2. Materials and methods

2.1. Viruses

The A/duck/Hokkaido/Vac-1/04 (H5N1) (Dk/Vac-1/04) virus belonging to the Eurasian lineage of a non-pathogenic avian influenza (AI) virus, generated as a reassortant virus between A/duck/Mongolia/54/01 (H5N2) and A/duck/Mongolia/47/01 (H7N1), was used for vaccine preparation [12].

A/chicken/Yamaguchi/7/04 (H5N1) (Ck/Yamaguchi/04) virus, isolated by the National Institute of Animal Health (Ibaraki, Japan) from a dead chicken during the HPAI outbreak in 2004 in Japan, was used as the challenge virus [18,19].

To prepare virus suspensions, the Dk/Vac-1/04 and Ck/Yamaguchi/04 viruses were inoculated into the allantoic cavity of embryonated chicken eggs and incubated at 34 C for 48 h and 35 °C for 24 h, respectively.

2.2. Vaccine preparation

A virus suspension of Dk/Vac-1/04 was inactivated by incubation with formalin at a 0.2% final concentration for 3 days at 4 °C. Virus inactivation was confirmed by inoculation of the formalin-treated samples into embryonated chicken eggs.

The inactivated Dk/Vac-1/04 virus suspension was diluted with phosphate-buffered saline (PBS) to appropriate concentrations based on hemagglutination (HA) titres. A 2.5 volume of viral suspension with HA titre of 1:256 was mixed with a 7.5 volume of oil adjuvant containing 3.9% anhydromannitol-octadecenoate-ether (AMOE) and sufficient light mineral oil to comprise the remaining volume. This mixture was homogenized using an ultra-homomixer (PRIMIX Co. Ltd.) to produce a water-in-oil type of adjuvant test vaccine. The virus concentration in the test vaccine was 640 HA units per dose [17].

2.3. Animals and serum sampling

Specific pathogen-free white leghorn chickens were obtained from Kyoto Biken Laboratories, Inc., Kyoto, Japan. Ten 4-week-old chickens were vaccinated intramuscularly in the lower thigh with 0.5 mL of the test vaccine, and 4 other 4-week-old chickens were used as non-vaccinated controls. The two groups of chickens were reared separately for 138 weeks after the vaccination. Five vaccinated chickens, however, kept separately, died due to air conditioning accident of rearing facility 44 weeks after vaccination. Thus we used the remaining 5 vaccinated chickens for the experiment. For the Ck/Yamaguchi/04 challenge test, the chickens were transported to a bio-safety level 3 facility at the Graduate School of Veterinary Medicine, Hokkaido University, Japan. All procedures were performed according to the animal experiment guidelines of Hokkaido University. A blood sample was obtained from each chicken every week after vaccination for 5 weeks, and then at 11–14-week intervals until 138 weeks. In addition, blood samples were obtained from all surviving chickens 2 weeks after the Ck/Yamaguchi/04 challenge.

2.4. Hemagglutination-inhibition (HI) test

The HI test was performed according to the Japanese Standards for Veterinary Biological Products. Briefly, 1 volume of each serum sample was mixed with 3 volumes of 10% chicken red blood cells (RBCs) and incubated overnight at 4 °C. The mixtures were centrifuged at 1000 × *g* for 5 min and the supernatants collected as four-fold diluted sera. One-hundred microliters of each supernatant were dispensed into wells in the first lane of a plastic V-bottomed microtitration plate. Fifty microliters of PBS was dispensed into all other wells, after which 50 μL of two-fold serial dilutions of the supernatants were added to the PBS-containing wells. The Dk/Vac-1/04 and Ck/Yamaguchi/04 virus suspensions were inactivated by incubation with formalin at a final concentration of 0.2% for 3 days at 4 °C and 0.1% for 7 days at 4 °C, respectively, followed by dilution of the antigen with PBS to adjust the HA titre to 1:8. Fifty microliters of each HA antigen was then dispensed into all wells of the plates and they were incubated for 30 min at room temperature. Finally, 100 μL of 0.5% chicken RBCs was dispensed into all previously prepared wells and the plates were incubated again for 60 min at room temperature. The HI antibody titres were expressed as the highest dilution of the serum sample that showed complete inhibition of hemagglutination.

2.5. Protection test of vaccinated chickens against HPAI virus challenge

All chickens were challenged intranasally with 100 times 50% chicken lethal dose (i.e. $10^{5.5}$ times 50% egg infectious dose) of Ck/Yamaguchi/04 at 138 weeks after the Dk/Vac-1/04 vaccination. Clinical signs, such as lethargy, loss of appetite and nervous symptoms, were monitored for 14 days post-challenge (p.c.). To detect virus shedding, laryngopharyngeal and cloacal swabs were individually collected from all surviving chickens on days 2 and 4 p.c. Laryngopharyngeal and cloacal swabs were also collected individually at the time of death or at euthanasia on day 14 p.c. Swabs were individually suspended in 1.0 mL of minimal essential medium. A 0.1-mL aliquot of each suspension was then inoculated into the allantoic cavity of embryonated chicken eggs. The infectivity titres of the swabs were calculated using the method of Reed and Muench [20], and expressed as 50% egg infectious dose per millilitre (EID_{50}/mL).

3. Results

The serum HI antibodies against Dk/Vac-1/04 reached a maximum geometric mean (GM) titre of 1:2048 at 4 weeks after vaccination, and then gradually decreased until reaching a GM titre level of 1:111 at 138 weeks after vaccination (Table 1). The serum HI titres of 5 vaccinated chickens that died due to an accident were basically the same as those of the 5 chickens in the present results at every points of time until the time of the accident (44 weeks after vaccination, data not shown).

During the challenge test, all chickens in the vaccinated group survived for 14 days p.c. without showing any clinical signs of HPAI (Table 2). In contrast, all chickens in the non-vaccinated group showed typical HPAI symptoms 1–2 days p.c. and died within 3 days p.c.

A marked secondary antibody response was observed 14 days p.c. in serum HI titres of chickens vaccinated with Dk/Vac-1/04 and challenged with Ck/Yamaguchi/04 viruses (Table 3). In addition, $10^{1.3}$–$10^{1.7}$ EID_{50}/mL of challenge virus were recovered from the laryngopharyngeal swabs from 3 of the 5 vaccinated chicken 2 days p.c. but there was no virus recovery at 4 and 14 days p.c. However, $10^{4.5}$–$10^{7.5}$ EID_{50}/mL of the challenge virus were recovered from laryngopharyngeal and cloacal swabs of the non-vaccinated chickens.

These findings demonstrated that all of the chickens vaccinated with Dk/Vac-1/04 survived the Ck/Yamaguchi/04 challenge without exhibiting any clinical signs. Three of those chickens shed small amounts of Ck/Yamaguchi/04 virus in their laryngopharyngeal swabs, only on day 2 p.c.

Table 1
HI antibody titres against Dk/Vac-1/04 in chickens during the 138 weeks following Dk/Vac-1/04 vaccination.

Group	Chicken No.	HI antibody titres and weeks after vaccination															
		1	2	3	4	5	16	28	40	52	64	76	88	100	112	124	138
Vaccinated	2	<4	128	2048	4096	2048	1024	512	512	512	256	256	256	256	256	256	256
	3	<4	256	1024	2048	2048	2048	1024	1024	1024	512	512	512	512	256	256	128
	6	<4	128	512	1024	1024	1024	256	256	256	128	128	128	128	64	64	64
	7	<4	128	512	2048	2048	256	128	128	128	128	128	128	128	64	64	64
	8	<4	512	1024	2048	2048	1024	512	512	512	512	512	512	256	128	128	128
GM[a]		<4	194	891	2048	1783	891	388	388	388	256	256	256	223	128	128	111
Non-vaccinated	9	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	10	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	11	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
	12	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4
GM[a]		<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4	<4

[a] GM: geometric mean.

Table 2
Clinical signs of influenza in vaccinated chickens after challenge with the HPAI virus Ck/Yamaguchi/04.

Group	Chicken No.	Serum HI antibody titre[a] and virus strain		Clinical signs on days following HPAI challenge													
		Dk/Vac-1/04	Ck/Yamaguchi/04	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Vaccinated	2	256	256	–[b]	–	–	–	–	–	–	–	–	–	–	–	–	–
	3	128	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	6	64	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	7	64	64	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	8	128	128	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Non-vaccinated	9	<4	<4	–	+[b]	D[b]											
	10	<4	<4	+	D												
	11	<4	<4	–	D												
	12	<4	<4	–	+	D											

[a] HI antibody titre at the time of challenge.
[b] '–' no abnormal signs; '+': typical clinical signs (lethargy, loss of appetite and nervous symptoms); D: death.

Table 3
Antibody response and virus isolation from laryngopharyngeal and cloacal swabs of chickens after challenge with the Ck/Yamaguchi/04 virus.

Group	Chicken no.	Serum HI antibody titre and virus strain				Virus titres[a] on the following days after challenge							
		Dk/Vac-1/04		Ck/Yamaguchi/04		2		3		4		14	
		Pre[b]	Post[b]	Pre	Post	L[c]	C[c]	L	C	L	C	L	C
Vaccinated	2	256	2048	256	2048	–[d]	–	NT[d]	NT	–	–	–	–
	3	128	8192	128	8192	1.7	–	NT	NT	–	–	–	–
	6	64	512	128	512	–	–	NT	NT	–	–	–	–
	7	64	2048	64	2048	1.5	–	NT	NT	–	–	–	–
	8	128	512	128	256	1.3	–	NT	NT	–	–	–	–
Non-vaccinated	9	<4	NT	<4	NT	6.8	5.5	6.0	4.5	NT	NT	NT	NT
	10	<4	NT	<4	NT	4.5	5.5	NT	NT	NT	NT	NT	NT
	11	<4	NT	<4	NT	6.5	4.8	NT	NT	NT	NT	NT	NT
	12	<4	NT	<4	NT	7.5	5.5	4.5	4.8	NT	NT	NT	NT

[a] Virus titre expressed as $\log_{10}$ EID_{50}/mL.
[b] Pre: at the time of challenge; Post: 14 days post-challenge.
[c] L: laryngopharynx; C: cloacal.
[d] '–': Indicates a virus recovery titre lower than 0.5 $\log_{10}$ EID_{50}/mL; NT: not tested.

4. Discussion

In the present study, we demonstrated that an avian influenza test vaccine produced using an oil adjuvant containing AMOE as a surfactant induced a high level of HI antibody response in chickens, lasting as long as 138 weeks after vaccination. Oda *et al.* reported that the surfactant contained in our oil adjuvant plays a key role in stimulation of an antibody response [21]. They also described that oligosaccharide oleate ester is the most important element in the adjuvant activity of AMOE. It is, thus, considered that AMOE is a potent adjuvant equivalent to QS-21 saponin or alum adjuvants in mice.

Other elements of AMOE may play an important role in the creation of stable water-in-oil emulsions of light mineral oil. Such emulsion stability may improve and prolong the adjuvant activity of AMOE by enhancing the potency of the vaccine as a foreign substance and by capturing the antigen at the injection site. Recently, Hikida *et al.* reported that phospholipase C-gamma 2 is essential for formation and maintenance of memory B cells [22]. It is possible that the long-lasting immunological memory and the marked

antibody production following the HPAI virus challenge in the present vaccinated chickens may have been regulated through similar immunological mechanisms.

Both Dk/Vac-1/04 and Ck/Yamaguchi/04 were viruses of the Eurasian lineage, and both showed similar antigenic cross reactivity in the HI test. This suggests that the potency of a vaccine prepared from a virus strain belonging to the same lineage as the outbreak virus may be higher than that of a vaccine prepared from a virus strain belonging to a heterologous lineage strain [12,13].

To assure the safety of vaccines in humans, ether-split influenza vaccines are widely adopted for human use. We chose formalin-inactivated whole virus particles as an antigen in order to maintain efficacy while reducing the cost of the vaccine. Recently, Hagenaars *et al.* reported that whole inactivated virus particles, containing all viral components, produced the best results in potency tests as compared to split, subunit and virosomes presented through intramuscular and intranasal routes [23]. Their report supports our approach of using whole virus particles for vaccine development. Furthermore, it is anticipated that a whole virus particle vaccine should be effective against H5 viruses, even on an antigenically drifted virus [14]. Thus, whole virus particles appear to be one of the best candidates for chicken vaccine development.

The present results show that the test vaccine constructed using an apparently optimum adjuvant composition and an appropriate lineage strain of whole inactivated virus particles induces long-lasting protective immunity in chickens.

It is possible that potency of the test vaccine may be different for SPF from conventional lines of chicken. Accordingly, studies to compare the immunological response to vaccination in SPF and conventional lines of chicken are under way.

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BRIEF REPORT

Characterization of H3N6 avian influenza virus isolated from a wild white pelican in Zambia

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Abstract We characterized an influenza virus isolated from a great white pelican in Zambia. Phylogenetic analysis showed that all of its gene segments belonged to the Eurasian lineage and that they appear to have evolved in distinct geographical regions in Europe, Asia, and Africa, suggesting reassortment of virus genes maintained in wild aquatic birds whose flyways overlap across these continents. It is notable that this virus might possess some genes of the same origin as those of highly pathogenic H7 and H5 viruses isolated in Eurasia. The present study underscores the need for continued monitoring of avian influenza viruses in Eurasia and Africa.

Aquatic birds of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds) are thought to constitute the major natural reservoir for avian influenza (AI) A virus [20, 24]. All known influenza A virus subtypes with respect to two surface glycoproteins, hemagglutinin (HA) (H1–H16) and neuraminidase (NA) (N1–N9) and most HA/NA combinations have been identified in wild birds and poultry [11, 24]. Influenza A viruses of avian origin have been implicated in outbreaks of influenza in other hosts [13, 20, 24], indicating that a vast influenza virus gene pool for future epidemics in other animal species, including human pandemics, exists in avian sources.

Highly pathogenic (HP) H5N1 AI virus has spread from Asia to other regions, including Europe, the Middle East, and Africa, causing outbreaks in domestic poultry and wild birds [5, 10, 15]. As of 17 June 2009, Egypt had recorded the highest number of H5N1 human infections in Africa: 78 confirmed cases with 27 fatalities [25]. The origins and transmission routes of HP H5N1 virus from Asia to Africa remain unclear. The potential spread of HP H5N1 virus by wild birds over large geographical regions and the direct

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Table 1 Antigenic characterization of Zb06 by HI and NI assays

Virus	Subtype	HI titer of chicken antiserum				Virus	Subtype	NI titer of chicken antiserum				
		Zb06	Aichi	Mem96	PR8			Zb06	Eng56	MD77	Czec56	PR8
Zb06	H3N6	**5,120**	320	<40	<40	Zb06	H3N6	**2,560**	2,560	640	320	<10
Aichi	H3N2	5,120	**10,240**	160	<40	Eng56	H11N6	1,280	**2,560**	320	320	<10
Mem96	H3N2	2,560	<40	**10,240**	<40	MD77	H13N6	5,120	2,560	**1,280**	640	<10
DHK836	H3N1	2,560	640	<40	<40	Czec56	H4N6	2,560	2,560	1,280	**1,280**	<10
PR8	H1N1	<40	<40	<40	**5,120**	PR8	H1N1	<10	<10	<10	<10	**1,280**

Homologous HI and NI titers are in boldface type and are underlined

zoonotic threat posed by several AI viruses of the Eurasian lineage underscore the need for more information on the ecology and evolution of AI A viruses circulating in the wild bird reservoir globally.

In attempting to narrow the knowledge gap that exists in the ecology of AI viruses circulating in wild birds in Africa, virologic surveillance studies were initiated in Zambian wetlands frequented by migratory birds. We report the characterization of the first influenza virus isolate from an avian host in Zambia.

In August 2006, 51 fresh fecal samples were collected from apparently healthy pelicans in Lochinvar National Park (15°40′S; 27°15′E), in Southern province of Zambia. Virus isolation was attempted in 10- to 11-day-old embryonated chicken eggs. One influenza virus isolate was obtained and designated A/pelican/Zambia/01/06 (H3N6) (Zb06) following subtyping by standard hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests using specific antisera to the reference strains of influenza viruses. We then prepared chicken antisera against Zb06. Briefly, purified virus was inactivated with 0.1% formalin at 4°C for 1 week. Three-month-old specific-pathogen-free chickens were immunized intramuscularly and subcutaneously with a 100-μl suspension containing 300 μg inactivated virus with complete Freund's adjuvant (DIFCO). The chickens were re-immunized 2 weeks later in a similar way, but with incomplete Freund's adjuvant. The chickens were given a third intravenous booster injection without adjuvant 3 weeks after the second immunization. One week after the final immunization, the chickens were killed to obtain serum. We used A/Puerto Rico/8/34 (H1N1) (PR8), A/duck/Hong Kong/836/80 (H3N1) (DHK836), A/Aichi/2/68 (H3N2) (Aichi), A/Memphis/1/96 (H3N2) (Mem96), A/Czeckoslovakia/56 (H4N6) (Czec56), A/duck/England/1/56 (H11N6) (Eng56), A/gull/Maryland/704/77 (H13N6) (MD77), and Zb06 for antigenic characterization by HI and NI assays. Chicken antiserum was raised against all of these viruses except DHK836. Chicken erythrocytes (0.5%) and fetuin (CALBIOCHEM) were used in the HI and NI assays, respectively.

In antigenic analysis, chicken antiserum raised against Zb06 showed high HI and NI titers, roughly equal to those of all the H3 and N6 influenza viruses tested, including the relatively recent human strain, Mem96 (Table 1), indicating that chicken antiserum raised against Zb06 has high cross-reactivity. The reason for the high cross-reactivity is unclear, but one possibility is that antibodies raised against Zb06 predominantly recognize the conserved epitopes of the surface glycoproteins of the viruses tested. Chicken antiserum raised against Zb06 could therefore be useful in diagnosis of H3 and N6 influenza viruses. On the other hand, Zb06 did not react with chicken antisera raised against Mem96 but reacted with antiserum against Aichi (HI titers <40 and 320, respectively), confirming antigenic drift, which has been observed since this virus was first introduced into the human population [2].

For genetic analysis, viral RNA was extracted and amplified by RT-PCR as described previously [12]. PCR products were purified from agarose gels and then sequenced directly using a BigDye terminator cycle sequencing ready reaction kit and analyzed on a 3130 Genetic Analyzer (Applied Biosystems). The nucleotide sequences obtained in this study will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB470293 to AB470300. Phylogenetic trees were constructed using the neighbor-joining bootstrap method (1,000 replicates) in MEGA4.

The entire genome of Zb06 was completely sequenced and analyzed with the basic local alignment search tool (BLAST) available from GenBank (Table 2). We found that the HA, PB2, and NS genes were highly similar (97–99%) to duck/South Africa/1108/04 (H3N8) (SAH3). The NP and PB1 genes showed 97% nucleotide similarity with H7N1 and H7N3 influenza viruses isolated from Italian poultry. The closest relative of the Zb06 M gene was duck/Mongolia/54/2001 (H5N2) (98% nucleotide similarity). The NA segment was closely related to mallard/Germany/Wv1806-09k/03 (H4N6), with 96% nucleotide identity. The PA gene showed close sequence identity (98%) to H5N3 virus, teal/Italy/3812/05.

Table 2 Influenza viruses with the highest nucleotide sequence similarity to Zb06

Gene (nucleotide positions of Zb06 compared)	Virus with highest degree of sequence identity	Subtype	Identity (%)	GenBank accession no.
HA (77–1,063)	A/duck/South Africa/1108/2004	H3N8	97	EF041487
NS (57–711)	A/duck/South Africa/1108/2004	H3N8	98	EF041491
PB2 (1,468–2,193)	A/duck/South Africa/1108/2004	H3N8	99	EF041493
NP (751–1,483)	A/turkey/Italy/3560/1999	H7N1	97	CY025168
PB1 (1,429–2,178)	A/turkey/Italy/9739/2002	H7N3	97	CY031617
M (197–868)	A/duck/Mongolia/54/2001	H5N2	98	AB301916
NA (38–1,264)	A/mallard/Germany/Wv1806-09k/2003	H4N6	96	AM933235
PA (1,456–2,149)	A/teal/Italy/3812/2005	H5N3	98	CY022650

The HA, NA, NP, and PA gene phylograms are shown in Fig. 1. Phylogenetic analysis of the HA gene of Zb06 showed the separation of the viruses into the Eurasian, American, and human-swine lineages (Fig. 1a). Sublineages 1–3 are distinguishable within the Eurasian lineage. The HA gene of Zb06 was closely related to that of SAH3, and belonged to the first sublineage, comprising viruses isolated mainly from the Far East and Europe. The much older virus, duck/Ukraine/1/63 (H3N8) and a swine isolate from Mongolia constituted the second Eurasian sublineage. The third sublineage is composed of H3N2 viruses isolated from fecal specimens collected from live poultry markets in Korea [21]. The NA gene tree of Zb06 revealed the assortment of viruses into the Eurasian, Eurasian–American, Oceania, and American lineages (Fig. 1b). Two sublineages were apparent within the Eurasian lineage: "contemporary" and 1970s and 1980s viruses (designated 1 and 2, respectively). The NA gene of Zb06 fell in the "contemporary" sublineage and was closely related to H4N6 viruses isolated from Germany and Norway. Aside from Zb06 and the two H4N6 European strains, all viruses of the "contemporary" sublineage were of Asian origin. Except for three viruses, Eng56, Czec56, and duck/Siberia/272/98 (H13N6), the Eurasian–American lineage was composed exclusively of shorebird and gull viruses isolated in America and Eurasia.

Phylograms of the internal protein genes (NP and PA) (Fig. 1c, d) of Zb06 showed the clustering of strains of the Eurasian lineage into sublineages as described previously [9]. In the NP phylogeny, four groups or sublineages are recognized. The first group consists of recent isolates from Europe, Asia and South Africa, including HP H5N1 viruses isolated from ducks and chickens in China, and from whooper swans in Japan. The NP gene of Zb06 belonged to the second group, consisting of "early" European strains represented by Dk/Potsdam/2216-4/84 (H5N6) and some recent isolates, including H7N1 Italian poultry viruses [1]. The third sublineage was composed of a single isolate, Dk/Hokkaido/120/01 (H6N2). The fourth group of the Eurasian lineage comprises 3 strains isolated from 1956 to 1961. The PA phylogram was topologically similar to that of the NP gene tree. In contrast to the NP gene, which clustered with H7N1 Italian poultry viruses, the PA gene of Zb06 was closely related to that of teal/Italy/3812/05 (H5N3) and grouped together with those of the Asian HP H5N1 viruses, suggesting a common source of the PA gene of these viruses.

The close relationship of the HA and internal (NS and PB2) protein genes (Supplementary Fig. S1) of Zb06 to those of wild bird isolates from South Africa suggests that some reassortment may have occurred within sub-Saharan Africa due to the interaction of wild birds through the intra-African flyways. Ring recoveries of water birds in southern Africa have shown that some waterbirds are migratory within southern Africa, while others show dispersal as far as central Africa [23]. Phylogenies of the NP and other internal (NS, PB1, and M) protein genes (Supplementary Fig. S1) of Zb06 showed that they were closely related to H7 influenza viruses isolated from Italian poultry in 1999, suggesting that viruses of the same origin as Zb06 may have contributed some internal protein genes to viruses that caused epidemics of AI of H7 viruses that have been observed in Europe since 1997 [1, 3, 4, 8]. Phylogenetic analysis of AI viruses isolated from wild ducks and domestic poultry in Italy revealed that the precursor H7 virus for AI in domestic poultry was introduced directly from migratory birds [4]. The close similarity of these genes of Zb06 to those of the viruses isolated in Italy leads us to speculate that these viruses may have infected their avian hosts on the Black Sea/Mediterranean flyway, which, together with the East Africa/West Asia flyway passes through Zambia. We acknowledge the need for caution in interpreting our data because only very limited sequence data from African wild birds are available in GenBank.

Until now, there has not been a report of influenza virus isolation from a great white pelican (*Pelecanus onocrotalus*). Influenza virus (H6N1) isolation from a great cormorant, a member of the order Pelecaniformes, has been

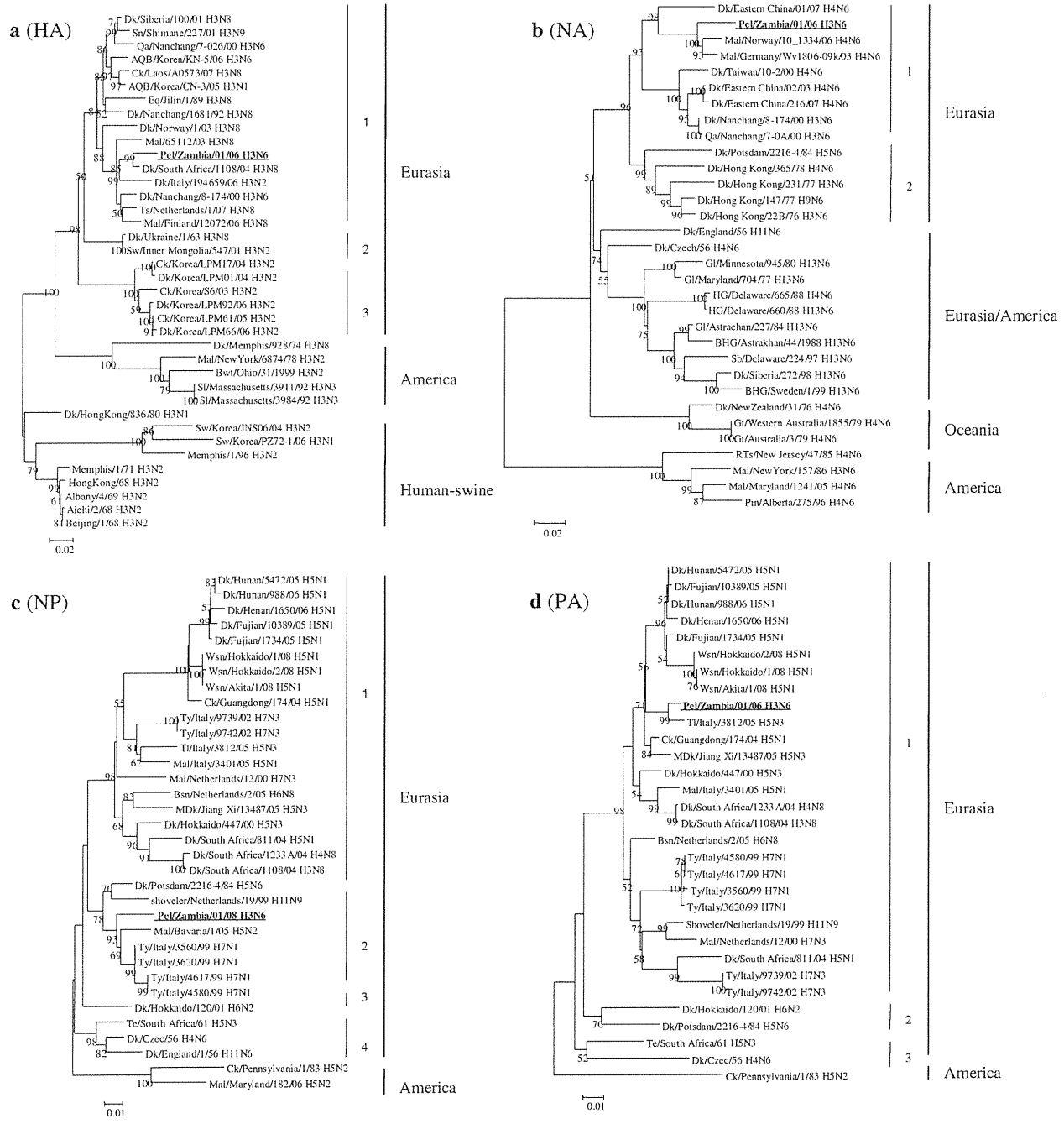

Fig. 1 Phylogenetic relationships of the HA (**a**), NA (**b**), NP (**c**), and PA (**d**) genes of Zb06. *Numbers* next to the branches indicate neighbor-joining bootstrap values of ≥50%. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The virus strain sequenced in this study is in *bold* and *underlined*. Analysis was based on the following nucleotides: HA (77–1,063), NA (38–1,264), NP (751–1,483), and PA (1,456–2,149). *AQB* aquatic bird, *BHG* black-headed gull, *Bsn* Bewick's swan, *Ck* chicken, *Dk* duck, *Eq* equine, *Gl* gull, *Gt* gray teal, *HG* herring gull, *MDk* migratory duck, *Mal* mallard, *Pel* pelican, *Pin* pintail, *Qa* quail, *RTs* ruddy turnstone, *Sb* shorebird, *Sl* seal, *Sn* swan, *Sw* swine, *Te* tern, *Ts* turnstone, *Ty* turkey, *Wsn* whooper swan

reported [22]. Other studies did not yield positive results of influenza virus isolation from this order [17, 19, 20]. The great white pelican is native to southern Africa. Limited breeding sites exist in the region, including two in South Africa and one in Namibia [6, 7]. Large colonies of white pelicans congregate in Lochinvar National Park, sharing the same habitat with other bird species in which AI viruses have been frequently isolated worldwide. The role of

"minor" bird reservoirs in influenza virus ecology is unclear. It remains to be determined in which of these species influenza viruses are endemic and in which ones the virus is a temporary pathogen [19, 20].

The available evidence suggests that the rapid spread of HP H5N1 virus from Qinghai Lake, China, to Europe and Africa may have involved migratory birds and possibly the poultry trade [14]. The close relationship of the PA gene of Zb06 to those of the Asian HP H5N1 viruses implies that wild birds could carry and spread, at least in part, genes of the same origin as those of HP AI viruses over large geographical regions. The overlap of multiple migratory flyways within Eurasia and Africa permits virus-infected birds of different bird populations to transmit pathogens to new hosts that may carry them to new areas [20].

While AI A viruses have evolved into two genetically distinct lineages, Eurasian and American, possibly due to long-term confinement of birds to distinct flyways [20, 24], transcontinental introduction of AI virus genes has been described between the two lineages [16, 18]. For instance, PB2 and PA genes of the American lineage were detected in H2 viruses isolated in Japan, and the H2 HA genes of Eurasian lineage was present in American birds. Our findings highlight that the gene segments of Zb06 appear to have been derived from multiple virus sources in Eurasia and Africa. Furthermore, our results indicate that wild waterfowl could play a role in the dissemination of genes of common origin as those of HP AI viruses over large geographical regions, thus underscoring the need for continued AI virus surveillance in Zambian wetlands as part of a global program.

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Factors responsible for plaque formation of A/duck/Siberia/272/1998 (H13N6) influenza virus on MDCK cells

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ABSTRACT

Many influenza A viruses form plaques on Madin-Darby canine kidney (MDCK) cells in the presence of trypsin. A/duck/Siberia/272/1998 (H13N6) (Sib272), however, does not form plaque on MDCK cells. After three blind passages of the strain on MDCK cells, plaque-forming variant was obtained and designated as A/duck/Siberia/272PF/1998 (H13N6) (Sib272PF). Genetic and functional analyses of Sib272 and Sib272PF revealed that amino acid substitutions, F3L of the HA2 subunit and T379K of the PB1, were responsible for plaque formation of Sib272PF by enhancing fusion and polymerase activities, respectively.

1. Introduction

Influenza A virus belongs to the family *Orthomyxoviridae* and has been isolated from birds and mammals, including humans. On the basis of the antigenic specificity of hemagglutinin (HA) and neuraminidase (NA), influenza A viruses is derived into subtypes H1–H16 and N1–N9, respectively (Fouchier et al., 2005; Hinshaw et al., 1982; Palese and Shaw, 2006; Webster et al., 1992). Influenza A virus contains eight segments of negative-stranded RNA genome, encoding eleven different proteins. Two glycoprotein spikes (HA and NA) and M2 protein are embedded in the lipid bilayer envelope underlain by the matrix protein (M1) (Palese and Shaw, 2006). In virus particles, genomic RNAs are associated with the RNA-dependent RNA polymerase complex consisting of polymerase basic proteins (PB1 and PB2) and acidic protein (PA), nucleoprotein (NP) and nonstructural protein 2 (NS2), together forming the ribonucleoprotein (RNP) complex (Akarsu et al., 2003; Kingsbury and Webster, 1969; Neumann et al., 2000). NS1 protein found in cells infected with influenza A viruses is multi-functional (Hatada and Fukuda, 1992; Lamb and Choppin, 1979). Recently, PB1-F2 which is encoded by a second open reading frame (+1) of PB1 gene was found as the protein that induces apoptosis (Chen et al., 2001).

Plaque assay is one of the most important procedures for the isolation and titration of viruses. Influenza A viruses form plaques on cell lines such as chicken embryo fibroblast cells, African green monkey kidney (Vero) cells and MDCK cells in the presence of trypsin (Babiker and Rott, 1968; Tobita et al., 1975). The role of influenza virus proteins in plaque formation has been studied. A/Udorn/72 (H3N2) formed plaques on MDCK cells, whereas the variant with mutant HA C562S and C565S failed to form plaque (Chen et al., 2005). Wagner et al. (2000) proposed that glycans flanking the receptor-binding pocket of the HA are potent regulators of virus growth in cell culture. Lack of oligosaccharides from Asn123 and Asn149 reduced virus growth and plaque size on MDCK cells. The M1 protein of A/WSN/33 was responsible for the determination of virus growth and plaque size in MDCK cells (Yasuda et al., 1994). The plaque size of a variant virus bearing mutant NS1 protein that loses binding activity to dsRNA was smaller than that of the original strain A/Udorn/72 (Min and Krug, 2006).

A/duck/Siberia/272/1998 (H13N6) (Sib272) does not form plaques on MDCK cells. After the blind passages of this strain in MDCK cells, a plaque-forming variant, Sib272PF was obtained. In the present study, the molecular basis of the plaque formation of Sib272PF was studied.

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2. Materials and methods

2.1. Cells and viruses

MDCK cells were maintained in Minimum Essential Medium (MEM) supplemented with 0.3 mg/ml L-glutamine, 10% calf serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 8 μg/ml gentamicin. Human embryonic kidney (293T) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.3 mg/ml L-glutamine, 10% fetal calf serum and antibiotics. A/duck/Siberia/272/1998 (H13N6) (Sib272), which does not form plaque on MDCK cells, was isolated from the fecal sample of a feral duck in Siberia (Okazaki et al., 2000). After three blind passages of the strain in MDCK cells, the plaque-forming variant was obtained and designated as A/duck/Siberia/272PF/1998 (H13N6) (Sib272PF). Sib272 and Sib272PF were propagated in 10-day-old embryonated chicken eggs at 35 °C for 48 h.

2.2. Plaque assay

Ten-fold dilutions of viruses were inoculated onto confluent monolayers of MDCK cells and incubated at 35 °C for 1 h. Unbound viruses were removed, and the cells were washed with MEM. Cells were then overlaid with MEM containing 0.7% bact-agar (Gibco) and 5 μg/ml acetyl trypsin (Sigma). After 48 h of incubation at 35 °C, cells were stained with 0.005% neutral red. Blind passaging of the virus was as follows: cells showing cytopathic effects were picked up by a sterile capillary pipette under a microscope and suspended in MEM. The cells were diluted 10-fold and inoculated into MDCK cells. After three successive passages, visible plaques were found. To evaluate plaque morphology, the cells were stained with 0.1% crystal violet solution with 10% formaldehyde 72 h, post-inoculation.

2.3. Sequence analysis

Viral RNAs were isolated from virus-containing allantoic fluid using Trizol-LS (Sigma) and ethanol. Universal primer sets for influenza A virus were used for RT-PCR (Hoffmann et al., 2001). PCR products were reacted with gene-specific primers and the CEQ Dye Terminator Cycle Sequencing Quick Start Kit (Beckman Coulter). Sequences of the DNA template were determined using CEQ 2000XL DNA analyzers (Beckman Coulter).

2.4. Preparation of mutant viruses derived from Sib272PF by reverse genetics system

Each of the PCR products of the eight gene segments of Sib272PF was cloned in TA vector. Mutations of Sib272, which are L3F of the HA2 and Q184H and K379T of the PB1, were introduced into cDNAs from Sib272PF by site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Eight segments of Sib272PF and mutated constructs were cloned into a dual-promoter plasmid, pHW2000 (Hoffmann et al., 2000b). MDCK and 293T cells were co-cultured and transfected with 1 μg of each of the eight plasmids and 16 μl of TransIT-293 (Mirus Bio) in 150 μl of OPTI-MEM (Gibco). After 30 h of incubation, 1 ml of OPTI-MEM containing 5 μg/ml acetyl trypsin was added to each well. After 48 h of incubation at 35 °C, the supernatant was collected, and 100 μl was injected into the allantoic cavity of 10-day-old embryonated chicken eggs. Using reverse genetics, rg-272, rg-272PF and other mutated variants were generated (Table 2). The infectivity of rg-viruses were determined as 50% egg infectious dose (EID_{50}) and 50% tissue culture infectious dose ($TCID_{50}$).

2.5. Growth curve of viruses

Mutant viruses generated by reverse genetics, rg-272, rg-HAF3L, rg-HAF3L/PB1T379K and rg-272PF, were inoculated onto the confluent monolayer of MDCK cells at a multiplicity of infection (MOI) of 0.01. After 1-h incubation at 35 °C, unbound viruses were washed away and MEM with 5 μg/ml acetyl trypsin was added. Cells were incubated at 35 °C, and the supernatant collected at 6, 9, 12, 24, 36 and 48 h after infection. EID_{50} of the supernatants were periodically monitored.

2.6. Hemolysis assay

Hemolysis assay was performed as described previously (Kida et al., 1983). Briefly, rg-272 and rg-272PF were centrifuged at 25,000 rpm for 1.5 h and the pellets were resuspended in phosphate-buffered saline, pH 7.2. Virus concentrates containing 200 HA units (50 μl) were added to 2.0 ml of 1% chicken erythrocytes in saline buffered with 0.1 M citric acid–sodium citrate, at 0 °C for 60 min, and then incubated at 37 °C for 60 min with mixing every 15 min. The cells were sedimented by centrifugation and the supernatants were measured for hemoglobin at 540 nm.

2.7. Polymerase assay

The luciferase assay was performed as described (Salomon et al., 2006). Subconfluent monolayers of 293T cells were transfected with 1 μg of luciferase reporter plasmid (EGFP open reading frame in pHW72-EGFP substituted with the luciferase gene (Hoffmann et al., 2000a)), renilla luciferase plasmid (Promega) and a mixture of PB2, PB1, PA, and NP plasmids in quantities of 1, 1, 1, and 2 μg, respectively. Forty-eight hours post-transfection, cell extracts were prepared in 250 μl of lysis buffer and luciferase levels were assayed with the Dual-Glo™ Luciferase Assay System (Promega) and read on LUMAT LB9507 (Berthold Technologies). Luciferase activities of the reporter gene were standardized by renilla luciferase activity. Polymerase activity was expressed as folds of luciferase activity.

3. Results

3.1. Comparison of Sib272 and plaque-forming variant, Sib272PF

In 1998, non-pathogenic influenza A virus strain Sib272 was isolated from the fecal sample of a feral duck in Siberia. This strain does not form plaques on MDCK cells (Fig. 1). After three blind passages of Sib272 in MDCK cells, the plaque-forming variant, Sib272PF was obtained. Each of the eight gene segments of the original virus and plaque-forming variant was analyzed and amino acid sequences were compared. An amino acid substitution of F to L at position 3 in the HA2 subunit and two amino acid substitutions of H to Q at position 184 and T to K at position 379 in the PB1 molecule were found (Table 1). Amino acid sequences of the other proteins including PB1-F2 of plaque-forming variant were the same as those of the original virus. It was confirmed that rg-272PF generated by reverse genetics formed plaque on MDCK cells but rg-272 did not (Table 2).

Table 1
Amino acid substitutions between Sib272 and Sib272PF.

Protein	Position	Amino acid	
		Sib272	Sib272PF
HA2	3	F	L
PB1	184	H	Q
	379	T	K

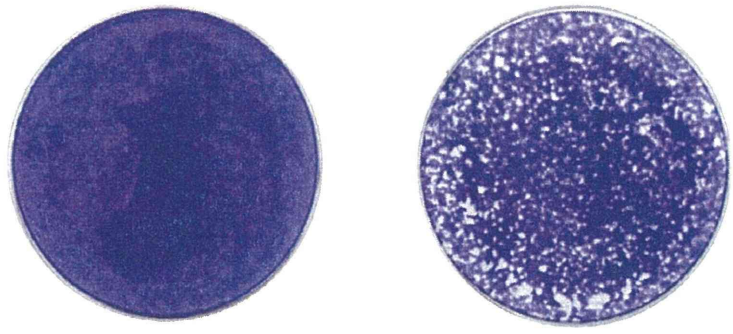

Fig. 1. Plaque formation of Sib272 and Sib272PF. Sib272 and Sib272PF were inoculated onto MDCK cells at 4.7 $\log_{10}$ EID_{50}/well, and incubated at 35 °C for 3 days on 0.7% bact-agar in MEM. Cells were stained with crystal violet after removal of overlay agar.

3.2. Amino acid residues responsible for plaque formation

To determine the amino acid residues responsible for plaque formation, recombinant vituses with HA and/or PB1 mutant proteins were generated. Of these recombinants, only rg-HAF3L/PB1T379K formed plaques (Table 2). Infectivity titers on MDCK cells of the viruses with HA2 3L (5.0–6.6 $\log_{10}$ $TCID_{50}$/100 μl) were 100–1000 times higher than those of the viruses with HA2 3F (2.8–3.8 $\log_{10}$ $TCID_{50}$/100 μl), while infectivity titers in eggs were similar. The present results indicate that substitution of F3L of the HA2 subunit is a critical factor for virus growth on MDCK cells, and T379K of the PB1 is also responsible for plaque formation.

3.3. Growth of viruses in MDCK cells

To assess the effect of amino acid substitutions of HA2 F3L and PB1 T379K, growth kinetics of rg-272, rg-HAF3L, rg-HAF3L/PB1T379K or rg-272PF were analyzed. As shown in Fig. 2, rg-272 showed poor growth in MDCK cells. The plaque-forming variant, rg-272PF, grew to titers 100–10000 times higher than rg-272 and showed rapid growth. Rg-HAF3L/PB1T379K, which also forms plaques, reached a high titer. Single mutant virus rg-HAF3L showed slower growth than plaque-forming variant.

3.4. Comparison of fusion activity of rg-272 and rg-272PF

Amino acid residue at position 3 of the HA2 subunit is on the fusion peptide which leads to the fusion process for virus genome entry. Fusion activity of virus was analyzed by hemolysis assay. The hemolysis activity of rg-272 and rg-272PF was examined between pH 5.0 and 7.2. Maximal hemolysis with rg-272 was observed at pH 5.5 (Fig. 3). Hemolysis activity of rg-272PF showed extensive

Table 2
Plaque formation of viruses generated by reverse genetics.

Virus	Amino acid			Plaque formation	EID_{50}[a]	$TCID_{50}$[b]
	HA2	PB1				
	3	184	379			
rg-272	F	H	T	–	6.7	3.0
rg-272PF	L	Q	K	+	6.7	6.6
rg-PB1H184Q	F	Q	T	–	6.0	2.8
rg-PB1T379K	F	H	K	–	7.5	3.8
rg-HAF3L	L	H	T	–	6.3	5.0
rg-PB1H184Q/T379K	F	Q	K	–	7.3	3.3
rg-HAF3L/PB1H184Q	L	Q	T	–	6.5	5.0
rg-HAF3L/PB1T379K	L	H	K	+	6.3	5.3

[a] $\log_{10}$ EID_{50}/100 μl.
[b] $\log_{10}$ $TCID_{50}$/100 μl.

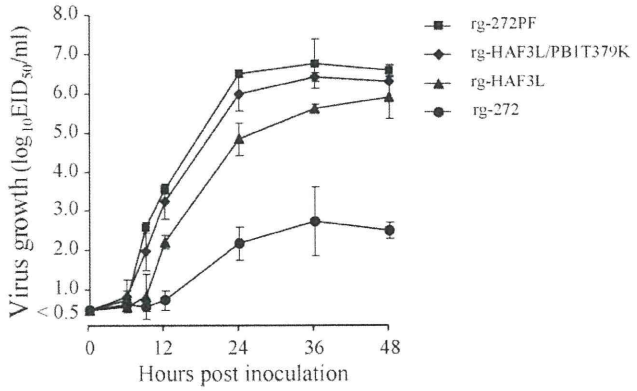

Fig. 2. Growth kinetics of viruses in MDCK cells. MDCK cells were inoculated with rg-272 (●), rg-HAF3L (▲), rg-HAF3L/PB1T379K (♦) and rg-272PF (■) at an MOI of 0.01. Infected cells were incubated at 35 °C and supernatants were collected at the indicated times. Virus titer of supernatants was expressed as EID_{50}. The results are the mean ± S.D. of three independent experiments.

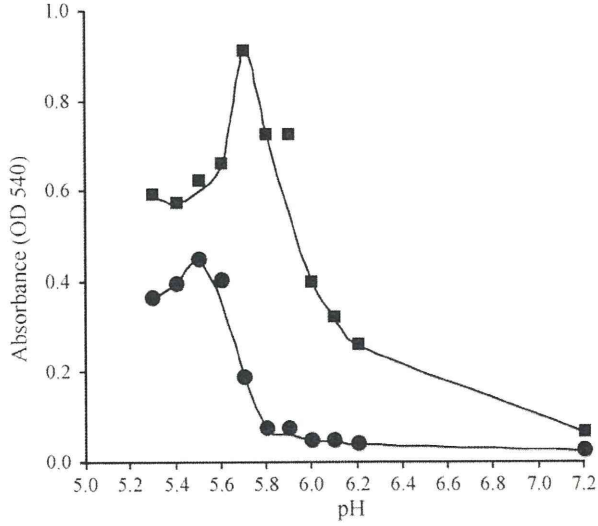

Fig. 3. Hemolysis assay of rg-272 and rg-272PF. Purified viruses rg-272 (●) and rg-272PF (■) were added to 2.0 ml of 1% chicken erythrocytes in saline buffer with 0.1 M citric acid–sodium citrate at various pHs, placed at 0 °C for 60 min, and then incubated at 37 °C for 60 min, mixing at every 15 min. The mixtures were then centrifuged and supernatants were measured at a wavelength of 540 nm.

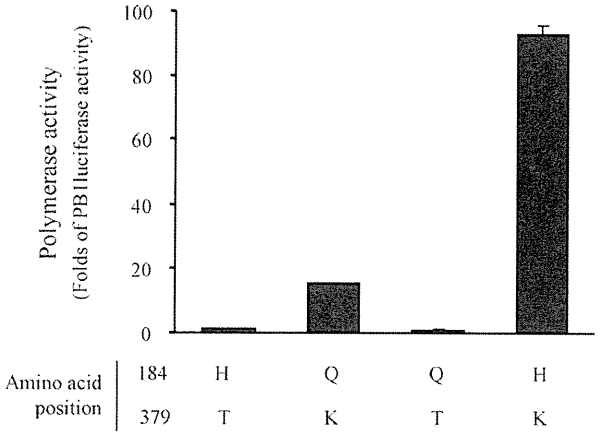

Fig. 4. Effects of PB1 mutations H184Q and T379K on polymerase activity. Polymerase activity was assayed by viral UTR-driven luciferase reporter genes. 293T cells were transfected with plasmids encoding PB1 or mutant PB1 gene with H184Q and/or T379K and PB2, PA and NP genes. After 48 h of incubation at 35 °C, cell extracts were tested for luciferase activity. Luciferase activity was standardized by renilla luciferase activity, and polymerase activity was expressed as folds of luciferase activity of PB1. The results are the mean + S.D. of triplicate samples in two different experiments.

enhancement than that of rg-272 and optimal pH for hemolysis was shifted to pH 5.7 from pH 5.5.

3.5. Polymerase activity of viruses with PB1 mutants

To compare the polymerase activity of variant viruses bearing mutant PB1 with that of the original virus, 293T cells were transfected with plasmids containing the PB1 or mutant PB1 gene with H184Q and/or T379K, and PB2, PA and NP genes. Polymerase complex with PB1 379K showed extensively high polymerase activity (Fig. 4).

4. Discussion

The plaque-forming variant, A/duck/Siberia/272PF/1998 (H13N6), was obtained by three successive passages from A/duck/Siberia/272/1998 (H13N6), which does not form plaque on MDCK cells. Genetic and functional analyses of Sib272 and Sib272PF revealed that amino acid substitutions of HA2 F3L and PB1 T379K were responsible for plaque formation of Sib272PF.

The major functions of HA are receptor binding and subsequent fusion of the virus envelope with the cellular endosomal membrane (Palese and Shaw, 2006). HA is synthesized as a single polypeptide (HA0) and cleaved into HA1 and HA2 subunits by trypsin-like endoprotease as an event in post-translational modification (Skehel and Wiley, 2000). The N-terminal region of HA2 polypeptide is highly conserved and is a critical component of the fusion process of virus entry (Cross et al., 2001). The substitution of glutamine for the glycine residue at the N-terminal of HA2 abolished fusion activity (Gething et al., 1986). Some mutants selected during passages in MDCK cells elevated the pH of membrane fusion (Lin et al., 1997; Rott et al., 1984). Amino acid substitution of G1V, W14A or F9A/I10A of HA2 of X31 (H3N2) influenza virus lost the stabilization of the boomerang structure of the fusion peptide (Lai et al., 2006; Lai and Tamm, 2007). In the present study, it was found that the amino acid substitution of HA2 F3L significantly enhanced fusion activity and induced effective entry of the virus genome into the cytoplasm of MDCK cells.

The RNA polymerase complex of the influenza virus consists of PA, PB1 and PB2 proteins, and PB1 is a key component of RNA polymerization. PB1 contains the polymerase motif in the amino acid region 298–484 and nuclear localization signal of the PB1 is identified between residues 180–195 and 202–252 (Kerry et al., 2008; Ohtsu et al., 2002; Poch et al., 1989). An amino acid substitution of PB1 T379K, which is located within the polymerase motif, increased polymerase activity. Polymerase proteins play important roles in virus replication, hence pathogenicity and host range (Gabriel et al., 2007; Hatta et al., 2001; Pappas et al., 2008; Salomon et al., 2006). On the other hand, increased polymerase activity does not necessarily correlate with the pathogenicity of viruses in vivo (Hulse-Post et al., 2007). Sib272 and even Sib272PF did not experimentally infect chickens (data not shown). The present results indicate that amino acid substitutions in Sib272PF affect the enhancement of replication in MDCK cells but not the infectiousness to chickens. Additionally, it is considered that the amino acid substitutions found in the HA and PB1 of Sib272PF may not constitute adaptation to mammalian hosts, since these amino acid residues are not typical in mammalian nor avian influenza viruses in data base.

It was concluded that two amino acid substitutions of HA2 F3L and PB1 T379K enhanced fusion and polymerase the activities of virus, respectively, resulting in plaque formation of Sib272PF on MDCK cells.

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Cross-Protective Potential of a Novel Monoclonal Antibody Directed against Antigenic Site B of the Hemagglutinin of Influenza A Viruses

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Abstract

The hemagglutinin (HA) of influenza A viruses has been classified into sixteen distinct subtypes (H1–H16) to date. The HA subtypes of influenza A viruses are principally defined as serotypes determined by neutralization or hemagglutination inhibition tests using polyclonal antisera to the respective HA subtypes, which have little cross-reactivity to the other HA subtypes. Thus, it is generally believed that the neutralizing antibodies are not broadly cross-reactive among HA subtypes. In this study, we generated a novel monoclonal antibody (MAb) specific to HA, designated MAb S139/1, which showed heterosubtypic cross-reactive neutralization and hemagglutination inhibition of influenza A viruses. This MAb was found to have broad reactivity to many other viruses (H1, H2, H3, H5, H9, and H13 subtypes) in enzyme-linked immunosorbent assays. We further found that MAb S139/1 showed neutralization and hemagglutination-inhibition activities against particular strains of H1, H2, H3, and H13 subtypes of influenza A viruses. Mutant viruses that escaped neutralization by MAb S139/1 were selected from the A/Aichi/2/68 (H3N2), A/Adachi/2/57 (H2N2), and A/WSN/33 (H1N1) strains, and sequence analysis of the HA genes of these escape mutants revealed amino acid substitutions at positions 156, 158, and 193 (H3 numbering). A molecular modeling study showed that these amino acids were located on the globular head of the HA and formed a novel conformational epitope adjacent to the receptor-binding domain of HA. Furthermore, passive immunization of mice with MAb S139/1 provided heterosubtypic protection. These results demonstrate that MAb S139/1 binds to a common antigenic site shared among a variety of HA subtypes and neutralizes viral infectivity in vitro and in vivo by affecting viral attachment to cells. The present study supports the notion that cross-reactive antibodies play some roles in heterosubtypic immunity against influenza A virus infection, and underscores the potential therapeutic utility of cross-reactive antibodies against influenza.

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Introduction

Neutralizing antibodies play a critical role in protection from influenza virus infection. Most neutralizing antibodies recognize hemagglutinin (HA), which is the major surface glycoprotein of influenza viruses. The HA of influenza A viruses has been classified into sixteen antigenically distinct subtypes (H1–H16) that are maintained in avian and mammalian species in nature [1,2].

HA is responsible for virus entry into target cells, virus binding to the host receptor, internalization of the virus, and subsequent membrane-fusion events. It is initially synthesized as a precursor polypeptide, HA0, that requires proteolytic cleavage into disulfide-linked HA1 and HA2 before it is functional and virus particles are infectious. The major part of HA1 forms the "globular head" region, which contains the necessary structure for binding to the sialic acid receptors. The "stem" region is mostly formed by HA2, which contains the fusion peptide and membrane anchor domain. It has been recognized that there is considerable amino acid variability (antigenic difference) in the globular head region among HA subtypes, whereas the structure of the stem region is relatively conserved.

The HA antigenic structure of the H3 subtype has been well characterized by using the sequence information on naturally occurring and laboratory-selected antigenic variants [3,4,5,6]. Five different antigenic sites have been identified and mapped mainly on the HA1 globular head region in the three-dimensional structure of the H3 HA molecule [3,4]. Antigenic sites of H1 [7] and H2 [8] subtypes were then characterized by the identification of amino acid substitutions found in the HA sequences of variants that escaped from neutralization by antibodies. Recently, it was suggested that the structures of antigenic sites of H5 [9,10] and H9 [11] subtypes were different from those of the H1, H2, and H3 subtypes.

Author Summary

Neutralizing antibodies play a critical role in protection from influenza A virus infection. Most neutralizing antibodies recognize hemagglutinin (HA), which is the major surface glycoprotein of influenza viruses. The HA has been classified into sixteen antigenically distinct subtypes. Since HA subtypes of influenza A viruses are principally defined as serotypes determined by neutralization or hemagglutination inhibition tests using polyclonal antisera to the respective HA subtypes, which have little cross-reactivity to the other HA subtypes, it is generally believed that the neutralizing antibodies are not broadly cross-reactive among HA subtypes. Herein we present a novel cross-neutralizing monoclonal antibody that reacts with a variety of HA subtypes in vitro and provides heterosubtypic protection against influenza A virus infections in mice. We demonstrate that this antibody recognizes a common epitope adjacent to the receptor binding region of HA and inhibits virus binding to the cells. The present study supports the notion that cross-reactive antibodies, as well as cytotoxic T lymphocytes, play some roles in heterosubtypic immunity against influenza A virus infection, and underscores the potential therapeutic utility of cross-reactive monoclonal antibodies for multivalent prophylaxis and treatment against infection with influenza A viruses, including the hypothetical new pandemic influenza viruses.

In general, HA subtypes of influenza A viruses are principally defined as serotypes determined by neutralization or hemagglutination inhibition (HI) tests using polyclonal antisera to the respective HA subtypes, which have little cross-reactivity to the other HA subtypes. Furthermore, since the structures of HA antigenic sites vary among not only different subtypes of viruses but also the same subtype, it is generally believed that the neutralizing antibodies are not broadly cross-reactive among HA subtypes. Therefore, studies on cross-reactive HA-specific antibodies to multiple HA subtypes have been limited [12,13,14].

Recently, it has been shown that intranasal immunization with inactivated viruses provided heterosubtypic protection in a mouse model, suggesting a role for cross-reactive antibodies in the heterosubtypic immunity against influenza viruses [15,16,17]. In this study, we generated a novel cross-neutralizing monoclonal antibody (MAb) that reacts with a variety of HA subtypes by intranasal immunization of mice. This antibody recognizes a common epitope on the globular head region of HA and inhibits virus binding to the sialic acid receptors. The present study suggests the further potential of antibodies in heterosubtypic immunity against influenza A virus infection.

Results

Characterization of MAb S139/1 in vitro

MAb S139/1 (IgG2a) was originally produced as an H3 HA-specific antibody. The cross-reactivity of MAb S139/1 to multiple subtypes of influenza A virus HAs was then tested by enzyme-linked immunosorbent assay (ELISA) using several H1, H2, H3, H5, H9, and H13 subtypes (Fig. 1). We found that MAb S139/1 reacted with all influenza A virus but not B virus strains tested, with higher binding activities to the A/WSN RG/33 (WSN) (H1), A/Adachi/2/57 (Adachi) (H2), A/Aichi/2/68 (Aichi) (H3), and A/gull/Maryland/704/77 (Maryland) (H13) strains than to the other strains. We confirmed the specificity of MAb S139/1 by Western blotting using purified viruses. MAb S139/1 bound to

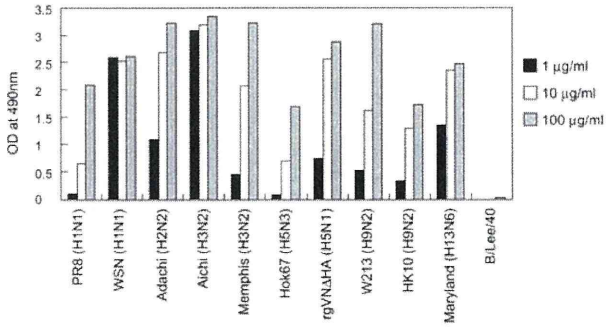

Figure 1. Reactivity of MAb S139/1 to various influenza virus strains. Binding activity of MAb S139/1 at the concentrations of 100 μg/ml (grey), 10 μg/ml (white), and 1 μg/ml (black) to the indicated virus strains was measured by ELISA as described in Materials and Methods.
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HA molecules under non-reducing conditions, whereas it bound very weakly to HA1 but not HA2 under reducing conditions (data not shown), suggesting that MAb S139/1 recognized a conformational epitope on the HA1 subunit of the HA molecule.

We next tested HI activity of MAb S139/1 to various influenza virus strains (Table 1). MAb S139/1 exhibited high HI titers to a particular H1 strain and most of the H3 strains tested, and moderate activity to H2 and H13 strains, but not to H5 and H9 or type B strains. Neutralizing activity of MAb S139/1 was then determined in vitro by a plaque reduction assay using Madin-Darby canine kidney (MDCK) cells (Fig. 2). Consistent with its reactivity profile in HI tests and ELISA, MAb S139/1 neutralized infectivity of WSN (H1), Adachi (H2), Aichi (H3), and Maryland (H13) strains. Relative neutralizing activities of MAb139/1 were also correlated with its reactivity to these viruses in the HI test and ELISA (i.e., MAb S139/1 neutralized Aichi (H3), WSN (H1), Adachi (H2), and Maryland (H13) in order of increasing activity). These results indicated that MAb S139/1 had a novel potential to neutralize the infectivity of multiple subtypes of influenza A viruses by inhibiting HA binding to the sialic acid receptors.

Protective Potential of MAb S139/1 in vivo

We then investigated the potential of MAb S139/1 to protect mice against influenza virus infection. Mice were passively immunized by intraperitoneal injection of purified MAb S139/1 one day before or after intranasal challenge with Aichi (H3) or WSN (H1). Control groups were given an irrelevant MAb (ZGP12/1.1) [18] or PBS alone. Protective efficacy was evaluated by titrating infectious virus in the lung tissues three days after challenge (Fig. 3). Mice pre-immunized with MAb S139/1 were almost completely protected from both Aichi (H3) and WSN (H1) infection, while these viruses were recovered from all the control mice at high titers (Fig. 3A). There was no significant difference between two control groups. Post-immunization with MAb S139/1 also significantly reduced both Aichi and WSN titers, as compared with control group ($p<0.05$ and $p<0.01$, respectively) (Fig. 3B). Importantly, virus was not detected from two of the five MAb S139/1-immunized mice infected with Aichi. These results indicate that passive immunization of mice with MAb S139/1 provided heterosubtypic protection against H1 and H3 viruses.

Amino Acid Substitutions of the Escape Mutants

To determine the epitope for MAb S139/1, escape mutants of Aichi (H3), WSN (H1), and Adachi (H2) were selected in the

Table 1. HI activity of MAb S139/1 with various influenza virus strains.

Virus	HI titer μg/ml [a]
A/PR/8/34 (H1N1)	>50 [b]
A/WSN RG/33 (H1N1)	1.56
A/Adachi/2/57 (H2N2)	12.5
A/Singapore/1/57 (H2N2)	6.25
A/duck/Hong Kong/836/80 (H3N1)	<0.39
A/Aichi/2/68 (H3N2)	0.78
A/Memphis/1/96 (H3N2)	>50
A/duck/Hokkaido/5/77 (H3N2)	12.5
A/chicken/Hong Kong/37/78 (H3N2)	<0.39
A/duck/Hokkaido/8/80 (H3N8)	<0.39
A/Hong Kong/483/97 (H5N1)	>50
A/rgViet Nam/1194ΔHA/2004 (H5N1)	>50
A/swan/Hokkaido/67/96 (H5N3)	>50
A/swine/Hong Kong/10/98 (H9N2)	>50
A/duck/Hong Kong/W213/97 (H9N2)	>50
A/duck/Hokkaido/49/98 (H9N2)	>50
A/gull/Maryland/704/77 (H13N6)	12.5
B/Lee/40	>50

[a]HI titers are expressed as the lowest concentrations of purified MAb S139/1 that completely inhibited hemagglutination.
[b]No detectable hemagglutination inhibition at 50 μg/ml by MAb S139/1.
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presence of this antibody as described in Materials and Methods. We confirmed that hemagglutination activities of these escape mutants were not inhibited by MAb S139/1 even at the concentration of 50 μg/ml. The nucleotide sequences of the HA genes of the parent strains and the escape mutants were determined and deduced amino acid sequences were compared among these viruses. We found amino acid substitutions at position 156, 158, or 193 (H3 numbering here and throughout the text) in these mutants (Table 2). Eleven and four escape mutants of WSN (H1) and Adachi (H2), respectively, all acquired the substitution at the same position, 193 (S193N and T193K, respectively). The amino acid residue at position 193 is located on the antigenic sites known as Sb and I-B of H1 [7] and H2 HAs [8], respectively, which correspond to HA antigenic site B of H3 HA [3]. On the other hand, the amino acid substitutions at position 156 (K156Q), 158 (G158E or G158R), or 193 (S193I or S193R) were found in the sixteen escape mutants of Aichi (H3). All these amino acid positions were involved in the conformation of HA antigenic site B [3].

Comparison of HA Amino Acid Sequences among Different HA Subtypes

We compared deduced amino acid sequences of the region including the MAb S139/1 epitope among H1, H2, H3, H5, H9, and H13 subtypes of HA (Fig. 4). Among these, WSN (H1), Adachi (H2), Aichi (H3), and Maryland (H13), which were neutralized by MAb S139/1, shared the amino acid sequence at positions 156, 158, and 193 (K, G and S/T, respectively), with one exception (N at position 158 in Maryland HA). By contrast, viruses that were not neutralized by MAb S139/1 possessed different sets of amino acids at positions 156, 158, and 193; A/PR/8/34 (PR8) (H1) (E, E and N), A/Memphis/1/96 (Memphis) (H3) (K, D, and T), A/Viet Nam/1194/2004 (VN1194) (H5) (K, N, and K), A/swan/Hokkaido/67/96 (Hok67) (H5) (K, N, and K) and, A/duck/Hong Kong/W213/97 (W213) (H9) (Q, N, and N), respectively.

Comparison of the Epitope Structure between Aichi and Other Viruses

In three-dimensional structural analysis of the Aichi (H3) HA molecule, positions of the amino acid substitutions found in the escape mutants were mapped in the globular head of the HA (Fig. 5A). We found that these three amino acids formed a conformational epitope that was adjacent to the receptor-binding site of the HA. We then compared the structure of this epitope among Aichi (H3), WSN (H1), Adachi (H2), PR8 (H1), and VN1194 (H5) HAs (Fig. 5). The epitopes formed by amino acid residues at positions 156, 158, and 193 were similar among Aichi (H3), WSN (H1), and Adachi (H2), whereas the structures of PR8

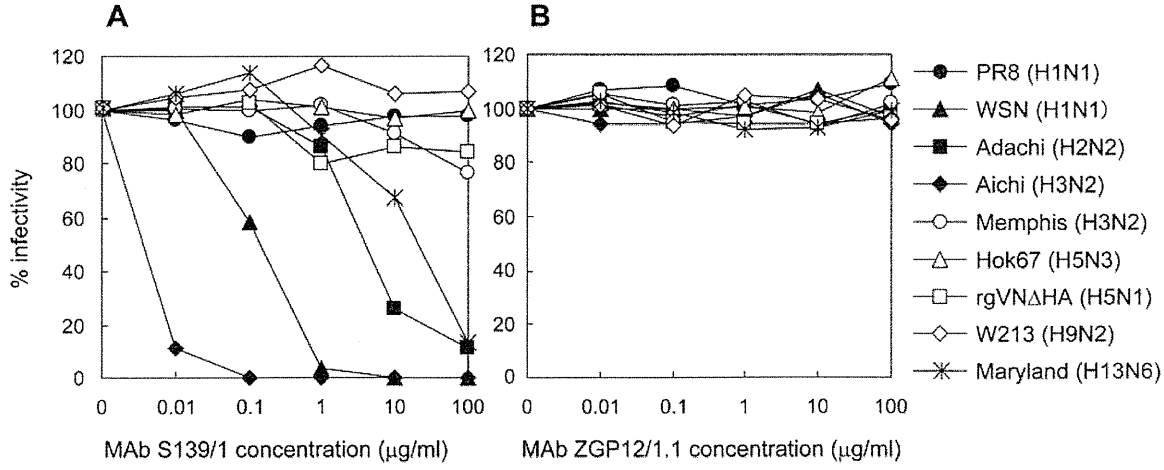

Figure 2. Neutralization activity of MAb S139/1 to various HA subtypes of influenza A virus strains. Viruses were mixed with indicated concentrations of the purified MAb S139/1 (A) or control IgG2a (ZGP12/1.1) [18] (B). Neutralization activities were evaluated by plaque reduction assays using MDCK cells.
doi:10.1371/journal.ppat.1000350.g002

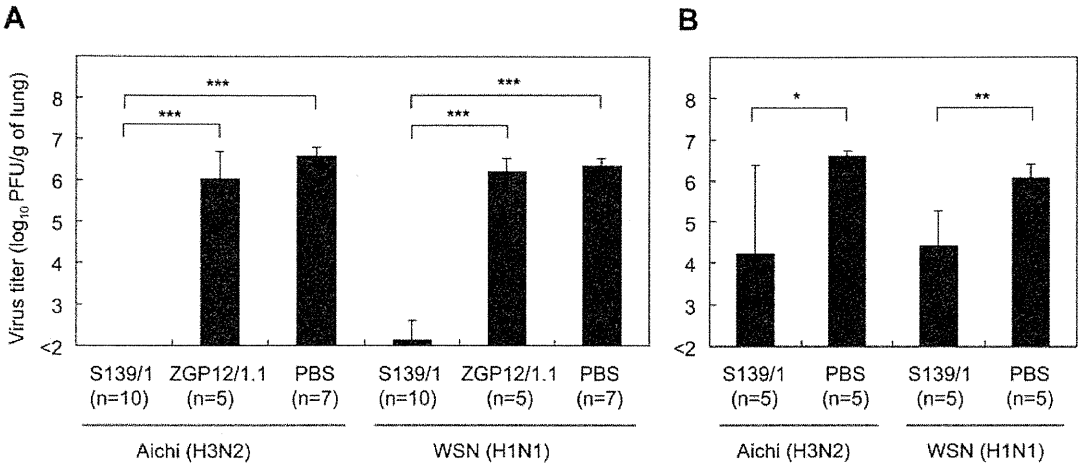

Figure 3. Protective efficacy of passive immunization with MAb S139/1 in mice. Mice were passively immunized before (A) or after (B) virus challenge with Aichi (H3) or WSN (H1). Control mice were given MAb ZGP12/1.1 or PBS. Virus titers of the lung were determined by a plaque assay. The means and standard deviations are shown. For statistical purposes, samples with undetectable virus titers were given the value 2.0 $\log_{10}$PFU/g. The data of pre-immunized mice were analyzed using the nonparametric Kruskal-Wallis ANOVA on ranks, followed by the Mann-Whitney U-test with the Bonferroni correction for multiple comparisons. The data of post-immunized mice were analyzed using the Mann-Whitney U-test. Two-sided p values less than 0.05 were considered statistically significant. Significant differences were indicated by asterisks (*** $p<0.001$, ** $p<0.01$, * $p<0.05$). All statistical analyses were performed with the computer program R (version 2.8.1).
doi:10.1371/journal.ppat.1000350.g003

(H1) and VN1194 (H5) epitopes were distinct in the amino acid properties or side-chain orientations at positions 156, 158, and 193. A significant difference of amino acid properties at positions 158 and 193 between PR8 (H1) and Aichi (H3) was that the molecular sizes of amino acid residues E and N (PR8) were larger than G and S (Aichi). In VN1194 (H5) HA, a significant difference was found in side chain orientation, which was presumably because of electrostatic repulsion between the positively charged K156 and K193 side chains.

Neutralization of PR8 Mutants Possessing the Modified Epitope for MAb S139/1

To confirm the importance of the amino acid residues at positions 156, 158, and 193 for binding of MAb S139/1, we generated recombinant PR8 (H1) mutant viruses with modified epitopes whose amino acid sequences at these positions were replaced with those of Aichi (H3), and tested neutralizing activities of MAb S139/1 to the mutant viruses (Fig. 6). Of the seven mutants generated, three had a single substitution (E156K, E158G, or N193S), three had double substitutions (E156K/E158G, E156K/N193S, or E158G/N193S), and one had triple substitutions (E156K/E158G/N193S). We found that only the triple mutant was neutralized by MAb S139/1 (Fig. 6). Accordingly, MAb S139/1 bound to the triple mutant more efficiently than to the other mutants and parent PR8 in ELISA (data not shown). These results suggest that all three amino acids, K, G, and S at positions 156, 158, and 193, respectively, are equally important components to form this epitope (i.e., MAb S139/1 recognizes this conformational epitope through interaction with all three of these amino acid residues).

Table 2. Amino acid substitutions found in HA of WSN, Adachi, and Aichi escape mutants.

Virus	Amino acid substitution
WSN (H1)	S193N (11/11) [a]
Adachi (H2)	T193K (4/4)
Aichi (H3)	K156Q (1/16)
	G158E (10/16)
	G158R (1/16)
	S193I (2/16)
	S193R (2/16)

[a]Total numbers of escape mutants obtained in 2 independent experiments are shown (No. of variants/total escape mutants cloned.)
doi:10.1371/journal.ppat.1000350.t002

Discussion

It has been generally known that hetrosubtypic immunity can be provided by subtype cross-reactive cytotoxic T lymphocytes that recognize conserved epitopes of viral internal proteins of influenza A viruses such as nucleoprotein and matrix protein [19]. However, recent studies in mouse models suggest that humoral immunity, B cells and antibodies, also contribute to heterosubtypic protection [15,16,17,20]. In the present study, we obtained a novel monoclonal antibody, MAb S139/1, which was broadly cross-reactive to a variety of HA subtypes of influenza A viruses. MAb S139/1 most likely neutralized the viral infectivity by blocking receptor binding of the virus, since hemagglutination of the viruses was also inhibited by this antibody. Influenza virus HA subtypes are determined as serotypes based on their distinct antigenicities, and thus there are a limited number of studies reporting such heterosubtypic and cross-reactive MAb to HA [12,13,14,21,22].

For example, MAb IVA1B10 [12,21] and MAb HA1-66 [13,22], both of which recognize the HA1 region, were shown to react with H3, H4, H11, and H13 strains, but did not have HI and neutralization activities. Some MAbs recognizing the HA2 region were also shown to be cross-reactive among influenza virus strains of the same subtypes, and even among various subtypes [12,21,22,23]. However, these antibodies neither prevented hemagglutination nor neutralized infectivity of the viruses

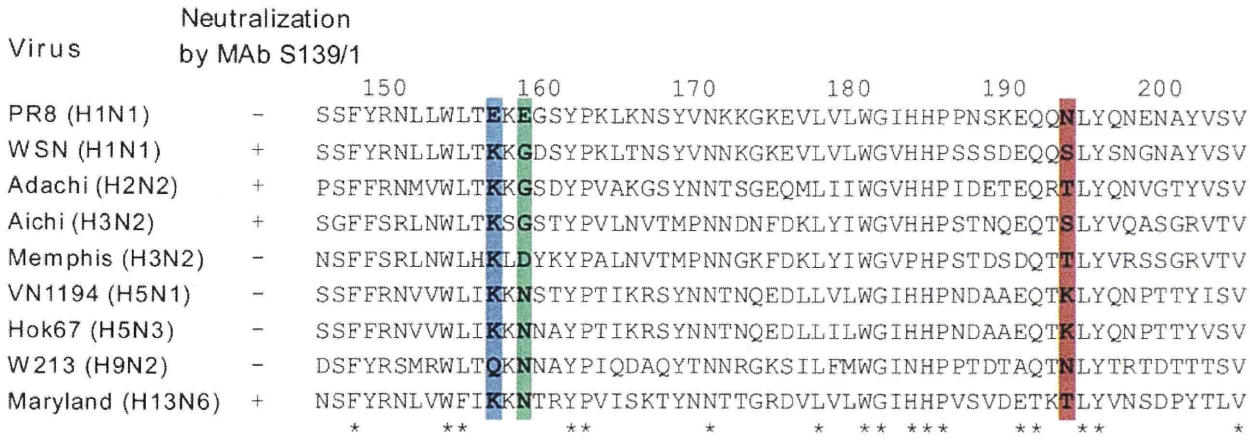

Figure 4. Comparison of the amino acid sequences of different subtypes of influenza A virus HAs. Amino acids at positions from 145 to 204 are shown. Boxed residues indicate the positions 156 (blue), 158 (green), and 193 (red). Asterisks indicate conserved amino acid residues among HA subtypes examined. Amino acid sequences for HAs, except Adachi (H2) and W213 (H9), were downloaded from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) [48]. The NCBI accession numbers are CAA24272 (PR8), AAA43209 (WSN), AB432938 (Adachi), CAA24269 (Aichi), AAB63708 (Memphis), AAT7327 (VN1194), BAE48688 (Hok67), AB432937 (W213), and BAF46906 (Maryland).
doi:10.1371/journal.ppat.1000350.g004

[12,21,22,23]. On the other hand, it was shown that cross-reactive MAb C179, specific to both H1 and H2 subtypes, neutralized viral infectivity [14], though this MAb did not show HI activity. It recognizes a conformational epitope consisting of HA1 and HA2 in the middle of the stem region, a conserved antigenic site among the subtypes, suggesting that inhibition of the low pH-induced conformational change followed by membrane fusion is the mechanism underlying the action of this antibody [14,24]. By contrast, this study for the first time demonstrates that there is a common epitope shared among multiple HA subtypes, which is recognized by a neutralizing antibody that prevents receptor binding of the virus.

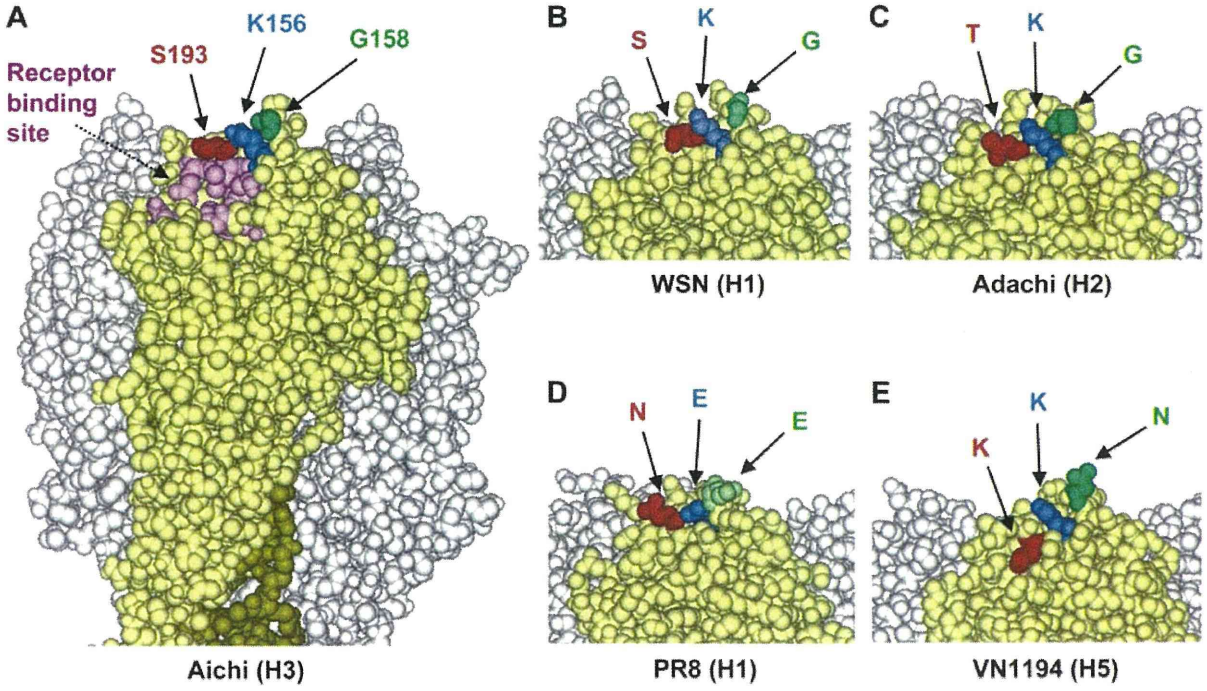

Figure 5. Structure of the MAb S139/1 epitope on the globular head of HA trimer models. Three-dimensional models of Aichi (H3) (A), PR8 (H1) (D), and VN1194 (H5) (E) HAs were constructed from the coordinates obtained from the Protein Data Bank (PDB codes: 1HGF, 1RVX, and 2IBX, respectively). The structures of WSN (H1) (B) and Adachi (H2) (C) were constructed by homology modeling as described in Materials and Methods. Images were prepared by using DS Visualizer (version 1.7, Accelrys, Inc.). Residue numbering is thoroughly on the basis of the H3 HA sequence.
doi:10.1371/journal.ppat.1000350.g005

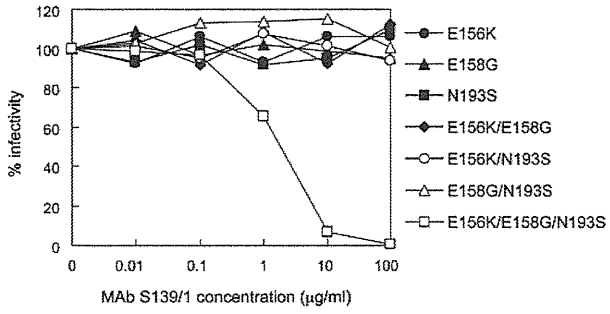

Figure 6. Neutralizing activity of MAb S139/1 to PR8 mutants with altered epitopes. Amino acids at positions 156, 158, and/or 193 of PR8 HA were substituted for by those at corresponding positions of the Aichi HA sequence. Other experimental conditions were described in Materials and Methods.
doi:10.1371/journal.ppat.1000350.g006

In the present study, we found that MAb S139/1 bound to all the strains of H1, H2, H3, H5, H9, and H13 subtypes tested by ELISA, whereas it showed neutralization and HI activities to some particular strains tested. Since we assume that MAb S139/1 recognizes a single epitope on the HA molecules of all the subtypes, it is likely that the different binding affinity of MAb S139/1 to each HA subtype influences the neutralization and HI activities. Indeed, our data demonstrated that there was an appreciable correlation between its binding affinities tested by ELISA and by HI or the neutralization test. Furthermore, our sequence analyses and reverse genetics approaches revealed the major contribution of the HA amino acid residues at positions 156, 158, and 193 to the binding capacity of MAb139/1.

We found three independent substitutions at positions 156, 158, or 193 in the Aichi mutant viruses that escaped from neutralization by MAb S139/1. Three-dimensional structural analyses revealed a conformational epitope consisting of these amino acid residues. Of these, the amino acid residue at position 193 of H3 HA was shown to interact with the host receptor molecule (i.e., sialic acid-linked oligosaccharide) [5,25,26,27,28], suggesting the contribution of this residue to receptor binding of the HA. Accordingly, Aichi escape mutants with a substitution at position 193 (S193I and S193R) formed significantly smaller plaques than the Aichi parent virus (data not shown), which might have resulted from the reduced HA function of these mutants.

Three-dimensional structural analyses based on HA molecules cocrystallized with a sialylated glycan receptor analogue (pentasaccharide) suggest that molecular contacts between HA and the sialylated glycan receptor are divided into base and extension regions which include contacts with the terminal sialylgalactose moiety and the subsequent sugar rings, respectively [29,30], and that the sialylated glycan molecules bind to H1 and H3 HAs in different conformations (i.e., the H1 HA glycan binding site in the extension region form different conformation from that of H3 HA) [27,29,30,31,32,33]. Since the amino acid residue at position 193 of H3 HA seems to directly interact with a specific sugar ring in the extension region [27,31,33], mutation at this position of the H3 HA likely influence its receptor binding properties. Consistent with this hypothesis, an Aichi (H3) escape mutant (S193I) had reduced ability to grow in cell culture and in mice (data not shown). On the other hand, it has been suggested that the amino acid residues not only at position 193 but also at position 190 of H1 HA play a key role in interaction with the glycan in the extension region [27,29,31,32]. Since the amino acid substitution at position 190 of H1 HA is believed to be responsible for the alteration of receptor binding properties [29,31], a single mutation at position 193 found in WSN (H1) escape mutants might have a limited effect on the overall receptor binding capacity of the HA.

It is well-known that HAs of avian and human influenza viruses bind preferentially to α2-3 and α2-6 sialylated (SA) glycan receptors, respectively. Although it has been generally believed that amino acid substitutions at positions 226 and 228 are primarily responsible for the differences in the receptor specificity between avian and human H3 viruses, several studies have reported that the mutation at position 193 of H3 HA might also alter the receptor binding properties [5,31,33,34]. For example, S193R substitution on prototype H3 human virus HA altered binding specificity by acquiring the ability to agglutinate erythrocytes containing SAα2-3Gal linkage [5], while a single S193R substitution of H3 HA in a recent human virus enhanced SAα2-6Gal but not SAα2-3Gal recognition [34]. Thus, it is conceivable that H3 escape mutants that are naturally selected by an antibody recognizing the S139 epitope may have reduced receptor binding capacity and/or altered receptor specificity. Moreover, amino acid positions 155, 159, 190, and 225 (H3 numbering) of H1 HA, most of which cluster around this epitope, have also been demonstrated to influence receptor specificity [31]. Thus, it might be of interest to clarify whether the reactivity of MAb S139/1 to multiple HA suptypes is affected by changes of HA receptor recognition associated with substitutions around this epitope.

Recently, passive transfer of MAbs specific to viral proteins has been tested in clinical studies, providing models for the use of MAbs for prophylaxis or treatment of infectious diseases. In fact, a humanized MAb specific to RSV F protein is already approved by the US Food and Drug Administration and used in clinical cases. It has been experimentally shown that this approach is effective for influenza virus infection in mice [24,35,36,37]. The present study further indicated that the MAb S139/1 provided heterosubtypic protection of mice from H1 and H3 influenza A virus infection. It may be one of the options in the event of pandemic influenza [36]. Although MAb S139/1 neutralizes only particular strains of H1, H2, H3, and H13 subtypes, this antibody has binding capacity to other virus strains of different subtypes (Fig. 1). Since in vitro neutralization activity was not necessarily linked to the protective potential in vivo (e.g., non-neutralizing MAbs such as anti-HA2 MAbs [22,37] and anti-M2 MAbs [38,39,40] protected mice from lethal influenza A virus infection), it may be interesting to estimate the broader heterosubtypic protective efficacy of passive transfer of MAb S139/1 in animal models.

Together with previous studies by others [22,24,36,37, 38,39,40], the present study supports the notion that cross-reactive antibodies, as well as cytotoxic T lymphocytes, play some roles in heterosubtypic immunity against influenza A virus infection, and underscores the potential therapeutic utility of cross-reactive MAbs for multivalent prophylaxis and treatment against infection with influenza A viruses, including the hypothetical new pandemic influenza viruses.

Materials and Methods

Viruses and Cells

Influenza virus strains, A/PR/8/34 (PR8) (H1N1), A/WSN RG/33 (WSN) (H1N1), A/Adachi/2/57 (Adachi) (H2N2), A/Singapore/1/57 (H2N2), A/duck/Hong Kong/836/80 (H3N1), A/Aichi/2/68 (Aichi) (H3N2), A/Memphis/1/96 (Memphis) (H3N2), A/duck/Hokkaido/5/77 (H3N2), A/chicken/Hong Kong/37/78 (H3N2), A/duck/Hokkaido/8/80 (H3N8), A/Hong Kong/483/97 (H5N1), A/rgViet Nam/1194ΔHA/2004 (rgVNΔHA)