Studies on the pathogenicity and vaccine development of H5N1 highly pathogenic avian influenza virus strains

(H5N1 高病原性鳥インフルエンザウイルスの病原性およびワクチン開発に関する研究)

Norikazu Isoda

Contents

Preface

Avian influenza is caused by a type A influenza virus of Orthomyxoviridae. Influenza A viruses infect swine, horses, seals, and a large variety of birds as well as humans. Each of the sixteen hemagglutinin (HA) and nine neuraminidase (NA) subtypes of influenza A virus has been isolated from aquatic birds. In summer, ducks are infected with the viruses on the lakes and marshes where they nest in the northern territories such as Siberia or Alaska. The viruses replicate in the simple columnar epithelial cells which form crypts in the colon of ducks and are shed in the feces. In autumn, the ducks leave the nesting areas for migration. Avirulent viruses which are circulating in natural hosts, ducks, transmit to poultry and domestic animals along the flyway of migration. Highly pathogenic viruses arise occasionally from avirulent virus pools. Especially, it is known that highly pathogenic avian influenza (HPAI) virus strains are selected when the viruses transmit to chickens through quails, turkeys, geese, or some waterfowls and are passaged among chicken populations. The pathogenicity of influenza viruses for chickens ranges from asymptomatic to systemic infections with low to high mortality. Apathogenic and low pathogenic avian influenza virus strains are in all HA subtypes (H1 -

H16). HPAI virus strains are restricted to subtypes H5 and H7, though not all viruses of these subtypes are HPAI viruses.

In 1997, outbreak of HPAI caused by H5N1 viruses occurred in three chicken farms in Hong Kong. In addition to the death of over 6,500 chickens by virus infection, 1.2 million chickens were slaughtered as control measures. This outbreak impacts not only the poultry industry but also public health since 18 people were infected and 6 died. In Japan, HPAI outbreak caused by H5N1 viruses also occurred in Yamaguchi, Oita, and Kyoto prefectures in 2004 and did in Miyazaki and Okayama prefectures in 2007. Although most of the HPAI viruses for chickens have not caused any disease signs in ducks [2, 19, 24], some of the H5N1 HPAI viruses, that have introduced back to feral water birds and been lethal for water birds, were isolated since 2004 [7, 30]. On the basis of phylogenic analysis, all H5N1 viruses isolated from dead water birds in Eurasia and Africa are identical to the isolates in China [27], indicating that this virus strain may spread worldwide by duck migration.

“Stamping-out” is the basic measure for the control of HPAI. Two outbreaks of HPAI occurred in Japan in 2004 and in 2008, were well controlled by the adequate measures. Vaccination may be an optional measures in cases where the disease spread widely in order to decrease the amount of infectious viruses in environment [38]. Since commercial vaccines have been prepared

from viruses belonging to the North American lineage in the USA and Mexico, they may be less effective for the control of current HPAI outbreaks in Asia. Antigenic analysis using a panel of monoclonal antibodies to the H5 non-pathogenic avian influenza viruses isolated from migratory ducks in Asia indicated that the HAs of highly pathogenic avian influenza viruses currently circulating in Asia were antigenically more closely related to that of isolates in Asia than those of viruses belonging to the North American lineage [46]. New inactivated influenza vaccines prepared from an H5N1 virus strain belonging to the Eurasian lineage are desired to be developed.

The present thesis is composed of two chapters. In chapter I, pathogenicity of two highly pathogenic avian influenza viruses, A/chicken/Yamaguchi/7/2004 (H5N1) and A/whooper swan/Mongolia/3/2005 (H5N1) in different species of birds and mammals is reported. In chapter II, an inactivated avian influenza vaccine prepared from a non-pathogenic H5N1 reassortant virus generated between isolates from migratory ducks in Asia is described.

Chapter I

Pathogenicity of highly pathogenic avian influenza virus strains, A/chicken/Yamaguchi/7 /2004 (H5N1), and A/whooper swan/Mongolia/3 /2005 (H5N1) in different species of birds and mammals

Introduction

A wide variety of species of birds and mammals are susceptible to influenza A virus infection. Viruses of all 16 hemagglutinin (HA) (H1-H16) and 9 neuraminidase (N1-N9) subtypes have been isolated from avian species [1, 12] Aquatic birds are the natural reservoirs of influenza A viruses [22]. Influenza viruses are perpetuated in nature by continuing to circulate in migratory ducks and frozen lake water [18]. Based on the severity of the disease they cause in chickens, avian influenza viruses are divided into two groups, highly pathogenic and low pathogenic [1]. Low pathogenic avian influenza (LPAI) viruses replicate in limited tissues where host proteases such as trypsin-like enzymes

are found. HPAI viruses possess inserted multiple basic amino acid residues at the site of cleavage of their HAs into HA1 and HA2 by ubiquitous proteases such as furin and PC6 [17, 49]. This cleavage confers infectivity to a greater number of tissues leading to a severe systemic disease, characterized by high mortality [25]. The HPAI viruses are restricted to subtypes H5 and H7, and viruses of these two subtypes had been believed to low pathogenic in the reservoir host, ducks, until HPAI H5N1 viruses were isolated from the bar-headed geese, brown-headed gulls, and black-headed gulls, 2005, in China [7, 30].

Outbreaks of HPAI in poultry such as chickens and quails around the world have caused high mortality and substantial economic losses, thereby, impacting negatively on the poultry industry [1, 53]. Outbreaks have occurred often in the last decade in North America, Europe and Asia. In Asia, H5N1 HPAI viruses have been recognized since 1996 [58]. In 1997, HPAI viruses were directly transmitted from birds to humans in Hong Kong, signaling the necessity to clarify the ecology of avian influenza viruses [51]. HPAI outbreaks again occurred during 2001-2002, in Hong Kong [45]. In 2004, HPAI outbreaks also occurred in Cambodia, China, Indonesia, Malaysia, Japan, Laos, South Korea, Thailand, and Vietnam [28]. The HPAI virus, A/chicken/Yamaguchi/7/2004 (H5N1), isolated in Japan, 2004, was lethal to chickens [35]. Although most of the HPAI viruses for chickens do not cause any disease signs in ducks [2, 19, 24], some of

the H5N1 HPAI viruses that had returned to feral water birds and been lethal for water birds, have been isolated since 2004 [7, 30]. In 2005 and 2006, dead aquatic birds (whooper swan, black-headed goose, and common golden eye) were found in the Lake Erhel and Khunt nuur, Mongolia and 4 H5N1 HPAI virus strains were isolated. Pathogenicity of these HPAI viruses in birds and in mammals is remaining unclear. In order to determine the pathogenicity of the two HPAI viruses, A/chicken/Yamaguchi/7/2004 (H5N1) and A/whooper swan/Mongolia/3/2005 (H5N1), which was isolated from dead whooper swan found at the lake Erhel nuur, in chickens, quails, starlings, budgerigars, ducklings, mice, and miniature pigs, and to compare the pathogenicity of these HPAI viruses in those animals in parallel with that of other H5N1 influenza viruses, experimental infection was carried out in the present study.

Materials and Methods

Viruses

Influenza virus strain, A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04) was isolated from a dead chicken during the first outbreak of HPAI in Japan and was provided by the National Institute of Animal Health

(Ibaraki, Japan) [35]. A/whooper swan/Mongolia/3/2005 (H5N1) (Swan/Mongolia/05) was isolated in our laboratory from the brain homogenates of a dead whooper swan found at the lake Erhel nuur, Mongolia. A/duck/Yokohama/aq-10/2003 (H5N1) (Dk/Yokohama/03), isolated from duck meat imported from China, was provided by the Animal Quarantine service (Kanagawa, Japan) [24, 34]. A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04) was a reassortant virus generated from A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) which were isolated in our laboratory from fecal samples of wild ducks in Mongolia [24]. These four viruses were propagated in 10-day-old embryonated chicken eggs at 35°C for 48 hours. The infectious allantoic fluid was used as inoculum for experimental infections of animals and for the preparation of purified virus.

Animals

Chickens (*Gallus gallus*), quails (*Coturnix japonica*), starlings (*Sturnus cineraceus*), budgerigars (*Melopsittacus undulatus*), ducklings (*Ahas platyhnchos*), mice (*Mus musculus*), and miniature pigs (*Sus scrofa domestic*) were used for the experimental infection study. Specific pathogen-free white leghorn chickens were hatched and raised for four weeks in our laboratory. One-month-old quails and three-month-old budgerigars were purchased from

pet shops. Wild starlings were captured by the Japan Hunting Association (Nagano, Japan). Three-day-old ducklings were purchased from a duck farm in Hokkaido, Japan. Six-week-old female BALB/c mice and two-month-old specific pathogen-free male miniature pigs were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Animal experiments

Viruses were inoculated intranasally, at a 50% egg infectious dose (EID_{50}) of $10^{8.0}$, into birds and mammals. For the birds and miniature pigs, 0.1 ml of each H5N1 virus containing $10^{8.0}$ EID_{50} was inoculated intranasally. For the mice, 0.03 ml of each H5N1 virus containing $10^{8.0}$ EID_{50} was inoculated intranasally. As a negative control, phosphate buffered saline (PH 7.2) (PBS) was given to the birds and mammals as much volume as virus suspension. Birds and mice were sacrificed on 3 and 14 days post-inoculation (p.i.). When animals were dead or sacrificed, respiratory organs (trachea and lung), liver, spleen (only mouse), kidneys, colon (except mouse), brain, heart, pancreas, and blood of each animals were collected aseptically and were used for the titration of virus and histopathological examination. For miniature pigs, nasal swabs were collected in minimal essential medium (MEM) daily from 1 to 7 days p.i., and were used for the titration of virus. Animals were housed in self-contained

isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL 3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Virus titration

The tissue homogenates from birds and mice were inoculated into 10-day-old chicken embryonated eggs and incubated at 35°C for 48 hours. The titers of virus were calculated by the method of Reed and Muench [43], and expressed as the EID_{50} per gram of tissue. Viral titers of the nasal swab samples of the miniature pigs were calculated as the 50% tissue culture infectious dose ($TCID_{50}$) per ml for swab and per gram for tissues in MDCK cells.

Antibody detection

Serum samples treated with beta-propiolactone (Wako Pure Chemicals Industries, Ltd., Japan) at 37°C for 3 hours were examined for the presence of antibodies against H5 influenza virus by ELISA. The purified Dk/Vac-1/04 (H5N1) virus was used as antigen for ELISA according to Kida et al. [20]. ELISA titers were expressed as reciprocals of serum dilutions.

Histopathological examination

The tissues of birds and mammals were fixed in 20% formalin in PBS,

sectioned, and stained with hematoxylin and eosin for microscopic examination. For the detection of influenza virus antigens in the tissues, all the sections were stained using the streptavidin-biotin immunoperoxidase complex method (Histofine SAB-PO ®kit, Nichirei Corp., Tokyo) with rabbit anti-A/duck/Pennsylvania/10218/1984 (H5N2) hyperimmune serum at a 1:1,000 dilution as the primary antibody.

Results

Chickens

All the chickens, inoculated with Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1), died on 2 days, and on between 2 and 4 days p.i., respectively, and virus was recovered from each of the tissues which were tested (respiratory organs, liver, kidneys, colon, and brain) (Table 1). Higher titers of viruses were detected in four out of the five tissues of chickens inoculated with Ck/Yamaguchi/04 (H5N1) than those with Dk/Yokohama/03 (H5N1). None of the chickens inoculated with Dk/Vac-1/04 (H5N1) died by 14 days p.i., and virus was not recovered from any of the tissues at day 3 and 14 p.i. Sero-conversion to H5 influenza virus was not detected in any birds, indicating

Table 1 Virus recovery and antibody response from chickens inoculated with H5N1 viruses

Virus	Days		Virus titer ($logEID_{50}$/g)						Antibody titers
			Respiratory organs	Liver	Kidneys	Colon	Brain	Blood[a]	
Ck/Yamaguchi/04	2	dead	8.0	7.5	7.8	7.5	6.8	ND[b]	ND
(H5N1)	2	dead	8.0	7.3	7.0	6.5	6.5	ND	ND
	2	dead	8.5	7.5	7.8	7.5	7.2	ND	ND
	2	dead	8.8	7.5	7.8	7.4	7.0	ND	ND
	2	dead	8.5	7.5	7.5	7.5	7.5	ND	ND
	2	dead	8.3	7.0	7.5	7.4	7.5	ND	ND
Dk/Yokohama/03	2	dead	6.3	5.8	6.8	5.8	6.3	ND	ND
(H5N1)	3	dead	6.5	6.5	6.8	6.8	7.5	ND	ND
	3	sacrificed	6.8	6.5	7.2	7.2	8.0	7.3	<40
	4	dead	7.8	5.2	5.5	5.5	8.5	ND	ND
	4	dead	8.2	7.2	7.2	5.5	7.8	ND	ND
	4	dead	6.8	4.5	5.5	5.5	8.5	ND	ND
Dk/Vac-1/04	3	sacrificed	-[c]	-	-	-	-	-	<40
(H5N1)	3	sacrificed	-	-	-	-	-	-	<40
	3	sacrificed	-	-	-	-	-	-	<40
	14	sacrificed	-	-	-	-	-	-	<40
	14	sacrificed	-	-	-	-	-	-	<40
	14	sacrificed	-	-	-	-	-	-	<40

[a] Blood samples were calculated as $logEID_{50}$/ml.

[b] Blood samples and antibodies were not corrected from the dead animals.

[c] - : <1.5 $logEID_{50}$ /g or <1.5 $logEID_{50}$ /ml

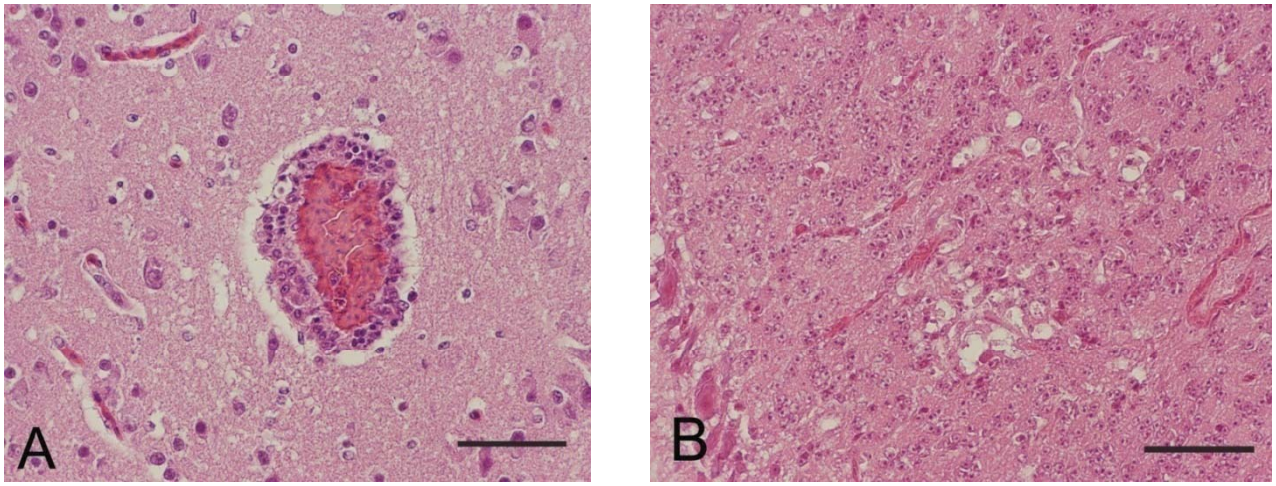

Fig.1. Histopathological examination in chickens (A) and quails (B) inoculated with Dk/Yokohama/03 (H5N1). Photomicrographs of hematoxylin and eosin-stained tissue sections. A: Perivascular cuffing, swelling of endothelial cells, infiltration and proliferation of microglia in the brain (cerebrum) of the chicken inoculated with Dk/Yokohama/03 (H5N1) on 4 days 4 post inoculation B: Laminar encephalomalaccia (nectosis) in the brain (cerebellum) of the quail inoculated with Dk/Yokohama/03 (H5N1) on 4 days post inoculation. Bar indicates 100 μm.

that the chickens were not infected with Dk/Vac-1/04 (H5N1). In the histopathological examination, influenza virus antigens were detected in the brain, liver, spleen, kidneys, heart, lungs, pancreas, and colon of chickens inoculated either with Ck/Yamaguchi/04 (H5N1) or with Dk/Yokohama/03 (H5N1). Since severe virus encephalitis with perivascular infiltration in the brain affected one chicken inoculated with Dk/Yokohama/03 (H5N1) (Fig. 1A) and higher titers were detected in the brains of chickens inoculated with Dk/Yokohama/03 (H5N1) than with Ck/Yamaguchi/04 (H5N1), it was found that infection with Dk/Yokohama/03 (H5N1) caused more severe lesions than infection with Ck/Yamaguchi/04 (H5N1) in the brain.

Quails

All the quails, inoculated with Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1), died by 3 and 4 days p.i. respectively, and virus was recovered from each of the tissues which were tested (Table 2). Disease signs characterized by severe nervous disorders were observed in 2 out of 6 quails inoculated with Dk/Yokohama/03 (H5N1). Higher titers of viruses were detected in all the tissues of quails inoculated with Ck/Yamaguchi/04 (H5N1) compared to those inoculated with Dk/Yokohama/03 (H5N1). None of the quails inoculated with Dk/Vac-1/04 (H5N1) died by 14 days p.i., and virus was

Table 2 Virus recovery and antibody response from quails inoculated with H5N1 viruses

Virus	Days		Virus titer ($logEID_{50}$/g)						Antibody titers
			Respiratory organs	Liver	Kidneys	Colon	Brain	Blood[a]	
Ck/Yamaguchi/04	2	dead	5.5	8.8	8.8	7.2	6.8	ND[b]	ND
(H5N1)	2	dead	7.8	7.5	9.2	7.5	8.5	ND	ND
	2	dead	7.2	6.8	8.5	6.2	8.2	ND	ND
	3	dead	6.8	6.2	8.8	7.5	9.2	ND	ND
	3	dead	7.5	6.5	8.5	7.5	8.5	ND	ND
	3	dead	8.8	6.8	8.8	7.8	8.5	ND	ND
	3	dead	8.5	7.2	9.2	6.8	9.2	ND	ND
Dk/Yokohama/03	3	dead	6.2	6.2	6.5	-	7.8	ND	ND
(H5N1)	3	sacrificed	-[c]	6.2	7.2	-	5.8	3.8	<40
	3	sacrificed	7.2	5.8	8.8	-	-	-	<40
	4	dead	6.8	3.8	-	6.5	8.5	ND	ND
	4	dead	6.8	3.2	4.8	6.2	8.8	ND	ND
	4	dead	7.5	4.5	-	6.5	8.2	ND	ND
Dk/Vac-1/04	3	sacrificed	-	-	-	-	-	-	<40
(H5N1)	3	sacrificed	-	-	-	-	-	-	<40
	3	sacrificed	-	-	-	-	-	-	<40
	14	sacrificed	-	-	-	-	-	-	160
	14	sacrificed	-	-	-	-	-	-	640

[a] Blood samples were calculated as $logEID_{50}$/ml.

[b] Blood samples and antibodies were not corrected from the dead animals.

[c] - : <1.5 $logEID_{50}$ /g or <1.5 $logEID_{50}$ /ml

not recovered from any of the tissues on 3 and 14 days p.i. Sero-conversion to H5 influenza virus was detected in the quails inoculated with Dk/Vac-1/04 (H5N1) on 14 days p.i., indicating that these quails were infected with Dk/Vac-1/04 (H5N1). In the histopathological examination, in the brain of the quail inoculated with Dk/Yokohama/03 (H5N1), severe virus encephalitis with laminar encephalomalacia (necrosis) was observed (Fig. 1B), and antigens to influenza viruses were detected in the brains and hearts of bird infected either with Ck/Yamaguchi/04 (H5N1) or with Dk/Yokohama/03 (H5N1).

Starlings

All the starlings, inoculated with Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1) died on between 2 and 8 days p.i., and virus was recovered from each of the tissues which were tested (Table 3). On the inoculation of Dk/Vac-1/04 (H5N1), all the starlings died by 7 days p.i., and infectous virus was recovered from the respiratory organs and colon. Incidentally, all starlings inoculated with PBS as a negative control, died by 3 days p.i. (data not shown), making it difficult to conclude whether these three H5N1 viruses have lethal pathogenicity in starlings or not.

Table 3 Virus recovery and antibody response from starlings inoculated with H5N1 viruses

Virus	Days		Virus titer ($logEID_{50}$/g)						Antibody titers
			Respiratory organs	Liver	Kidneys	Colon	Brain	Blood[a]	
Ck/Yamaguchi/04	2	dead	3.3	3.3	<2.0	<1.7	-	ND[b]	ND
(H5N1)	2	dead	<2.0	4.5	2.5	<2.5	-	ND	ND
	3	dead	6.3	5.5	4.5	4.5	<2.5	ND	ND
	3	dead	6.5	6.5	6.5	5.5	4.7	ND	ND
	5	dead	7.3	6.5	5.3	6.7	5.0	ND	ND
	6	dead	4.5	3.5	5.7	2.7	6.3	ND	ND
Dk/Yokohama/03	2	dead	3.3	4.5	3.5	3.5	-	ND	ND
(H5N1)	2	dead	5.5	5.5	3.7	3.7	3.3	ND	ND
	3	dead	3.7	4.3	5.5	5.5	3.7	ND	ND
	4	dead	5.3	5.3	6.5	5.3	4.5	ND	ND
	6	dead	5.7	5.5	-	4.7	-	ND	ND
	8	dead	6.5	3.0	4.3	-	6.5	ND	ND
Dk/Vac-1/04	2	dead	3.3	-	-	-	-	ND	ND
(H5N1)	2	dead	2.7	-	-	<2.3	-	ND	ND
	2	dead	4.5	-	-	-	-	ND	ND
	2	dead	-[c]	-	-	3.5	-	ND	ND
	2	dead	-	-	-	<1.7	-	ND	ND
	7	dead	-	-	-	-	-	ND	ND

[a] Blood samples were calculated as $logEID_{50}$/ml.

[b] Blood samples and antibodies were not corrected from the dead animals.

[c] - : <1.5 $logEID_{50}$ /g or <1.5 $logEID_{50}$ /ml

Budgerigars

All the budgerigars, inoculated either with Ck/Yamaguchi/04 (H5N1) or with Dk/Yokohama/03 (H5N1), died by 5 days p.i., and the virus was recovered from each of the tissues which were tested (Table 4). Disease signs such as severe nervous disorders were observed in 3 out of 7 budgerigars infected with Dk/Yokohama/03 (H5N1). None of the budgerigars inoculated with Dk/Vac-1/04 (H5N1) died by 14 days p.i., and virus was not recovered from any of the tissues on 3 and 14 days p.i. Sero-conversion to H5 influenza virus was not detected in any budgerigars inoculated with Dk/Vac-1/04 (H5N1) on 14 days p.i., indicating that the budgerigars were not infected with Dk/Vac-1/04 (H5N1).

Ducklings

Two of the ducklings inoculated with Ck/Yamaguchi/04 (H5N1) died on 6 and 7 days p.i., and virus was recovered from each of the tissues including the brain (Table 5). One of the ducklings survived for 14 days, and from this duckling, specific serum antibodies against H5 influenza virus were detected. All the ducklings inoculated either with Dk/Yokohama/03 (H5N1) or Swan/Mongolia/05 (H5N1) died by 4 and by 2 days p.i., respectively, and the virus was recovered from each tissues which were tested. None of the ducklings inoculated with Dk/Vac-1/04 (H5N1) died by 14 days p.i., and virus

Table 4 Virus recovery and antibody response from budgerigars inoculated with H5N1 viruses

Virus	Days		Virus titer ($logEID_{50}$/g)						Antibody titers
			Respiratory organs	Liver	Kidneys	Colon	Brain	Blood[a]	
Ck/Yamaguchi/04	3	dead	6.7	5.5	6.8	3.5	6.3	ND[b]	ND
(H5N1)	3	dead	7.5	6.8	8.8	5.5	7.3	ND	ND
	3	dead	7.5	2.8	7.8	2.5	6.3	ND	ND
	3	dead	6.5	2.8	7.2	-	6.8	ND	ND
	3	dead	5.5	3.5	6.8	-	8.8	ND	ND
	3	dead	7.0	4.2	5.8	-	8.5	ND	ND
	4	dead	5.5	4.8	6.3	-	7.8	ND	ND
	5	dead	5.5	5.5	4.8	-	6.5	ND	ND
Dk/Yokohama/03	3	sacrificed	4.3	3.2	3.8	2.2	4.8	ND	ND
(H5N1)	3	sacrificed	4.8	3.8	4.8	3.5	6.8	ND	ND
	3	sacrificed	5.8	-	6.5	3.5	6.8	ND	ND
	3	sacrificed	5.0	3.5	6.5	2.5	6.5	ND	ND
	5	dead	5.3	2.5	3.2	2.5	8.2	ND	ND
	5	dead	4.0	2.2	7.8	-	8.2	ND	ND
	5	dead	6.5	3.2	3.8	3.2	7.5	ND	ND
Dk/Vac-1/04	1	dead	-[c]	-	-	-	-	ND	ND
(H5N1)	3	sacrificed	-	-	-	-	-	ND	ND
	3	sacrificed	-	-	-	-	-	ND	ND
	3	sacrificed	-	-	-	-	-	ND	ND
	14	sacrificed	-	-	-	-	-	ND	<40
	14	sacrificed	-	-	-	-	-	ND	<40
	14	sacrificed	-	-	-	-	-	ND	<40

[a] Blood samples were calculated as $logEID_{50}$/ml.

[b] Blood samples and antibodies were not corrected from the dead animals.

[c] - : <1.5 $logEID_{50}$ /g or <1.5 $logEID_{50}$ /ml

Table 5 Virus recovery and antibody response from ducklings inoculated with H5N1 viruses

virus	days		Virus titer ($logEID_{50}$/g)					Antibody titers[a]
			Respiratory organs	Liver	Kidneys	Colon	Brain	
Ck/Yamaguchi/04 (H5N1)	3	sacrificed	5.5	5.7	5.5	3.5	-	<40
	3	sacrificed	5.5	4.5	4.3	2.5	-	<40
	3	sacrificed	6.5	5.7	5.7	3.0	-	<40
	6	dead	4.3	-	5.7	2.5	5.3	ND
	7	dead	3.5	5.5	-	-	-	ND
	14	sacrificed	-	-	-	-	-	10240
Dk/Yokohama/03 (H5N1)	3	dead	7.5	6.3	6.3	5.5	7.5	ND
	3	dead	7.0	8.5	5.3	5.5	8.5	ND
	3	dead	7.0	7.5	6.7	3.0	7.5	<40
	4	dead	6.7	5.5	5.7	2.7	7.7	ND
	4	dead	6.7	6.7	5.3	6.3	8.7	ND
	4	dead	7.5	4.7	4.7	5.3	8.5	ND
Swan/Mongolia/05 (H5N1)	2	dead	6.5	6.8	7.5	4.5	7.3	ND
	2	dead	7.5	8.3	7.8	6.3	8.3	ND
	2	dead	7.5	7.3	8.5	6.5	9.0	ND
	2	dead	6.8	6.3	7.8	6.8	9.5	ND
	2	dead	7.8	7.8	7.5	7.5	9.3	ND
	2	dead	6.8	7.5	7.3	7.5	8.8	ND
Dk/Vac-1/04 (H5N1)	3	sacrificed	-[b]	-	-	-	-	<40
	3	sacrificed	-	-	-	-	-	<40
	3	sacrificed	-	-	-	-	-	<40
	14	sacrificed	-	-	-	-	-	40
	14	sacrificed	-	-	-	-	-	40
	14	sacrificed	-	-	-	-	-	160

[a] Antibodies were not corrected from the dead animals.

[b] - : <1.5 $logEID_{50}$/g.

was not recovered from any of the tissues on 3 and 14 days p.i. Sero-conversion to H5 influenza virus was detected in the ducklings inoculated with Dk/Vac-1/04 (H5N1) on 14 days p.i.

Mice

Three of the mice inoculated with Ck/Yamaguchi/04 (H5N1) died on 3 and 4 days p.i. (Table 6). Virus was recovered only from the respiratory organs in all the mice except one, which died on 4 days p.i. In this mouse, the virus was recovered not only from the respiratory organs but also from the spleen and kidneys. The other mice survived for 14 days and specific antibodies against H5 influenza virus were detected. All the mice inoculated with Dk/Yokohama/03 (H5N1) and Dk/Vac-1/04 (H5N1) survived for 14 days. The virus was recovered only from the respiratory organs of the mice on 3 days p.i. It was found that the pathogenicity of these two viruses in mice was relatively low. On the other hand, high titer of virus was recovered from the each organs of mice inoculated with Swan/Mongolia/05 (H5N1).

Miniature pigs

All the miniature pigs inoculated with the four H5N1 viruses survived for 14 days (Table 7). No virus was detected in the nasal swabs of the miniature pigs

Table 6 Virus recovery and antibody response from mice inoculated with H5N1 viruses

virus	days		Virus titer ($logEID_{50}$/g)					Antibody titers[a]
			Respiratory organs	Liver	Spleen	Kidneys	Brain	
Ck/Yamaguchi/04 (H5N1)	3	dead	6.8	-	-	-	-	ND
	3	dead	7.0	-	-	-	-	ND
	3	sacrificed	5.8	-	-	-	-	<40
	3	sacrificed	6.8	-	-	-	-	<40
	4	dead	6.5	-	3.3	2.3	-	ND
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-[b]	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	40960
Dk/Yokohama/03 (H5N1)	3	sacrificed	-	-	-	-	-	ND
	3	sacrificed	4.5	-	-	-	-	ND
	3	sacrificed	4.8	-	-	-	-	ND
	3	sacrificed	4.3	-	-	-	-	ND
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	10240
	14	sacrificed	-	-	-	-	-	40960
	14	sacrificed	-	-	-	-	-	40960
Swan/Mongolia/05 (H5N1)	3	sacrificed	7.8	3.5	3.8	2.8	3.3	ND
	3	sacrificed	7.5	-	3.3	3.3	2.3	ND
	3	sacrificed	8.0	2.5	4.3	4.3	3.5	ND
	3	sacrificed	8.0	4.0	4.5	4.5	1.7	ND
	4	dead	6.8	2.5	3.5	3.5	3.5	ND
	5	dead	7.5	3.3	3.3	3.3	3.5	ND
	5	dead	7.8	3.5	4.3	4.3	3.2	ND
	5	dead	7.5	4.3	3.8	3.8	3.8	ND
Dk/Vac-1/04 (H5N1)	3	sacrificed	5.3	-	ND	-	-	ND
	3	sacrificed	5.5	-	ND	-	-	ND
	3	sacrificed	2.8	-	ND	-	-	ND
	3	dead	3.0	-	ND	-	-	ND
	14	sacrificed	-	-	ND	-	-	ND
	14	sacrificed	-	-	ND	-	-	ND
	14	sacrificed	-	-	ND	-	-	ND

[a] Antibodies were not corrected from the dead animals.

[b] - : <1.5 $logEID_{50}$/g.

Table 7 Virus recovery and antibody response from miniature pigs inoculated with H5 viruses

virus	Virus titer ($logTCID_{50}$/ml)							Antibody titers
	1day	2day	3day	4day	5day	6day	7day	
Ck/Yamaguchi/04 (H5N1)	- [a]	-	-	-	-	-	-	<40
Dk/Yokohama/03 (H5N1)	-	-	-	-	-	-	-	<40
Swan/Mongolia/05 (H5N1)	3.3 3.3	3.8 4.3	2.0 2.8	2.6 -	3.8 -	2.6 -	- -	2560 2560
Dk/Vac-1/04 (H5N1)	-	2.7	2.5	1.7	-	-	-	2560

[a] - : <1.5$logTCID_{50}$/ml

inoculated either with Ck/Yamaguchi/04 (H5N1) or Dk/Yokohama/03 (H5N1) from 1 to 7 days p.i. (Table 7). In these two miniature pigs, sero-conversion to H5 influenza virus was not detected on 14 days p.i. In another experiment with miniature pigs inoculated with Ck/Yamaguchi/04 (H5N1), the virus was not recovered from any of the tissues on 3 and 14 days p.i. (data not shown). These results indicated that miniature pigs were not infected with Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1). Although there was no disease signs in the miniature pigs inoculated either with Swan/Mongolia/05 (H5N1) or with Dk/Vac-1/04 (H5N1), viruses were recovered from the nasal swabs. Sero-conversion to H5 influenza virus was detected in these three miniature pigs.

Discussion

The present study was conducted to determine the pathogenicity of Ck/Yamaguchi/04 (H5N1) and Swan/Mongolia/05 (H5N1) in birds and mammals. Two H5N1 avian influenza viruses, Dk/Yokokama/03 (H5N1) and Dk/Vac-1/04 (H5N1), were compared in terms of pathogenicity with Ck/Yamaguchi/04 (H5N1) and Swan/Mongolia/05 (H5N1). The intravenous pathogenicity index (IVPI) in

6-week-old chickens for Ck/Yamaguchi/04 (H5N1), Dk/Yokohama/03 (H5N1), Swan/Mongolia/05 (H5N1) and Dk/Vac-1/04 (H5N1) was 3.00, 2.70, 2.95, and 0.00, respectively (data not shown). Based on the present results, Ck/Yamaguchi/04 (H5N1), Dk/Yokohama/03 (H5N1), and Swan/Mongolia/05 (H5N1) were classified as HPAI viruses and Dk/Vac-1/04 (H5N1) as a non-pathogenic virus on the OIE criteria [38]. Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1) caused systemic infections in birds, but should little or no pathogenic in mammals. On the other hand, Swan/Mongolia/05 (H5N1) was highly pathogenic not only to ducklings but also to mice and miniature pigs. The slightly longer mean death time in chickens inoculated with Dk/Yokohama/03 (H5N1) allowed for the development of cyanosis of the wattle, typical signs of HPAI. It was shown the tendency that virus of higher titer was recovered from chickens inoculated with Ck/Yamaguchi/04 (H5N1) than those inoculated with Dk/Yokohama/03 (H5N1).

The pathogenicity of Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1) in quails and budgerigars was as high as that of the HPAI virus, A/chicken/Hong Kong/220/1997 (H5N1) which caused an acute and lethal infection [41]. Notably, the pathogenicity of Ck/Yamaguchi/04 (H5N1) in the quails seemed to be higher than that of Dk/Yokohama/03 (H5N1), as evidenced by the mean death times (Ck/Yamaguchi/04 (H5N1) vs Dk/Yokohama/03 (H5N1),

P=0.05) and the tissues from which the viruses were recovered. This difference may be due to the adaptation of isolated HPAI viruses from different hosts (chicken and duck) to quails. The greater susceptibility of quails to the virus originating from duck than from chickens is consistent with previous reports [33].

During the outbreaks of H7N7 HPAI in Australia, 1985, viruses were also isolated from starlings. In experimental infection, A/chicken/Victoria/1/1985 (H7N7) caused a systemic infection in starlings and was recovered even from the brain with high mortality [37]. In the present study, all starlings inoculated with Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1) developed systemic infections involving the brain, though it is difficult to evaluate the mortality of starlings infected with H5N1 HPAI viruses since those inoculated with PBS also died unexpectedly by 8 days p.i. (data not shown). Nevertheless, the fact that starlings were highly susceptible to the H5N1 HPAI virus indicates that feral birds, including starlings, could play a role as intermediates in virus transmission among poultry flocks, thereby contributing to the spread of avian influenza virus. During the outbreaks of H5N1 HPAI in Japan, 2004, viruses were isolated not only from chickens but dead crows [35]. The possibility that avian influenza viruses are spread by the contact of wild birds to poultry, is remaining.

Swan/Mongolia/05 (H5N1) was highly pathogenic not only to chickens (IVPI=2.95, data not shown) but also ducklings. Swan/Mongolia/05 (H5N1) was isolated from the dead whooper swan, indicating that this virus strain was adatpted to migratory birds after acquiring high pathogenicity to poultry. The pathogenicity of Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1) to five-week-old ducks was not high compared to that to chickens [24]. In the present study, virus was recovered from multiple tissues of three-day-old ducklings inoculated either with Ck/Yamaguchi/04 (H5N1) or with Dk/Yokohama/03 (H5N1), and some of these ducklings died, indicating that the pathogenicity of these two viruses in three-day-old ducklings was high. It was considered that Dk/Yokohama/03 (H5N1) replicated more rapidly and efficiently in the multiple organs than Ck/Yamaguchi/04 (H5N1) in ducks [24].

In the present study, virus was recovered from multiple tissues of only one mouse which died on 4 days p.i. Three mice died after the inoculation of Ck/Yamaguchi/04 (H5N1) at an EID_{50} of $10^{8.0}$ and the mortality rate of mice was only 30% (n=10). In the another publication, the 50% lethal dose of the same strain in mice was $5 \times 10^{5.0}$ EID_{50} under the same conditions (6-week-old female BALB/c mice via the intranasal route) and virus was also recovered from the brain [35]. The difference in pathogenicity may be due to the passage history of Ck/Yamaguchi/04 (H5N1) since the virus obtained from the National Institute of

Animal Health (Japan) was propagated twice in embryonated chicken eggs before the present animal experiments. The pathogenicity of the H5N1 viruses isolated from humans in Hong Kong, 1997, in mice was extremely high [9, 13]. In the present study, more than half of mice inoculated with Ck/Yamaguchi/04 (H5N1) survived from the infection, indicating that the 50% mouse lethal dose was over $10^{8.0}$ EID_{50}. The pathogenicity of Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1) in mice was much lower than that of the H5N1 viruses isolated from humans in Hong Kong, 1997. On the other hand, all the mice inoculated with Swan/Mongolia/05 (H5N1) died by 5 days p.i. except sacrificed ones. The mortality rate of mice inoculated with Swan/Mongolia/05 (H5N1) at an EID_{50} of $10^{8.0}$ was 100%. It is well known that pathogenicity of H5N1 HPAI viruses to mice were associated with amino acid at position 627 of the PB2 protein [15]. The amino acid at position 627 of the PB2 protein of Ck/Yamaguchi/04 (H5N1), Swan/Mongolia/05 (H5N1), Dk/Yokohama/03 (H5N1) were glutamic acid, lysine, and glutamic Acid, respectively. According to Hatta et al., these sequence data must support the results of mortality rate of mice inoculated with H5N1 viruses in the present study [15].

Miniature pigs showed susceptibility to influenza virus, similarly to domestic pigs [3]. Miniature pigs were not susceptible either to Ck/Yamaguchi/04 (H5N1) or to Dk/Yokohama/03 (H5N1), but viral replication was observed in upper

respiratory tissues in the miniature pigs inoculated either with Swan/Mongolia/05 (H5N1) or with Dk/Vac-1/04 (H5N1). Therefore, the pigs may not play a major role in the maintenance and spread of Ck/Yamaguchi/04 (H5N1) and Dk/Yokohama/03 (H5N1). In contrast, H5N1 viruses isolated in 1997 from a boy (A/Hong Kong/156/1997 (H5N1)) and chicken (A/chicken/Hong Kong/258/1997 (H5N1)) replicated in pigs, though transmission through contact was not detected [45]. Since H5N1 viruses, which were spread worldwide such as Swan/Mongolia/05 (H5N1) are identical to the isolates at the lake Qinghai, China, the great concern is still remaining in terms of public health. Susceptibility of pigs to avian influenza viruses has no relation to pathogenicity of the strains to chickens or their subtypes, indicating that possible factors involved in host range restriction may be located in some gene segment(s) other than the HA gene [21, 44].

In conclusion, Ck/Yamaguchi/04 (H5N1) is highly pathogenic to the birds and cause systemic infection, including the brain. The results indicate that the susceptibility of pigs to this HPAI virus is very low, and that the possibility of genetic reassortments with this HPAI virus in pigs is not a concern. However, the pathogenicity of Swan/Mongolia/05 (H5N1) in mammals was high. We need to develop new countermeasures to control the new type of H5N1 HPAI viruses.

Brief Summary

Outbreaks of HPAI have been occurring in domestic poultry in Asia since 1996. In the beginning of 2004, HPAI outbreaks were caused by H5N1 virus in two farms and a group of pet chickens in different areas of Japan. In 2005, HPAI viruses were isolated from aquatic birds, which had been assumed as natural host, in Mongolia. In the present study, the pathogenicity of A/chicken/Yamaguchi/7/2004 (H5N1), which had been isolated from a dead chicken during the first outbreak in Japan, was assessed in chickens, quails, budgerigars, ducklings, mice, and miniature pigs by experimental infection. The pathogenicity of A/whooper swan/Mongolia/3/2005 (H5N1), which had been isolated from a dead whooper swan in Mongolia, also assessed in ducklings, mice, and miniature pigs. These two virus strains were highly pathogenic to all the birds tested. Mice were susceptible to infection with a low mortality rate and miniature pigs were resistant to infection with A/chicken/Yamaguchi/7/2004 (H5N1). On the other hand, the mortality rate of mice inoculated with A/whooper swan/Mongolia/3/2005 (H5N1) was high, and miniature pigs were susceptible to infection with A/whooper swan/Mongolia/3/2005 (H5N1).

Chapter II

Development of an inactivated avian influenza vaccine prepared from a non-pathogenic H5N1 reassortant virus generated between isolates from migratory ducks in Asia

Introduction

Outbreaks of HPAI caused by H5N1 viruses have spread worldwide to 62 countries from southeast Asia since 1996 [58] . More than 300 million poultry have died or been culled, impacting not only on the poultry industry but also on public health, since 18 people were infected with HPAI viruses and 6 died in 1997 during the first large scale outbreak in Hong Kong [8], and now more than 380 cases of human infections with 60 % death rates have been reported in 14 countries [56].

On the basis of antigenic specificity, influenza A viruses are classified into 16 HA (H1-H16) and 9 NA (N1-N9) subtypes [1, 12]. Based on the severity of the disease in chickens, avian influenza viruses are divided into three groups:

non-pathogenic, low pathogenic, and HPAI viruses [1]. Non-pathogenic avian influenza viruses are circulating among migratory ducks and the water of the lakes where they nest in summer [18]. Some virus isolates from migratory ducks did not experimentally transmit to chickens [23], and these viruses generally transmit to chicken population through domestic waterfowls, quails, or turkeys [6, 19, 33, 40, 53]. In chickens, low pathogenic avian influenza viruses replicate in a limited range of tissues where trypsin-like endoproteases are expressed. HPAI viruses possess HAs with insertion of multiple basic amino acid residues at the site of cleavage into HA1 and HA2 subunits by ubiquitous proteases such as furin and PC6 [17, 49]. The cleavage activation of the HA enables the virus to replicate at multiple tissues of the host, thereby leading to a severe systemic infection with high mortality [25]. It is known that in addition to poultry, pet birds and wild birds are also susceptible to H5 HPAI virus infection [24].

“Stamping-out” is the basic measure for the control of HPAI. Vaccination may be an optional measure in cases where the disease spreads widely in addition to test and culling [38]. A number of the evaluation of not only inactivated avian influenza vaccine but also new generation vaccine such as vectored vaccine was reported [5, 10, 14, 29, 48, 52, 55]. Since commercial vaccines have been prepared from viruses of the North American lineage in the

USA and Mexico, they may be less effective for the control of current HPAI outbreaks caused by the infection with the viruses of the Eurasian lineage in Asia. Inactivated influenza vaccines for the control of the present HPAI had better, therefore, being prepared from an H5N1 virus strain belonging to the Eurasian lineage. Potency of the vaccine developed from virus strain belonging to the homologous lineage was higher than that from the strain of the heterologous lineage were different in the term of reduction of virus shedding [3]. Swayne et al. found that an oil adjuvanted vaccine prepared from inactivated H5N1 virus of the North American lineage protect chickens, but infectious viruses were recovered from 30% of vaccinated chickens after challenge with HPAI viruses of the Eurasian lineage [52, 55]. It was also reported that amount of the virus shedding of chickens contacting with a bird challenged with an HPAI virus strain belonging to the Eurasian lineage was different between the group of the chickens vaccinated with the Eurasian strain and the North American strain [4].

In the present study, a non-pathogenic H5N1 reassortant influenza virus with high yield in embryonated chicken eggs was generated between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) strains which were isolated from the natural host, migratory ducks in Asia. The resulting reassortant virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) was

propagated in embryonated chicken eggs. The yielded virus was inactivated with formalin and adjuvanted with mineral oil. Potency of the vaccine was evaluated by challenging with H5 HPAI virus strains which belong to the Eurasian lineage but antigenicity was different each other.

Materials and Methods

Viruses

A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04) isolated from a dead chicken during the outbreak of HPAI in Japan [35] was provided by the National Institute of Animal Health (Ibaraki, Japan). A/whooper swan/Mongolia /3/2005 (H5N1) (Swan/Mongolia/05) was isolated in our laboratory from the brain homogenates of a dead whooper swan found at the lake Erhel nuur, Mongolia. A/duck/Mongolia/54/2001 (H5N2) (Dk/Mong/54/01) and A/duck /Mongolia/47/2001 (H7N1) (Dk/Mong/47/01) were isolated in our laboratory from fecal samples of migratory ducks in Mongolia. All four viruses were propagated in 10-day-old embryonated chicken eggs at 35 ^{o}C for 48 hours and the infectious allantoic fluids were used as viruses.

Evaluation of pathogenicity of avian influenza viruses to chickens

The intravenous pathogenicity test for influenza viruses were carried out according to the World Health Organization (WHO) manual [57]. Briefly, each of eight six-week-old specific pathogen free chickens was inoculated with 0.1 ml of the infectious allantoic fluids by intravenous route. The birds were observed for their disease manifestation at intervals of 24 hours by a ten day period, and each bird was scored 0 if normal, 1 if sick, 2 if severely sick (including paralyze), 3 if dead, at each observation. The intravenous pathogenicity index (IVPI) was the mean score per bird per observation by the 10 days post inoculation.

To calculate the 50% chicken lethal dose (CLD_{50}) of Ck/Yamaguchi/04 (H5N1) and Swan/Mongolia/05 (H5N1), six groups of 4 seven-week-old chickens were challenged intranasally with serial dilutions 1 : 10^2 - 10^7 of infectious allantoic fluid of either viruses. Birds were observed for mortality for 14 days post challenge (p.c.). The titers of CLD_{50} were calculated by the method of Reed and Muench [43].

Vaccine

Dk/Mong/54/01 (H5N2) and Dk/Mong/47/01 (H7N1) were mixed and inoculated into 10-day-old embryonated chicken eggs and incubated at 35 ^{o}C for 48 hours. For the selection of H5N1 reassortant viruses, chicken antiserum to

A/duck/Hong Kong/301/1978 (H7N1) was used. Viruses in the allantoic fluids were plaque-cloned in MDCK cells. From these virus clones, H5N1 subtype influenza viruses were selected by hemaggultination-inhibition and neuraminidase-inhibition assays according to the WHO manual [57]. One of these clones, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04), was inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs and propagated at 34 ^{o}C for 48 hours. The yielded virus in the allantoic fluids (256 HAU) was inactivated with 0.2% formalin at 4 ^{o}C for 3 days. To test virus inactivation with formalin, the virus suspension was inoculated into the allantoic cavities of embryonated chicken eggs. After 48 hours at 35 ^{o}C incubation, the allantoic fluids were examined by hemaggutination test. The inactivated virus suspension was diluted with PBS. A 2.5 volume of the viral suspension with 256 HAU was mixed with 7.5 volume of oil adjuvant containing 3.9 v/v % of anhydromannitol-octadecenoate-ether and light mineral oil as remaining volume. The mixture was homogenized using an ultra-homomixer to make water-in-oil type vaccine. This vaccine contains 0.64 ug/0.5 ml HA protein, from the analysis of the detection the content of the HA protein; after scanning the SDS-PAGE gel, the protein of the HA band was quantified by Western blotting analysis using the serum of the SPF chicken immunized with Dk/Vac-1/04 (H5N1).

Sequencing

Virus RNAs were extracted from infectious allantoic fluids using TRIzol LS reagent (Invitrogen). After reverse transcription with M-MLV reverse transcripase (Invitrogen) using Uni12 primer: 5'-AGC AAA AGC AGG-3' [16], cDNAs were amplified by polymerase chain reaction (PCR) with the RNA LA PCR Kit Ver. 1.1 (TaKaRa) and PTC-200 thermal cycler (Bio-Rad). The primers used for RT-PCR in the present study were gene-specific primer sets [16]. Nucleotide sequences were determined from these RT-PCR products using a CEQ2000 automated DNA sequencer (Beckman Coulter) according to the Dye Terminator Cycle Sequence Chemistry Protocol (Beckman Coulter). Sequence data were analyzed and compared using GENETYX Version 7.0 (GENETYX).

Hemaggulutination-inhibition (HI) test

Antibodies in the serum samples were detected by HI test. Test sera treated with 10% chicken erythrocytes before HI tests according to the protocol set by Japanese Standards for Veterinary Biological Products were serially 2-fold diluted with PBS in 96 well microplates. Twenty five μl serum dilutions were mixed with the same volume of 8 HAU of virus antigen, and incubated at room temperature for 30 minutes. Fifty μl of 0.5% chicken erythrocytes were mixed with antigen-serum mixtures and incubated at room temperature for 60 minutes.

HI titers were expressed as reciprocals of the highest serum dilutions that showed complete hemaggutination inhibition.

Antigenic analysis of H5 influenza viruses

Antigenic analysis was examined by HI test. Anti-Ck/Yamaguchi/04 (H5N1) and anti-Swan/Mongolia/05 (H5N1) chicken serum were produced in our laboratory. Dk/Vac-1/04 (H5N1), Ck/Yamaguchi/04 (H5N1), A/Viet Nam/1194 /2004 (H5N1) (VN/04), A/Hong Kong/483/97 (H5N1) (HK/483), and Swan /Mongolia/05 (H5N1) were propagated in 10-day-old embryonated chicken eggs at 35 ^{o}C for 48 hours and yielded viruses were inactivated by 0.1% formalin and were used as the antigens of HI test.

Serological test of vaccinated chickens to the vaccine strain

Ten SPF chickens of white leghorn were hatched and raised exclusively in Kyoto Biken Laboratories, Inc. These chickens were used for the animal experiments of antibody response in chickens. Half ml of the vaccine was injected into the lower thigh muscle of the 10 chickens at 4-week-old. Serum samples of the vaccinated chickens collected every three weeks were examined for antibodies to the vaccine strain, Dk/Vac-1/04 (H5N1) by HI test.

Onset of protective immunity in chickens post vaccination

Twenty-one SPF chickens of white leghorn were hatched and raised exclusively in our laboratory and used for the animal experiments of onset of protective immunity in chickens post vaccination (p.v.). Half ml of the vaccine was injected into the lower thigh muscle of the six groups of 3 chickens at 36-day-old (14 days p.v.), 40-day-old (10 days p.v.), 42-day-old (8 days p.v.), 44-day-old (6 days p.v.), 46-day-old (4 days p.v.), and 48-day-old (2 days p.v.), respectively. At 50-day-old, all the vaccinated chickens and 3 non-vaccinated chickens were challenged intranasally with 100 CLD_{50} of Ck/Yamaguchi/04 (H5N1). Serum samples were examined for the presence of antibodies against Ck/Yamaguchi/04 (H5N1) at the challenge by HI test. Each group of three chickens was housed in a self-contained isolator unit (Tokiwa Kagaku Kikai) in BSL 3 biosafety facilities at the Graduate School of Veterinary Medicine, Hokkaido University, Japan.

Potency test of the present vaccine in chickens

Eighty-five SPF chickens of white leghorn were hatched and raised exclusively in our laboratory and used for the animal experiments of potency of the test vaccine in chickens. Half ml of the vaccine was injected into the lower thigh muscle the 58 chickens.

Thirty-four four-week-old chickens were vaccinated with the present vaccine in order to investigate the survival rates of chickens after challenge either with Ck/Yamaguchi/04 (H5N1) or Swan/Mongolia/03 (H5N1). Three weeks later, 24 vaccinated chickens and 12 seven-week-old non-vaccinated chickens were challenged intranasally with 100 CLD_{50} of Ck/Yamaguchi/04 (H5N1). The other 10 vaccinated chickens and 3 seven-week-old non-vaccinated chickens were challenged intranasally with $10^{4.5}$ CLD_{50} of Swan/Mongolia/05 (H5N1). All chickens were observed for disease signs and death daily for 14 days after challenge. Serum samples were corrected from all chickens at challenge and on 14 days p.c., to examine for the presence of antibodies against Dk/Vac-1/04 (H5N1), Ck/Yamaguchi/04 (H5N1), and Swan/Mongolia/05 (H5N1) by HI test.

Twenty-four chickens of four-week-old were immunized with the present vaccine. Three weeks later, to check shedding of the challenge viruses, 12 vaccinated chickens and 6 non-vaccinated birds were challenged intranasally with 100 CLD_{50} of Ck/Yamaguchi/04 (H5N1) at seven-week-old, and the other 12 vaccinated chickens and 6 non-vaccinated birds were challenged intranasally with $10^{4.5}$ CLD_{50} of Swan/Mongolia/05 (H5N1) at seven-week-old. Six chickens in each challenged group were sacrificed on day 2 and 4 p.c. When chickens died or sacrificed, swabs (trachea and cloaca) of them were collected in minimal

essential medium (MEM) and tissues (trachea, colon, and kidneys) of them were collected aseptically. To make a 10% suspension with MEM, the collected tissue samples were homogenized by Multi Beads Shocker (Yasui Kikai). These suspensions were serially 10-fold diluted with PBS and were inoculated into 10-day-old embryonated chicken eggs and incubated at 35 ^{o}C for 48 hours. The titers of virus were calculated by the method of Reed and Muench [43], and expressed as EID_{50} per gram and ml of tissue and swab respectively. Serum samples were examined for the presence of antibodies against vaccine strain and challenge virus strains by HI test.

Detection of the antibodies to the anti-NS1 protein of challenge viruses

Detection of the antibodies to the anti-nonstructural protein (NS1) of challenge viruses were performed as described previously [39]. Briefly, the sera collected from the vaccinated chickens which were challenged with 100 CLD_{50} of Ck/Yamaguchi/04 (H5N1) at 14 days p.c. Anti-NS1 antibodies were detected by ELISA using the recombinant NS1 protein derived from A/equine/Miami/1/1963 (H3N8) as antigens [20].

Results

Generation of a non-pathogenic H5N1 vaccine strain

From the allantoic fluid of embryonated eggs inoculated with the mixure of Dk/Mong/54/01 (H5N2) and Dk/Mong/47/01 (H7N1), 3 H5N1 virus clones were obtained. One of the three virus clones that showed the highest HA titer was selected as a vaccine strain and was designated as A/duck/Hokkaido/Vac-1/2004 (H5N1). It was confirmed that H5HA protein of Dk/Vac-1/04 (H5N1), equal to that of Dk/Mong/54/01 (H5N2), was classified into the Eurasian lineage.

Derivation of the internal protein genes (PB2, PB1, PA, NP, M, NS) of Dk/Vac-1/04 (H5N1) was determined by sequencing (Fig. 2.). NA and NS genes of Dk/Vac-1/04 (H5N1) were derived from Dk/Mon/47/01 (H7N1), and the other genes were from Dk/Mon/54/01 (H5N2). Nucleotide sequences of the eight genes of Dk/Vac-1/04 (H5N1) were submitted to the DNA Data Bank of Japan under accession numbers; AB253760 (PB2), AB253761 (PB1), AB257726 (PA), AB263192 (HA), AB263193 (NP), AB263194 (NA), AB263195 (M), and AB263196 (NS).

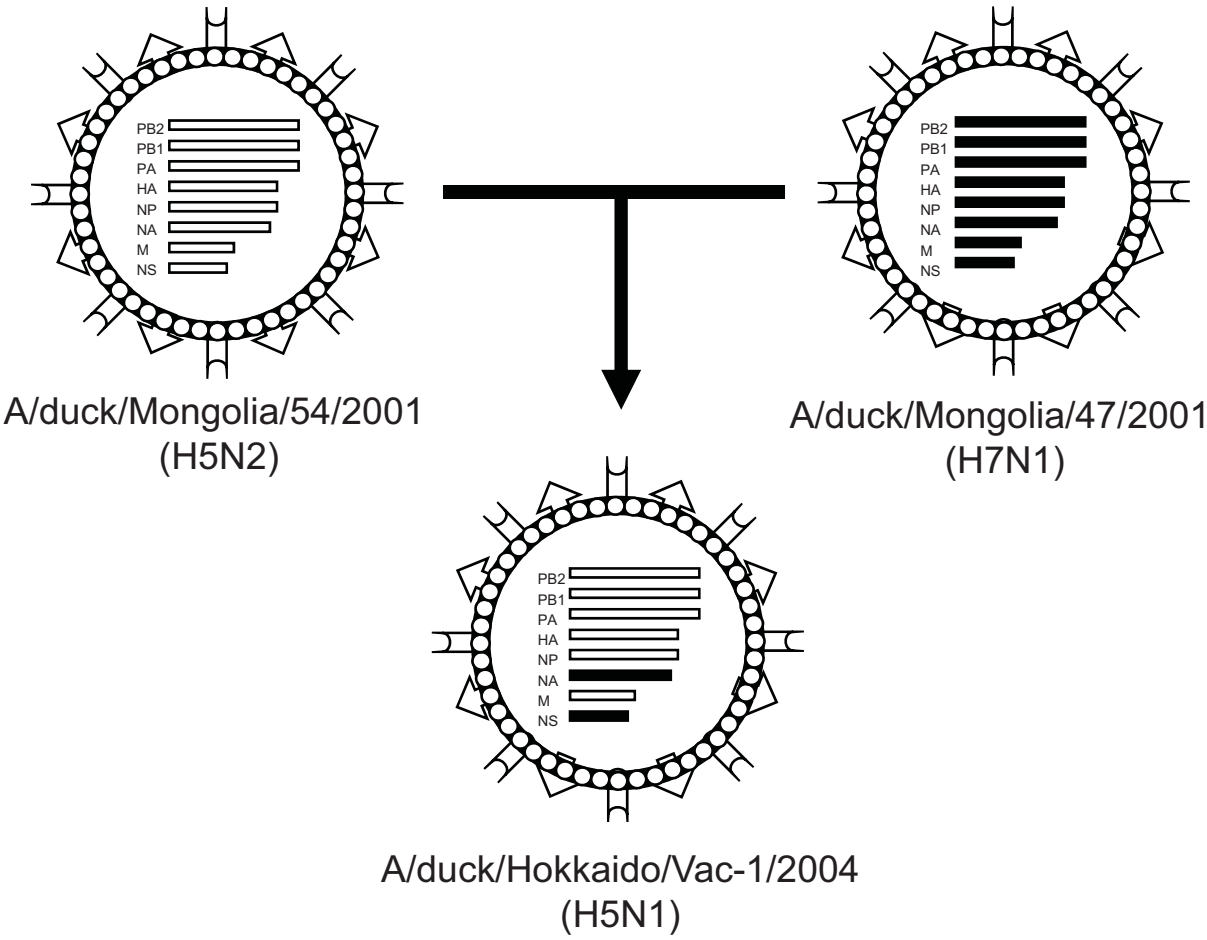

Fig. 2. Establishment of H5 avian influenza vaccine strain generated as a reassortant virus. H5 avian influenza vaccine strain, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04) was generated from two non-pathogenic avian influenza virus, A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) , which were isolated from fecal samples in our laboratory. PB2, PB1, PA, HA, NP, and M genes of Dk/Vac-1/04 (H5N1) were derived from A/duck/Mongolia/54/2001 (H5N2) and the other genes were derived from A/duck/Mongolia/47/2001 (H7N1).

Antigenic analysis of H5 influenza viruses

In HI test, Ck/Yamaguchi/04 (H5N1), and VN/04 (H5N1) had the same reactivity pattern as Dk/Vac-1/04 (H5N1), high reactivity to antiserum against Dk/Vac-1/04 (H5N1) but low reactivity to that against Swan/Mongolia/05 (H5N1) (Table 8). HK/483 (H5N1) had sufficient reactivity to both antisera against Dk/Vac-1/04 (H5N1) and Swan/Mongolia/05 (H5N1). Reactivity of Dk/Vac-1/04 (H5N1) to two polyclonal antisera was apparently different to that of Swan/Mongolia/05 (H5N1), indicating that an antigenic difference was confirmed between Dk/Vac-1/04 (H5N1) and Swan/Mongolia/05 (H5N1).

Antibody response of vaccinated chickens to the vaccine strain

Serum HI antibody titers to the vaccine strain in the vaccinated chickens were 512 to 1,024 in 3 weeks p.v. (Fig. 3). At 6 weeks p.v., titers reached 1,024 to 2,048, and serum antibodies were detected in the birds by 28 weeks p.v.

The IVPI and CLD_{50}

All of the chickens challenged with Ck/Yamaguchi/04 (H5N1) by intravenous route died within 24 hours p.c. (date not shown). Seven of eight chickens challenged with Swan/Mongolia/05 (H5N1) died within 24 hours p.c. and the other bird died by 2 days p.c. (date not shown). These results indicate

Table 8. Antigenic analysis between H5N1 virus strains

Anti-sera	HI titers against following H5N1 virus strains				
	Dk/Vac-1/04 (H5N1)	Ck/Yamaguchi/04 (H5N1)	VN/04 (H5N1)	HK/483 (H5N1)	Swan/Mongolia/05 (H5N1)
Anti-Dk/Vac-1/04 (H5N1)	512	256	256	64	16
Anti-Swan/Mongolia/05 (H5N1)	8	8	8	64	256

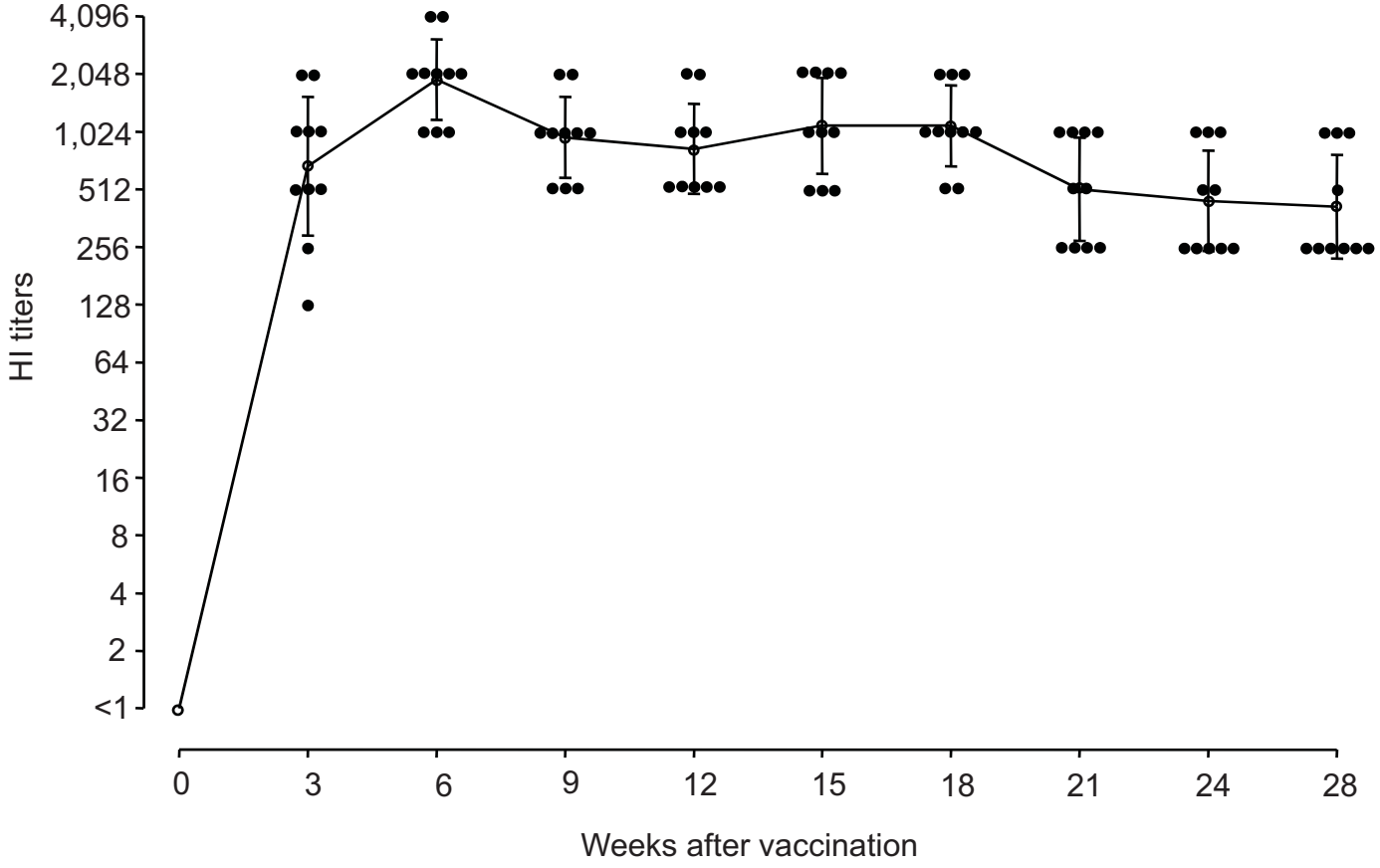

Fig. 3. Serum HI antibody titer in chickens after injection with the present vaccine. Ten four-week-old chickens were vaccinated intramuscularly with the present vaccine. Serum samples collected at 3, 6, 9, 12, 15, 18, 21, 24, and 28 weeks were examined for their antibodies to the Dk/Vac-1/04 (H5N1). White circle blots indicate the geometric mean HI antibody titers of 10 vaccinated chickens on each week. The bars indicate the standard error.

that the IVPIs for Ck/Yamaguchi/04 (H5N1) and Swan/Mongolia/05 (H5N1) are 3.00 and 2.95, respectively. On the other hand, no disease signs were observed in any of chickens challenged with Dk/Vac-1/04 (H5N1) by intravenous route through 10 days after challenge, resulting in that the IVPI for Dk/Vac-1/04 (H5N1) is 0.00 (date not shown).

To prepare the challenge viruses for the evaluation study of the present vaccine, the CLD_{50}s of Ck/Yamaguchi/04 (H5N1) and Swan/Mongolia/05 (H5N1) were determined. It was determined that $10^{3.3}$ EID_{50} of Ck/Yamaguchi/04 (H5N1) was equal to one CLD_{50}, and that $10^{3.0}$ EID_{50} of Swan/Mongolia/05 (H5N1) was equal to one CLD_{50} (data not shown).

Onset of protective immunity in chickens post vaccination

Six groups of 3 vaccinated chickens and 3 non-immunized birds were challenged intranasally at seven-week-old with Ck/Yamaguchi/04 (H5N1) on 14, 10, 8, 6, 4, and 2 days p.v.

Serum HI antibodies to Ck/Yamaguchi/04 (H5N1) were not detected in any of the chickens challenged on 6, 4, and 2 days p.v., and all of these birds died by day 3 p.c. (Fig. 4). On the other hand, all chickens challenged with Ck/Yamaguchi/04 (H5N1) on 14, 10, and 8 days p.v. survived without showing any disease signs, while serum HI antibodies were not detected in two out of the

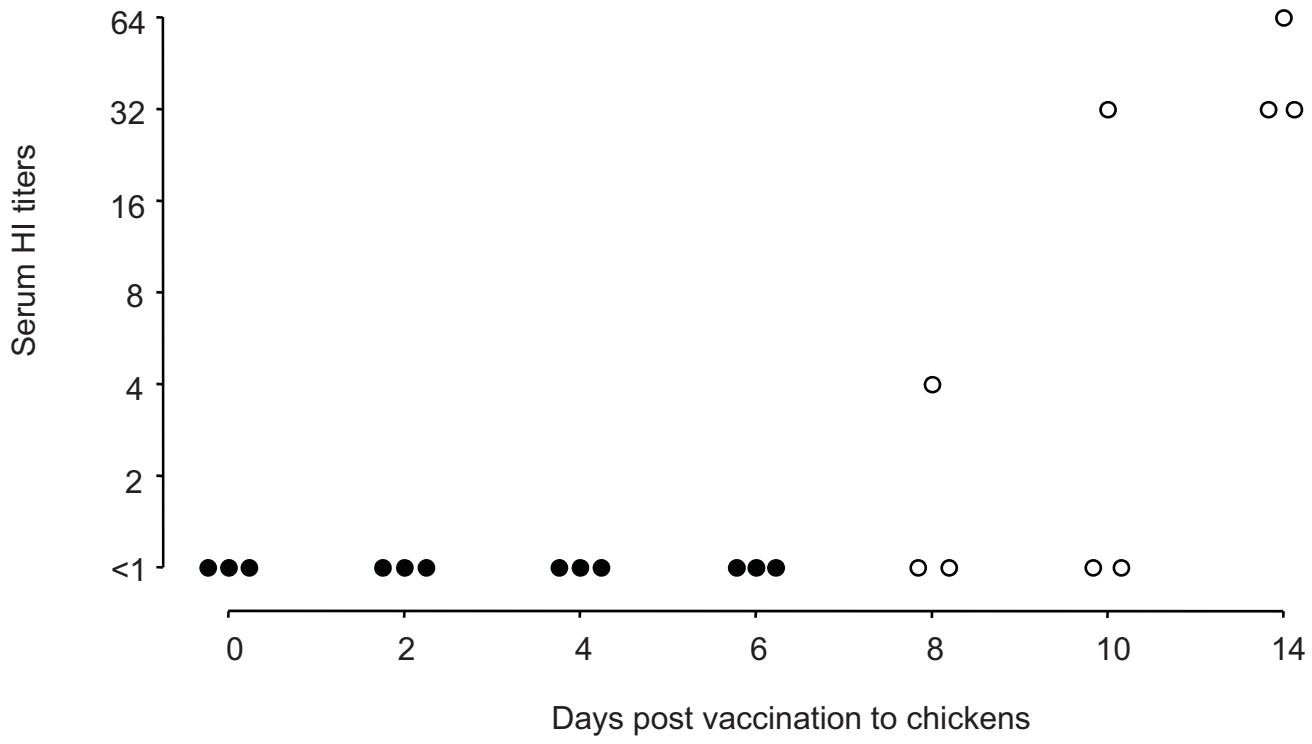

Fig. 4. Onset of protective immunity in chickens post vaccination. Six groups of 3 vaccinated chickens and 3 non-vaccinated chickens were challenged with Ck/Yamaguchi/04 (H5N1) at 14, 10, 8, 6, 4, and 2 days post vaccination. Serum antibodies to Ck/Yamaguchi/04 (H5N1) at challenge were examined by HI test. Black circles indicate the birds died by 3 days post challenge. White circles indicate the birds had survived for 14 days post challenge.

three chickens challenged on 8 days p.v.

Potency of the present vaccine in chickens

Twenty-four vaccinated chickens and 12 seven-week-old non-vaccinated chickens were challenged intranasally with Ck/Yamaguchi/04 (H5N1), and 10 vaccinated chickens and 3 seven-week-old non-vaccinated chickens were challenged intranasally with Swan/Mongolia/05 (H5N1). The geometric mean serum HI titers of the vaccinated chickens were 64 with the challenge virus strains. Survival rates of the chickens challenged with each of the two HPAI viruses are shown in Fig. 5. All vaccinated chickens survived without showing any disease signs after challenge either with Ck/Yamaguchi/04 (H5N1) or with Swan/Mongolia/05 (H5N1), whereas all the control chickens died within 2 days p.c. Antibody responses of vaccinated chickens after challenge with each of the two HPAI viruses are shown in Table 9. Average serum HI titers of the vaccinated chickens against the challenge virus strain increased approximately 4 fold. The other 10 chickens were vaccinated and investigated their serum HI antibodies at 3 and 5 weeks p.v. Average serum HI titers of the non-challenged chickens also increased approximately 4 fold.

In order to investigate the virus shedding from vaccinated chickens, the seven-week-old vaccinated chickens and non-vaccinated chickens were

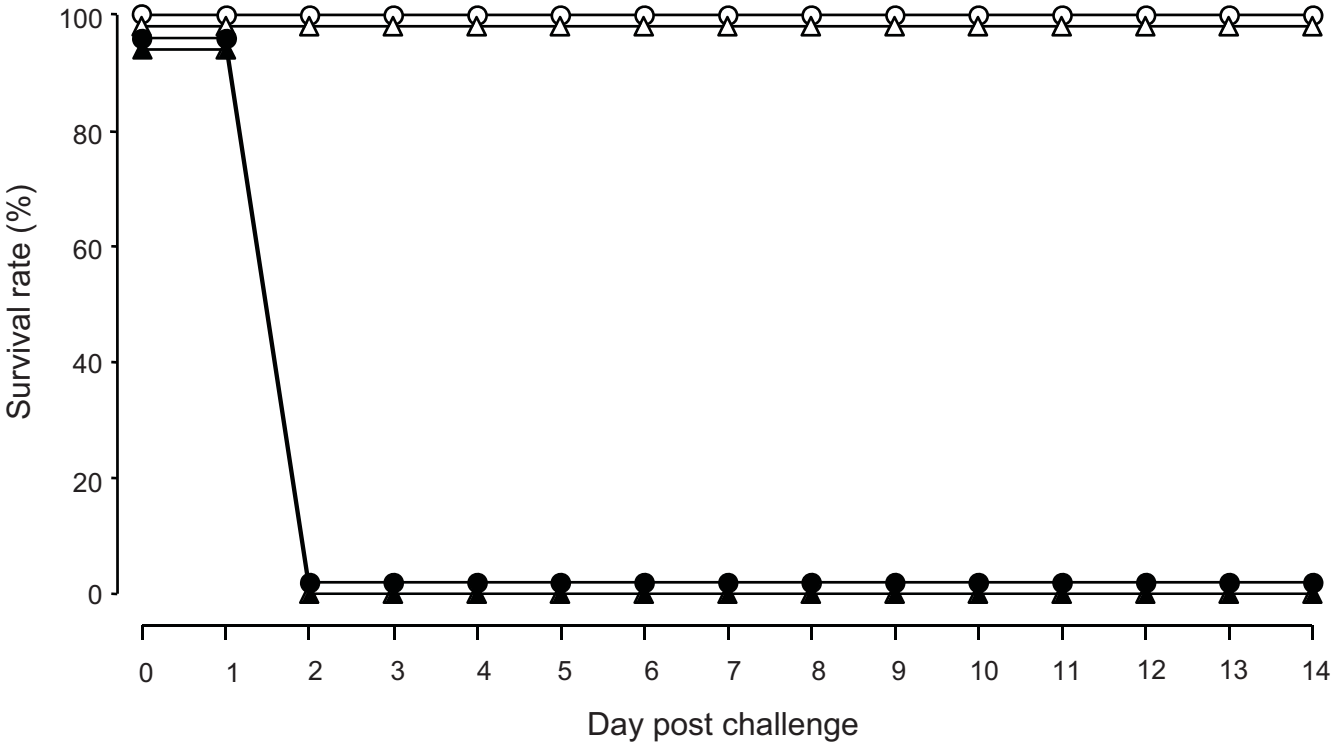

Fig. 5. Survival rates of chickens after challenge either with Ck/Yamaguchi/04 (H5N1) or Swan/Mongolia/05 (H5N1). Four-week-old chickens were vaccinated intramuscularly with 0.5 ml of the test vaccine. Three weeks later, all the vaccinated chickens were challenged intranasally with either Ck/Yamaguchi/04 or Swan/Mongolia/05. Symbols: 24 vaccinated chickens after challenge with Ck/Yamaguchi/04 (○), 10 vaccinated chickens after challenge with Swan/Mongolia/05 (△), 12 non-vaccinated chickens after challenge with Ck/Yamaguchi/04(●), 3 non-vaccinated chickens after challenged with Swan/Mongolia/05 (▲).

Table 9. Antibody response of vaccinated chickens challenged with each of two HPAI viruses

Challenge virus	Number of chickens	Serum HI titers of chickens against the following virus strain [a]					
		Dk/Vac-1/04 (H5N1)		Ck/Yamaguchi/04 (H5N1)		Swan/Mongolia/05 (H5N1)	
		Pre-challenge	14 days p.c.	Pre-challenge	14 days p.c.	Pre-challenge	14 days p.c.
Ck/Yamaguchi/04 (H5N1)	24	64 – 1,024 (338)	256 – 2,048 (776)	16 – 512 (79)	256 – 2,048 (512)	ND	ND
Swan/Mongolia/05 (H5N1)	10	256 – 512 (337)	512 – 2,048 (1,176)	ND	ND	8 – 64 (32)	64 – 128 (111)
PBS	10	128 – 1,024 (315)	256 – 4,096 (1448)	16 – 512 (137)	256 – 2,048 (549)	8 – 128 (52)	64 – 512 (128)

[a] Digit : range of HI titers. Parenthesis : geometric means of HI titers

challenged intranasally with Ck/Yamaguchi/04 (H5N1) or Swan/Mongolia/05 (H5N1). From non-vaccinated chickens, viruses with high titers were recovered from each of the swab and tissues tested (Table 10). Viruses were not recovered from the swabs of any of the vaccinated chickens after challenge with Ck/Yamaguchi/04 (H5N1). However, infectious viruses were recovered from the colons of two vaccinated chickens after challenge with Ck/Yamaguchi/04 (H5N1) on 2 days p.c., though titers of viruses recovered from these birds were lower than those from non-vaccinated chickens. The HI titers of two vaccinated chickens from which the viruses were recovered at the challenge were 64 and 256. Viruses were recovered from trachea swabs and tissues of vaccinated chickens after challenge with Swan/Mongolia/05 (H5N1) on 2 and 4 days p.c. The viruses were recovered from five vaccinated chickens which possessed low titer of HI antibodies at the challenge against challenge viruses, Swan/Mongolia/05 (H5N1).

Detection of the antibodies to the NS1 protein of challenge viruses

All the titer of HI antibodies of the vaccinated chickens after challenge were increased (Table 11). Since the vaccinated chickens were challenged with the HPAI virus at 3 weeks p.v., it was unclear that increase of HI antibody titers was concerned due to the challenge virus infection to the host. Since it has been

Table 10. Virus recovery in vaccinated chickens

	Challenge virus	No. of chickens	HI titer[a] (GM value)	Virus recovery[b]					
				Days p.c.[c]	No.of the chickens each virus was recovered [GM value of the virus titer (log10)]				
					Swab (logEID$_{50}$ /ml)		Organs (logEID$_{50}$ /g)		
					Trachea	Cloaca	Trachea	cloaca	Kidneys
Vaccinated chickens	Ck/Yamaguchi/04	6	32 – 256 (73)	2	0	0	0	2 (2.0)	0
	(H5N1)	6	32 – 1,024 (97)	4	0	0	0	0	0
	Swan/Mongolia/05	6	64 – 256 (97)	2	2 (1.3)	0	2 (1.8)	0	0
	(H5N1)	6	32 – 64 (39)	4	2 (0.8)	0	2 (1.8)	0	0
No immunized chickens	Ck/Yamaguchi/04 (H5N1)	6	< 2	2+	6 (5.7)	6 (5.3)	6 (6.9)	6 (7.3)	6 (8.4)
	Swan/Mongolia/05	4	< 2	2+	4 (6.0)	4 (5.7)	4 (6.6)	4 (7.2)	4 (8.2)
	(H5N1)	2	< 2	3+	2 (7.0)	2 (5.4)	2 (5.2)	2 (5.4)	2 (7.3)

[a] Challenge virus strain was used as the HI antigens. Digit : range of HI titers. Parenthesis : geometric means of HI titers

[b] Digit: number of chickens in which each virus was isolated. Parenthesis: average of virus titers (logEID$_{50}$ /ml) and (logEID$_{50}$ /g).
0 indicates no virus was isolated from chickens.

[c] Number indicates the sampling day at p.c., "+" indicates that chickens were dead at that day.

Table 11. Antibody response in vaccinated chicken challenged with Ck/Yamaguchi/04 (H5N1)

chicken	HI titer [a]		ELISA titer of anti-NS1 protein	
	Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
1	16	512	- [b]	-
2	32	512	-	-
3	64	1,024	-	40
4	64	1,024	-	-
5	64	1,024	-	-
6	128	2,048	-	640
7	32	256	-	-
8	32	256	-	-
9	64	512	-	-
10	128	1,024	-	-
11	128	1,024	-	-
12	128	1,024	-	-
13	32	512	-	-
14	64	256	-	-
15	64	256	-	-
16	64	256	-	-
17	64	256	-	-
18	64	256	-	-
19	128	512	-	-
20	128	512	-	-
21	512	2.048	-	-
22	128	256	-	-
23	128	256	-	-
24	128	256	-	-

[a] Allantoic fluid of Dk/Vac-1/04 (H5N1) was used for HI test antigens.
[b] - : < 40

known that a large amount of the NS1 protein is expressed in virus-infected cells but not detected in virions [26], anti-NS1 protein antibodies were recognized as one of the indicator of virus infection. Anti-NS1 antibodies were not detected from most of the vaccinated chickens after challenge except two birds. It seems difficult to give complete protection by inactivated vaccine.

Discussion

It is known that low pathogenic avian influenza viruses of H5 or H7 subtypes acquire pathogenicity during repeated passages among chicken population [19]. Although most of the HPAI viruses for chickens do not cause any disease signs in ducks [2, 19, 24], some of the H5N1 HPAI viruses that had returned to feral water birds and been lethal for water birds, have been isolated since 2004 [7, 30]. On the basis of phylogenetic analysis, most of the H5N1 viruses isolated from dead water birds in Eurasia and Africa are classified into Clade 2.2, indicating that these HPAI viruses are nearly identical to the isolates from dead birds at the Lake Qinghai, China, 2005 [27]. Since it is concerned that the H5N1 HPAI virus strains brought by the infected birds from the south may perpetuate in the lakes where migratory birds nest in summer, intensive

virological surveillance of avian influenza in feral water birds flying from their northern territories in Siberia has been carried out in Mongolia and Japan. The results of the surveillance study during 2004-2007, so far, showed that no HPAI H5N1 virus has been isolated, indicating that the H5N1 HPAI virus strains have not perpetuated [42]. Potency of the vaccine developed from the homologous lineage strain and that from the heterologous lineage strain were different in the term of reduction of virus shedding [3], indicating that virus strains belonging to the Eurasian lineage are preferable to those to the North American lineage for vaccine strains for the control of HPAI in Asia. Since a non-pathogenic H5N1 avian influenza virus had not been isolated from water birds, an H5N1 influenza virus strain was generated by genetic reassortment of non-pathogenic H5N2 and H7N1 isolates from migratory ducks in embryonated chicken eggs and designated as A/duck/Hokkaido/Vac-1/2004 (H5N1). The IVPI for Dk/Vac-1/04 (H5N1) was 0.00 and chickens were not susceptible to infection with that strain, indicating that the resulting reassortant virus strain is non-pathogenic in chickens [47].

In the present study, high level of antibodies was induced and maintained at least for seven months in chickens by a single shot of the present vaccine. On the other hand, few studies have been performed for onset of effective immunity in laboratory setting [36], and in field setting [11]. Most of the

chickens which had serum antibodies with low HI titer (below 10) died in 14 days after challenge [36]. Although we did not examine the cellular immune response in chickens injected with the present vaccine, and the lack of detectable HI antibodies, all of the vaccinated chickens survived when challenged on 8 days p.v., indicating that not only by humoral immunity but also cellular immunity may contribute the survival of the chickens. The mean HI antibody titer of chickens on 21 days post single shot of the present vaccine was equivalent to that of birds 4 weeks after the second injection of the commercial vaccine, though the experimental conditions, such as age of birds, vaccine strains, and adjuvant of the present study are different from those of the previous study [31]. A single intramuscular injection of the present vaccine, thus, conferred sufficient protective immunity to chickens to prevent the manifestation of disease signs and reduce the amount of virus shedding caused by infection with currently prevalent H5 HPAI viruses. The present vaccine, therefore, should be useful in the emergency case.

The present vaccine was indicated not to protect birds from virus challenge. In order to support the notion that the effects of inactivated vaccine are limited, anti-NS1 protein antibodies were detected from vaccinated chickens after virus challenge. Anti-NS1 antibodies were detected from 2 of 24 vaccinated chickens after challenge, though the infectious viruses were recovered from 2 of

12 birds at either 2 or 4 day p.c. It has been reported that the detection of antibodies to the NS1 protein is useful to differentiate infected from vaccinated animals (DIVA) [39, 54]. Since the NS1 protein is expressed in virus-infected cells but not detected in virions [26], antibodies to the NS1 protein are not induced in the animals injected with inactivated virus vaccine but induced by only when virus replication occurs. Although the NS genes of influenza A viruses are phylogenically divided into two lineages, alleles A and B, the NS1 protein is antigenically conserved among influenza A viruses [32, 50]. The use of "NS1-ELISA" for the detection of antibodies to the NS1 protein, is thus, recommended as the DIVA system in the surveillance of influenza in chickens inoculated with the present vaccine.

Non-vaccinated chickens died within 2 days after challenge with 100 CLD_{50} of Ck/Yamaguchi/04 (H5N1), indicating that the challenge virus titer is enough to evaluate the vaccine potency. In addition, the present vaccine conferred protective immunity in chickens from manifestation of disease signs against challenge with $10^{4.5}$ CLD_{50} of Swan/Mongolia/05 (H5N1). Infectious viruses were recovered from the colon of vaccinated chickens after challenge with Ck/Yamaguchi/04 (H5N1), resulting that the present vaccine did not confer abolition but reduction of infectious virus shedding to the birds. In the outbreak of HPAI caused by H5N1 viruses in 2002 – 2003 in Hong Kong, infectious

viruses were spread to the vaccinated poultry with deaths although the mortality rate was low [11]. We, therefore, conclude that inactivated vaccines confer immunity to hosts to prevent from manifestation of disease signs, but not from infection. This limitation of inactivated vaccines is supported by Swayne et al.[52] . It should be, thus, noted that improper use of inactivated vaccine may lead silent spread of HPAI virus in poultry population and vaccine should be used under the well controlled setting.

Brief Summary

A reassortant influenza virus, A/duck/Hokkaido/Vac-1/2004 (H5N1) (Dk/Vac-1/04) was generated between non-pathogenic avian influenza viruses isolated from migratory ducks in Asia. Dk/Vac-1/04 (H5N1) virus particles propagated in embryonated chicken eggs were inactivated with formalin and adjuvanted with mineral oil to form a water-in-oil emulsion. The resulting vaccine was injected intramuscularly into chickens. The chickens were challenged with either of highly pathogenic avian influenza virus strains, A/chicken/Yamaguchi/7/2004 (H5N1) or A/whooper swan/Mongolia/3/2005 (H5N1) on 21 days p.v. when the geometric mean serum HI titers of the birds was 64 with the challenge virus strains. The vaccinated chickens were protected from manifestation of disease signs upon the challenge with either of the highly pathogenic avian influenza viruses. However, challenge virus was recovered at low titers from the birds on 2 and 4 days p.c. All 3 chickens challenged on 6 days p.v. died, whereas 3 chickens challenged on 8 days p.v. survived. These results indicate that the present vaccine confers clinical protection and reduction of virus shedding against highly pathogenic avian influenza virus challenges and should be useful as an optional tool in the emergency case.

Conclusion

Since 1997, outbreaks of highly pathogenic avian influenza caused by H5N1 viruses have occurred. In the present study, pathogenicity of two H5N1 HPAI virus strains, A/chicken/Yamaguchi/7/2004 (H5N1) (Ck/Yamaguchi/04), which was isolated from a dead chicken during the first outbreak in Japan, and A/whooper swan/Mongolia/3/2005 (H5N1) (Swan/Mongolia/05), which was isolated from a dead whooper swan which had found at the lake Erhel nuur, Mongolia, was assessed in avian species and mammalians by experimental infection study.

Ck/Yamaguchi/04 (H5N1) was highly pathogenic to the birds and cause systemic infection, including the brain. On the other hand, mice were susceptible to infection with Ck/Yamaguchi/04 (H5N1) but with a mild pathogenicity. In contrast, miniature pigs were not susceptible to Ck/Yamaguchi/04 (H5N1). Swan/Mongolia/03 (H5N1) was highly pathogenic to ducklings and mice and infected to miniature pigs. These results indicated that the pathogenicity of Swan/Mongolia/05 (H5N1) was higher than that of Ck/Yamaguchi/04 (H5N1) in avian species and mammalians. The susceptibility of pigs to Swan/Mongolia/03 (H5N1) was confirmed, indicating that the possibility of genetic reassortments with this strain in pigs is a concern.

Inactivated avian influenza vaccine with high efficacy prepared from a non-pathogenic H5N1 virus was developed and potency of it was evaluated by animal experiments. Although, “stamping-out” is the basic measure for the control of HPAI, vaccination may be an optional measure in cases where the disease spread widely. In the present study, a non-pathogenic H5N1 reassortant influenza virus with high proliferation in embryonated chicken eggs was generated between A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1) strains which were isolated from the migratory ducks in Asia. High titers of antibody were induced in the chickens injected with inactivated vaccine prepared from A/duck/Hokkaido/Vac-1/2004 (H5N1) three weeks post vaccination and then all the vaccinated chickens survived without showing any disease signs after challenge either with Ck/Yamaguchi/04 (H5N1) or with Swan/Mongolia/05 (H5N1). All 3 chickens challenged on 6 days post vaccination, died, whereas 3 chickens challenged on 8 days post vaccination survived. These results indicate that the present vaccine confers clinical protection and reduction of virus shedding against highly pathogenic avian influenza virus challenges and should be useful as an optional tool in the emergency case.

Acknowledgements

The author would like to acknowledge Prof. Hiroshi Kida, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, for his guidance and encouragement during the course of this work.

Great appreciation is extended to Dr. Takashi Umemura, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Dr. Ayato Takada, Department of Global Epidemiology, Research Center for Zoonosis Control, Hokkaido University.

The author is grateful to Dr. Yoshihiro Sakoda, and Dr. Masatoshi Okamatsu, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, for his valuable advice, support and encouragement throughout this study.

The author thanks to the National Institute of Animal Health (Ibaraki, Japan) and the Animal Quarantine Service (Kanagawa, Japan) for providing the A/chicken/Yamaguchi/7/2004 (H5N1) and A/duck/Yokohama/aq-10/2003 (H5N1) influenza viruses, respectively.

The author thanks to Dr. R. Sodnomdarjan, State Central Veterinary Laboratory, Ulaanbaatar, Mongolia for providing the samples of dead whooper swans which were found at the lake Erhel nuur.

The author heartily thanks to Dr. Syuichi Ota, Japan Veterinary Products Association, Dr. Toshiaki Ohgitani, Dr. Norihide Kokumai, Dr. Takashi Sasaki, Avian Biologics Department, Kyoto Biken Laboratories, Inc., Dr. Masashi Sakaguchi, Dr. Takashi Imamura, Dr. Ryuichi Sakamoto, Division 2, Second Research Department, The Chemo-Sero-Therapeutic Research Institute, Dr Akira Sawata, Dr. Katsumi Kume, Dr. Kazue Saijo, Research Center for Biologicals, The Kitasato Institute, Dr. Zhifeng Lin, Dr. Natsumi Takeyama, Dr. Junko Hagiwara, Research Department, Nippon Institute for Biological Science, to product the inactivated avian influenza vaccine with high efficacy.

Finally, the author greatly appreciates all colleagues of Laboratories of Microbiology and Comparative Pathology for their great cooperation.

References

1. Alexander DJ (2000) A review of avian influenza in different bird species. Vet Microbiol 74: 3-13
2. Alexander DJ, G.Parsons, and R.J.Manvell (1986) Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtypes for chickens, turkeys, ducks, and quail. Avian Pathol 15: 647-662
3. Bai GR, Sakoda Y, Mweene AS, Kishida N, Yamada T, Minakawa H, Kida H (2005) Evaluation of the ESPLINE INFLUENZA A&B-N Kit for the diagnosis of avian and swine influenza. Microbiol Immunol 49: 1063-1067
4. Bublot M, Le Gros FX, Nieddu D, Pritchard N, Mickle TR, Swayne DE (2007) Efficacy of two H5N9-inactivated vaccines against challenge with a recent H5N1 highly pathogenic avian influenza isolate from a chicken in Thailand. Avian Dis 51: 332-337
5. Bublot M, Pritchard N, Cruz JS, Mickle TR, Selleck P, Swayne DE (2007) Efficacy of a fowlpox-vectored avian influenza H5 vaccine against Asian H5N1 highly pathogenic avian influenza virus challenge. Avian Dis 51: 498-500

6. Chen H, Deng G, Li Z, Tian G, Li Y, Jiao P, Zhang L, Liu Z, Webster RG, Yu K (2004) The evolution of H5N1 influenza viruses in ducks in southern China. Proc Natl Acad Sci U S A 101: 10452-10457

7. Chen H, Smith GJ, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peiris JS, Guan Y (2005) Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 436: 191-192

8. Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG (1998) Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 351: 472-477

9. Dybing JK, Schultz-Cherry S, Swayne DE, Suarez DL, Perdue ML (2000) Distinct pathogenesis of hong kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. J Virol 74: 1443-1450

10. EFSA (2007) Opinion of the scientific panel on animal health and welfare (AHAW) related with the vaccination against avian influenza of H5 and H7 subtypes in domestic poultry and captive birds". The EFSA Journal 489

11. Ellis TM, Leung CY, Chow MK, Bissett LA, Wong W, Guan Y, Malik Peiris JS (2004) Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol 33:

405-412

12. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD (2005) Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 79: 2814-2822

13. Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y (1999) Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 73: 3184-3189

14. Gao W, Soloff AC, Lu X, Montecalvo A, Nguyen DC, Matsuoka Y, Robbins PD, Swayne DE, Donis RO, Katz JM, Barratt-Boyes SM, Gambotto A (2006) Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. J Virol 80: 1959-1964

15. Hatta M, Gao P, Halfmann P, Kawaoka Y (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 293: 1840-1842

16. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146: 2275-2289

17. Horimoto T, Nakayama K, Smeekens SP, Kawaoka Y (1994)

Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J Virol 68: 6074-6078

18. Ito T, Okazaki K, Kawaoka Y, Takada A, Webster RG, Kida H (1995) Perpetuation of influenza A viruses in Alaskan waterfowl reservoirs. Arch Virol 140: 1163-1172

19. Ito T, Goto H, Yamamoto E, Tanaka H, Takeuchi M, Kuwayama M, Kawaoka Y, Otsuki K (2001) Generation of a highly pathogenic avian influenza A virus from an avirulent field isolate by passaging in chickens. J Virol 75: 4439-4443

20. Kida H, Brown LE, Webster RG (1982) Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology 122: 38-47

21. Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka Y, Webster RG (1994) Potential for transmission of avian influenza viruses to pigs. J Gen Virol 75 (Pt 9): 2183-2188

22. Kida H YR, Matsuoka Y (1980) Duck influenza lacking evidence of disease signs and immune response. Infect Immun 30: 547-553

23. Kishida N, Sakoda Y, Eto M, Sunaga Y, Kida H (2004) Co-infection of Staphylococcus aureus or Haemophilus paragallinarum exacerbates

H9N2 influenza A virus infection in chickens. Arch Virol 149: 2095-2104

24. Kishida N, Sakoda Y, Isoda N, Matsuda K, Eto M, Sunaga Y, Umemura T, Kida H (2005) Pathogenicity of H5 influenza viruses for ducks. Arch Virol 150: 1383-1392

25. Klenk HD, Garten W (1994) Host cell proteases controlling virus pathogenicity. Trends Microbiol 2: 39-43

26. Krug RM, Etkind PR (1973) Cytoplasmic and nuclear virus-specific proteins in influenza virus-infected MDCK cells. Virology 56: 334-348

27. Lee MS, Deng MC, Lin YJ, Chang CY, Shieh HK, Shiau JZ, Huang CC (2007) Characterization of an H5N1 avian influenza virus from Taiwan. Vet Microbiol 124: 193-201

28. Li KS, Guan Y, Wang J, Smith GJ, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepangestie AT, Chaisingh A, Auewarakul P, Long HT, Hanh NT, Webby RJ, Poon LL, Chen H, Shortridge KF, Yuen KY, Webster RG, Peiris JS (2004) Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature 430: 209-213

29. Lierz M, Hafez HM, Klopfleisch R, Luschow D, Prusas C, Teifke JP, Rudolf M, Grund C, Kalthoff D, Mettenleiter T, Beer M, Hardert T (2007) Protection and virus shedding of falcons vaccinated against highly

pathogenic avian influenza A virus (H5N1). Emerg Infect Dis 13: 1667-1674

30. Liu J, Xiao H, Lei F, Zhu Q, Qin K, Zhang XW, Zhang XL, Zhao D, Wang G, Feng Y, Ma J, Liu W, Wang J, Gao GF (2005) Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 309: 1206

31. Liu M, Wood JM, Ellis T, Krauss S, Seiler P, Johnson C, Hoffmann E, Humberd J, Hulse D, Zhang Y, Webster RG, Perez DR (2003) Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. Virology 314: 580-590

32. Ludwig S, Schultz U, Mandler J, Fitch WM, Scholtissek C (1991) Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. Virology 183: 566-577

33. Makarova NV, Ozaki H, Kida H, Webster RG, Perez DR (2003) Replication and transmission of influenza viruses in Japanese quail. Virology 310: 8-15

34. Mase M, Eto M, Tanimura N, Imai K, Tsukamoto K, Horimoto T, Kawaoka Y, Yamaguchi S (2005) Isolation of a genotypically unique H5N1 influenza virus from duck meat imported into Japan from China. Virology 339: 101-109

35. Mase M, Tsukamoto K, Imada T, Imai K, Tanimura N, Nakamura K,

Yamamoto Y, Hitomi T, Kira T, Nakai T, Kiso M, Horimoto T, Kawaoka Y, Yamaguchi S (2005) Characterization of H5N1 influenza A viruses isolated during the 2003-2004 influenza outbreaks in Japan. Virology 332: 167-176

36. Middleton D, Bingham J, Selleck P, Lowther S, Gleeson L, Lehrbach P, Robinson S, Rodenberg J, Kumar M, Andrew M (2007) Efficacy of inactivated vaccines against H5N1 avian influenza infection in ducks. Virology 359: 66-71

37. Nestorowicz A, Kawaoka Y, Bean WJ, Webster RG (1987) Molecular analysis of the hemagglutinin genes of Australian H7N7 influenza viruses: role of passerine birds in maintenance or transmission? Virology 160: 411-418

38. OIE (2004) OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th edn, Paris

39. Ozaki H, Sugiura T, Sugita S, Imagawa H, Kida H (2001) Detection of antibodies to the nonstructural protein (NS1) of influenza A virus allows distinction between vaccinated and infected horses. Vet Microbiol 82: 111-119

40. Perez DR, Lim W, Seiler JP, Yi G, Peiris M, Shortridge KF, Webster RG (2003) Role of quail in the interspecies transmission of H9 influenza A

viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. J Virol 77: 3148-3156

41. Perkins LE, Swayne DE (2002) Pathogenicity of a Hong Kong-origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. Avian Dis 46: 53-63

42. Rashid Manzoor YS, Aaron Mweene, Yoshimi Tsuda, Hiroshi Kida (2008) Phylogenic Analysis of the M genes of influenza viruses isolated from free-flying water birds from their northern territory to Hokkaido, Japan. Virus Genes

43. Reed LJ, Muench H (1938) A simple method for estimating fifty percent endpoints. Am J Hyg 37: 493-497

44. Scholtissek C, Burger H, Kistner O, Shortridge KF (1985) The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology 147: 287-294

45. Sims LD, Ellis TM, Liu KK, Dyrting K, Wong H, Peiris M, Guan Y, Shortridge KF (2003) Avian influenza in Hong Kong 1997-2002. Avian Dis 47: 832-838

46. Soda K, Ozaki H, Sakoda Y, Isoda N, Haraguchi Y, Sakabe S, Kuboki N, Kishida N, Takada A, Kida H (2008) Antigenic and genetic analysis of H5 influenza viruses isolated from water birds for the purpose of vaccine use.

Arch Virol

47. Soda K, Sakoda Y, Isoda N, Kajihara M, Haraguchi Y, Shibuya H, Yoshida H, Sasaki T, Sakamoto R, Saijo K, Hagiwara J, Kida H (2008) Development of vaccine strains of H5 and H7 influenza viruses. Jpn J Vet Res

48. Steel J, Burmakina SV, Thomas C, Spackman E, Garcia-Sastre A, Swayne DE, Palese P (2008) A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. Vaccine 26: 522-531

49. Stieneke-Grober A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W (1992) Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. Embo J 11: 2407-2414

50. Suarez DL, Perdue ML (1998) Multiple alignment comparison of the non-structural genes of influenza A viruses. Virus Res 54: 59-69

51. Suarez DL, Perdue ML, Cox N, Rowe T, Bender C, Huang J, Swayne DE (1998) Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 72: 6678-6688

52. Swayne DE, Lee CW, Spackman E (2006) Inactivated North American and European H5N2 avian influenza virus vaccines protect chickens from Asian H5N1 high pathogenicity avian influenza virus. Avian Pathol 35:

141-146

53. Tumpey TM, Kapczynski DR, Swayne DE (2004) Comparative susceptibility of chickens and turkeys to avian influenza A H7N2 virus infection and protective efficacy of a commercial avian influenza H7N2 virus vaccine. Avian Dis 48: 167-176

54. Tumpey TM, Alvarez R, Swayne DE, Suarez DL (2005) Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the nonstructural protein of influenza A virus. J Clin Microbiol 43: 676-683

55. Webster RG, Webby RJ, Hoffmann E, Rodenberg J, Kumar M, Seiler P, Krauss S, Songserm T (2006) The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. Virology

56. WHO Cumulative number of confirmed human cases of avian influenza A/(H5N1)

57. WHO (2002) WHO manual on animal influenza diagnosis and surveillance., Geneva

58. Xu X, Subbarao, Cox NJ, Guo Y (1999) Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks

in Hong Kong. Virology 261: 15-19

和文要旨

1997 年香港で発生して以来、H5N1 ウイルスによる高病原性鳥インフルエンザの発生が続いている。筆者は 2004 年、日本で発生した高病原性鳥インフルエンザの病原ウイルスである A/chicken/Yamaguchi/7/2004 (H5N1) (山口株) および、2005 年モンゴルの Erkhel 湖で発見されたオオハクチョウの斃死体から分離された高病原性鳥インフルエンザウイルス、A/whooper swan/Mongolia/3/2005 (H5N1) (モンゴル株) の鳥類および哺乳動物に対する病原性を実験室内で確認した。

山口株は調べた 4 つの鳥類に対して高い病原性を示し、全身感染を起こすことが分かった。しかし、マウスに対する病原性は低く、ミニブタには感染しなかった。モンゴル株は、山口株に対する感受性が低かった幼ガモおよびマウスに高い病原性を示し、さらにミニブタには感染することが確認された。これらの結果から、山口株は鳥類に対して非常に高い病原性を示すが、哺乳類には病原性が低いものと考えられる。またモンゴル株は山口株よりも多くの種類の動物に対して病原性を示し、ミニブタに感染することから、公衆衛生上非常に重要であることが示唆された。

次にH5 ウイルスによる高病原性鳥インフルエンザに対して有効なワクチンの開発およびその評価を行った。高病原性鳥インフルエンザの防疫の基本は摘発淘汰であるが、防圧困難な非常時に備え、高力価のワクチンを開発および備

蓄することが必要である。そこで筆者は H5N2 および H7N1 亜型の 2 株の非病原性ウイルスから H5N1 亜型の遺伝子再集合ウイルスを実験室内で作出し、それをワクチン株とした。ワクチン株を鶏胚尿膜腔内に接種して得た尿液のウイルスを不活化し、256～512HA/0.1ml 相当の油中水型ワクチンを試製した。ワクチン 0.5ml をニワトリ 4 週齢のニワトリの下脚部筋肉内に 1 回注射し、免疫 3 週後にワクチン株と抗原性が類似する山口株または抗原性が異なるモンゴル株で攻撃したところ、いずれの場合もニワトリは臨床症状を示すことなく 14 日間耐過した。さらに、免疫後の日数が異なるニワトリに、HPAI ウイルス株にて攻撃したところ、ワクチン接種後 6 日以内のニワトリは攻撃ウイルスにより全て死亡したが、ワクチン接種 8 日目のニワトリは HI 抗体が検出されないにも関わらず、HPAI ウイルスの攻撃に対して耐過した。これらの結果から、本ワクチンはアジアで近年流行している高病原性鳥インフルエンザの病原ウイルスに有効であり、発症防御効果も接種 8 日目から確認されたことから、緊急用ワクチンとして有用であることが判った。