ORIGINAL ARTICLE

Antigenic and genetic analysis of H5 influenza viruses isolated from water birds for the purpose of vaccine use

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Abstract In order to prepare H5N1 influenza virus vaccine, the hemagglutinins (HAs) of 14 H5 virus isolates from water birds in Asia were antigenically and genetically analyzed. Phylogenetic analysis of the H5 HA genes revealed that 13 isolates belong to Eurasian and the other one to North American lineages. Each of the deduced amino acid sequences of the HAs indicated a non-pathogenic profile. Antigenic analysis using a panel of monoclonal antibodies recognizing six different epitopes on the HA of A/duck/Pennsylvania/10218/1984 (H5N2) and chicken antiserum to an H5N1 reassortant strain generated between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), [R(Dk/Mong-Dk/Mong)(H5N1)] showed that the HAs of highly pathogenic avian influenza (HPAI) viruses currently circulating in Asia were antigenically closely related to those of the present isolates from water birds. Mice subcutaneously injected with formalin-inactivated R(Dk/Mong-Dk/Mong) were protected from challenge with 100 mouse lethal dose of A/Viet Nam/1194/2004 (H5N1). The present results support the notion that the H5 isolates and the reassortant H5N1 strain should be useful for vaccine preparation.

Introduction

Outbreaks of highly pathogenic avian influenza (HPAI) caused by H5N1 strains have occurred in many countries, leading to serious economic losses in the poultry industry. In addition, the H5N1 HPAI virus returned to the natural host, migratory water birds, and spread to Asia, the Middle East, Europe, and Africa [2, 14, 29]. These incidents have increased the possibility of further spread of HPAI viruses in poultry and transmission to humans. In Japan, there had been no outbreak since 1925, when HPAI caused by H7N7 virus occurred in Chiba prefecture [24]. In January 2004, HPAI outbreaks caused by H5N1 virus occurred in Yamaguchi, Oita, and Kyoto prefectures in Japan [6, 15]. H5N1 virus infection then reoccurred in chicken farms in Miyazaki and Okayama prefectures in January 2007. The depopulation of chickens in the relevant farms and control measures successfully prevented the spread of the HPAI. The source of infection is unclear, although it was revealed that the H5N1 virus isolates from the affected chickens in Japan in 2004 were phylogenetically identical to HPAI viruses isolated in Guangdong Province of China in 2003 [15]. It was also suggested that the H5N1 viruses isolated in 2007 outbreaks in Japan showed a close phylogenetic relationship, with similarity to viruses isolated from water birds in Qinghai lake, Mongolia, Korea, and even in Nigeria during 2005–2007.

As described above, the standard measures for the control of HPAI outbreaks are testing and culling of all of the chickens in the farm. But, in addition, it has been

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suggested by OIE that when outbreaks spread to a broad area and become uncontrollable, ring vaccination would be an optional measure in addition to stamping out to reduce the virus concentration and hence to suppress the spread of viruses [1].

It has been established that influenza viruses are perpetuated between ducks and the water of the lakes where they nest in summer [7, 27]. Viruses of 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified in avian species [4]. Viruses have been highly conserved in water birds, antigenically and genetically [11]. Phylogenetic analysis has revealed that each of the influenza A viruses of birds and mammalian hosts including humans originated from water bird reservoirs [27]. Thus, continuous surveillance of avian influenza is essential for the preparedness against the emergence of HPAI and human pandemics. To provide information on the precursor genes of future pandemic influenza viruses, we have been conducting global surveillance of avian influenza since 1977 in Alaska, Australia, China, Japan, Mongolia, Siberia, and Taiwan [7, 9, 18, 21]. Virus isolates from birds in the surveillance have been stored in the influenza virus strain library in our laboratory for vaccine and diagnostic use [12].

The aim of the present study is to assess the applicability of the virus strain library for vaccine preparation. Here, we report the usefulness of the library by choosing an H5N1 virus strain for vaccine preparation as a model.

Materials and methods

Viruses

A total of 10,549 fecal samples were collected from water birds in 1996–2007 in Siberia, Mongolia, China, Australia, and Japan. The samples were kept cool, transported to Hokkaido University, and stored at 4°C or frozen at −80°C until assayed. Each sample was inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs. Subtype identification of influenza virus isolates was done by hemagglutination-inhibition (HI) and neuraminidase-inhibition (NI) tests using a series of standard antisera to the reference strains of influenza viruses [9].

A/duck/Pennsylvania/10218/1984 (H5N2) [17] was kindly provided by Dr. R. G. Webster, St. Jude Children's Research Hospital (Memphis, TN, USA). A/Hong Kong/156/1997 (H5N1) and A/Hong Kong/483/1997 (H5N1) [23] were obtained from Dr. K. F. Shortridge, University of Hong Kong (Hong Kong, Special Administrative Region, China). A/duck/Yokohama/aq-10/2003 (H5N1) [13] was received from Dr. M. Eto, Animal Quarantine Service (Yokohama, Kanagawa, Japan). A/chicken/Yamaguchi/7/ 2004 (H5N1) [15] and A/chicken/Ibaraki/1/2005 (H5N2) were obtained from the National Institute of Animal Health (Tsukuba, Ibaraki, Japan). A/Viet Nam/1194/2004 (H5N1) [Vietnam/1194] [16] was received from Dr. Y. Kawaoka, University of Tokyo (Tokyo, Japan). R(Duck/Mongolia/54/ 2001-Duck/Mongolia/47/2001) (H5N1) [R(Dk/Mong-Dk/ Mong)] was generated in our laboratory by the standard genetic reassortment procedure [6] as described below.

Generation of genetic reassortant virus

To generate the H5N1 reassortant virus, parental viruses, A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1), were mixed and inoculated into the allantoic cavity of 10-day-old chicken embryos. After incubation of the chicken embryos at 35°C for 48 h, the allantoic fluids were collected, mixed with chicken antiserum raised against A/duck/Hong Kong/301/1978 (H7N2), and incubated at 37°C for 1 h prior to plaque cloning of the viruses in MDCK cells. Cloned viruses were subtyped by HI and NI tests.

Phylogenetic analysis

To evaluate the genetic relationships among H5 influenza virus strains, nucleotide sequences of the HA genes (position 54–1,012) were determined and compared with those of H5 viruses from the NCBI database (http://www.ncbi.nlm.nih.gov/). Viral RNA was extracted from the allantoic fluid of chicken embryos infected with viruses by using a commercial kit (TRIzol LS Reagent, Invitrogen, Carlsbad, CA, USA) and reverse-transcribed with the Uni12 primer [5] and M-MLV reverse transcriptase (Invitrogen). Polymerase chain reaction (PCR)-based amplification of the coding regions of the HA genes was performed with gene-specific primer sets. The following primers were designed on the basis of published nucleotide sequences of influenza virus HA genes: forward primer (BmHA-1) 5′-TATTCGTCTCAGGGAGCAAAAGCAGG GG-3′ [5] and reverse primer (H5-1695R) 5′-CGATCC ATTGGAGCACATCC-3′. Direct sequencing of the HA gene was performed by using an autosequencer CEQ2000 (Beckman Coulter, Fullerton, CA, USA). The nucleotide sequences were proofread using GENETYX, version 7.0 (Genetyx, Tokyo, Japan). For phylogenetic analysis, sequence data of the genes together with those from Gen-Bank were analyzed by the neighbor-joining method [20]. The 959-bp fragments of the HA of each isolate were aligned using the CLUSTAL X version 1.83 program [26]. The transition/transversion rates were calculated using the PUZZLE 5.2 program [22]. Bootstrapping values were calculated using the modules SEQBOOT (random number seed: 123; 1,000 replicates), DNADIST (distance

estimation: maximum likelihood; analysis of 1,000 data sets), NEIGHBOR (neighbor-joining method; random number seed: 99; analysis of 1,000 data sets) and CONSENSE from the PHYLIP package, version 3.67 [3]. The phylogenetic trees were computed with the DNADIST and NEIGHBOR modules with the same parameters as above. For visualization of the trees, TREEVIEW version 1.6.6 was utilized [19].

The nucleotide sequences of H5 isolates obtained in the present study have been registered in GenBank (accession numbers: AB233320, AB241614, AB241616-AB241626, AB284068-AB284073, AB298276, AB299162, AB299181, AB299377-AB299378, AB299802-AB299832, AB300036-AB300050, AB300223-AB300050, AB300223-AB300235, AB300434-AB300441, AB301913-AB301917, AB302086, AB378682, and AB378690).

Monoclonal antibodies

Monoclonal antibodies (MAbs) to the HA of A/duck/Pennsylvania/10218/1984 (H5N2) were prepared [10]. Briefly, a BALB/c mouse (CLEA Japan Inc., Tokyo, Japan) was immunized with two intraperitoneal injections of purified influenza virus (100 µg protein each) 2 weeks apart. Two weeks after the second injection, the same antigen (50 µg) was intravenously administered, and 3 days later, the spleen cells were fused with myeloma SP2/0-Ag14 cells. Hybridoma cells producing antibodies specific for the HA were selected on the basis of the result of immunoprecipitation and enzyme-linked immunosorbent assay (ELISA) as described [10]. The hybridoma cells were then cloned in soft agar (Bacto Agar, Difco, Sparks, MD, USA) and grown as ascites in BALB/c mice. To select escape mutants, a 1:10 dilution of ascites containing MAbs was mixed with an equal volume of serial 10-fold dilutions of the parental viruses. After incubation for 60 min at room temperature, the mixture was inoculated into the allantoic cavities of 10-day-old embryonated chicken eggs. Virus that grew in the presence of MAbs was cloned by plaque formation in MDCK cells, and its nucleotide sequence was determined and compared to that of the wild-type strain.

Antigenic analysis

The antigenic specificity of H5 influenza viruses was assessed by the fluorescent antibody method with MAbs and a neutralization test. MDCK cells infected with each of 24 H5 influenza viruses were fixed with 100% acetone 8 h post-inoculation. The reactivity patterns of the MAbs to H5 strains were investigated by the immunofluorescent method with a FITC-conjugated goat IgG to mouse IgG (ICN Biomedicals, Inc., Costa Mesa, CA, USA). For the

neutralization test, a polyclonal chicken antiserum raised against R(Dk/Mong-Dk/Mong), including a water-in-oil adjuvant provided by Kyoto Biken Laboratories, Inc. (Uji, Kyoto, Japan), and 100 $TCID_{50}$ of test viruses were mixed and incubated for 1 h at room temperature. This mixture was inoculated onto MDCK cells in 96-well tissue culture plates and incubated for 1 h at 35°C. Then the cells were washed with PBS and incubated in MEM (MEM, Nissui Pharmaceutical, Tokyo, Japan) containing 5 µg/ml trypsin (Sigma-Aldrich, Inc., St. Louis, MO, USA) without serum for 2 days at 35°C. The cytopathic effect was observed, and neutralization titers were expressed as reciprocals of the highest dilution of serum sample that showed complete neutralization.

Immunization and challenge of mice

To assess the potency of R(Dk/Mong-Dk/Mong) as a H5N1 vaccine strain, the test whole virus vaccine was prepared as described previously [25]. Five 4-week-old female C57BL/6 mice (CLEA Japan Inc., Tokyo, Japan) were injected subcutaneously with 100, 20, 4, and 0.8 µg proteins of inactivated R(Dk/Mong-Dk/Mong) whole virus vaccine, respectively. Two weeks later, the mice were boosted by subcutaneous injection with the vaccine. Control mice were injected with PBS as well. One week after the second vaccination, five mice in each group were challenged intranasally with 30 µl of 100 MLD_{50} (50% mouse lethal dose) of Vietnam/1194 under anesthesia. Challenge study was carried out in self-contained isolator units (Tokiwa Kagaku, Tokyo, Japan) at a BSL3 biosafety facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. Serum samples were obtained from mice before challenge. Serum samples were pooled by each group and tested by HI test.

Results

Isolation of influenza viruses from fecal samples of water birds

During 1996–2007, 524 influenza virus strains were isolated from 10,549 fecal samples of migratory ducks and swans that flew from their nesting lakes in Siberia to Mongolia, China, and Japan in autumn each year. Fourteen of them were H5 influenza viruses, as shown in Table 1.

Generation of H5N1 genetic reassortant virus

To prepare non-pathogenic H5N1 influenza viruses for vaccine production, H5N1 genetic reassortants were

Table 1 H5 avian influenza virus isolates examined in this study

Isolates	Year of isolation	Amino acid sequence at the cleavage site	Pathotype
Ohnuma Pond, Wakkanai, Hokkaido, Japan			
Swan/Hokkaido/4/1996 (H5N3)	October 1996	RETR/G	Non-pathogenic
Swan/Hokkaido/51/1996 (H5N3)	October 1996	RETR/G	Non-pathogenic
Swan/Hokkaido/67/1996 (H5N3)	October 1996	RETR/G	Non-pathogenic
Duck/Hokkaido/69/2000 (H5N3)	October 2000	RETR/G	Non-pathogenic
Duck/Hokkaido/447/2000 (H5N3)	October 2000	RETR/G	Non-pathogenic
Ohno Pond, Sapporo, Hokkaido, Japan			
Duck/Hokkaido/84/2002 (H5N3)	September 2002	KETR/G	Non-pathogenic
Duck/Hokkaido/101/2004 (H5N3)	October 2004	RETR/G	Non-pathogenic
Duck/Hokkaido/193/2004 (H5N3)	October 2004	RETR/G	Non-pathogenic
Duck/Hokkaido/299/2004 (H5N3)	November 2004	RETR/G	Non-pathogenic
Duck/Hokkaido/167/2007 (H5N3)	October 2007	RETR/G	Non-pathogenic
Duck/Hokkaido/201/2007 (H5N3)	November 2007	RETR/G	Non-pathogenic
Ugii nuur, Arkhangai, Mongolia			
Duck/Mongolia/54/2001 (H5N2)	September 2001	RETR/G	Non-pathogenic
Duck/Mongolia/500/2001 (H5N3)	September 2001	RETR/G	Non-pathogenic
Duck/Mongolia/596/2001 (H5N3)	September 2001	RETR/G	Non-pathogenic

generated between A/duck/Mongolia/54/2001 (H5N2) and A/duck/Mongolia/47/2001 (H7N1) isolated from migratory ducks, and the origin of the internal protein genes was determined. PB2, PB1, PA, HA, NP, and M gene segments of generated H5N1 virus, R(Dk/Mong-Dk/Mong), were derived from A/duck/Mongolia/54/2001 (H5N2), which was one of the non-pathogenic strains isolated from a water bird in this study (Table 1), and NA and NS gene segments from A/duck/Mongolia/47/2001 (H7N1).

Phylogenic analysis of the H5 isolates

The HA genes of the 14 H5 isolates were sequenced and analyzed by the neighbor-joining method along with those of other H5 strains, including HPAI viruses presently circulating in Asia. The amino acid sequence at the cleavage site of the HA was deduced from the nucleotide sequence of the corresponding gene of each of the isolates. As shown in Table 1, HAs of all isolates had RETR or KETR sequences at the cleavage sites, which are typically found in the HA of viruses that are non-pathogenic for chickens. The HAs of 13 out of 14 isolates were of the Eurasian type and the HA of A/duck/Hokkaido/84/2002 (H5N3) [Dk/Hok/84/02] was of the North American type by phylogenetic analysis (Fig. 1). Sequence analysis of Dk/Hok/84/02 revealed that the other seven gene segments were classified into the Eurasian lineage (data not shown). The 13 isolates of the Eurasian type constituted a different cluster from that of HPAI viruses isolated in Asia.

Antigenic comparison of H5 influenza virus isolates

For the antigenic analysis of H5 influenza viruses, a panel of MAbs with neutralizing activities toward the HA of A/duck/Pennsylvania/10218/1984 (H5N2) was prepared (Table 2). By sequence analysis of the HA genes of escape mutants selected in the presence of these seven MAbs, the epitopes recognized by these MAbs were mapped to the globular head of the H5 HA molecule (data not shown), and the MAbs were divided into six groups (Groups I–VI) accordingly. Reactivity of 24 H5 influenza virus strains with the panel of MAbs was analyzed by the immunofluorescent assay. Although there was some difference in their patterns of reactivity with MAbs, all of the isolates and HPAI viruses were neutralized by the polyclonal chicken antiserum raised against R(Dk/Mong-Dk/Mong) with a water-in-oil adjuvant (Table 2).

Protective effect of the test vaccine in mice against H5 HPAI virus challenge

The test inactivated vaccine was prepared from R(Dk/Mong-Dk-Mong). To assess the potency of the vaccine against H5 HPAI virus infection, mice vaccinated subcutaneously with inactivated R(Dk/Mong-Dk/Mong) were challenged intranasally with a lethal dose of virus strain Vietnam/1194. Survival numbers of the mice after virus challenge are shown in Fig. 2. All the control mice died within 9 days after challenge, while all of the mice
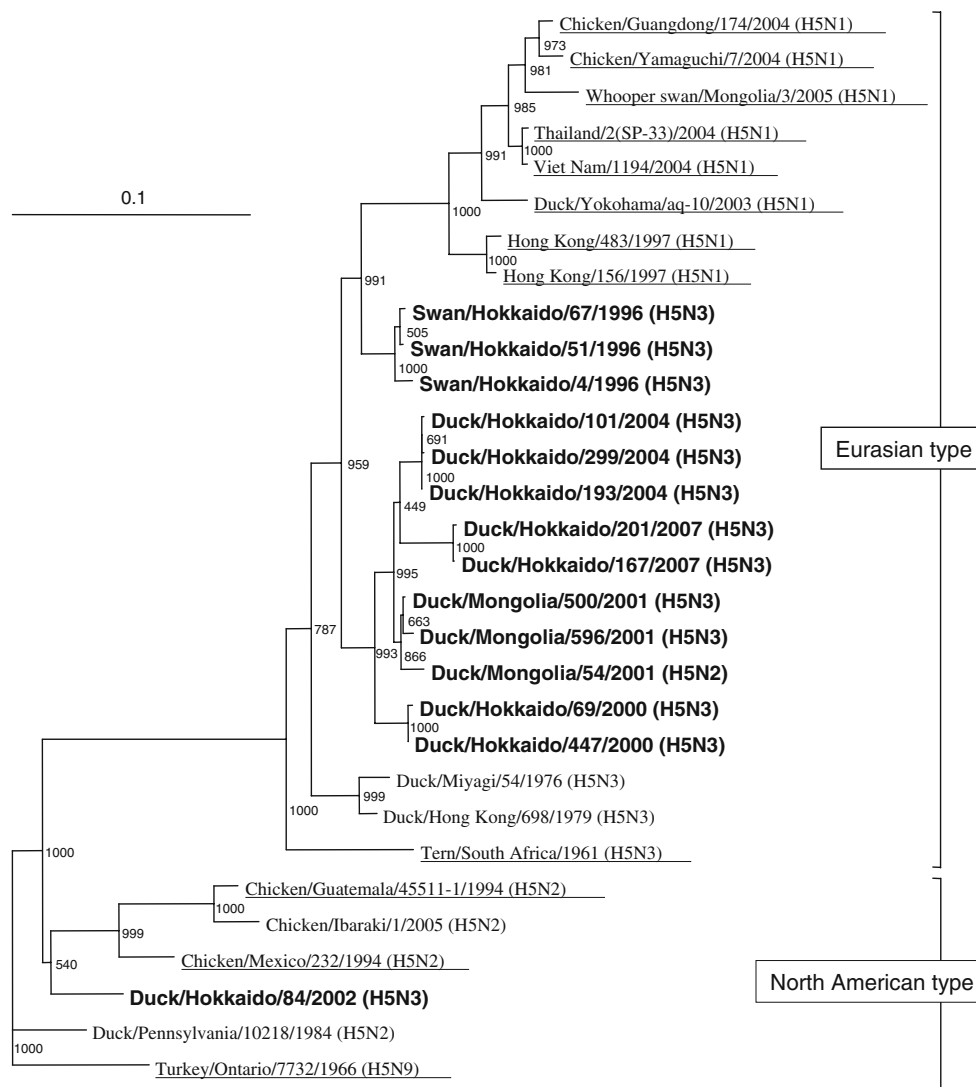
Fig. 1 Phylogenic tree of the HA genes of H5 influenza viruses. Nucleotides 54–1032 (979 bases) of the H5 HA genes were used for the analysis. Horizontal distances are proportional to the minimum number of nucleotide differences required to join nodes and sequences. *Numbers* at the nodes indicate confidence levels in a bootstrap analysis with 1,000 replications. Viruses isolated in this study are in *bold*. HPAI viruses are *underlined*

immunized with 100 μg vaccine survived 14 days without showing any disease signs. The survival rate was correlated to the dose of vaccine. The mice that died of virus challenge showed clinical signs including ruffled fur, inactivity, and depression, starting 8 days post-inoculation. The mean HI titers against the vaccine strain and challenge strain of mice immunized with 100, 20, 4, and 0.8 μg protein before challenge were 1:128, 64, 32, and 8 for R(Dk/Mong-Dk/Mong) and 1:16, 16, 8, and <8 for Vietnam/1194.

Discussion

The results of the phylogenetic analysis of the H5 HAs of 14 isolates revealed that 13 belong to the Eurasian lineage

and the other one, Dk/Hok/84/02, to the North American lineage. Since other gene segments of Dk/Hok/84/02 were classified into the Eurasian lineage, Dk/Hok/84/02 should be a reassortant virus whose HA gene originated from a virus strain that was introduced into the Eurasian area by migrating water birds from their nesting lakes in North America.

The pathogenicity of avian influenza virus has been shown to be associated with the presence of multiple basic amino acids at the cleavage site of the HA molecule [8]. As shown in Table 1, all of the H5HAs of the isolates were of the non-pathogenic type, possessing only two basic amino acid residues at their cleavage sites. The homology in the amino acid sequence of the HA1 among the 13 isolates of Eurasian lineage was over 95% (data not shown),

Table 2 Antigenic analysis of H5 influenza viruses

Viruses	Monoclonal antibodies[a]							Polyclonal antibodies[c]
	I (88[b])	II (145)	III (157)	IV (168)		V (169)	VI (205)	α-R(Dk/Mong-Dk/Mong)
	D101/1	A310/39	64/1	B9/5	B220/1	B59/5	25/2	
LPAIV								
Duck/Pennsylvania/10218/1984 (H5N2)	+	+	+	+	+	+	+	512
Swan/Hokkaido/4/1996 (H5N3)	+	+	+	+	+	+	+	128
Swan/Hokkaido/51/1996 (H5N3)	+	+	+	+	+	+	+	256
Swan/Hokkaido/67/1996 (H5N3)	+	+	+	+	+	+	+	256
Duck/Hokkaido/69/2000 (H5N3)	+	+	+	+	+	+	+	128
Duck/Hokkaido/447/2000 (H5N3)	+	+	+	+	+	+	+	512
Duck/Mongolia/54/2001 (H5N2)	+	+	+	+	+	+	+	256
Duck/Mongolia/500/2001 (H5N3)	+	+	+	+	+	+	+	32
Duck/Mongolia/596/2001 (H5N3)	+	+	+	+	+	+	+	256
Duck/Hokkaido/84/2002 (H5N3)	+	+	+	+	+	+	+	512
Duck/Hokkaido/101/2004 (H5N3)	+	+	+	+	+	+	+	64
Duck/Hokkaido/193/2004 (H5N3)	+	+	+	+	+	+	+	128
Duck/Hokkaido/299/2004 (H5N3)	+	+	+	+	+	+	+	128
Chicken/Ibaraki/1/2005 (H5N2)	−	−	−	−	−	−	−	256
Duck/Hokkaido/167/2007 (H5N3)	+	+	+	+	+	+	+	1,280
Duck/Hokkaido/201/2007 (H5N3)	+	+	+	+	+	+	+	80
R(Dk/Mong-Dk-Mong) (H5N1)	+	+	+	+	+	+	+	256
HPAIV								
Tern/South Africa/1961 (H5N3)	+	−	+	−	−	+	+	256
Hong Kong/156/1997 (H5N1)	−	+	+	+	+	+	+	64
Hong Kong/483/1997 (H5N1)	−	+	+	+	+	+	+	64
Duck/Yokohama/aq-10/2003 (H5N1)	−	−	+	+	+	+	+	256
Chicken/Yamaguchi/7/2004 (H5N1)	−	+	+	+	+	−	+	256
Viet Nam/1194/2004 (H5N1)	+	+	+	+	+	−	+	128
Whooper Swan/Mongolia/3/2005 (H5N1)	+	−	+	+	+	−	+	256

[a] Fluorescent antibody methods were performed with monoclonal antibodies to the HA of A/duck/Pennsylvania/10218/1984 (H5N2)

[b] Location of amino acid substitutions in antigenic variants selected in the presence of respective monoclonal antibodies

[c] Neutralizing antibody titers

indicating that the HA genes have been well conserved in natural hosts. A previous study has already indicated that ddY mice immunized with a formalin-inactivated vaccine prepared from A/swan/Hokkaido/67/1996 (H5N3) [Swan/Hok/67/96], one of the isolates belonging to Eurasian lineage, survived a challenge with HPAI viruses A/Hong Kong/156/1997 (H5N1) and A/Hong Kong/483/1997 (H5N1) [25]. Therefore, the 13 isolates of the Eurasian type including Swan/Hok/67/96 could be useful as potential vaccine strains for HPAI caused by H5 strains.

Antigenic analysis of H5 influenza viruses with the panel of MAbs to the HA molecule of A/duck/Pennsylvania/10218/1984 (H5N2) confirmed that H5 isolates from water birds and the HPAI viruses share epitopes at the globular heads of the HAs, although there were some differences in reactivity with MAbs (Table 2). In addition, all

H5 HPAI viruses were neutralized by the chicken polyclonal antiserum to R(Dk/Mong-Dk/Mong). Thus, it was confirmed that there was little difference in antigenicity among the H5 influenza viruses.

HI titers of mice against R(Dk/Mong-Dk/Mong) before HPAI virus challenge were positively correlated with the dose of vaccine. Mice vaccinated subcutaneously with 100 μg of inactivated R(Dk/Mong-Dk/Mong) were protected from lethal infection with Vietnam/1194 (Fig. 2). The HI titer of the pooled sera of mice against R(Dk/Mong-Dk/Mong) was 1:128 before challenge. Thus, it was speculated that an HI antibody titer of 1:128 was a prerequisite for complete protection of mice from manifestation of disease signs. All seven H5 HPAI viruses tested in this study were neutralized by the chicken polyclonal antiserum to R(Dk/Mong-Dk/Mong), suggesting that
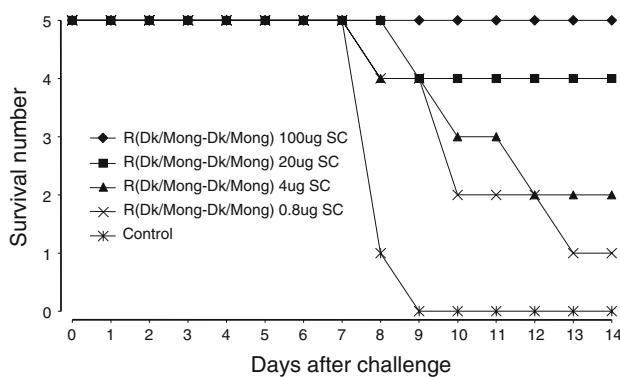
Fig. 2 Survival numbers of mice after challenge with Vietnam/1194. Five 4-week-old female C57BL/6 mice were vaccinated subcutaneously with inactivated R(Dk/Mong-Dk/Mong) virus particles. The mice were boosted 2 weeks later. One week after the second vaccination, five mice in each group were challenged intranasally with 100 LD_{50} of Vietnam/1194. PBS was inoculated subcutaneously into the control mice

the test vaccine could be protective against HPAI virus of clade 1, such as Vietnam/1194, as well as clade 2 and 3 viruses such as A/chicken/Yamaguchi/7/2004 (H5N1) and A/Hong Kong/483/1997 (H5N1) [28]. These findings support the notion that the R(Dk/Mong-Dk/Mong) virus vaccine is potent against infection with H5N1 HPAI virus strains presently circulating in the world. The vaccine prepared from the present R(Dk/Mong-Dk-Mong) strain has been produced in collaboration with vaccine producers, and its protective effect is under investigation.

None of the 16 HA and 9 NA subtypes can be ruled out as potential candidates for causing a future influenza pandemic. To provide vaccine strains as seeds for vaccines, it is important to establish a library of vaccine strain candidates of isolates from water birds [12]. The present results demonstrate that the library of a panel of influenza virus strains isolated from natural hosts in the global surveillance of avian influenza is useful for preparedness for future pandemics.

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