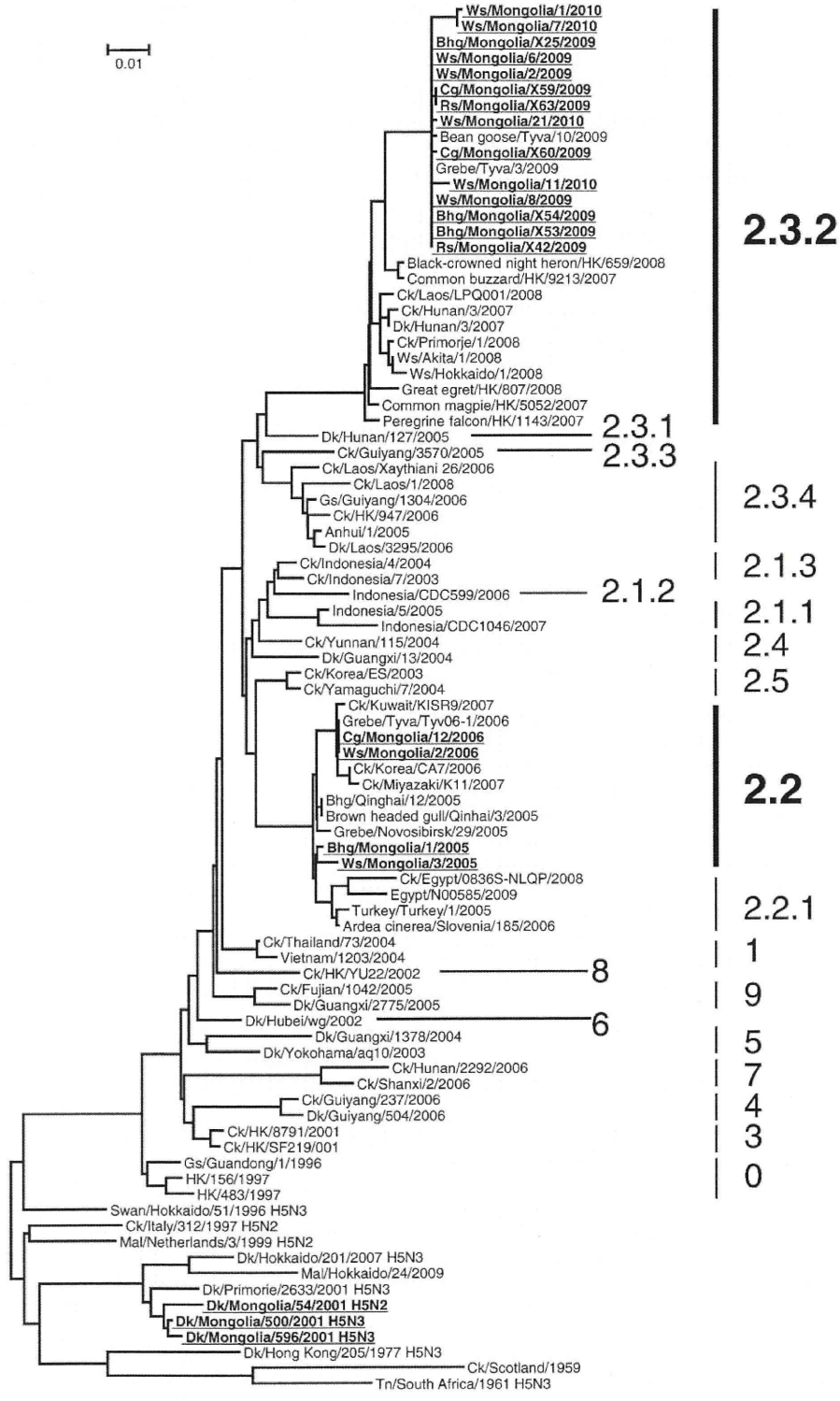

Fig. 1. Phylogenetic trees of the HA genes of H5 influenza viruses. Nucleotide sequences (976 bp) of the HA genes of H5 avian influenza viruses isolated in Mongolia (shown in bold and underlined) and the sequence information of other related viruses were cited from the public database for phylogenetic analysis. The sequence data of Dk/Mongolia/54/01 (H5N2), Dk/Mongolia/500/01 (H5N3), and Dk/Mongolia/596/01 (H5N3) were determined in our previous study (Soda et al., 2008). Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. Genetic classification (clades 0 to 9) was indicated for recent H5N1 HPAI viruses. HA and NA subtypes were eliminated for the names of H5N1 viruses. Abbreviations: Bhg (bar-headed goose), Ws (whooper swan), Cg (common goldeneye), Rs (ruddy shelduck), Ck (chicken), Dk (duck), Gs (goose), Mal (mallard), Tn (tern), and HK (Hong Kong).

Table 2
Experimental infection of H5N1 HPAI viruses in 4-week-old SPF pigs and virus recovery from nasal swabs.

Inoculated viruses	Virus titers on the dpi (log EID_{50}/ml)							
	0	1	2	3	4	5	6	7
Ws/Mongolia/3/05 (H5N1)	–[a]	3.3	3.8	2.0	2.6	3.8	2.6	–
	–	3.3	4.3	2.8	–	–	–	–
Ws/Mongolia/2/06 (H5N1)	–	0.8	2.8	3.0	3.3	4.5	5.0	3.8
	–	–	1.8	2.0	4.3	4.3	4.5	3.3
Ws/Mongolia/6/09 (H5N1)	–	3.5	2.8	–	≦1.3	1.5	–	–
	–	4.3	2.8	–	–	–	–	–

[a] –: <0.5 log EID50/ml.

intranasally inoculated at $10^{8.0}$ EID_{50} into six 4-week-old ducks (Table 3). Viruses were recovered from each of the tested samples of the ducks euthanized on 3 days post-inoculation (dpi). The titers of tissue samples from ducks infected with Ws/Mongolia/6/09 were relatively higher than those with Ws/Mongolia/3/05 or Ws/Mongolia/3/06. Three ducks of each group were kept for 14 days to observe the clinical signs of infected ducks. One of the 3 ducks infected with Ws/Mongolia/3/05 died on 9 dpi. Viruses were recovered only from the brain homogenate of this duck. Several neurological signs, such as depression, blindness, and intermittent head-shaking, were observed from 5 dpi onward in all ducks infected with Ws/Mongolia/3/05, and two recovered and survived on 14 dpi. For ducks infected with Ws/Mongolia/2/06, all three ducks survived during the experiment without showing any typical clinical signs. In contrast to the isolates in 2005 and 2006, the ducks infected with Ws/Mongolia/6/09 died earlier, on 4, 5, and 8 dpi, and showed depression and intermittent head-shaking before their death. Viruses were recovered from each of the tested tissues of dead ducks. Notably, the titers of tissue samples from 2 ducks that died on 4 and 5 dpi were clearly higher ($10^{7.5}$-$10^{9.5}$ EID_{50}/g) than those of the others.

Identification of avian influenza virus isolates from fecal samples of wild waterfowl

Since 2001 we have conducted surveillance studies on avian influenza in wild waterfowl in autumn at several lakes in Mongolia, including Khunt, Erkhel, Doityn Tsagaan, and Doroo Lakes, where migrating waterfowl congregate and H5N1 HPAI viruses were isolated from dead carcasses in 2005, 2006, and 2009. By 2009, 6,211 fecal samples of waterfowl had been collected and inoculated into chicken embryos. As a result, 338 avian influenza viruses of 10 different HA subtypes (H1, H2, H3, H4, H5, H7, H8, H9, H10, H12) were isolated, as shown in Table 4. A/duck/Mongolia/54/2001 (H5N2), A/duck/Mongolia/500/2001 (H5N3), and A/duck/Mongolia/596/2001 (H5N3) (underlined in Table 4) were isolated as H5 viruses in 2001. From sequence data of these isolates obtained previously (Soda et al., 2008), it was clear that the cleavage site of the HA of these H5 viruses had a low pathogenic profile without a pair of dibasic amino acid residues and these H5 viruses were genetically different from H5N1 HPAI virus isolates in Mongolia in phylogenetic analysis (Fig. 1). The results indicate that H5N1 HPAI viruses have not so far perpetuated at their nesting lakes in Siberia until 2009, since H5N1 HPAI viruses were isolated from migratory waterfowl only on their way back to their northern territory, not from those flying south from Siberia in autumn.

Discussion

Since 2005, numerous cases of H5N1 HPAI virus infection in wild birds have been found in Eurasian and African countries. The viruses of clades 2.2 and 2.2.1 are still epidemic in Asian and African countries in poultry and wild birds (WHO/OIE/FAO H5N1 Evolution Working Group, 2009). This suggests that H5N1 viruses prevailing in domestic birds have transmitted to wild migratory waterfowl by water-borne transmission repeatedly and it was a concern that these H5N1 viruses may perpetuate among migratory waterfowl and in their nesting lake water in nature. In Mongolia, H5N1 viruses of clade 2.2 were isolated from waterfowl spontaneously in 2005 and 2006 after the infections in Qinghai Lake, China. In May and July 2009, H5N1 viruses of clade 2.3.2 were isolated from whooper swans, bar-headed goose, common goldeneye, and ruddy shelduck at Doityn Tsagaan and Doroo Lakes. Furthermore, H5N1 viruses of clade 2.3.2 were isolated again from whooper swans at Ganga Lake in May 2010. H5N1 viruses of clade 2.3.2 were first identified from ducks, geese and other mammals in China and Vietnam in 2005 (Chen et al., 2006; Roberton et al., 2006). In addition, H5N1 viruses of clades 2.3.2 and 2.3.4 were isolated from wild birds in Hong Kong (Ellis et al., 2009; Smith et al., 2009). H5N1 viruses of clade 2.3.2 were also isolated in Japan, Korea, and Russia in 2008 from whooper swan (L'Vov et al., 2008; Uchida et al., 2008). In the present study, genetic analyses indicate that H5N1 isolates in Mongolia in 2009 and 2010 were closely related with those in Russia, China, Laos, and Japan. In particular, the homologies of nucleotides of each segment between Ws/Mongolia/6/09 and A/grebe/Tyva/3/2009 (H5N1), which was isolated in Russia (accession No. GQ386142–GQ386149), ranged from 99.8% to 99.9%. It is clear that these waterfowl were infected with the same H5N1 viruses in southern areas and flew north since the place and date of outbreaks were closely related according to information from the OIE (2009b). In addition, waterfowl were infected again with the similar H5N1 viruses of clade 2.3.2 in southern areas and flied to the north in 2010. H5N1 viruses isolated from wild birds in Hong Kong in 2007 and 2008 also showed high homology with H5N1 isolates in Mongolia in 2009 and 2010, suggesting that the origin of these viruses was H5N1 viruses prevailing in domestic poultry in China, and those progeny viruses must have transmitted to wild migratory waterfowl by water-borne transmission every year.

Table 3
Experimental infection of H5N1 HPAI viruses in 4-week-old domestic ducks and virus recovery from organs.

Inoculated viruses	No. of ducks	dpi (Health status)	Virus titers of organs (log EID_{50}/g)				
			Brain	Trachea	Lungs	Kidneys	Colon
Ws/Mongolia/3/05 (H5N1)	3	3 (sacrificed)	4.5, 5.3, 6.3	4.0, 5.5, 6.3	4.0, 5.5, 6.3	5.8, 6.3, 6.5	4.3, 4.6, 4.8
	1[a]	9 (dead)	3.3	–[b]	–	–	–
	2[a]	14 (sacrificed)	–,–	–,–	–,–	–,–	–,–
Ws/Mongolia/2/06 (H5N1)	3	3 (sacrificed)	2.3, 3.8, 3.8	4.0, 4.3, 4.3	4.0, 4.3, 4.3	3.5, 4.3, 5.0	3.5, 4.0, 4.0
	3[c]	14 (sacrificed)	–,–,–	–,–,–	–,–,–	–,–,–	–,–,–
Ws/Mongolia/6/09 (H5N1)	3	3 (sacrificed)	4.3, 7.3, 7.3	5.7, 6.8, 8.5	6.5, 6.8, 7.8	6.0, 7.5, 8.3	4.8, 5.8, 7.6
	1[a]	4 (dead)	9.5	9.3	8.5	8.5	8.5
	1[a]	5 (dead)	8.3	7.5	9.5	8.3	8.3
	1[a]	8 (dead)	3.8	5.5	4.5	3.0	3.5

[a] Each duck showed depression, blindness, and head-shaking.
[b] <1.5 log EID50/g.
[c] One of the three ducks showed depression and blindness at 5-8 dpi and survived for 14 days.

Table 4
Isolation of avian influenza viruses from fecal samples of migratory waterfowl in Mongolia.

Sampling date	Name of lakes	Isolated viruses/Total samples	Subtypes of viruses[a] (No. of isolates)
Sep., 2001	Ugii, Doityn tsagaan,	37/725	H1N1 (1), H3N2 (1), H3N6 (3), H3N8 (11), H4N2 (1), H4N6 (12), H5N2 (1), H5N3 (2), H7N1 (1), H10N3 (4)
Sep., 2002	Erkhel, Ugii	109/959	H1N1 (3), H3N3 (2), H3N6 (20), H3N8 (53), H4N6 (12), H4N7 (1), H4N8 (1), H7N1 (1), H7N7 (9), H8N4 (5), H10N7 (1), H12N5 (1)
Sep., 2003	Ugii,	68/750	H1N1 (1), H2N3 (1), H3N6 (6), H3N8 (28), H4N2 (1), H4N6 (25), H9N2 (1), H10N5 (5)
Sep., 2005	Ugii,	32/476	H3N2 (1), H3N6 (2), H3N8 (10), H4N6 (6), H8N4 (1), H10N3 (11), H10N7 (5)
Aug., 2006	Khunt, Ugii, Borgin, Shorvog, Baga Tsaisam, Duut, Ikh Tsaidam, Doityn tsagaan	18/545	H2N2 (1), H3N8 (8), H4N6 (9)
Aug., 2007	Khunt, Ugii, Dunt, Ikh Tsaidam, Doityn tsagaan	20/943	H3N8 (14), H4N3(1), H7N6 (1), H7N7 (4)
Aug., 2008	Khunt, Ugii, Dunt, Ikh Tsaidam, Doityn tsagaan	40/792	H3N6 (3), H3N8 (23), H4N6 (8), H4N8 (3), H7N9 (3)
Aug., 2009	Ugii, Doityn tsagaan, Khunt Doroo, Sharga	9/1021	H1N8 (1), H3N8 (2), H4N6 (3), H8N4 (3)

[a] H5 isolates, A/duck/Mongolia/54/2001 (H5N2), A/duck/Mongolia/500/2001 (H5N3), and A/duck/Mongolia/596/2001 (H5N3), were underlined.

All cases of H5N virus infection in 2005, 2006, 2009, and 2010 were in May and July, when wild waterfowl migrate from the southern Asia to their nesting lakes in Siberia. In addition, H5N1 viruses genetically related to the isolates in Mongolia were prevailing in domestic poultry in the southern Asia, although no outbreak of HPAI was so far reported in poultry in Mongolia. Furthermore, the results of intensive surveillance of avian influenza in migratory waterfowl flying from their nesting lakes in Siberia to Mongolia in every autumn indicate that no HPAI virus has been isolated from wild waterfowl flying from their nesting lakes until 2009. These results demonstrate that wild waterfowl were sporadically infected with H5N1 HPAI viruses prevailing in domestic poultry in the southern Asia and died in Mongolia on the way back to their northern territory in spring and no HPAI virus has perpetuated at their nesting lakes in Siberia until 2009. To reduce the risk of the perpetuation of HPAI viruses among migratory waterfowl at their nesting lakes in Siberia, HPAI viruses should be contained within poultry in the southern Asia by a stamping-out strategy, the basic control measure of HPAI.

It was proposed that the expression of sialic acid receptors for human and avian influenza viruses on epithelial cells of the trachea renders pigs susceptible to infection with both types of influenza viruses (Ito et al., 1998). From the previous experience of pandemic influenza, pigs play an important role as a "mixing vessels" to generate pandemic influenza virus as a genetic reassortant between avian and human influenza viruses (Kida et al., 1988, 1994). In this experiment, all 3 H5N1 viruses replicated in pigs, but the titers of nasal swabs and the period of virus shedding were lower than the infections with swine influenza viruses (Bai et al., 2005). Although the susceptibility of domestic pigs to H5N1 avian influenza viruses is not high (Isoda et al., 2006; Lipatov et al., 2008), natural pig-to-pig infections with H5N1 avian influenza viruses have been found (Choi et al., 2005; Takano et al., 2009). A surveillance study of influenza virus infection in pigs should be promoted to assess the prevalence of H5N1 viruses in pigs and the pathogenicity of these isolates in mammals and birds for future pandemics in humans.

Originally, non-pathogenic avian influenza viruses isolated from migratory waterfowl replicated only in columnar epithelial cells, forming crypts in the large intestine, and were excreted in the fecal materials (Kida et al., 1980; Webster et al., 1978). In the case of H5N1 HPAI viruses, recent isolates acquired lethal pathogenicity in waterfowl, although previous H5N1 isolates also replicated systemically and did not show lethal clinical signs in ducks (Chen et al., 2004; Hulse-Post et al., 2005; Kim et al., 2008; Pantin-Jackwood et al., 2007; Sturm-Ramirez et al., 2005). In the present study, we examined the pathogenicity of Ws/Mongolia/3/05 (clade 2.2), Ws/Mongolia/2/06 (clade 2.2), and Ws/Mongolia/6/09 (clade 2.3.2) of H5N1 viruses in domestic ducks. To assess the pathogenicity of avian influenza in ducks, the age and strain of ducks, infectivity titers of the inocula, and the route of inoculation influence the results (Keawcharoen et al., 2008; Kim et al., 2008; Pantin-Jackwood et al., 2007). In our studies, including previous experiments (Kishida et al., 2005), H5 avian influenza viruses of $10^{8.0}$ EID_{50} were inoculated intranasally into 4-week-old domestic ducks of Chelly Valley strain. It is noted that systemic replication with low mortality of Ws/Mongolia/3/05 and Ws/Mongolia/2/06, and high mortality of Ws/Mongolia/6/09 was observed as compared with previous reports (Brown et al., 2006; Kishida et al., 2005; Pfeiffer et al., 2009). The present results support that H5N1 influenza viruses have evolved to cause lethal infection in ducks since multiple infections of domestic ducks and wild birds with these viruses have continued in epidemic areas. Further investigation on the H5N1 virus infections in wild birds is needed in addition to the recent studies (Hulse-Post et al., 2007; Reed et al., 2010) since they are not sufficient to understand on the molecular basis of the pathogenicity of these H5N1 isolates in ducks.

In conclusion, H5N1 HPAI viruses were isolated from migratory waterfowl only on their way back to their northern territory, and not from those flying to the south from Siberia in autumn, suggesting that H5N1 HPAI viruses have not perpetuated at their nesting lakes in Siberia until 2009. For the control of influenza virus infection in birds and mammals, the global surveillance to understand the ecology of influenza viruses and stamping out policy to contain the HPAI viruses in the domestic poultry are essential.

Materials and methods

Isolation and identification of viruses

Virus isolation was carried out from the homogenate of the brain, lungs, spleen of bar-headed goose (*Anser indicus*), whooper swan (*Cygnus cygnus*), common goldeneye (*Bucephala clangula*), and ruddy shelduck (*Tadorna ferruginea*), which were found as carcasses in the Khunt, Erkhel, Doityn Tsagaan, Doroo, and Ganga Lakes, Mongolia in July 2005, May 2006, May 2009, July 2009, and May 2010 (Table 1). Ten percent organ homogenates were inoculated into the allantoic cavities of 10-day-old chicken embryos. Subtypes of influenza virus isolates were identified by hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests using antisera to the reference strains of influenza viruses (Kida and Yanagawa, 1979).

A total of 6,211 fecal samples was collected from waterfowl in 2001–2009 in Mongolia. Each sample was mixed with minimum essential medium (MEM) containing antibiotics and inoculated into the allantoic cavities of 10-day-old chicken embryos. Subtypes of influenza virus isolates were identified by HI and NI tests as described above.

Sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluid of chicken embryos infected with viruses by TRIzol LS Reagent (Invitrogen) and reverse-transcribed with the Uni12 primer (Hoffmann et al., 2001) and M-MLV Reverse Transcriptase (Invitrogen). The full-length genome of each gene segment was amplified by polymerase chain reaction with gene-specific primer sets (Hoffmann et al., 2001). Direct sequencing of each gene segment was performed using an auto sequencer, CEQ 2000XL (Beckman Coulter). The nucleotide sequences of H5 isolates obtained in the present study have been registered in GenBank/EMBL/DDBJ, as shown in Table 1.

To assess genetic relationship among H5 influenza virus strains, the sequence of 976 bp of the HA gene of each isolate was compared with those of H5 viruses from our previous study (Soda et al., 2008) and the public database. Phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987).

Experimental infection of chickens, pigs, and domestic ducks with H5N1 isolates

To assess the pathogenicity of H5N1 isolates, each virus was inoculated into chickens (*Gallus gallus*), pigs (*Sus scrofa domestic*), and domestic ducks (*Anas platyrhynchos var. domesuticus*), respectively. For the intravenous pathogenicity index (IVPI) test, 0.1 ml of 1:10 dilutions of infectious allantoic fluids were inoculated intravenously into ten 6- or 7-week-old chickens (Boris brown, Japan). The IVPI was calculated according to the standard protocol (OIE, 2009a).

For the pathogenicity test in pigs, 1 ml of each H5N1 isolate containing $10^{8.0}$ EID_{50} was inoculated intranasally into two 4-week-old specific pathogen-free pigs (Sankyo Lab Service, Japan) and nasal swabs of each pig were collected daily in 2 ml MEM containing antibiotics from 1 to 7 dpi for virus recovery.

For the pathogenicity test in ducks, 0.1 ml of each H5N1 isolate containing $10^{8.0}$ EID_{50} was inoculated intranasally into six 4-week-old ducks (Chelly Valley, Japan). Three of the ducks were euthanized on 3 dpi and the brain, trachea, lungs, kidneys and colon were collected aseptically for virus recovery. The remaining 3 ducks were observed clinically for 14 days after inoculation. On the death of ducks, their tissues were collected for virus recovery. The sera and organs were collected from survived ducks for antibody response and virus recovery. Swab samples of pigs and tissue homogenates from ducks were inoculated into 10-day-old embryonated chicken eggs and virus titers were calculated and expressed as the EID_{50} per ml (swab) or gram (tissue). For the evaluation of immune response, specific antibodies were detected by hemagglutination-inhibition test in 0.025 ml of collected duck sera according to the standard protocol (OIE, 2009a).

Each animal was housed in a self-contained isolator unit (Tokiwa Kagaku, Japan) at a BSL-3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

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Antigenic, genetic, and pathogenic characterization of H5N1 highly pathogenic avian influenza viruses isolated from dead whooper swans (*Cygnus cygnus*) found in northern Japan in 2008

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Abstract In April and May 2008, whooper swans (*Cygnus cygnus*) were found dead in Hokkaido in Japan. In this study, an adult whooper swan found dead beside Lake Saroma was pathologically examined and the identified H5N1 influenza virus isolates were genetically and antigenically analyzed. Pathological findings indicate that the swan died of severe congestive edema in the lungs. Phylogenetic analysis of the HA genes of the isolates revealed that they are the progeny viruses of isolates from poultry and wild birds in China, Russia, Korea, and Hong Kong. Antigenic analyses indicated that the viruses are distinguished from the H5N1 viruses isolated from wild birds and poultry before 2007. The chickens vaccinated with A/duck/Hokkaido/Vac-1/2004 (H5N1) survived for 14 days after challenge with A/whooper swan/Hokkaido/1/2008 (H5N1), although a small amount of the challenge virus was recovered from the tissues of the birds. These findings indicate that H5N1 highly pathogenic avian influenza viruses are circulating in wild birds in addition to domestic poultry in Asia and exhibit antigenic variation that may be due to vaccination.

Keywords H5N1 highly pathogenic avian influenza virus · Whooper swan · Antigenic variation

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Introduction

Since 2003, H5N1 highly pathogenic avian influenza viruses (HPAIVs) have spread to 63 countries in Asia, Europe, and Africa. Japan and some other countries where H5N1 virus infection occurred in poultry flocks were successful in rapid eradication of the infection by an aggressive stamping-out policy [1, 2]. However, the virus still persists in Asian and North African countries. Thousands of migratory birds of several species died due to H5N1 HPAIV infection at Qinghai Lake in China in 2005 [3, 4]. Viruses similar to the Qinghai-virus spread to Asia, Europe, and Africa [5–7], raising concerns that migratory birds may transmit HPAIVs to poultry and even to humans.

The responses to infection with the H5N1 HPAIV vary in different wild water birds. Ducks inoculated with HPAIV survived and showed neurological signs with the replication of the virus in the brain [8, 9]. On the other hand, highly susceptible species such as swans (*Cygnus* spp.) showed high mortality by infection with HPAIV [10]. There are few reports on the pathology of swans naturally infected with H5N1 HPAIV [11, 12].

The long-term endemic of H5N1 virus in poultry has been known to generate antigenically and genetically diversified viruses in some countries. A broadly cross-protective vaccine for antigenic variants of H5N1 viruses may be a useful option as a tool for the control of avian influenza [13]. Previously, we developed avian influenza vaccine prepared

from non-pathogenic avian influenza viruses isolated from migratory ducks [14]. The vaccine conferred protective immunity to suppress the manifestation of disease signs and reduction of virus shed in chickens and monkeys (*Cynomolgus macaques*) against H5N1 viruses isolated in 2004 and 2005 [15, 16].

In this study, a whooper swan found dead beside Lake Saroma was pathologically examined and the H5N1 virus isolate was compared genetically and antigenically with other isolates from swans found dead in Japan in 2008 [20, 21]. An inactivated avian influenza vaccine prepared from A/duck/Hokkaido/Vac-1/2004 (H5N1) [15] was also assessed for its potency to suppress the manifestation of disease signs.

Materials and methods

Viruses

A/whooper swan/Hokkaido/1/2008 (H5N1) (Ws/Hok/1/08) and A/whooper swan/Hokkaido/2/2008 (H5N1) (Ws/Hok/2/08) were isolated from trachea of whooper swans found dead at Notsuke Peninsula and at Lake Saroma, respectively, in Hokkaido Prefecture, Japan. All viruses used in this study were propagated in 10-day-old embryonated chicken eggs at 35°C for 30 to 48 h and stored at −80°C until use.

Sequencing and phylogenetic analysis

Viral RNAs were extracted with TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) from allantoic fluids. Nucleotide sequences of all eight gene segments were determined after RT-PCR as described previously [14]. The sequence data were analyzed using GENETYX ver. 9.1 (GENETYX Corporation, Tokyo, Japan). Phylogenetic analysis of HA gene was performed using BioEdit ver. 7.0 and MEGA 4 by the neighbor-joining method with 1000 bootstraps. The nucleotide sequences obtained in this study are available from DDBJ/EMBL/GenBank under accession numbers AB436547–AB436554 and AB436899–AB436906.

Intravenous pathogenicity test in chickens

The intravenous pathogenicity test of chickens for influenza viruses was carried out according to the OIE standard method [13]. Briefly, 1/10 dilutions of infectious allantoic fluids containing the viruses were intravenously inoculated into eight 7-week-old chickens. These chickens were housed for 10 days in self-contained isolator units (Tokiwa Kagaku Kikai Co., Ltd., Tokyo, Japan) at a BSL 3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. All animal experiments were conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan.

Histopathology and immunohistochemistry

An adult male whooper swan found dead beside Lake Saroma on 5th May 2008, was pathologically examined. The tissues of the swan were fixed in 10% formalin in PBS (pH 7.2). Paraffin-embedded sections were processed for hematoxylin and eosin staining and immunohistochemistry. For the detection of influenza virus antigens in the tissues, the sections were incubated with rabbit anti-A/whistling swan/Shimane/499/1983 (H5N3) hyper-immune serum at 1:1000 dilution. Bound antibodies were detected by the peroxidase-labeled streptoavidin–biotin method (Histofine SAB-PO rabbit kit: Nichirei, Tokyo, Japan).

Antigenic analysis of the viruses

Hemagglutination-inhibition (HI) test was performed as described by Sever [17]. A panel of monoclonal antibodies to H5 HA of A/duck/Pennsylvania/10218/1984 (H5N2) was used as previously described [14]. Hyper-immune antisera against Ws/Hok/1/08, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04), and A/tern/South Africa/1961 (H5N3) were prepared according to Kida and Yanagawa [18].

Potency test of vaccine efficacy in chickens against Ws/Hok/1/08

The inactivated avian influenza virus Dk/Vac-1/04 vaccine was intramuscularly inoculated to chickens as described previously [15, 19]. Briefly, Dk/Vac-1/04 was inactivated with 0.1% formalin and mixed with oil adjuvant containing anhydromannitol-octadecenoate-ether (AMOE). Eleven four-week-old chickens were intramuscularly immunized and, 3 weeks later, challenged intranasally with a dose 100-fold that of 50% chicken lethal dose (CLD_{50}) of Ws/Hok/1/08. Clinical signs were monitored for 14 days post-challenge (p.c.) and chickens were sacrificed on day 2 and 4 p.c. When chickens died or were sacrificed, tracheal and cloacal swabs and their tissues (trachea, lung, kidney, and colon) were collected. Virus titers were measured by 50% egg infectious dose (EID_{50}).

Results

Pathological findings of the whooper swan

A whooper swan found dead beside Lake Saroma on 5th May 2008 presented as well-nourished with sufficient body fat reserves. Gross lesions were not found except for some

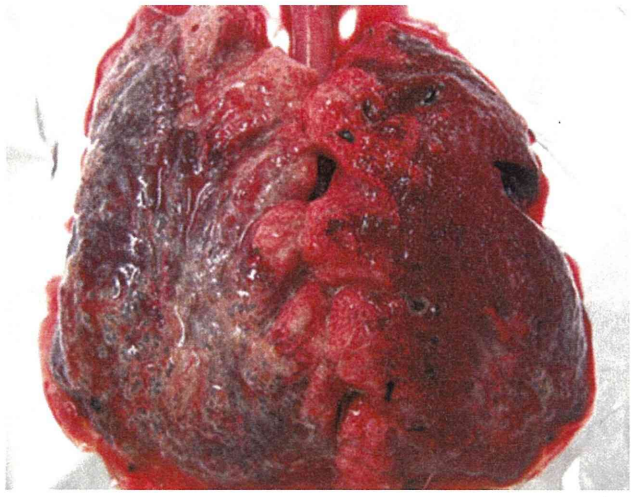

Fig. 1 Gross appearance of the lungs of whooper swan found dead beside Lake Saloma. The lungs show diffuse congestive edema. The pleura is edematously thickened

damage to its head and neck, which may have been due to bites by a wild animal. At necropsy, the swan showed diffuse severe congestive edema of the lungs with thickening of the pleura (Fig. 1). Echymotic hemorrhage was scattered in the pancreas and epicardium. A whooper swan found dead in Notsuke Peninsula on 24th April 2008 was not pathologically examined since the body had already decomposed when it arrived.

The predominant histological lesions were found exclusively in the brain, pancreas, and lungs. In the cerebrum and cerebellum, glial nodules were scattered with spongiform change of the neuropil and with necrosis of nerve and glial cells (Fig. 2a). Small necrotic foci of acinar cells were observed in the pancreas (Fig. 2c). Only a small number of heterophils and macrophages were infiltrated in the cerebral and pancreatic lesions. The lungs were severely congested with diffuse moderate edema of interlobular and peribronchial connective tissues. Small amounts of fibrin and heterophils exuded into parabronchi and infundibula. By the immunohistochemical examination, influenza virus antigens were found in the brain, pancreas, lungs, and trachea. In the cerebrum and cerebellum, nerve and glial cells within and around the glial nodules were stained positive by hyperimmune serum to A/whistling swan/Shimane/499/1983 (H5N3) (Fig. 2b). In the necrotic areas of the pancreas, some necrotic and degenerative acinar cells were stained positive (Fig. 2d). In the lungs and trachea, the antigen was detected in only a few respiratory and mucosal epithelial cells.

Pathogenicity of the isolates in chickens

Ws/Hok/1/08 (H5N1) and Ws/Hok/2/08 (H5N1) were inoculated intravenously into eight 7-week-old chickens, respectively. Within 2 days post-inoculation, all chickens died. This result was consistent with a prediction based on the amino acid sequence at the cleavage site of the HA protein of the isolates in Hokkaido (PQRERRRKR/GLF).

Genetic analysis of virus isolates from whooper swans

To elucidate the genetic relationships of the isolates with other H5N1 influenza virus isolates, all eight gene segments were compared. It was revealed that the all gene segments of the isolates were closely related to each other and to the H5N1 HPAIVs isolated from whooper swans in Akita and Aomori Prefectures in 2008 (more than 99.0% similarity in all genes) [20, 21]. It was also revealed that all isolates found in Japan in 2008 were closely related to those of isolates found in Korea in 2008 (personal communication). Phylogenetic analysis of the HA genes showed that these isolates belonged to Clade 2.3.2 and formed a unique branch with isolates found in Hong Kong in 2007-08 and those found in Russia in 2008 (Fig. 3).

Antigenic characterization of the HA of the isolates

Antigenic analysis of the HA of the isolates with antisera to H5 of influenza viruses and monoclonal antibodies to the HA of A/duck/Pennsylvania/10218/1984 (H5N2) was performed by HI test. The antigenicities of the HA of the isolates in 2008 were similar to each other but different from those of Dk/Vac-1/04, which is the reassortant virus generated from the isolates from fecal samples of wild ducks, and H5N1 HPAIVs isolated from chickens and whooper swans in Asia (Table 1).

Potency of the vaccine against the isolate in chickens

Ws/Hok/1/08 (H5N1) was selected as the challenge strain for the vaccine potency test since the isolates from whooper swans were genetically and antigenically identical. Eleven chickens intramuscularly inoculated with the vaccine prepared from Dk/Vac-1/04 and 3 non-vaccinated chickens were challenged intranasally with Ws/Hok/1/08 on 3 weeks after vaccination. The HI titers of the sera of the vaccinated chickens were 1:128–512 and 1:4–8 with the vaccine strain and with the isolate, respectively. All vaccinated chickens survived without showing any disease signs after challenge, whereas all of the control chickens died within 2 days p.c. Viruses were not recovered from the swabs of any of the vaccinated chickens after challenge. Low titers of infectious virus were recovered from the trachea, lungs, kidneys, and colon of three of the four vaccinated birds on day 2 p.c. (Table 2).

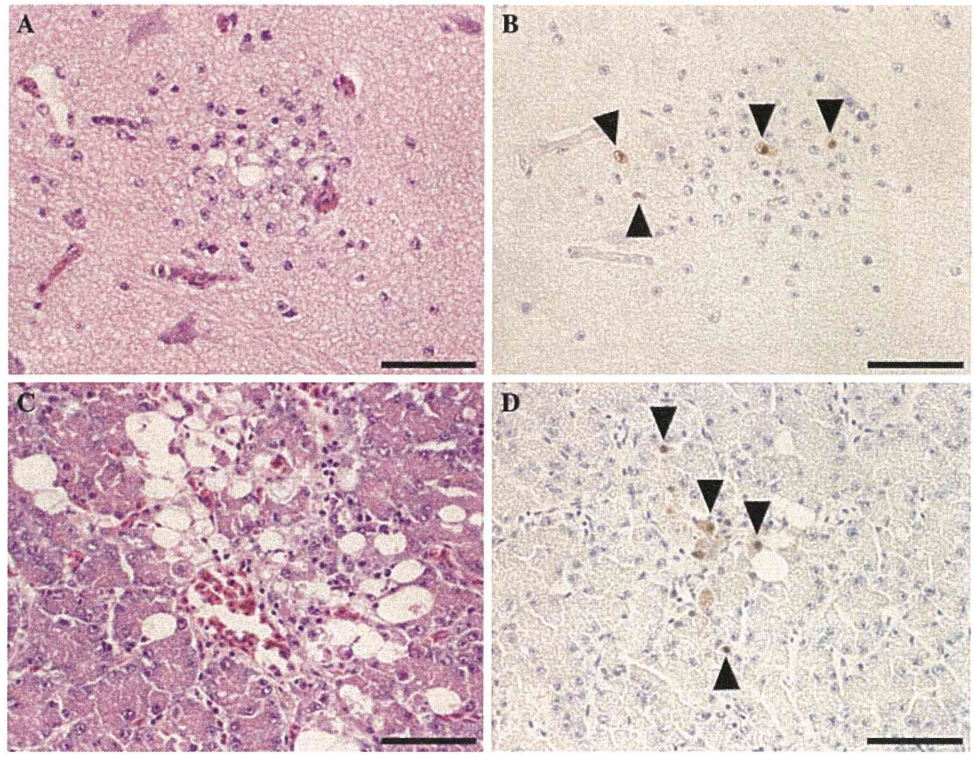

Fig. 2 Histopathological and immunohistochemical findings of the whooper swan. **a** Glial nodule with spongiform change of neuropile. Cerebrum, HE stain. **b** Nerve and glial cells in the glial nodule are positively stained for influenza virus antigen (*arrowheads*). **c** Focal necrosis of acinar cells. Pancreas, HE stain. **d** Necrotic and degenerative acinar cells show positive staining for influenza virus antigen (*arrowheads*). *Bars* = 50 μm

Discussion

In this study, genetic analysis of the H5N1 viruses isolated from the swans in Hokkaido in Japan revealed that they belonged to Clade 2.3.2. The results also indicate that whooper swans were infected with HPAIV though water-borne transmission somewhere in a lake or pond where feral water birds, who were probably infected with HPAIV in Southern China, congregated on the way back to the north in spring. Although viruses belonging to Clade 2.2, Qinghai-like viruses, had been spread in Asia, Europe, and Africa by wild water birds [5–7, 22], the present results indicate that the viruses belonging to Clade 2.3.2, which differ from Qinghai-like viruses, were also spread by wild water birds. Actually, the number of case reports of infections of wild birds with H5N1 HPAIV belonging to Clades 2.3.2 and 2.3.4 have been increasing since 2008 [23].

High mortality in wild water birds infected with HPAIV was not recognized before 2005. However, swans and geese are apparently most commonly infected with the recent H5N1 virus strains [10, 12, 24]. In this study, pathological changes of dead whooper swan with HPAIV were confined to the central nervous system (CNS), pancreas, and lungs. Inflammatory reaction of the wild water birds infected with H5N1 HPAIV was limited. The present findings indicate that the whooper swan died of severe congestive edema of the lungs at an early stage of systemic infection with HPAIV. Neither myocardial necrosis nor influenza virus antigen was found in the heart of the swan. These findings coincide with those of the gross lesions of mute swans and whooper swans that were identified as multifocal pancreatic necrosis, hemorrhage, and lung edema during an outbreak in Germany in 2006 [10].

In the poultry population in Asia, antigenic variants of H5N1 HPAIV have been selected, indicating that these wild birds were infected with the H5N1 viruses prevailing in domestic poultry [25]. Antigenic analysis revealed that the isolates were different from the virus isolates from poultry and wild water birds in Japan, Mongolia, and China including the vaccine strain, Dk/Vac-1/04, that we previously developed [15]. It is suggested that the antigenicity of H5N1 HPAIVs has changed more during circulation in the chicken population since 2007. Given this notion, chickens inoculated with the vaccine that we previously developed were challenged with the present HPAIV isolate. In the challenge study to vaccinated chickens, higher titers of the challenge viruses were recovered from various tissues of the chickens than those from birds challenged with A/chicken/Yamaguchi/7/2004 (H5N1) strain in a previous study [15], although all of the vaccinated chickens were survived for 14 days after the challenge with Ws/Hok/1/08 (H5N1). This may be influenced by an antigenic difference between the vaccine strain and the challenge

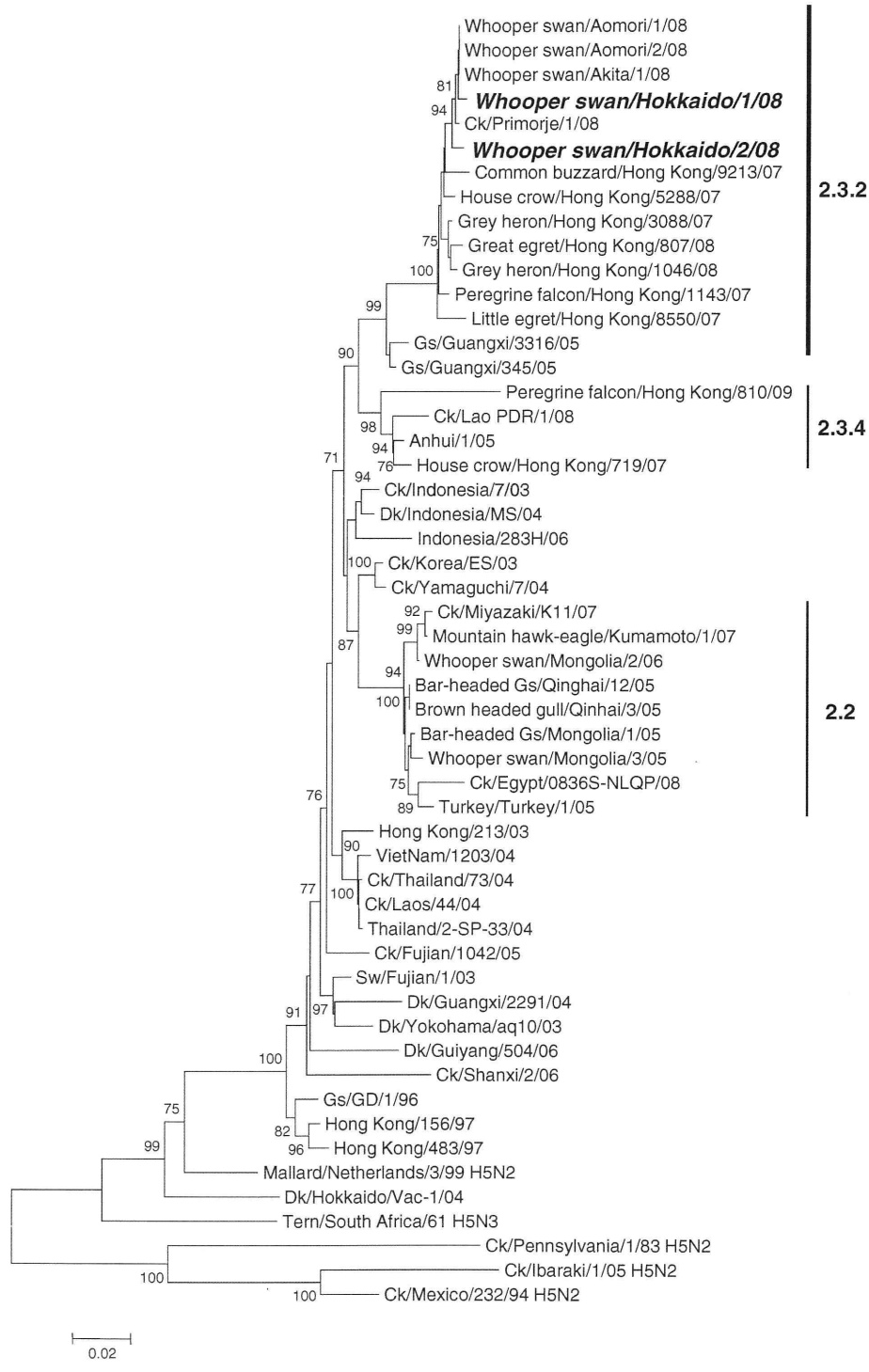

Fig. 3 Phylogenetic tree of the HA genes of H5 influenza viruses. Nucleotide sequences of the HA genes of H5 influenza viruses isolated in the present study (shown in *bold italic*) and the sequence information of other related viruses were cited from the public database. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. *Numbers* at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. *Ck* chicken, *Gs* goose, *Dk* duck, *Qa* quail, *Sw* swine

virus. In poultry, avian influenza viruses have not been under constant immunological selection pressure induced by vaccines. Since vaccine use for poultry has increased in several countries, antigenic variation could occur in H5N1 HPAIV as it did for H5N2 viruses in the 1990s in Mexico [26]. It is strongly emphasized that stamping-out measures without misuse of vaccine is best way in eradication of HPAI. For control of HPAI, continuing surveillance to understand influenza virus infection in birds and mammals and preparation for the diagnosis of influenza virus infection, such as technical training, making antiserum, and sharing information are essential.

Table 1 Antigenic property of influenza viruses isolated in Japan in 2008

Virus	Clade	Polyclonal antiserum (hyper-immune)			Monoclonal antibodies[a]		
		Ws/Hok/1	Dk/Vac-1/04	Tn/SA	A310/39	64/2	25/2
Whooper swan/Hokkaido/1/08 (H5N1)	2.3.2	1280	40	40	<	<	<
Whooper swan/Hokkaido/2/08 (H5N1)	2.3.2	1280	40	40	<	<	<
Whooper swan/Akita/1/08 (H5N1)	2.3.2	1280	20	40	<	<	<
Whooper swan/Akita/2/08 (H5N1)	2.3.2	1280	40	40	<	<	<
Viet Nam/1194/04 (H5N1)	1	80	160	160	40	1280	80
Whooper swan/Mongolia/3/05 (H5N1)	2.2	320	320	160	<	320	320
Whooper swan/Mongolia/2/06 (H5N1)	2.2	80	320	160	<	1280	80
Chicken/Yamaguchi/7/04 (H5N1)	2.5	320	640	320	40	640	160
Duck/Hokkaido/Vac-1/04 (H5N1)	Classical	40	640	160	320	320	40
Tern/South Africa/61 (H5N3)	Classical	40	320	640	<	160	20
Chicken/Ibaraki/1/05 (H5N2)	American	20	80	40	<	<	<

Homologous titer of the antiserum is *underlined*

< = The HI titer was lower than 1:20

[a] Monoclonal antibodies against Dk/Pennsylvania/84 (H5N2)

Table 2 Antibody titers and virus recovery in chickens

	Days p.c.	HI titer (0 dpc)		HI titer (14 dpc)		Virus recovery					
		Dk/Vac-1/04	Ws/Hok/08	Dk/Vac-1/04	Ws/Hok/08	Swabs (log EID_{50}/ml)		Tissues (log EID_{50}/g)			
						Trachea	Cloaca	Trachea	Lung	Kidney	Colon
Vaccinated chickens	2	256	8	NT	NT	–	–	2.0	2.5	2.5	3.5
	2	256	4	NT	NT	–	–	1.8	–	–	2.7
	2	256	8	NT	NT	–	–	–	–	2.5	–
	2	512	8	NT	NT	–	–	–	–	–	–
	4	128	4	NT	NT	–	–	–	3.3	–	–
	4	256	4	NT	NT	–	–	–	–	–	–
	4	256	8	NT	NT	–	–	–	–	–	–
	14	256	4	512	64	NT	NT	NT	NT	NT	NT
	14	256	4	512	64	NT	NT	NT	NT	NT	NT
	14	256	8	512	64	NT	NT	NT	NT	NT	NT
	14	128	4	1024	128	NT	NT	NT	NT	NT	NT
Non-vaccinated chickens	2[a]	<2	<2	NT	NT	4.5	4.3	8.5	7.5	7.3	9.8
	2[a]	<2	<2	NT	NT	6.3	4.8	9.3	7.8	7.3	7.8
	2[a]	<2	<2	NT	NT	6.8	4.5	7.8	7.5	9.5	8.8

– The titer of the virus recovery lower than 0.5 (swabs) or 1.5 (tissues), *NT* not tested

[a] Chicken died

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Intranasal Administration of Adjuvant-Combined Vaccine Protects Monkeys From Challenge With the Highly Pathogenic Influenza A H5N1 Virus

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The effectiveness in cynomolgus macaques of intranasal administration of an influenza A H5N1 pre-pandemic vaccine combined with synthetic double-stranded RNA (polyI/polyC12U) as an adjuvant was examined. The monkeys were immunized with the adjuvant-combined vaccine on weeks 0, 3, and 5, and challenged with the homologous virus 2 weeks after the third immunization. After the second immunization, the immunization induced vaccine-specific salivary IgA and serum IgG antibodies, as detected by ELISA. The serum IgG antibodies present 2 weeks after the third immunization not only had high neutralizing activity against the homologous virus, they also neutralized significantly heterologous influenza A H5N1 viruses. The vaccinated animals were protected completely from the challenge infection with the homologous virus. These results suggest that intranasal immunization with the Double stranded RNA-combined influenza A H5N1 vaccine induce mucosal IgA and serum IgG antibodies which could protect humans from homologous influenza A H5N1 viruses which have a pandemic potential.
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KEY WORDS: H5N1 influenza A virus; cynomolgus macaques; adjuvant; intranasal vaccine; IgA

INTRODUCTION

The highly pathogenic avian influenza A subtype H5N1 virus was first discovered to have the capacity to infect humans in Hong Kong in 1997 [Claas et al., 1998; Subbarao et al., 1998]. Influenza A H5N1 virus strains re-emerged subsequently in Southeast Asia, after which they spread across Asia to the Middle East, Europe, and Africa. The World Health Organization has recorded 473 laboratory-confirmed H5N1-infected human cases (including 282 deaths) from January 2003 to February 2010. If influenza A H5N1 viruses can spread from person to person, a pandemic could result. Therefore, the development of effective vaccines against the highly pathogenic avian influenza A H5N1 virus is an urgent public health need.

H5 vaccines, which are prepared from reference strains and delivered by intramuscular injection, will be less effective if the vaccine strains differ from the future pandemic virus strain [Horimoto et al., 2004]. This is because intramuscular injection of a vaccine only induces anti-vaccine IgG antibodies, which are highly protective against homologous virus infections, but less protective against heterologous virus infections [Hasegawa et al., 2007]. This disadvantage of intramuscular vaccination could be circumvented by employing intranasal vaccination, which induces respiratory tract IgA antibodies that are capable of providing protection

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against not only homologous virus infection, but also heterologous virus infections [Asahi et al., 2002; Hasegawa et al., 2007]. Indeed, it has been showed previously in BALB/c mice that intranasal co-administration of an influenza A H5N1 vaccine and synthetic double-stranded RNA (polyI/polyC12U, Ampligen®) as an adjuvant induced both nasal IgA and serum IgG antibodies, and provided protection against infection with not only the homologous influenza A H5N1 virus, but also variant virus strains [Ichinohe et al., 2007a,b]. However, while these experiments using the mouse influenza model provided insights into the effectiveness of intranasal administration of a vaccine against H5 virus infection, primate model experiments would show more directly that the intranasally delivered H5 vaccine could be effective in humans as well. It has been already demonstrated that infection of cynomolgus macaques with influenza A H5N1 viruses can serve as a model for these infections in humans [Rimmelzwaan et al., 2001].

In the present study, the protective effects of intranasal administration of an influenza A H5N1 vaccine together with Amligen as an adjuvant was examined in cynomolgus macaques (*Macaca fascicularis*). The influenza A H5N1 vaccine used was NIBRG14, which is derived from the pathogenic influenza virus A/Vietnam/1194/2004 that had been isolated from a patient with H5N1 influenza. The results suggest that intranasal immunization with the Ampligen-combined influenza A H5N1 vaccine can provide monkeys with cross-protective immunity against influenza A H5N1 virus challenge.

MATERIALS AND METHODS

Influenza Viruses and Animals

The influenza A H5N1 viruses used in this study were A/Vietnam/1194/2004, A/Hong Kong/483/97, and A/Indonesia/6/2005 (A/Indonesia/6/05), all of which were isolated from patients with influenza A H5N1 disease [Gao et al., 1999]. The viruses were propagated in 10-day-old embryonated chicken eggs for 2 days at 37°C and then stored at −80°C before use.

Two male and four female cymomolgus monkeys (*Macaca fascicularis*), aged 3–4 years and weighing 2,130–4,180 g, were used in the experiments (Table I). These monkeys were born and raised in the Tsukuba Primate Center for Medical Science, National Institute of Infectious Diseases, Tukuba, Japan, and were maintained in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases. They were assigned to two groups as shown in Table I. The naïve group consisted of three unvaccinated monkeys (ID numbers 4668, 4669, and 4672), while the immunized group consisted of three vaccinated monkeys (ID numbers 4670, 4671, and 4673). All monkeys were then challenged intranasally with A/Vietnam/1194/04 under Biosafety level 3 containment according to the Guidelines for Animal Experiments Performed at National Institute of Infectious Diseases. All procedures used in this study complied with federal guidelines and were approved by the Institutional Animal Care and Use Committee of National Institute of Infectious Diseases.

Preparation of Vaccine and Adjuvants

Formalin-inactivated whole virus vaccine (NIBRG14) was derived from a recombinant avirulent avian virus that contains a modified hemagglutinin and neuraminidase from the highly pathogenic avian influenza virus strain A/Vietnam/1194/2004 along with other viral proteins from influenza A/PuertoRico/8/34 (A/PR8, H1N1) [Nicolson et al., 2005]. Poly I/Poly $C_{12}U$ (Ampligen®) was provided by Hemispherx Biopharma (Philadelphia, PA).

Immunization With the Vaccine and Virus Challenge

On weeks 0, 3, and 5, the monkeys were anaesthetized with ketamine (0.1 ml/kg) and immunized intranasally with 90 μg of the NIBRG14 vaccine mixed with 500 μg of Ampligen in 0.5 ml of PBS or PBS alone using a spray (0.25 ml in each nostril, DIA, Keytron, Ichikawa, Japan). Two weeks after the final immunization, all

TABLE I. Characteristic Influenza A H5N1 Virus-Associated Symptoms in Mock-Immunized Monkeys and Those Immunized With NIBRG14 and Ampligen

Group	ID	Vaccination	Sex (wt, g)	Challenge virus strain (dose, PFU)	Virus-associated symptoms	Outcome
Mock	4668	Mock	F (2,780)	A/Vietnam/1194/04	Tachypnea, diarrhea	Survival
	4669		F (2,130)	(3×10^5)	Nasal discharge, cough, tachypnea, diarrhea, intention tremor	Survival
	4672		M (3,280)		Nasal discharge	Survival
Vaccine	4670	NIBRG14 (90 μg) + Ampligen (500 μg)	F (3,080)	A/Vietnam/1194/04	None	Survival
	4671		F (2,540)	(3×10^5)	None	Survival
	4673		M (4,180)		None	Survival

ID, monkey identification number; F, female; M, male.

monkeys were infected with 3×10^5 plaque-forming units (PFU) of A/Vietnam/1194/2004, which was suspended in 3 ml of PBS. Of this virus suspension volume, 2.5 ml were applied intratracheally with a catheter (7Fr, Atom Medical, Tokyo, Japan) and 0.5 ml was applied intranasally (0.25 ml into each nostril) with a spray (Keytron, Inc., Chiba, Japan).

Serological Assays

Serum and saliva were collected 0, 1, 2, 3, 4, 5, 6, and 7 weeks after immunization and 0, 2, 5, 9, 12, and 14 days post-infection to measure the levels of Abs specific for the NIBRG14 vaccine. The IgA and IgG antibodies against the NIBRG14 vaccine were measured by an enzyme-linked immunosorbent assay (ELISA) [Ichinohe et al., 2007a]. Briefly, ELISA was conducted on the solid phase (EIA plate; Costar, Cambridge, MA) with the following series of reagents: first, NIBRG14 vaccine; second, serum or saliva; third, goat anti-monkey IgG (γ-chain specific; Alpha Diagnostic Intl., Inc., San Antonio, TX) or goat anti-monkey IgA (α-chain specific; Rockland, Inc.) conjugated with alkaline phosphatase; and fourth, *p*-nitrophenylphosphate. The chromogen produced was measured by determining the absorbance at 405 nm with an ELISA reader.

The virus neutralization activity of the antisera was determined as described previously [Kida et al., 1982]. Briefly, 2-fold serial dilutions of receptor-destroying enzyme (RDE(II); Denka Seiken Co. Ltd, Tokyo, Japan)-treated serum samples (10-fold diluted samples) were mixed with 10^2 $TCID_{50}$ of the virus and incubated at 37°C for 1 hr. The virus and serum mixtures were then inoculated onto confluent Madin-Darby Canine Kidney (MDCK) cell monolayers in 96-well plates and incubated at 37°C. After 1 hr, the inocula were removed and 100 µl of MEM was added to each well. The cells were incubated at 37°C for 4 days and the neutralization titer was determined as the reciprocal of the serum dilution that inhibited the cytopathic effect of the virus by 50%.

Virus Isolation and Virus Titer Measurement

Nasal, throat, and rectal swabs were collected at 0, 2, 5, 9, 12, and 14 dpi in 1 ml MEM containing 2% FBS and antibiotics. Frontal lobe, vertex, cerebellum, brain stem, trigeminal nerve, lung, and ileum tissue samples were collected at 14 dpi and 10% (w/v) tissue homogenates were prepared by using a bead homogenizer in MEM containing 2% FBS and antibiotics. For virus isolation, the swabs and the supernatants of 10% tissue homogenates were inoculated onto MDCK cells in 24-well plates. cytopathic effects were determined under a microscope 3 days later. The samples were considered to be negative for infectious virus when no virus-specific cytopathic effects were observed in the culture.

The virus titer of each swab sample was measured according to the method of Tobita [1975] and Tobita et al. [1975]. Briefly, 200 µl aliquots of serial 10-fold dilutions of the swabs were added to confluent MDCK cell monolayer cultures in six-well plates. After 1 hr of adsorption, each well was overlaid with 2 ml of agar medium. The plate was incubated for 48 hr in a CO_2 incubator and the number of plaques in each well was counted. The virus titers were expressed as the mean PFU/ml ± standard deviations (SD) of triplicate swab samples from each monkey.

Histopathological and Immunohistochemical Analyses

The upper jaw, including the nasal cavity, tonsils, lymph nodes, lung, heart, kidney, liver, spleen, small and large intestine, brain, and spinal cord, was fixed with 10% neutral-buffered formalin, and the upper jaw, including the nasal cavity, was decalcified in EDTA solution. After fixation, the tissues were embedded in paraffin by conventional methods and stained with hematoxylin and eosin (H&E), or subjected to immunohistochemical staining with antiserum against the nucleoprotein from the influenza A/PR8 virus. The specificity of the anti-nucleoprotein Ab and its reactivity to influenza A H5N1 influenza virus have been confirmed previously [Nishimura et al., 2000; Asahi-Ozaki et al., 2006]. Immunohistochemical staining was performed by the biotin-streptavidin-peroxidase method using 3,3′-diaminobenzidine as a substrate.

Statistical Analysis

Comparisons between experimental groups were performed by two-tailed Student's *t*-tests. Values of $P < 0.05$ were considered significant unless otherwise indicated.

RESULTS

Antibody Responses Induced by Intranasal Administration of the Ampligen-Combined Influenza A H5N1 Vaccine

Cynomolgus macaques (*Macaca fascicularis*) were immunized by nasal spraying with the adjuvant-combined vaccine (0.5 ml PBS containing 90 µg of the NIBRG14 whole particle vaccine derived from the Vietnam/1194/2004 strain and 500 µg of Ampligen) on weeks 0, 3, and 5, after which they were challenged by infection with the homologous virus 2 weeks after the third immunization. Their vaccine-specific serum IgG and saliva IgA antibody responses were assayed by ELISA and virus neutralization tests (Fig. 1 and Table II). Serum IgG-ELISA antibody responses became detectable 1 week after the second immunization, peaked 2 weeks after the third immunization, and were maintained at that high level after the challenge infection (Fig. 1A). The mock-immunized control monkeys, which had also been infected with the homologous virus, produced only low IgG antibody responses, which became detectable 1 week after infection (Fig. 1A). In addition, when assayed 2 weeks after the third immunization, the immunized monkeys also evinced serum neutralization antibody activity in neutralization tests

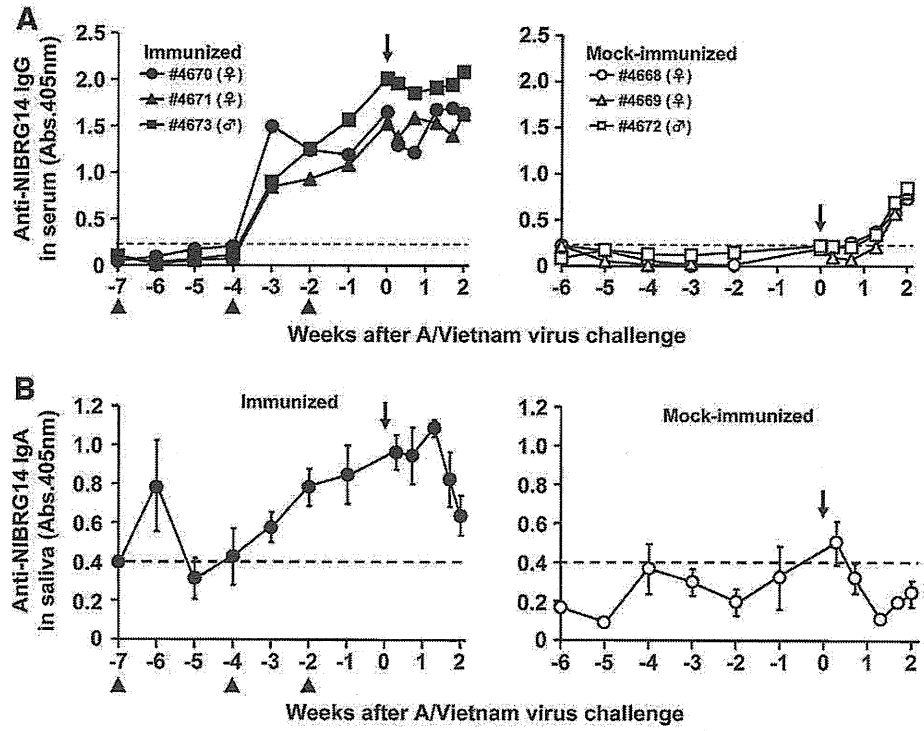

Fig. 1. Changes in the anti-NIBRG14-specific serum IgG (**A**) and saliva IgA (**B**) titers of vaccinated and mock-immunized monkeys after intranasal immunization with the Ampligen-combined NIBRG14 vaccine. The vaccinated (closed symbols) and mock-immunized (open symbols) monkeys were challenged with A/Vietnam/1194/04 virus 2 weeks after the third immunization. Arrowheads below the time scale of each graph indicate the immunization times, while the black arrow in each graph indicates the challenge infection time. The broken line in each graph indicates the background IgG or IgA antibody levels. Each point in the figures in (B) represents the mean ± SE of the combined salivary IgA antibody titers of three monkeys.

using not only the homologous virus A/Vietnam/1194/04, but also heterologous viruses, namely, A/Indonesia/6/05 and A/Hong Kong/483/97 (Table II). The neutralization activity against the homologous and heterologous viruses was high and low, respectively. In contrast, the neutralization activity of the mock-immunized naïve monkeys was below the limit of detection (<10).

Salivary IgA-ELISA antibody responses became detectable 1 week after the second immunization, continued to increase up to the time of challenge, peaked about 1 week after the challenge infection, and decreased thereafter (Fig. 1A). The mock-immunized control monkeys did not produce any detectable saliva IgA-ELISA Ab responses (Fig. 1A). The neutralization activities of the salivary antibodies from the immunized monkeys were below the limit of detection (data not shown).

TABLE II. Serum Neutralizing Antibody Reciprocal Titers Specific for Influenza A H5N1 Viruses

Influenza A H5N1 virus strains	Group	
	Mock-immunized	Vaccine
A/Vietnam/1194/04	<10	40
A/Hong Kong/483/97	<10	10
A/Indonesia/6/05	<10	10

Protection From Challenge With the A/Vietnam/1194/2004 Virus

Next, the ability of intranasal immunization with the Ampligen-combined influenza A H5N1 vaccine to protect the monkeys from challenge with the homologous virus was examined. A monkey was considered protected if the virus could not be isolated from the 0, 2, 5, 9, 12, and 14 dpi nasal, throat or rectal swabs, as determined by testing for cytopathic effects or measuring the virus titer. Table III shows the results of the cytopathic effect assays. The A/Vietnam/1194/2004 virus was not isolated from any of the swabs from the vaccinated monkeys, but was isolated 2 days dpi from the throat swabs of two mock-vaccinated monkeys and from the nose, throat, and rectal swabs of the remaining mock-vaccinated monkey. The virus could also be isolated from the 5 dpi nasal swab of the latter monkey. The results of viral titration were consistent with the cytopathic assay data since they showed that the A/Vietnam/1194/2004 virus could not be isolated from the nasal and throat swabs of any of the vaccinated monkeys, but could be isolated from the 2 and 5 dpi nasal swabs of one mock-vaccinated monkey (Fig. 2A) and from the 2 dpi throat swabs of the other two mock-vaccinated monkeys (Fig. 2B). However, the virus could not be detected in any of the 14 dpi frontal lobe, vertex, cerebellum, brain stem, trigeminal nerve, lung, and

TABLE III. Detection of Influenza Virus in Swabs Obtained From Monkeys Infected With Influenza Virus A/Vietnam/1194/04

Group	Monkey no.	0 dpi			2 dpi			5 dpi			9 dpi			12 dpi			14 dpi		
		N	T	R	N	T	R	N	T	R	N	T	R	N	T	R	N	T	R
Mock	4668	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	4669	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
	4672	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−
Vaccine	4670	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	4671	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	4673	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

N, nasal swab; T, throat swab; R, rectal swab.

ileum tissue samples from vaccinated and mock-vaccinated monkeys (data not shown). Thus, intranasal immunization with the Ampligen-combined influenza A H5N1 vaccine protects monkeys from homologous H5 virus infection.

Pathological Signs of A/Vietnam/1194/2004 Virus Infection

The pathological changes in the lungs of the mock-vaccinated monkeys 14 days after H5 virus infection were compared to those in the lungs of the immunized monkeys. All mock-immunized monkeys developed pneumonia characterized by the destruction of alveoli, lymphocyte infiltration, and proliferation of type II alveolar cells (Fig. 3). In contrast, none of the vaccinated monkeys exhibited active pneumonia, with only scars from the initial infection being observed. Viral antigen was not detected by immunohistochemical staining in the lungs of either the mock-vaccinated or vaccinated monkeys 14 days after infection (data not shown).

To assess clinical signs of infection, the food consumption patterns of the vaccinated and mock-vaccinated monkeys after A/Vietnam/1194/04 virus infection were examined. The mock-immunized monkeys consumed significantly less food on days 1 and 4 after infection ($P < 0.05$) than the vaccinated monkeys (data not shown). Furthermore, the mock-immunized monkeys exhibited significant leukopenia until 5 days post-infection, but this effect was not notable in any of the vaccinated monkeys ($P < 0.001$, data not shown). In addition, the mock-immunized monkeys developed symptoms of tachypnea, diarrhea, nasal discharge, cough, and intention tremor, unlike the vaccinated animals (Table I). However, the body weights and body temperature of all monkeys did not change significantly after A/Vietnam/1194/04 virus infection (data not shown).

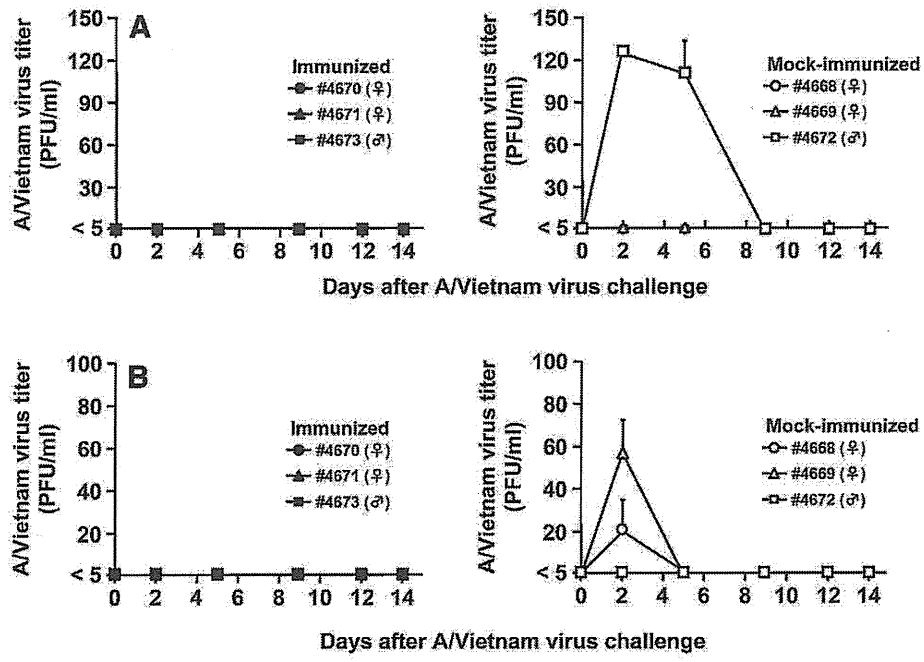

Fig. 2. Changes in the A/Vietnam/1194/04 virus titers in nasal (A) or throat swabs (B) after challenge with the A/Vietnam/1194/04 virus in mock-vaccinated monkeys (open symbols) and in monkeys immunized intranasally with Ampligen-combined NIBRG14 vaccine (filled symbols). Each point represents the mean virus titer (PFU/ml) ± SD of triplicate swab samples from each monkey.

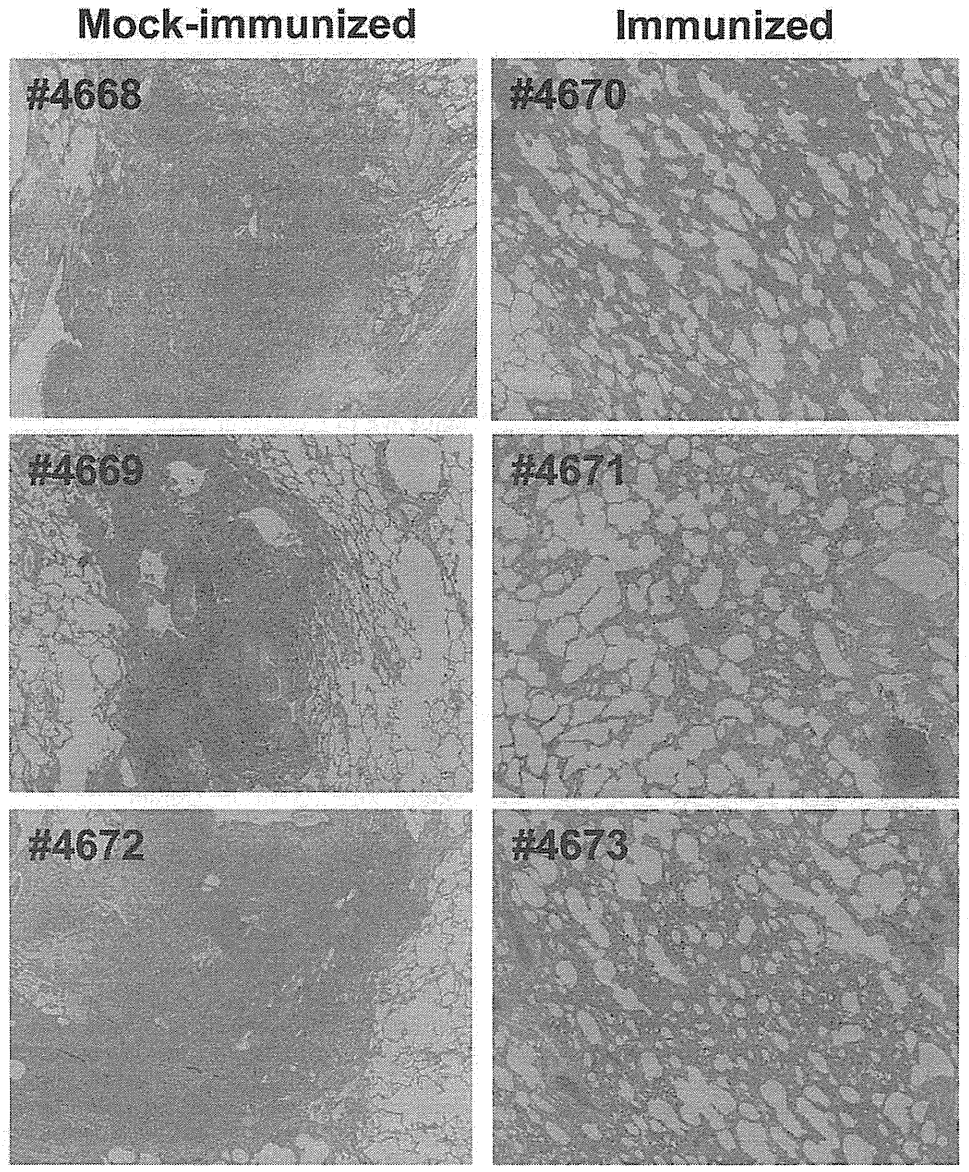

Fig. 3. Histopathological changes in the lungs from monkeys immunized intranasally with the Ampligen-combined NIBRG14 vaccine (#4670, #4671, and #4673) or from mock-immunized control monkeys (#4668, #4669, and #4672) 14 days after challenge with A/Vietnam/1194/04 virus (40×, H&E).

DISCUSSION

In this study, the effectiveness of the influenza A H5N1 vaccine (NIBRG14) in cynomolgus macaques was examined when it was delivered intranasally together with Ampligen. Intranasal vaccinations are known to induce mucosal immune responses by respiratory tract mucosa (which is the initial site of virus infection) and thus could be the most effective immunization strategy to deliver protection from influenza virus infection [Tamura et al., 2005]. However, such a vaccine is more likely to induce effective mucosal antibody responses if it is combined with a potent mucosal adjuvant. While cholera toxin and *Escherichia coli* heat-labile toxin are potent adjuvants that can enhance mucosal immune responses [Tamura et al., 2005], they have several undesirable side-effects in humans, including VIIth cranial nerve dysfunction [Mutsch et al., 2004]. Therefore, for intranasal influenza vaccines in humans, other adjuvants that are both clinically safe and effective should be developed. In this study, Ampligen®, which is a synthetic double-stranded RNA polyI/polyC_{12}U that has both a good safety profile as shown by clinical trials [Unknown, 2004] and good mucosal adjuvant activity in mice when co-administered intranasally with NIBRG14 [Ichinohe et al., 2007a,b] was used.

When administered to the monkeys, the adjuvant-combined vaccine elicited salivary IgA and serum IgG

antibody responses, as detected by ELISA (Fig. 1). Significantly, these vaccine-induced serum antibodies have neutralizing activity against both homologous and heterologous influenza A H5N1 viruses (Table II). Previously, it has been demonstrated in the influenza model mouse that this intranasal vaccination induces both cross-reactive mucosal antibodies and less cross-reactive serum antibodies [Ichinohe et al., 2005, 2007b], and that the ability of the mucosal antibodies to cross-react with various strains of influenza virus could be attributed to the secretory IgA antibodies [Ichinohe et al., 2007a]. These observations, together with the fact that cross-reactive neutralizing antibodies were detected in the serum of the vaccinated monkeys (Table II), suggest that more cross-reactive mucosal antibodies may also have been induced in the vaccinated monkeys. The cross-reactive neutralization activity was not detected in the salivary IgA antibodies, but this may have been due to the low concentration of the IgA antibodies (data not shown). Somewhat surprisingly, IgA antibody in saliva of both vaccinated and mock-immunized monkeys decreased quickly after 9 and 2 days post infection, respectively (Fig. 1B). The decrease of IgA antibody in mock-immunized monkey might be a background level (below broken line). However, the immunized monkeys sustained significant levels of salivary IgA antibody responses after the infection. Since saliva is so sticky and impure, it is required to optimize collection of saliva samples for IgA-ELISA to reduce background levels. Further work will be required to determine whether the vaccine also induces mucosal antibodies in monkeys that have greater cross-reactive neutralization activity than the serum antibodies.

Concomitant with these antibody responses, the vaccinated monkeys were protected completely from a challenge infection with the homologous virus, as shown by the inability to isolate the A/Vietnam/1194/04 virus from the vaccinated monkeys. In contrast, this virus was isolated readily from the nasal and throat swabs of the mock-immunized monkeys (Fig. 2 and Table III). Notably, it has been shown that intranasal administration of mice with NIBRG14 combined with Ampligen elicited protective immunity against both the homologous virus (A/Vietnam/1194/2004) and heterologous viruses, namely, A/Hong Kong/483/97 and the recent A/Indonesia/6/2005 virus [Ichinohe et al., 2007a]. These observations suggest that the monkeys that were immunized intranasally with the Ampligen-combined influenza A H5N1 vaccine may also have developed cross-protective immunity against influenza A H5N1 virus challenge.

Cynomologous macaques have been used as non-human primate models for studying influenza virus infection [Rimmelzwaan et al., 2001]. They demonstrated that when cynomologus monkeys are infected intratracheally with the A/Hong Kong/156/97 (H5N1) virus, they develop acute respiratory distress syndrome along with fever, and the virus can be isolated 4 days after infection from tissue samples of the trachea, lung, tracheobronchial lymph nodes, and heart [Rimmelzwaan et al., 2001]. In the present experiments, the results showed that when the mock-immunized monkeys were infected by the A/Vietnam/1194/2004 (H5N1) virus delivered intranasally and intratracheally, they developed pneumonia (Fig. 3), the virus-associated symptoms of tachypnea, diarrhea, nasal discharge, cough, and intention tremor (Table I), and lost their appetite, although their body weights and body temperatures did not change significantly (data not shown). Furthermore, the viruses were isolated from the nasal, throat or rectal swabs of the mock-immunized monkeys at 5 and/or 2 days post infection (Table III and Fig. 2), although the virus could not be isolated from the 14 dpi tissue samples of the frontal lobe, vertex, cerebellum, brain stem, trigeminal nerve, lung, and ileum (data not shown). However, although Rimmelzwaan et al. [2001] could also detect viral antigen (influenza virus nucleoprotein) in the lung on 4 and 7 days post-infection by immunohistochemistry, the viral antigen was not detected by immunohistochemical staining of the lungs of either mock-immunized or immunized monkeys at 14 days post infection (data not shown). The differences between the study of Rimmelzwaan et al. and present study in terms of the detection of influenza virus in tissue samples and the clinical signs may be due to the time point of virus collection (4 and 14 days after challenge, respectively), the virus strains (A/Hong Kong/156/97 and A/Vietnam/1194/04, respectively), and the infecting influenza virus dose (2.5×10^4 50% tissue culture infective dose and 3×10^5 PFU, respectively).

Itoh et al. [2008] demonstrated clearly that intranasal vaccination of cynomologus monkeys with a formalin-inactivated vaccine prepared from a non-pathogenic influenza A H5N1 virus conferred protective immunity against highly pathogenic influenza A H5N1 virus infection. However, in their experiments, the monkeys were given a high dose of whole virus vaccine (1 mg/dose). In the present experiments, the monkeys were immunized three times intranasally with 90 μg/dose (less than 10% of the whole virus vaccine dose) of NIBRG14, which is a medicinal product for human use, together with Ampligen as an adjuvant, and this regimen protected the monkeys from highly pathogenic influenza A H5N1 influenza. This suggests that this adjuvant-combined intranasal vaccine may overcome the problem of a limited supply of influenza A H5N1 virus vaccine.

In summary, nonhuman primates immunized intranasally with an Ampligen-combined NIBRG14 vaccine derived from a highly pathogenic influenza virus clinical isolate developed mucosal and systemic immunity that protected them from homologous A/Vietnam/1194/04 influenza virus infection. The intranasal administration of NIBRG14 and Ampligen was well tolerated. The vaccinated monkeys did not exhibit any clinical signs after challenge. Although the safety of Ampligen when administered intranasally with an influenza vaccine should be examined further, previous results using an intravenous protocol suggest that Ampligen may also be

useful in nasal vaccines destined for humans [2004]. Further clinical studies are needed to clarify these issues.

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