an inactivated whole virion vaccine induced a broad spectrum of heterosubtypic immunity [25]. The stronger immunogenicity of the inactivated whole virion vaccine in mice was likely due to the stimulation of innate immunity by genomic single stranded RNA, via TLR7 [95,96]. Since most viruses produce dsRNA during replication [97], synthetic dsRNA can likely act as a partial molecular mimic of viral infection.

This has been confirmed in a study where intranasal administration of an ether-split vaccine from PR8 (a H1N1 type influenza strain) and poly(I:C) adjuvant induced a strong anti-HA IgA and IgG response in nasal washes and serum, respectively, while vaccination without poly(I:C) induced very little response. In addition, administration of either an A/Beijing (H1N1) or A/Yamagata (H1N1) vaccine which are antigenically different from A/PR8, in the presence of poly(I:C) conferred complete protection against A/PR8 virus challenge in a mouse model of nasal infection, suggesting that intranasal vaccination with poly(I:C) adjuvant confers cross-protection against variant viruses [15]. Clinically safe when administered intravenously, with intranasal administration currently in the pre-clinical phase and recently shown to be a potent

inducer of innate immune responses upon subcutaneous administration [98], dsRNA, poly(I:C_{12}U)(Ampligen), was investigated as a dsRNA adjuvant for intranasal avian influenza vaccines [22].

The stronger immunogenicity of the live virus compared to the whole inactivated vaccine may be caused by many mechanisms other than stimulation of TLR7 or 3, such as additional receptors involved or a different biodistribution or kinetic profile of live virus compared to inactivated vaccines. The former might be mimicked by using a ligand for those receptors as an adjuvant, the latter two might possibly be mimicked by the use of different carriers for the antigens that will influence kinetics as well as biodistribution [99].

While we are currently still learning from influenza virus infections, ultimately we would like to design vaccines that outclass natural infections. This might be achieved by careful selection of highly conservative parts of influenza membrane proteins, in combination with several adjuvants that together will activate the required broad spectrum of tissues and cells.

A very promising combination might be nanoparticles, mostly associated with enhanced CD8+ T-cell responses, and TLR ligands, that together can induce very strong and broad humoral responses via induction of GC formation and expansion of Tfh cells [100]. For the rational design of effective vaccines directed against different pathogens, increased understanding of the mechanisms of single as well as combinations of adjuvants in great detail [98,100] will be indispensable.

Notably, recent clinical trials revealed that the intranasal administration of a whole inactivated influenza virus to healthy human subjects, without adjuvant but with a prime-boost regimen, induced high levels of nasal neutralizing antibodies that consisted primarily of polymeric IgA (unpublished data). Whether the absence of adjuvant was less important in human subjects because of the pre-existence of (cross-)protective memory due to a history of infections and/or vaccinations, we are currently investigating.

In conclusion, the induction of IgA will broaden the immune response induced by vaccines, by introducing local immune responses, adding to cross-protection, balancing pro-inflammatory responses and making memory similarly more diverse (Fig. 2). That IgA alone will most probably not be able to induce full protection in case of a heterosubtypic infection could actually be an advantage, since partial protection by IgA will reduce the viral load, while leaving enough space for the cellular immune system to get primed. This way, innate, humoral and cellular responses will all be activated, resulting in the strongest renewal of the immunological memory and ensuring the best possible preparedness for the next influenza virus that will be encountered.

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Characterization of Neutralizing Antibodies in Adults After Intranasal Vaccination With an Inactivated Influenza Vaccine

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The levels and properties of neutralizing antibodies in nasal wash and serum collected from five healthy adults were examined after intranasal administration of an A/Uruguay/716/2007 (H3N2) split vaccine (45 μg hemagglutinin (HA) per dose; five doses, with an interval of 3 weeks between each dose). Prior to the assays, nasal wash samples were concentrated so that the total amount of antibodies was equivalent to about 1/10 of that found in the natural nasal mucus. Vaccination induced virus-specific neutralizing antibody responses, which increased with the number of vaccine doses given. Neutralizing antibodies were produced more efficiently in the nasal passages than in the serum: A ≥4-fold increase in nasal neutralization titres was observed after the second vaccination in four out of five subjects, whereas a rise in serum neutralization titres was observed only after the fifth vaccination. Nasal and serum neutralizing antibodies were mainly found in the polymeric IgA and monomeric IgG fractions, respectively, after gel filtration. Taken together, these results suggest that intranasal administration of an inactivated split vaccine induces high levels of nasal neutralizing antibodies (primarily polymeric IgA) and low levels of serum neutralizing antibodies (primarily monomeric IgG). ***J. Med. Virol. 84:336–344, 2012.***

KEY WORDS: influenza; vaccine; neutralizing antibody

INTRODUCTION

To prevent influenza, protective immunity must be induced in advance by administration of a vaccine. Currently available inactivated vaccines, detergent disrupted split-viruses, or purified glycoproteins (surface antigen vaccines) are given via parenteral injection [Murphy and Webster, 1996]. Parenteral vaccination, that is, vaccination via the non-mucosal route, induces serum IgG antibodies, which are highly protective against homologous virus infection, but less effective against heterologous virus infection. Thus, intramuscular vaccination of seasonal influenza vaccine would be less effective in protecting against a heterologous virus epidemic.

A large number of studies show that the protective immunity induced by influenza virus infection is mainly mediated by secretory IgA (S-IgA) and IgG antibodies within the respiratory tract. S-IgA is carried to the mucus by transepithelial transport, while serum IgG is transported from the serum to the mucus by diffusion [Murphy and Clements, 1989; Brandtzag et al., 1994; Murphy, 1994; Asahi et al., 2002; Asahi-Ozaki et al., 2004]. S-IgA in the upper respiratory tract prevents viral infection, while IgG supports S-IgA-mediated protection by neutralizing newly-generated viruses [Ito et al., 2003; Renegar et al., 2004]. IgG is the main antibody involved in anti-viral protection in the lungs [Ramphal et al., 1979; Palladino et al., 1995; Renegar et al., 1998; Ito et al., 2003]. Also, polymeric S-IgA neutralizes viruses more effectively than monomeric IgA or IgG [Taylor

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and Dimmock, 1985; Renegar et al., 1998]. The polymeric nature of S-IgA also explains why S-IgA cross-reacts with variant influenza viruses to a greater extent than serum IgG [Tamura et al., 1990, 1991, 1992; Asahi-Ozaki et al., 2004]. Thus, intranasal administration of an inactivated influenza vaccine is advocated to elicit S-IgA and IgG responses and improve the protective efficacy of current vaccination procedures [Tamura and Kurata, 2004; Tamura et al., 2005, 2010].

Several clinical trials have examined the induction of both S-IgA and IgG following intranasal administration of inactivated influenza vaccines, either with or without adjuvant [Kuno-Sakai et al., 1994; Hashigucci et al., 1996; Muszkat et al., 2000; Greenbaum et al., 2002; Durrer et al., 2003; Treanor et al., 2006; Atmar et al., 2007]. The antibody responses after intranasal administration of inactivated influenza vaccines were assessed by measuring hemagglutination inhibition (HI) titres in the serum, and anti-hemagglutinin (HA) IgA and IgG titres in nasal wash samples. They did not measure the titre of neutralizing antibodies, which is considered to be a better criterion for functional protective antibodies. Neutralization titres can directly inhibit the complex process involved in virus replication, which include virus attachment and entry to the host cells, and release of newly-synthesized virus from the infected cells in tissue culture. In addition, a previous study found that HI titres were lower, or higher, than the corresponding neutralization titres, depending on a strain of influenza A or B virus used for the assay [Okuno et al., 1990], whereas other studies show that anti-H5 HI antibodies fail to detect H5N1 viruses [Lu et al., 1982; Rowe et al., 1999]. Thus, neutralizing antibody responses following intranasal administration of an inactivated influenza vaccine remain to be fully characterized.

Therefore, the aim of the present study was to examine the levels and properties of neutralizing-antibodies in nasal wash and serum samples from healthy adults after intranasal administration of an inactivated vaccine (five doses, with an interval of 3 weeks between each dose). The inactivated vaccine used in this study was a concentrated split-virus vaccine (containing 45 μg HA per dose), prepared from the A/Uruguay/716/2007 (H3N2) strain. A concentrated split-virus vaccine was chosen because the vaccine has already been shown to induce mucosal antibody responses after intranasal vaccination [Kuno-Sakai et al., 1994]. To ensure that neutralization titres specific for the A/Uruguay/716/2007 virus were assayed at equivalent levels in both serum and nasal wash samples, the neutralization titres were measured using concentrated nasal wash samples (1 mg/ml total protein) that contained approximately 1/10 of the IgA found in undiluted mucus [Kurono and Mogi, 1987]. The properties of the neutralizing IgA and IgG antibodies induced by intranasal vaccination were then examined, and their relative levels and molecular size were determined.

MATERIALS AND METHODS

Subjects

Five healthy male subjects (P1, P2, P3, P4, and P5) were enrolled in the study (aged 22, 32, 42, 42, and 68 years, respectively, at the time of the first vaccination). All participants had already acquired some degree of immunity to H1N1 and H3N2 influenza A virus subtypes after previous exposure to these viruses and/or as a result of previous vaccinations. Each subject provided informed consent and the study protocol and other relevant documentation were reviewed and approved by the Ethics Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

Virus and Vaccine

The A/Uruguay/716/2007 (A/Uruguay; H3N2) influenza virus strain was propagated in the allantoic cavity of 10-day-old embryonated hen's eggs and purified from the allantoic fluid. The $TCID_{50}$ (50% infectious dose in tissue culture) of the virus was estimated as described previously [Tobita et al., 1975; Kadowaki et al., 2000]. In brief, 10-fold serial dilutions of the allantoic fluid containing the virus were inoculated into Madin-Darby canine kidney (MDCK) cells (ATCC No. CCL-34) cells in 96-well culture plates and incubated for 4 days at 37°C in a 5% CO_2 humidified atmosphere. The cytopathic effects in the virus-containing wells were monitored under a microscope and the $TCID_{50}$ was calculated using the Reed–Muench method. The split product virus vaccine was supplied by the Research Foundation for Microbial Disease of Osaka University (BIKEN, Kanonji, Japan). The vaccine was prepared from purified viruses, which were sedimented through a linear sucrose gradient according to the manufacturer's protocol. The viruses were then treated with ether and formalin according to the manufacturer's protocol, which was based on the method of Davenport et al. [1964]. The concentrated split vaccine containing 45 μg HA was the product of a process used to prepare a trivalent vaccine comprising A/H1N1, A/H3N2, and B type vaccines, each containing 15 μg HA.

Vaccinations

All participants were immunized intranasally with a threefold concentrated split H3N2 virus vaccine (A/Uruguay, containing 45 μg HA). Each received five doses, with an interval of 3 weeks between each dose. Intranasal vaccination was performed by spraying 0.25 ml of the split vaccine into each nostril (0.5 ml total) using an atomizer (Keytron, Ichikawa, Japan). The mean droplet diameter was 56.5 μm, ranging in size between 10 μm and 90 μm.

Nasal Wash and Serum Samples

About 100 ml of nasal wash was collected from each participant in polypropylene tubes by washing the

nasal cavity several times using a nose irrigation device (Hananoa; Kobayashi Pharmaceutical, Osaka, Japan) filled with saline solution according to the manufacturer's instructions. Pieces of dental cotton (Dental Cotton Roll; B.S.A. Sakurai, Nagoya, Japan) were then immersed in the collected nasal washes. Dental cotton pieces (containing a combined absorbed volume of about 25 ml of nasal wash) were then placed into a filter insert (Oxi Fil filter insert; TOHO, Tokyo, Japan) with bottoms drilled to create several pores, and placed in 50 ml polypropylene centrifuge tubes. Clean nasal wash was separated from mucopolysaccharides and other debris by centrifugation at 2,200*g* for 5 min at room temperature. This procedure was repeated for the entire 100 ml nasal wash sample from each participant. The pooled, clean nasal wash was then concentrated to a final volume of approximately 0.5 ml using Vivaspin centrifugal concentrators (Vivaspin 20, MWCO = 30,000; Sartorius Stedim Biotech, Aubagne, France). The concentrated nasal wash was stored at −80°C before use.

Quantitation of IgA, IgG and IgM Antibodies and Other Proteins

The levels of human IgA, IgG, and IgM antibodies in the nasal wash and serum samples were estimated using human IgA, IgG, or IgM ELISA kits (Bethyl Laboratories, Montgomery, USA). The level of human serum albumin in the nasal wash samples was estimated using a Human Albumin ELISA kit (Bethyl Laboratories). The protein concentration in the samples was measured using either a BCA Protein Assay Kit, or a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Yokohama, Japan) according to the manufacturer's instructions.

Neutralization Assays

The level of serum antibodies against the vaccine viruses was examined using micro-neutralization assays as previously described [Belshe et al., 2000; Kadowaki et al., 2000] with minor modifications. In brief, serum samples were treated with a receptor-destroying enzyme (RDE(II); Denka Seiken, Tokyo, Japan) overnight at 37°C and heat-inactivated for 30 min at 56°C before use. The first dilution tested in the assays was 1:10. The concentrated nasal wash samples [1 mg/ml total protein, corresponding to about 1/10 of the total IgA found in nasal mucus (2.20 mg/ml)] [Kurono and Mogi, 1987] were also treated with RDE(II) and heat-inactivated before use. The first dilution tested in the nasal wash assays was 1:20. Twofold serial dilutions of the serum samples were mixed with an equal volume (50 μl) of diluent containing influenza virus equivalent to 100 $TCID_{50}$. Each mixture was added to the wells of a 96-well plate containing a monolayer of MDCK cells. Four control wells were included on each plate and contained either virus or diluent alone. The plates were then incubated for 4 days at 37°C in a 5% CO_2-humidified atmosphere. The monolayer in each well was observed for the presence or absence of cytopathic effects, fixed with 10% formalin for more than 5 min at room temperature, and stained with Naphthol blue black. After the plates were washed and dried, the stained cells were solubilized with 0.1 M NaOH and the absorbance (A) was measured at 630 nm. The average $A_{630\ nm}$ value was determined from quadruplicate virus-infected wells (A_{virus}) and cell culture-only controls (A_{cell}). All values above 50% of the specific signal, calculated using the formula: $X = (1/2) \times (A_{cell} - A_{virus}) + A_{virus}$, were considered positive for neutralization. The titres recorded were the reciprocal of the highest dilution, where A_{630} was >X.

Hemagglutination Inhibition

The antibody responses to the vaccine viruses were examined in serum and nasal washes using HI antibody assays incorporating a microtiter method as described elsewhere [Hierholzer et al., 1969]. All samples were pre-treated with RDE(II) at 37°C for 18 hr, subsequently inactivated at 56°C for 30 min, and mixed with packed red blood cells to remove any nonspecific inhibitors. The starting material for the assays was a 1:10 dilution for the serum samples and a 1:40 dilution for the nasal wash samples.

Fractionation of Nasal and Serum Samples

The concentrated nasal wash samples (100 μl, 6 mg/ml) and diluted serum samples (10-fold dilution, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 10/300 GL gel filtration column using an FPLC-AKTA chromatography system (GE Healthcare, Little Chalfont, UK). The concentrated nasal wash sample was treated with 1 μg/ml of lysozyme (Sigma-Aldrich, St. Louis, MO) for 1 hr at 37°C to decrease the viscosity and then centrifuged using Vivaspin to remove the lysozyme prior to gel filtration. Fractions (each 500 μl) were collected in PBS at a flow rate of 0.1 ml/min; little or no change in the fractionation pattern of the antibodies in the concentrated nasal wash samples was observed following lysozyme treatment. Molecular weight marker proteins (Kit for Molecular Weights 29,000–700,000 Da; Sigma-Aldrich) were eluted under the same conditions to determine the size of each fraction.

RESULTS

Measurement of Neutralization and HI Titres in Concentrated Nasal Wash Samples

The total protein level and the levels of IgA, IgG, and IgM and human serum albumin in 100 ml of unconcentrated nasal wash and in approximately 0.5 ml of concentrated nasal wash are shown in Table I. About 70% of the total nasal wash proteins were lost during the concentration process. Also, a fraction of the higher molecular weight (MW) proteins and lower MW proteins (less than 30 kDa) was lost by

TABLE I. Concentration of IgA, IgG, IgM and HSA in 0.5 ml of Solution Concentrated From 100 ml of Nasal Wash (n = 10)*

Unit	Concentration: Mean ± SD				
	Total protein	IgA	IgG	IgM	HSA
Nasal wash (n = 10)					
Unconcentrated mg/100 ml	5.875 ± 1.856	1.132 ± 0.678	0.125 ± 0.057	0.032 ± 0.021	0.531 ± 0.280
Concentrated mg/0.43 ± 0.06 ml	1.647 ± 0.549	0.375 ± 0.193	0.093 ± 0.044	0.007 ± 0.006	0.292 ± 0.214
Concentration calculated in terms of total protein (mg/ml)	1.00	0.217	0.057	0.004	0.177

*The concentration was calculated using two nasal wash samples collected from five participants (with a 1 week interval).

adsorption to the cotton and during Vivaspin centrifugation, respectively. However, better recovery was observed for IgA and IgG. When the concentration of the enriched nasal washes was adjusted to 1 mg/ml total protein, the amount of IgA was 0.217 mg/ml. This amount of IgA in the concentrated nasal wash corresponded to about 1/10 of the levels of total IgA recovered from nasal mucus (2.20 mg/ml) by aspiration as reported by Kurono and Mogi [1987] (Table I). In subsequent experiments, neutralization and HI titres in the nasal wash samples were measured using concentrated nasal wash proteins (1 mg/ml of total protein), which contained 1/10 of the IgA found in mucus, to ensure that the nasal and serum neutralization titres were assayed at equivalent levels.

The amount of total IgA and total IgG in the nasal wash samples from each participant varied slightly at each sampling time. Also, the level of total IgA and IgG antibodies did not increase significantly between pre-vaccination and post-vaccination in any of the participants. Thus, the average amount of total IgA or total IgG in the nasal wash samples from the five participants was relatively constant (data not shown).

Neutralizing Antibody Responses in Nasal Wash and Serum Samples

Next, antibody responses in the nasal wash and serum samples were examined in all five study participants. The responses are presented as neutralization titres against the A/Uruguay (H3N2) virus in Table II. The responses recorded in the four young adults (between 18- and 50-years-old) are also shown as geometric neutralization titres (Fig. 1). The nasal wash and serum neutralization titres increased in all participants as the number of vaccinations increased, although the degree of increase differed between participants. In addition, nasal wash neutralization titres increased more rapidly than serum titres. The nasal wash titres showed at least a fourfold increase after the second vaccination in the four young participants (all of whom had a nasal wash neutralization titre of 1:20 or 1:40 before vaccination). By contrast, a fourfold increase in the serum titre was observed only after the fifth vaccination in three of the participants (all of whom had serum titres of <1:10, 1:20, or 1:40 before vaccination). Participant P5, who was 67 years old, showed at least a fourfold increase in nasal wash titre after the fourth vaccination, but no significant

TABLE II. Neutralizing Antibody Responses in Subjects who Received the Threefold Concentrated A/Uruguay/716/2007 (H3N2) Split Vaccine

Weeks (vaccination)	Neutralization titre against A/Uruguay virus (H3N2)[a]									
	P1		P2		P3		P4		P5	
	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum	Nasal wash	Serum
0 (1st)	20 (1)	40 (2)	20 (1)	<10 (<0)	40 (2)	20 (1)	20 (1)	<10 (<0)	20 (1)	<10 (<0)
3 (2nd)	80 (3)	160 (4)	20 (1)	<10 (<0)	80 (3)	20 (1)	20 (1)	<10 (<0)	40 (2)	<10 (<0)
6 (3rd)	160 (4)	160 (4)	80 (3)	10 (0)	320 (5)	20 (1)	80 (3)	<10 (<0)	40 (2)	<10 (<0)
9 (4th)	320 (5)	160 (4)	160 (4)	20 (1)	1280 (7)	40 (2)	160 (4)	10 (0)	40 (2)	<10 (<0)
12 (5th)	320 (5)	160 (4)	320 (5)	40 (2)	2560 (8)	80 (3)	80 (3)	10 (0)	80 (3)	<10 (<0)
15	1280 (7)	160 (4)	320 (5)	40 (2)	2560 (8)	80 (3)	160 (4)	20 (1)	160 (4)	10 (0)
28	640 (6)	160 (4)	160 (4)	40 (2)	1280 (7)	80 (3)	N.D.	N.D.	80 (3)	10 (0)

N.D., not done.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

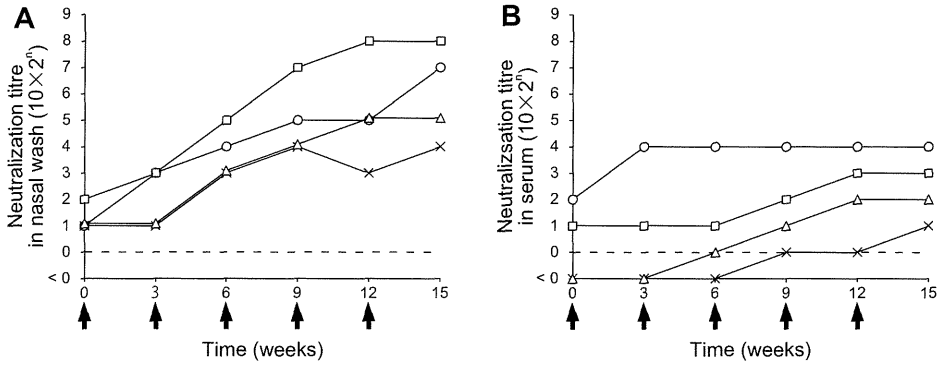

Fig. 1. Nasal wash and serum sample neutralization titres against A/Uruguay (H3N2) (pre- and post-intranasal immunization). Neutralization titres against the A/Uruguay virus in nasal washes (**A**) and serum (**B**) were determined in samples collected from four participants (18–60 years old; P1, open circle; P2, open triangle; P3, open square; and P4, cross). The participants were given five doses of the threefold concentrated A/Uruguay split influenza vaccine intranasally with an interval of 3 weeks between doses (each arrow indicates a point of vaccination). The neutralization titre shown is the geometric titre.

increase was observed in serum titre after five vaccinations. In all participants, the nasal wash and serum titres were largely maintained, even at 16 weeks after the fifth vaccination, at which point the nasal wash titre decreased only slightly, while no decrease was observed in the serum titre (Table II).

HI Antibody Responses in Nasal Wash and Serum Samples

Antibody responses were also examined by measuring the HI titre against the A/Uruguay (H3N2) virus. Table III shows the pre-vaccination HI titres of the nasal wash and serum samples from two participants, and the HI titres 3 weeks after each of the five vaccinations. For each participant, the HI titres were lower than the neutralization titres shown in Table II. The HI titres were approximately 1/4–1/8 the level of the neutralization titres. Statistical correlation analysis of the data presented in Tables II and III showed a strong correlation between the HI titres and the neutralization titres ($r = 0.8699$). Thus, the HI titre correlated with the neutralization titre, although it was less sensitive than the neutralization titre.

TABLE III. Hemagglutinin Inhibition (HI) Antibody Responses in Subjects who Received the Threefold Concentrated A/Uruguay/716/2007 (H3N2) Split Vaccine

	HI titre against A/Uruguay virus (H3N2)[a]			
	P1		P2	
Weeks (vaccination)	Nasal wash	Serum	Nasal wash	Serum
0 (1st)	N.D.	10 (0)	<40 (<2)	<10 (<0)
3 (2nd)	<40 (<2)	20 (1)	<40 (<2)	<10 (<0)
6 (3rd)	<40 (<2)	20 (1)	<40 (<2)	<10 (<0)
9 (4th)	40 (2)	20 (1)	<40 (<2)	10 (0)
12 (5th)	40 (2)	20 (1)	80 (3)	20 (1)
15	160 (4)	40 (2)	80 (3)	20 (1)

N.D., not done.
[a]Respective values are a reciprocal titre and a geometric titre (10×2^n) in a parenthesis.

Fractionation of The Nasal Wash and Serum Samples

The types of antibody present in the nasal wash and serum samples were examined after fractionation on a gel filtration column. The concentrated nasal wash samples (100 μl, about 6 mg/ml) and diluted serum samples (10-fold diluted sera, 100 μl, about 6 mg/ml) were fractionated on a Superose 6 column in PBS. The antibody concentration in each fraction was then measured by ELISA. Figure 2 shows the profiles for IgM, IgA, and IgG antibodies, together with the absorbance values for the total protein in each fraction. The nasal wash samples contained IgM, which comprised less than 1% of the total protein and showed a peak MW of 970 kD; IgA, which comprised about 20% of the total protein and showed a peak MW of about 660 kD; and IgG, which comprised about 6% of the total protein and showed a peak MW of 150 kD. The MW of the nasal IgA (150 kD–900 kD, with a peak MW of 660 kD) appeared to correspond to that of tetrameric IgA (the MW of dimeric IgA is estimated to be about 360 kD). The maximum absorbance value observed in the protein profile (at around 66 kD) was due to the presence of human serum albumin (Fig. 2A).

The serum samples contained IgM, which comprised about 3% of the total protein and showed a peak MW of 970 kD; IgA, which comprised about 6% of the total protein and showed a peak MW of about 150 kD; and IgG, which comprised about 23% of the total protein and showed a peak MW of 150 kD