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

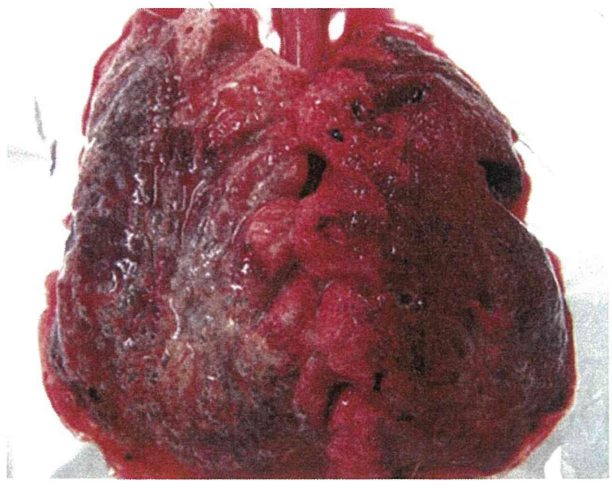

Fig. 1 Gross appearance of the lungs of whooper swan found dead beside Lake Saloma. The lungs show diffuse congestive edema. The pleura is edematously thickened

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).

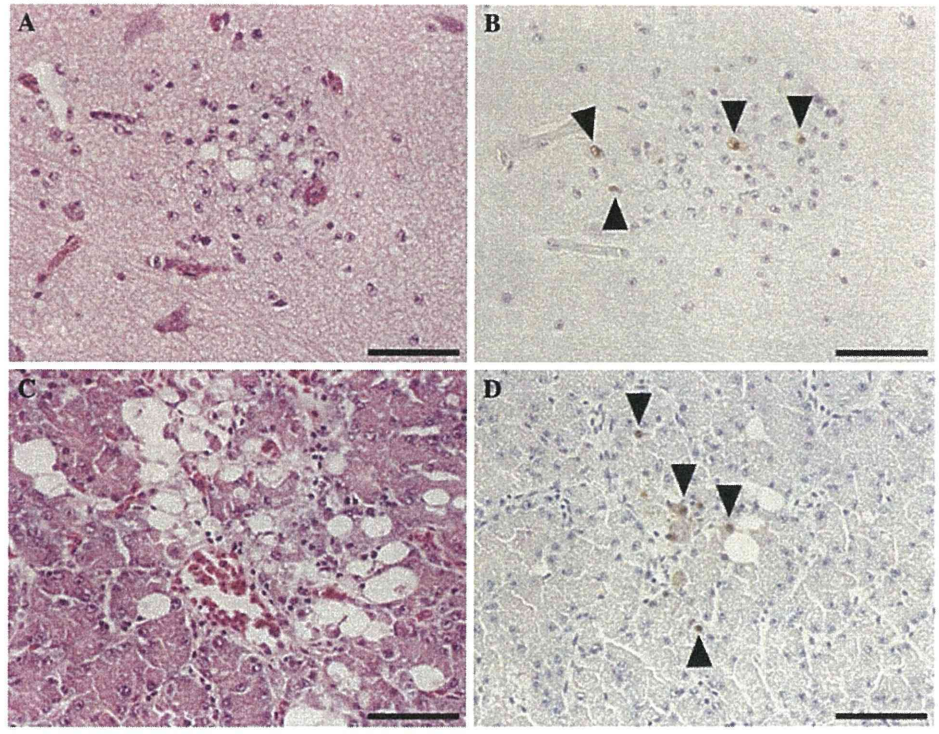

Fig. 2 Histopathological and immunohistochemical findings of the whooper swan. **a** Glial nodule with spongiform change of neuropile. Cerebrum, HE stain. **b** Nerve and glial cells in the glial nodule are positively stained for influenza virus antigen (*arrowheads*). **c** Focal necrosis of acinar 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

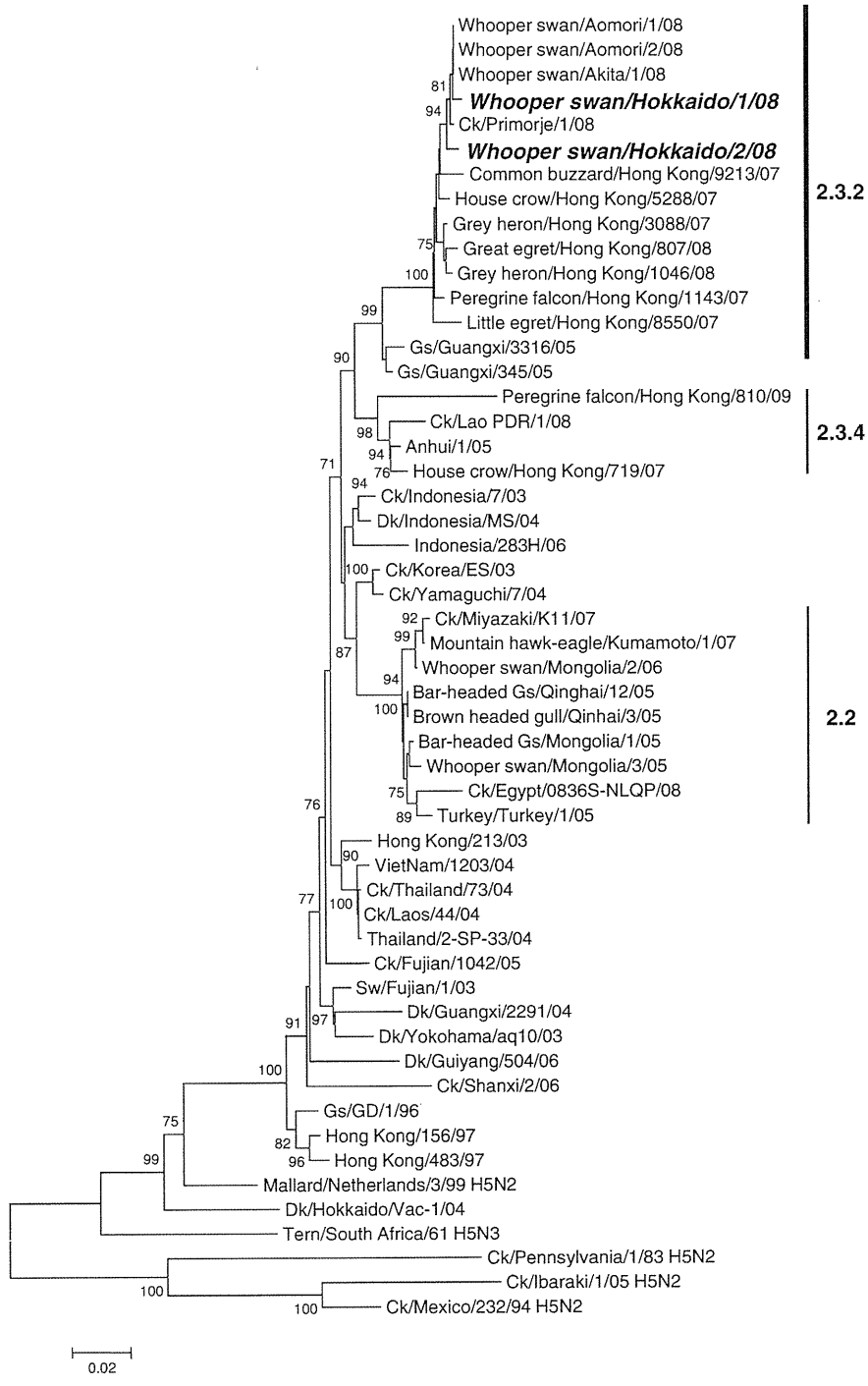

Fig. 3 Phylogenetic tree of the HA genes of H5 influenza viruses. Nucleotide sequences of the HA genes of H5 influenza viruses isolated in the present study (shown in *bold italic*) and the sequence information of other related viruses were cited from the public database. Horizontal distances are proportional 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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Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory

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ABSTRACT

H5N1 highly pathogenic avian influenza (HPAI) viruses were isolated from dead wild waterfowl at Khunt, Erkhel, Doityn Tsagaan, Doroo, and Ganga Lakes in Mongolia in July 2005, May 2006, May 2009, July 2009, and May 2010, respectively. The isolates in 2005 and 2006 were classified into genetic clade 2.2, and those in 2009 and 2010 into clade 2.3.2. A/whooper swan/Mongolia/6/2009 (H5N1) experimentally infected ducks and replicated systemically with higher mortality than that of the isolates in 2005 and 2006. Intensive surveillance of avian influenza in migratory waterfowl flying from their nesting lakes in Siberia to Mongolia in every autumn indicate that HPAI viruses have not perpetuated at their nesting lakes until 2009. The present 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.

Introduction

H5N1 highly pathogenic avian influenza (HPAI) virus infections have 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). H5N1 virus infections have become endemic at poultry farms in some countries and cause accidental transmissions to humans, so H5N1 viruses are recognized as the most likely candidate for the next pandemic (Li et al., 2004; Peiris et al., 2007). The widespread presence of H5N1 HPAI viruses in poultry, especially in domestic ducks reared in free range, has inevitably resulted in the transmission of viruses to wild bird populations. Domestic ducks and geese infected with HPAI virus shed progeny viruses in feces at the ponds in the farms, where migratory waterfowl visit. Thus, water-borne transmission easily occurs from domestic waterfowl to migratory waterfowl. In the past, such infections had been restricted to wild birds found dead in the vicinity of infected poultry farms, but there are concerns that infections of wild birds in which HPAI virus has caused mild or no clinical signs (e.g., ducks) could result in spread of the virus over large areas and long distances (Kim et al., 2009). Infections with HPAI viruses in many wild bird species at 2 waterfowl parks in Hong Kong were recorded in 2002 (Ellis et al., 2004) and further, more significant outbreaks in wild waterfowl were found at Lake Qinghai in Western China (Chen et al., 2005). H5N1 HPAI virus infections in poultry and wild birds now spread in Asia, Europe, and Africa, and it has been suggested that the H5N1 virus could spread by migratory waterfowl to the west and south, since genetically closely related H5N1 viruses (clade 2.2) were isolated in several countries from 2005 to 2006 (Monne et al., 2008; Salzberg et al., 2007; Starick et al., 2008). From intensive surveillance in China, 2 antigenically distinct virus groups, clade 2.3.2 and clade 2.3.4, were characterized as the dominant isolates in wild birds (Kou et al., 2009; Smith et al., 2009).

A natural reservoir of influenza A virus is wild waterfowl (Kida et al., 1980, 1987; Webster et al., 1978). In previous studies, influenza A viruses of different subtypes were isolated from water of the lakes where migratory waterfowl nest in summer, even in autumn when waterfowl had left for the south for migration, suggesting that influenza A viruses are preserved in frozen lake water each year while

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Table 1
Identification of H5N1 isolates in Mongolia.

Date of isolation	Place	Isolates[a]	Amino acid sequence of HA cleavage site[b]	Intravenous pathogenicity index in chicken	Database accession no.
July, 2005	Khunt Lake, Bulgan	Bhg/Mongolia/1/05 (H5N1)	GERRRKKR/G	2.95	AB239300–AB239302, AB233319, AB239303–AB239306
	Erkhel Lake, Khuvsugul	Ws/Mongolia/3/05 (H5N1)	GERRRKKR/G	2.90	AB239307–AB239309, AB233320, AB239310–AB239313
May, 2006	Khunt Lake, Bulgan	Ws/Mongolia/2/06 (H5N1)	GERRRKKR/G	2.71	AB264769–AB264770, AB263751–AB263753, AB265202–AB265204
	Erkhel Lake, Khuvsugul	Cg/Mongolia/12/06 (H5N1)	GERRRKKR/G	2.80	AB284321–AB284328
May, 2009	Doityn Tsagaan Lake, Arkhangai	Ws/Mongolia/2/09 (H5N1)	RERRRKR/G	ND[c]	AB517665–AB517666
		Ws/Mongolia/6/09 (H5N1)	RERRRKR/G	2.97	AB520705–AB520712
		Ws/Mongolia/8/09 (H5N1)	RERRRKR/G	ND	AB517667–AB517668
July, 2009	Doroo Lake, Arkhangai	Bhg/Mongolia/X25/09 (H5N1)	RERRRKR/G	ND	AB521999, AB522000
		Bhg/Mongolia/X53/09 (H5N1)	RERRRKR/G	3.00	AB523764–AB523771
		Bhg/Mongolia/X54/09 (H5N1)	RERRRKR/G	ND	AB523366, AB523367
		Rs/Mongolia/X42/09 (H5N1)	RERRRKR/G	ND	AB523756–AB523763
		Rs/Mongolia/X63/09 (H5N1)	RERRRKR/G	ND	AB523368, AB523369
		Cg/Mongolia/X59/09 (H5N1)	RERRRKR/G	ND	AB522001, AB522002
		Cg/Mongolia/X60/09 (H5N1)	RERRRKR/G	ND	AB523772–AB523779
May, 2010	Ganga Lake, Sukhbaatar	Ws/Mongolia/1/10 (H5N1)	RERRRKR/G	3.00	AB569345–AB569352
		Ws/Mongolia/7/10 (H5N1)	RERRRKR/G	ND	AB569353, AB569354
		Ws/Mongolia/11/10 (H5N1)	RERRRKR/G	ND	AB569607, AB569608
		Ws/Mongolia/21/10 (H5N1)	RERRRKR/G	ND	AB569609, AB569610

[a] Abbreviated name of birds of each isolate: Bhg: bar-headed goose, Ws: whooper swan, Cg: common goldeneye, Rs: ruddy shelduck.
[b] A pair of dibasic amino acid residues was underlined.
[c] ND: not determined.

the waterfowl are absent (Ito et al., 1995; Okazaki et al., 2000). To monitor whether these HPAI viruses perpetuate in nature, virological surveillance of avian influenza has been carried out in the lakes in Mongolia where ducks congregate on their migration path from Siberia to the south since 2001.

In July 2005, May 2006, May 2009, July 2009, and May 2010, H5N1 HPAI viruses were isolated from whooper swans and other migratory waterfowl in Mongolia on the way back to their northern territory, although no outbreak was so far reported in poultry in Mongolia. In the present study, influenza A viruses isolated from dead waterfowl and fecal samples in the intensive surveillance of avian influenza in Mongolia were antigenically and genetically characterized. Pathogenicity of the isolated H5N1 viruses in chickens, pigs, and domestic ducks were investigated by experimental infection studies. The present results strongly support the notion that the global surveillance is essential to understand the ecology of influenza viruses for the control of influenza virus infection in birds and mammals.

Results

Isolation and identification of H5N1 HPAI viruses from dead waterfowl

Virus isolation was carried out for tissue samples of dead waterfowl, a bar-headed goose, whooper swan, common goldeneye, and ruddy shelduck, which were found at Khunt, Erkhel, Doityn Tsagaan, Doroo, and Ganga Lakes in 2005, 2006, 2009, and 2010 (Table 1). In July 2005, H5N1 viruses were isolated from tissue homogenates and swab samples of a bar-headed goose and a whooper swan in Khunt and Erkhel Lakes. Similarly, H5N1 viruses were isolated from a whooper swan and a common goldeneye in May 2006 in Khunt and Erkhel Lakes. In May 2009, H5N1 viruses were isolated from 3 whooper swans in Doityn Tsagaan Lake. In late July 2009, H5N1 viruses were also isolated from dead wild birds, 3 bar-headed geese, 2 ruddy shelducks, and 2 common goldeneyes in Doroo Lake. In May 2010, H5N1 viruses were isolated from 4 whooper swans in Ganga Lake. From sequence analysis of the cleavage site of the hemagglutinin (HA), the C-terminus of HA1 had a pair of dibasic amino acid residues, which is a characteristic of HPAI viruses according to the manual of World Organization for Animal Health (OIE, 2009a). Furthermore, representative isolates of each year were highly pathogenic in chickens on intravenous inoculation and IVPIs of each isolate ranged from 2.71 to 3.00 (Table 1). Complete sequences of the HA, neuraminidase (NA), and other segments were deposited in the GenBank/EMBL/DDBJ as accession numbers described in Table 1.

Phylogenetic analysis of H5N1 isolates

The HA genes of H5N1 isolates were analyzed by the neighbor-joining method along with those of other H5 strains containing HPAI viruses recently isolated in the world (Fig. 1). The HA genes of the isolates in 2005 and 2006 were classified into clade 2.2, as Qinghai Lake-type viruses. Isolates from the same year, A/bar-headed goose/Mongolia/1/2005 and A/whooper swan/Mongolia/3/2005 (Ws/Mongolia/3/05), A/whooper swan/Mongolia/2/2006 (Ws/Mongolia/2/06) and A/common goldeneye/Mongolia/12/2006, were closely related and showed the highest homology. The 3 isolates in May 2009, 7 isolates in July 2009, and 4 isolates in May 2010 were classified into clade 2.3.2, the prototype of this clade was isolates from Hong Kong, China, and Vietnam in 2005. A/whooper swan/Mongolia/6/2009 (Ws/Mongolia/6/09) and other 13 isolates were closely related, having high homology with previous isolates from wild birds and chickens in Russia, China, Laos, and Japan.

Pathogenicity of H5N1 influenza viruses in pigs

To assess the pathogenicity of H5N1 isolates in pigs, each of Ws/Mongolia/3/05, Ws/Mongolia/2/06, and Ws/Mongolia/6/09 was inoculated intranasally at $10^{8.0}$ EID_{50} into two 4-week-old SPF pigs. Viruses were recovered from nasal swabs of all pigs infected with each H5N1 virus although apparent clinical signs were not observed in pigs for the 14 days study (Table 2). The periods of virus shedding in the pigs infected with Ws/Mongolia/2/06 were longer than in the pigs infected with Ws/Mongolia/3/05 or Ws/Mongolia/6/09.

Pathogenicity of H5N1 influenza viruses in ducks

To assess the pathogenicity of H5N1 isolates in ducks, each of Ws/Mongolia/3/05, Ws/Mongolia/2/06, and Ws/Mongolia/6/09 was

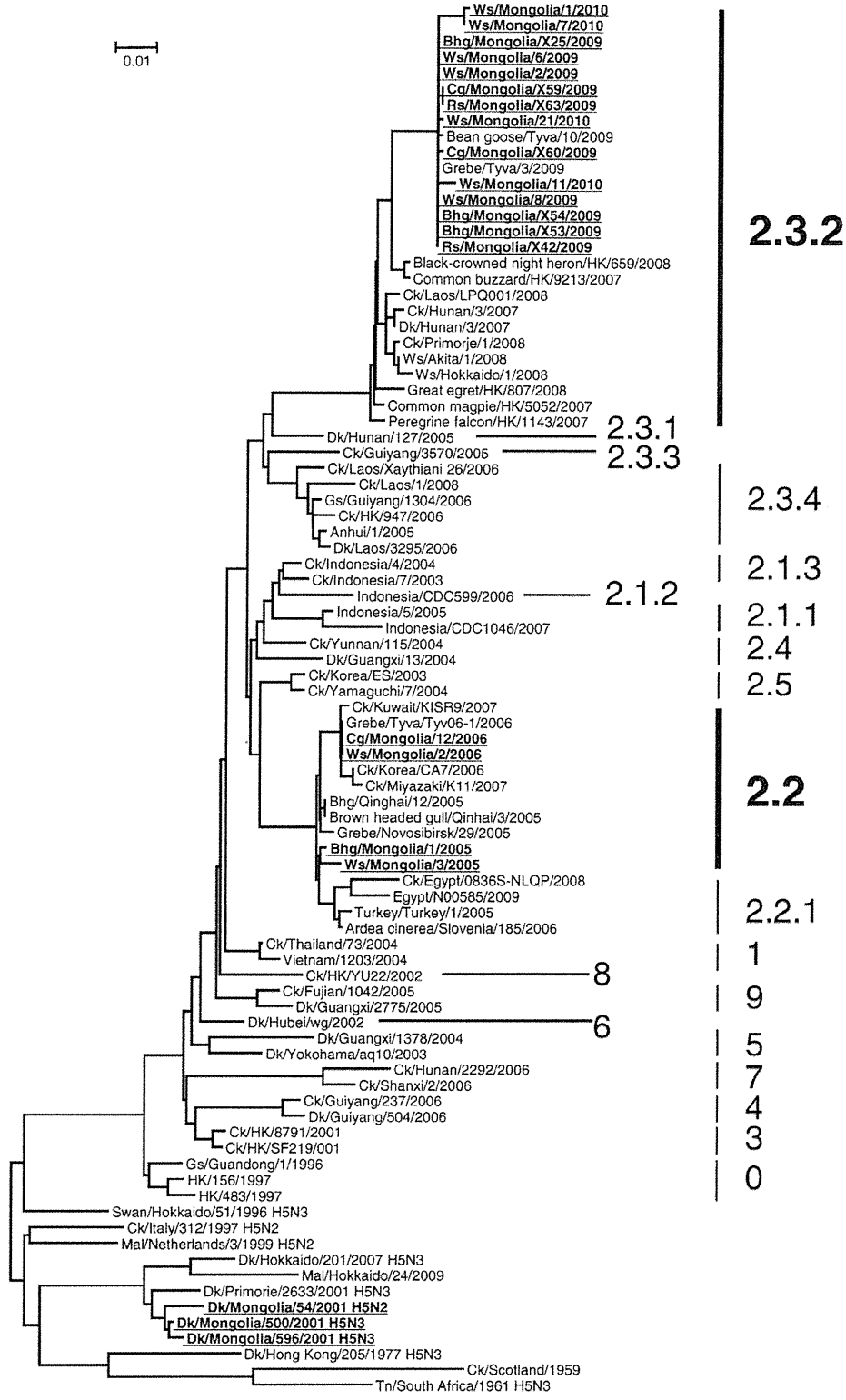

Fig. 1. Phylogenetic trees of the HA genes of H5 influenza viruses. Nucleotide sequences (976 bp) of the HA genes of H5 avian influenza viruses isolated in Mongolia (shown in bold and underlined) and the sequence information of other related viruses were cited from the public database for phylogenetic 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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Lipopolysaccharide treatment and inoculation of influenza A virus results in influenza virus–associated encephalopathy–like changes in neonatal mice

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Influenza virus–associated encephalopathy (IAE) is a highly mortal neural complication of influenza A virus (IAV) infection, mostly affecting children younger than 5 years old, and the brain pathology of IAE is characterized by peracute brain edema with evidence of an impaired blood-brain barrier. The pathogenesis of IAE is unknown, but hypercytokinemia of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 is suspected of playing a central role in the development of IAE. Because the brain pathology of IAE is similar to that of septic encephalopathy due to endotoxemia, the effect of combined treatment of IAV and lipopolysaccharide (LPS) was tested using suckling mice. The results show that pulmonary infection with non-neurotropic IAV enhanced the neuropathogenicity of LPS and induced encephalopathy that was similar to IAE with respect to the occurrence of central nervous system (CNS) histopathology and the absence of direct infection of IAV in the brain. Influenza A virus also increased blood-brain barrier (BBB) permeability and induced inflammatory cytokines in the blood. These results suggested that the mice treated with IAV+LPS are possible animal models of IAE, and that hypercytokinemia and/or the involvement of endotoxemia in IAV infection are possible causes of IAE. *Journal of NeuroVirology* (2010) **16**, 125–132.

Keywords: encephalopathy; inflammatory cytokines; influenza virus; mouse model

Introduction

Influenza virus-associated encephalopathy (IAE) is a rare but highly mortal neural complication of influenza virus infection, mostly affecting children younger than 5 years old (Morishima *et al*, 2002). It has been reported around the world (Toovey, 2008), but patients have been found most frequently in Japan. According to etiological research, IAE is typically associated with non-neurotropic H3N2 influenza A virus (IAV) infection (Morishima *et al*, 2002). Postmortem microscopic analyses on the patients demonstrated hyalinization of blood vessels and extravascular leakage of plasma proteins in the brains, which suggest the occurrence of vasogenic edema due to damage of vascular endothelial cells (Morishima *et al*, 2002). The encephalopathy is often followed by disseminated intravascular coagulation and multiple organ failure (Togashi *et al*, 2004; Yokota *et al*, 2000).

The pathological mechanism of the IAE is unknown. In IAE patients, IAV infects mucosal epithelial cells of the respiratory tract, and an isolation of the virus from the central nervous system (CNS), including the cerebrospinal fluid (CSF), is usually negative (Ito *et al*, 1999; Smidt *et al*, 2004). Therefore, the direct invasion of the virus into the CNS is irrelevant as the cause of IAE. Miniplasmin, a hemagglutinin processing protease, accumulates in the cerebral capillaries of mice with abnormal

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mitochondrial β-oxidation after non-neurotropic IAV infection (Yao *et al*, 2004). The accumulation of miniplasmin allows non-neurotropic IAV to infect the cerebral vascular endothelial cells, which triggers the increased permeability of the blood-brain barrier (BBB). However, it may not be the cause of IAE, since IAV has not usually been demonstrated in the CNS of IAE patients and this animal model is accompanied by a fatty change in hepatocytes similar to Reyes' syndrome (Okita *et al*, 1996). Elevated concentrations of several cytokines, including tumor necrosis factor (TNF)-α, soluble TNF receptor 1, interleukin (IL)-6, and IL-1β, have been reported in the serum and CSF of IAE patients (Ichiyama *et al*, 2003; Ito *et al*, 1999), and the concentrations of these inflammatory cytokines were correlated with the severity of CNS dysfunction (Aiba *et al*, 2001; Toovey, 2008). From these results, hypercytokinemia is suspected of playing an important role in the development of IAE. However, there are arguments on whether the hypercytokinemia is the cause or result of IAE, since the involvement of hypercytokinemia to IAE has not been experimentally confirmed yet and the direct cause of hypercytokinemia in IAE is unknown.

Sepsis is a condition characterized by uncontrolled bacterial infection and affects many organs, including the CNS. Septic encephalopathy is one of the common complications of sepsis and is characterized by diffuse or multifocal neural dysfunction as a result of an inflammatory response with or without direct bacterial invasion into the brain (Papadopoulos *et al*, 2000). Administration of lipopolysaccharide (LPS), a structural component of the outer membrane of gram-negative bacteria, induces pathological changes that mimic the process of sepsis (Alexander *et al*, 2008; Papadopoulos *et al*, 2000). The predominant microscopic findings of the encephalopathy are vasogenic cerebral edema and neuronal damage (Bogdanski *et al*, 2000; Stolp *et al*, 2005). Although the mechanism of septic encephalopathy is not fully elucidated, TNF might play an important role in the pathogenicity (Alexander *et al*, 2008). Therefore, septic encephalopathy is similar to IAE regarding the involvement of inflammatory cytokines as well as the CNS histopathology.

In this study, we demonstrate that pulmonary infection of non-neurotropic IAV enhances neuropathogenicity of LPS and induces encephalopathy similar to IAE in regards to the occurrence of CNS histopathology with the absence of direct infection of IAV in the brain, an increase of BBB permeability, and the induction of inflammatory cytokines in the blood.

Results

General statuses and survival rates of each group of mice

We gave inoculations of IAV, LPS, or both (IAV+LPS) to 1-week-old ICR mice. The mice of the IAV group showed depression, rough fur, and emaciation at 3 to 4 days post infection (dpi), which progressively worsened. Meanwhile, LPS and IAV+LPS group mice showed similar symptoms as well as diarrhea and a sluggish response to manual stimulations within several hours of the LPS inoculations. The general symptoms appeared severer in the IAV+LPS group than in the LPS group. All the mice in the LPS group survived, whereas 93% and 68% of mice in the IAV and IAV +LPS groups survived, respectively (Figure 1).

Histopathological changes in mice

The mice of the IAV and IAV+LPS groups showed multifocal bronchointerstitial pneumonia in the lungs (Figure 2A). The lesions were mainly located around the bronchioles, and the bronchiolar and alveolar walls were thickened by infiltration of mononuclear cells and neutrophils. Bronchiolar lumina and alveoli also contained a small number of sloughed epithelial cells. There were no obvious differences between the mice in the IAV and IAV +LPS groups regarding the quality, distribution, and severity of the pulmonary lesions. Although there were no any significant lesions in the brains of the IAV group, multifocal microbleeding, irregular dilation of perivascular spaces, and neutrophilic infiltration were found in the brains of the mice in the LPS and IAV+LPS groups (Figure 2B, C). These brain lesions were usually located at the cerebral cortex and brain stem, and were sometimes accompanied by mild spongiform change of neuropils and Alzheimer type 2–like degenerated astrocytes (Figure 2D). These cerebral lesions appeared 24 h after the second inoculation of LPS, and were more prominent in the IAV+LPS group than the LPS group 48 h after this inoculation. Other organs were unremarkable, except that there was a depletion of thymic lymphocytes in the LPS and IAV+LPS groups. There were no significant differences in the histopathological semiquantitative scores on thymic lymphocytic depletion and bronchointerstitial pneumonia between IAV and IAV +LPS groups. Cerebral lesions in the IAV+LPS group were more severe than those in the LPS group,

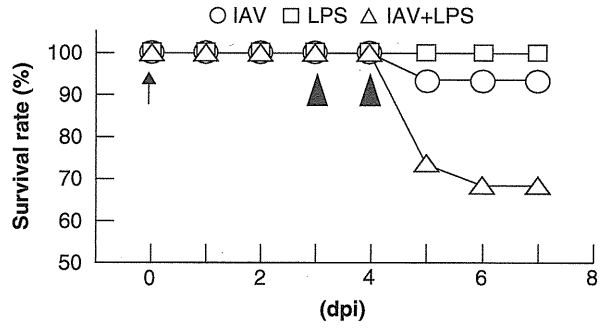

Figure 1 Survival rates of IAV, LPS, and IAV+LPS group mice. The mice were inoculated with IAV (IAV and IAV+LPS groups) or saline (LPS group) at 0 dpi (arrow). Then, the mice received inoculations of LPS (LPS and IAV+LPS mice) or saline (IAV mice) at 3 and 4 dpi (arrow heads).

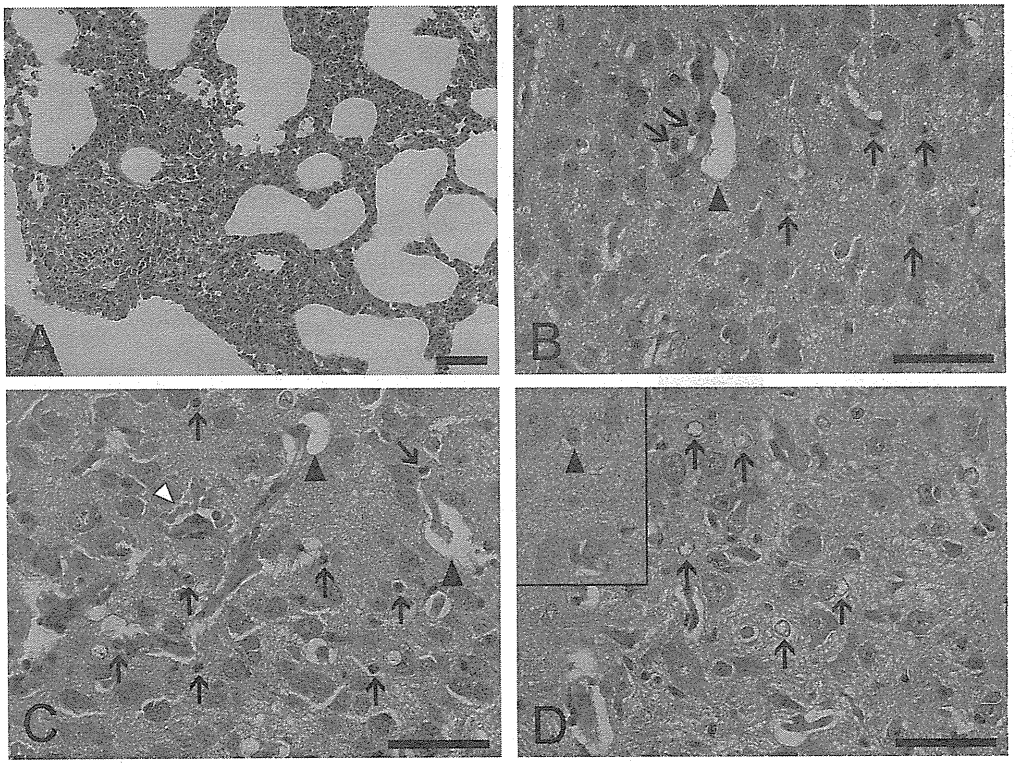

Figure 2 Representative histopathology of the lungs (**A**) and brain (B-D) from IAV+LPS group mice at 5 dpi. (A) Bronchointerstitial pneumonia due to IAV infection. (**B**) Cerebral microbleeding (arrowhead) and irregular dilation of perivascular spaces (arrows). (**C**) Neutrophils (arrows) infiltrating the cerebral cortex. (**D**) Mild spongiform change of neurophil and degenerated astrocytes having vacuolated and swollen nuclei. Hematoxylin and eosin stain. Bars = 50 μm.

especially regarding the severity of cerebral microbleeding (Table 1).

The estimation of cerebrovascular permeability

To evaluate cerebrovascular permeability, we administered Evans blue (EB) dye to the abdominal cavity of mice, and then compared the concentration of the dye in the brains from the IAV, LPS, and IAV+LPS groups. Taken as a whole, the color of the brains from the IAV group was normal, whereas the brains from the LPS and IAV+LPS groups had a noticeable bluish tinge that appeared deeper in the IAV+LPS group than in the LPS group (Figure 3A). To quantify the concentrations of EB dye, the densities of the dye in the brains were measured by absorption spectrometry (Figure 3B). The brains from the IAV+LPS group contained significantly higher levels of the dye than those from the LPS group. These results suggest that the integrity of the BBB significantly deteriorated in the IAV+LPS group, and the results were consistent with histopathological data in which the mice of the IAV+LPS group showed severer cerebral lesions than the other groups.

The induction of cytokines in plasma

In previous studies, it has been reported that the infection of IAV enhanced the inductions of inflammatory cytokines in human leukocytes (Lundemose *et al*, 1993; Nain *et al*, 1990), and inflammatory cytokines were demonstrated to have important roles

Table 1 Scores of microscopic changes

Groups	Time since 2nd LPS injections	Thymus Lymphocytic depletion	Lungs Interstitial pneumonia	Brain		
				Microbleeding	Neutrophilic infiltration	Edema
IAV		0 (0–0)[a]	2 (2–2)	0 (0–0)	0 (0–0)	0 (0–0)
LPS	48 h	2 (1.25–2)	0 (0–0)	1.5 (1–2)	2.5 (1.25–3)	2 (1.25–2)
	72 h	2 (2–2)	0 (0–0)	0 (0–0)	2 (1.5–2)	2 (0.5–2)
IAV+LPS	48 h	3 (3–3)	1.5 (1–2)	3 (3–3)*	3 (3–3)	2 (2–3)
	72 h	2 (1.5–2)	2 (2–2)	0 (0–0)	2 (1.5–2)	1 (1–1.5)

[a]Values mean median of scores (25–75 percentile) in this order. 0 = no obvious change; 1 = focal mild change; 2 = multifocal moderate change; 3 = diffuse, moderate to severe change. *$P < .05$. Differences were analyzed using Mann-Whitney U test.

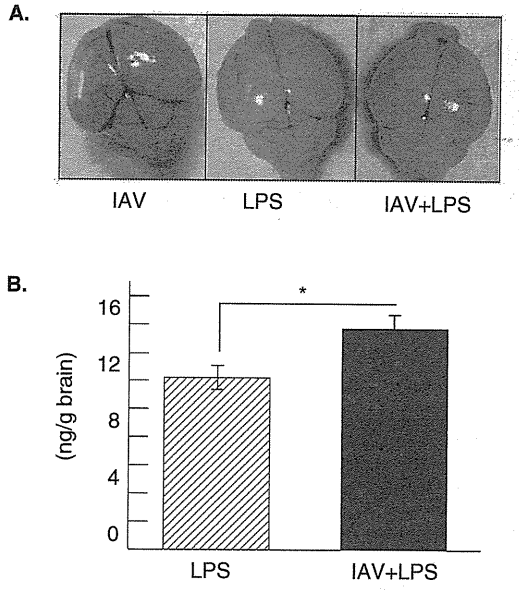

Figure 3 Comparison of BBB integrity. (**A**) Photographs of the brains from the IAV, LPS, and IAV+LPS groups injected intraperitoneally with EB dye. The brains were collected after 48 h of LPS inoculations. (**B**) Quantification of EB dye extracted from the brains. Each error bar means standard error. The statistical comparison was performed using Student's *t* test (*$P < .05$).

as pathogenic agents of LPS (Alexander *et al*, 2008; Papadopoulos *et al*, 2000). Therefore, we measured the levels of plasma cytokines in order to clarify whether IAV infection affects the inductions of inflammatory cytokines by LPS as well as the severity of the encephalopathy. Cytokine levels in the plasma of each group were measured 6, 12, and 24 h after the LPS inoculations. Six hours after the LPS inoculations, the induction of most inflammatory cytokines was upgraded in the LPS and IAV +LPS groups (Figure 4A–D). The serum concentrations of TNF-α and IL-6 in the IAV+LPS group were significantly higher than those in the IAV and LPS groups (Figure 4A, C). In addition, the concentrations of monocyte chemoattractant protein (MCP)-1 and IL-10 in the IAV+LPS group were significantly higher than those in the IAV group (Figure 4B, D). In contrast, the interferon (IFN)-γ level of the IAV group tended to be higher without statistical significance than those of the other groups (Figure 4E). The high cytokine levels in the LPS and IAV+LPS groups dropped to normal or to lower levels than normal until 24 h after of the LPS stimulations (data not shown). Serum concentrations of IL-12p70 were unchanged in all groups throughout the experiments (data not shown).

The distribution of viral antigens and viral titer measurement

Viral antigens were detected by immunohistochemistry in the lungs from IAV and IAV+LPS groups. The IAV antigens were located in bronchiolar epithelial cells and alveolar epithelial cells (Figure 5A). In the bronchioli, many bronchiolar epithelial cells showed positive reaction along the walls, and alveolar

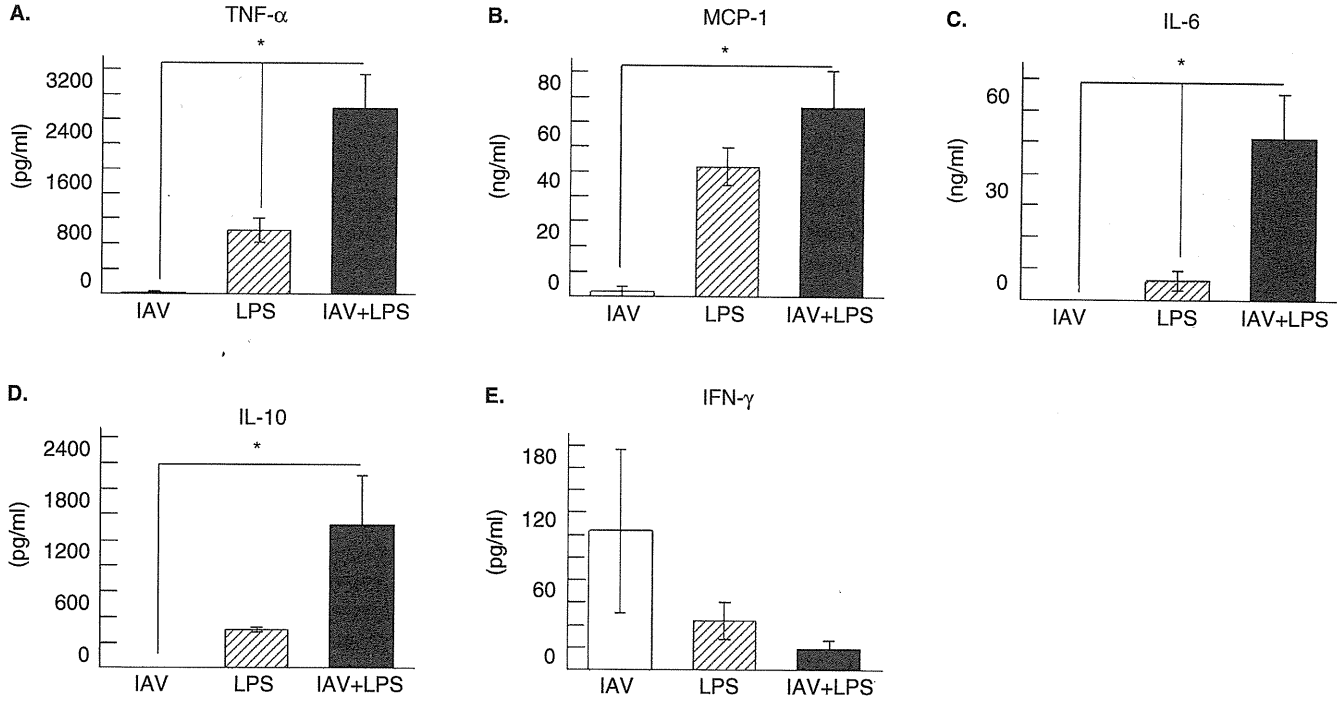

Figure 4 Quantitative analysis of the production of TNF-α (**A**), MCP-1 (**B**), IL-6 (**C**), IL-10 (**D**), and IFN-γ (**E**) in plasma from the IAV, LPS, and IAV+LPS groups. The plasma was collected after 6 h of LPS (or saline) inoculations. Each error bar means standard error. Differences among means were statistically analyzed using Tukey-Kramer multiple comparison tests (*$P < .01$).

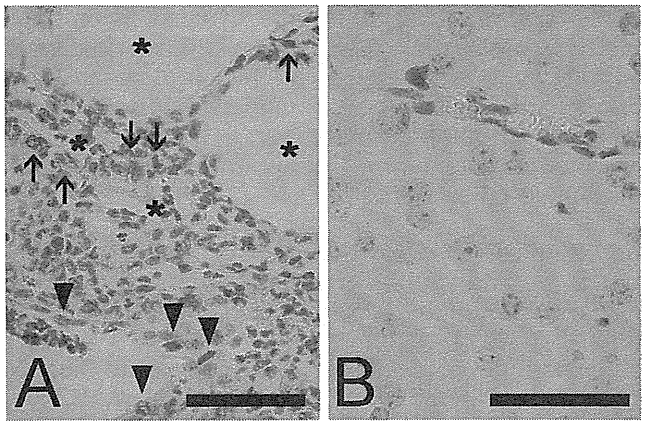

Figure 5 Representative photographs of immunohistochemistry for IAV antigens. (**A**) In the lung from the IAV+LPS group mouse, some bronchiolar and alveolar epithelial cells show positive reactions (arrows). (**B**) The brain of the IAV+LPS mouse is entirely negative for virus antigen. The tissues were collected at 5 dpi of LPS. Bars = 50 μm.

epithelial cells which had oval or flattened shapes showed positive reaction in some thickened alveolar walls. There were no obvious differences in the localization and amounts of the antigen between both groups. However, organs such as the liver, spleen, kidneys, heart, and brain (Figure 5B) stained negative for IAV antigens in the IAV and IAV+LPS groups. We measured viral titers of the lungs and brain by plaque assay. Viral titers in the lungs of the IAV and IAV+LPS groups were 6.62 ± 0.08 and 6.42 ± 0.13 (mean ± SEM: $\log_{10}$ plaque-forming units [PFU]/g), respectively. IAV was not isolated from brain of the IAV and IAV+LPS groups by plaque assay.

Discussion

Early diagnosis, prevention, and therapy for IAE are still to be established because the pathological mechanisms of the disease are unknown. The disease usually occurs in children younger than 5 years of age, and we used neonatal mice to produce IAE in the present experiment. IAV infection induced bronchointerstitial pneumonia in neonatal mice with survival rate of 93% in the IAV group. There were no differences in the viral titers and localization of viral antigen between the IAV and IAV+LPS groups. Survival rates of the IAV+LPS and LPS groups were 68% and 100%, respectively, and only the CNS involvement was severer in the IAV +LPS group than the LPS group. Therefore, the CNS damage was considered to be the direct cause of the decreased survival rate in the IAV+LPS group.

The microscopic cerebral lesions, including microbleeding and dilation of perivascular space, suggest the impairment of cerebral blood vessels and vasogenic edema. This was consistent with the findings of LPS-induced encephalopathy in previous reports (Bogdanski *et al*, 2000; Stolp *et al*, 2005). Scattered neutrophilic infiltration was likely to be the secondary reaction to the brain damage. In our experiment, IAV infection aggravated LPS-induced encephalopathy. Similarly, an infection of infant ferrets with IAV enhanced their susceptibility to the lethal effects of endotoxin (Jakeman *et al*, 1991). The pathological mechanism of LPS encephalopathy remains to be elucidated, but previous studies reported the participation of multiple factors, including inflammatory cells and their mediators, reduced cerebral blood flow, and disruption of the BBB (Papadopoulos *et al*, 2000). In the present study, we did not find any intravascular thrombi in all mice and it was unlikely that microvascular infarction caused cerebral damage in the LPS and IAV+LPS groups. On the other hand, IAV infection enhanced an increase of BBB permeability and the production of TNF-α and IL-6 in plasma of the IAV+LPS mice. The enhanced TNF-α induction may contribute to cerebral vascular damage because TNF-α has a critical role as a pathogenic agent of LPS-induced encephalopathy (Alexander *et al*, 2008). Previous *in vitro* studies using human peripheral blood leukocytes revealed that the production of TNF-α, IL-1, and IL-6 was enhanced by the combined treatment of IAV and LPS (Lundemose *et al*, 1993; Nain *et al*, 1990). Although the mechanism of cytokine induction by IAV and LPS treatment was not elucidated completely, it was suggested that IAV and LPS concurrently activate several common transcription factors responsible for cytokine gene expression, including nuclear factor (NF)-κB, interferon regulatory factor (IRF), and activator protein (AP)-1 (Julkunen *et al*, 2000; Kawai and Akira, 2006). Additionally, LPS is thought to potentiate cytokine synthesis at the posttranscriptional level (Han *et al*, 1990; Nain *et al*, 1990). In the present experiment, the level of plasma TNF-α and the severity of brain lesions were positively correlated. Therefore, the infection of neonatal mice with non-neurotropic IAV might enhance LPS-induced encephalopathy by means of overinduction of cytokines such as TNF-α and IL-6, which contributed to cerebral vascular damage and increased permeability of BBB.

Pathological entity of IAE is vasogenic brain edema due to the damage of vascular endothelial cells without direct invasion of IAV to the brain (Morishima *et al*, 2002). Elevated concentrations of cytokines such as TNF-α and IL-6 may be the cause of the disease (Aiba *et al*, 2001; Ichiyama *et al*, 2003; Ito *et al*, 1999; Toovey, 2008). In the present experiment, IAV+LPS mice showed the IAE-like encephalopathy in respect to the vasogenic brain disorders and the dynamics of plasma inflammatory cytokines without direct infection of IAV to the brain. From these results, it may be concluded that the mice treated with IAV+LPS are a potential animal model of IAE, and that hypercytokinemia and/or the involvement of endotoxemia in IAV infection are possible causes of IAE.

Materials and methods

Virus and mice

Influenza A virus strain A/Aichi/2/68 (H3N2) was propagated in 10-day-old embryonated chicken eggs at 35°C for 48 h (Isoda *et al*, 2006). The virus-containing allantoic fluid was stored at −80°C and used as the source of virus. The infectivity of the virulent fluid was determined using hamagglutinin assay and plaque assay.

Pregnant ICR mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). The 7-day-old suckling mice were submitted to the following animal experiment. To minimize the difference between maternal mice, the number of neonatal mice was kept around 12 per female mouse. All animal studies were carried out with the approval of the committee of Laboratory Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University, and are consistent with the Association for Assessment and Accreditation of Laboratory Animal Care International standards.

Inoculation of influenza virus and LPS to mice

Seven-day-old mice were anesthetized by isoflurane inhalation and inoculated in both nostrils with 10 μl (1×10^5 PFU) of IAV. Then, the mice were inoculated with LPS of *Esherichia coli* O55:B5 (Sigma, St. Louis, MO, USA) twice. Briefly, the mice received 0.5 μg/g of the first LPS injection to the hindlimb muscle at 3 dpi of the virus inoculation, followed by the second injection of 20 μg/g LPS to the peritoneal cavity 24 h after the first injection. Control mice were inoculated with equivalent volumes of sterile saline in place of IAV or LPS. Each mouse was monitored for 7 days, or was submitted to the collection of whole blood under anesthesia and necropsied during 4 to 7 dpi at 6- or 12-h intervals.

Histopathological analysis and semiquantitative evaluation of microscopic changes

At necropsy, tissue samples from the liver, spleen, kidneys, heart, lungs, thymus, and brain were collected from mice, fixed in 20% neutral-buffered formalin solution, and embedded in paraffin wax. The tissue samples were sectioned at a thickness of 4 μm and stained with hematoxylin-eosin stain for light microscopy. Histopathological changes of thymus, lungs, and brains were semiquantitatively scored from score 0 (absent) to score 3 (severe) on the basis of the distribution and severity. Briefly, we made sections from predetermined areas of thymus, lungs and brain. For the evaluation of pulmonary lesion, we obtained four sections from lungs (two sections from the left lung and two sections from the anterior and caudal lobes of the right lung). For the evaluation of brain lesions, we obtained five sections from brain (from each level of frontal lobe, diencephalon, occipital lobe, brain stem, and medulla oblongata). The criteria for scoring were as follows: 0 = no significant lesion; 1 = localized and very mild lesions; 2 = moderate lesions multifocally distributed on more than half of the sections; 3 = moderate to severe lesions diffusely distributed on almost all sections.

Immunohistochemical detection of viral antigen

Serial sections were stained with the streptavidin-biotin immunoperoxidase complex method using Histofine SAB-PO kit (Nichirei, Tokyo, Japan). To restore antigens, the sections were treated with 0.01 M phosphate-buffered saline (PBS) containing 0.1% trypsin (Becton Dickinson, Mountain View, CA, USA) for 20 min at 37°C. As the primary antibody, a diluted rabbit anti-strain A/Whistling swan/Shimane/499/83 (H5N3) hyperimmuno serum (produced in our laboratory) was applied for approximately 12 h at 4°C (Matsuda *et al*, 2004). The chromogenic reaction was performed by 0.05 M Tris-HCl buffer (pH 7.6) containing 0.02% 3,3′-diaminobenzidinetetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan), 0.005% H_2O_2, and 0.01 M imidazole (Sigma). The sections were counterstained with Mayer's hematoxylin. The primary antibody was replaced by 0.01 M PBS for negative controls.

The evaluation of BBB integrity

The integrity of BBB was evaluated by Evans blue (EB; Kanto Chemical, Tokyo, Japan) extravasations, as described previously (Bigdeli and Khoshbaten, 2008; Fukui *et al*, 2003). Briefly, the mice of the IAV group (n = 3), LPS group (n = 4), and IAV +LPS group (n = 4) were injected intraperitoneally with 50 μl of filtered 2% EB solution in sterile saline after 48 h of the second LPS inoculation. The mice were anesthetized after 2 h of EB injection and the brains were rapidly removed. The EB dye in the brains was extracted with 500 μl formamide for 24 h at 38°C. The amount of EB in the supernatants was measured against 90% formamide solution in saline at 630 nm using a plate reader (Multiscan Ascent; Thermo Labsystems, Franklin, MA, USA). EB levels were expressed as ng/g of brain tissue against a standard curve.

The determination of plasma cytokine concentrations

Whole blood was taken into blood collection tubes containing EDTA (Terumo Medical, Elkton, MD, USA). Supernatant plasma was separated by centrifugation at 3500 × g for 2 min and frozen under −80°C until assayed for cytokines. The analysis of plasma cytokine levels was conducted using a mouse inflammation cytometric bead array kit and a FACSArray bioanalyzer (Becton Dickinson, USA) (Li *et al*, 2007; Morgan *et al*, 2004). The experiment was conducted following manufacturer's instructions. Standard curves were determined for each cytokine

from the range of 20 to 5000 pg/ml. The following cytokines were measured: IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70.

Plaque assay

To evaluate viral titer, we performed a plaque assay as previously described (Tsuda *et al*, 2009). Briefly, Mardin-Darby canine kidney (MDCK) cells were grown in Eagle's minimal essential medium (EMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% inactivated fetal bovine serum and 2 mM L-glutamine. Pieces of mouse tissues were homogenized in 10% concentration with EMEM under 4°C. The 10-fold dilutions of the homogenized sample were inoculated to confluent monolayers of MDCK cells and incubated for 1 h at 37°C. The dilutions were removed, and the cells were washed with PBS. Cells were overlaid with EMEM containing 1% Bacto Agar (Becton Dickinson) and 0.0005% acetyl trypsin. After 48 h of inoculation at 37°C, cells were stained with 0.005% neutral red for 12 h and visible plaques were counted.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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