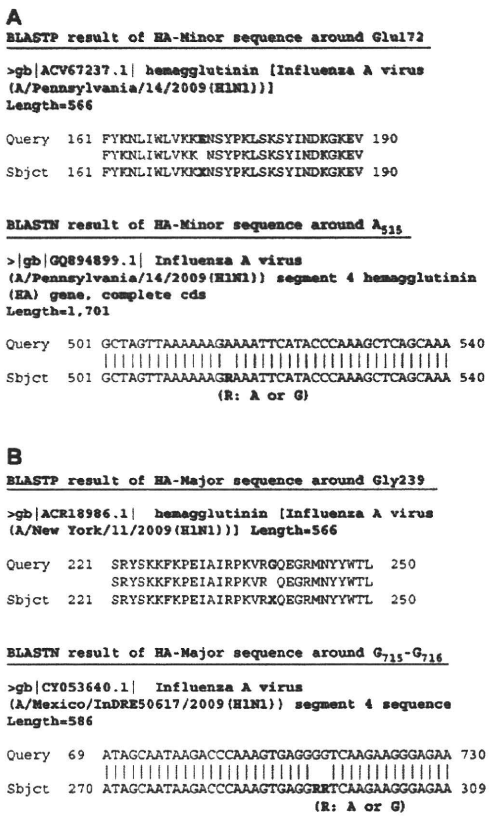

Figure 5. BLAST homology search of the HA sequences against the nr or nt databases. (**A**) Glu172 of HA-Minor. (**B**) Gly239 of HA-Major. R: A or G.
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investigation facilitates the identification of influenza A viral heterogeneity during infection. The possibility of mixed infections with variants remains to be elucidated in this case, but worldwide sequencing efforts suggest that quasispecies of the A/H1N1/2009 virus evidently appear and are observed. To better characterize the currently emerging A/H1N1/2009 virus and prevent worse pandemics in the near future, unbiased *de novo* sequencing techniques will be indispensable for the primary investigations of emerging infectious diseases.

Materials and Methods

Ethics Statement

The study protocol was approved by the institutional medical ethical committee, National Institute of Infectious Diseases, Japan (Approval No.236), and the study was conducted according to the Declaration of Helsinki Principles. In the autopsy case, a written consent for autopsy was obtained from relatives.

Total RNA and cDNA preparation from autopsy human lung

Information for the patient was previously reported [15]. Briefly, in August 2009, a 33-year-old male patient with chronic heart failure due to dilated cardiomyopathy, mild diabetes mellitus, atopic dermatitis, asthma, and obesity (BMI: 38) died from respiratory failure and multiple organ dysfunction syndrome. A diagnosis of pandemic influenza A virus (A/H1N1/2009) infection was determined using RT-PCR testing in a clinical laboratory. Total RNA was prepared from a 5-mm cube of the autopsy lung tissue using ISOGEN (NipponGene, Japan), followed by Ambion TurboDNase treatment (Ambion, Austin, TX USA). Double-stranded cDNA was prepared from 5 μg of total RNA using the random priming method with SuperScript Choice System for cDNA synthesis (Invitrogen, Carlsbad, CA, USA). cDNA was purified using a QIAquick PCR Purification kit (QIAGEN, Hilden, Germany).

Short-read DNA sequencing using the Illumina Genome Analyzer II

An approximately 300-bp length cDNA library was prepared using a genomic DNA sample prep kit (Illumina, San Diego, CA, USA), and DNA clusters were generated on a slide using a Cluster Generation kit (ver. 2) on an Illumina cluster station (Illumina), according to the manufacturer's instructions. To obtain $\sim 1.0 \times 10^7$ clusters for one lane, the general procedure as described in the standard recipe (Illumina) was performed as follows: template hybridization, isothermal amplification, linearization, blocking, denaturation, and hybridization of the sequencing primer (Illumina). All sequencing runs for 40 mers were performed with GA II using the Illumina Sequencing kit (ver. 3). Fluorescent images were analyzed using the Illumina base-calling pipeline 1.4.0 to obtain FASTQ formatted sequence data.

Homology search analysis

The obtained DNA sequences were investigated using a BLAST search as shown in Fig. 1A. The results of the BLASTN search were analyzed and visualized using MEGAN v3.7.4 [25] with the following parameters: minimum support, 5; minimum score, 35.0.

de novo assembly of short reads

Prior to *de novo* assembly, all obtained 40-mer reads were trimmed based on the *phred* quality value obtained using the Euler-SR *qualitytrimmer* command [26]. Such trimmed read sequences were assembled using Velvet v0.7.55 [27] or Euler-SR v1.0 [26] with the default parameters (Velvet: hash length, 25; Euler-SR: vertex size, 25).

Read mapping

To obtain consensus sequences for the respective 8 segments of influenza A virus, 40-mer short reads were aligned to A/Tronto/T0106/2009(H1N1) sequences (gb|CY045951.1 – .8) as reference sequences with Maq software (ver. 0.7.1) [28] using the *easyrun* Perl-command. The consensus sequences were extracted as a "cns.fq" file for each segment, and deposited in the DNA Data Bank of Japan (DDBJ; accession numbers: AB538386 to AB538393 for the 8 segments of A/Nagano/RC1-L/2009(H1N1), and AB538394 for segment 4 encoding the HA-Minor sequence). Read coverage at every nucleotide was obtained using Maq software (ver. 0.7.1) with the *pileup* command. Read alignment for the validation of SNPs was performed using the MapView graphical alignment viewer [29].

qRT-PCR analysis

qRT-PCR was performed using 100 ng of total RNA, HA variant-specific primers (forward common primer: pdmFlu09-HA-F, 5′–CGAACAAAGGTGTAACGGCAGCAT–3′; HA-Major-specific reverse primer: pdmFlu-HA-R_Major, 5′–ATAGTT-CATTCTCCCTTCTTGACC–3′; HA-Minor-specific reverse primer: 5′–ATAGTTCATTCTCCCTTCTTGATT–3′), and

the SuperScript III Platinum SYBR Green One-Step qRT-PCR kit with ROX (Invitrogen), and analyzed using the ABI PRISM 7900HT Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following qRT-PCR program was used: RT reaction, 50°C for 3 min; initial denaturation, 95°C for 5 min; 2 steps of amplification (×40 cycles), 95°C for 15 sec and 60°C for 30 sec. The human β-actin gene was used as the internal control. PCR products were resolved by 5% polyacrylamide gel electrophoresis, followed by Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Virus isolation

The A/H1N1/2009 virus was isolated from MDCK cells passaged once with trypsin.

Supporting Information

Text S1 Fastq file of the 40-mer short reads with similarity to influenza A virus extracted from whole obtained short reads.
Found at: doi:10.1371/journal.pone.0010256.s001 (11.19 MB PDF)

Text S2 De novo assembly of the influenza A virus using Euler-SR v1.0 [26] with the default parameters (vertex size, 25).
Found at: doi:10.1371/journal.pone.0010256.s002 (0.04 MB RTF)

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Author Contributions

Conceived and designed the experiments: MK TS. Performed the experiments: MK HK TS YS. Analyzed the data: MK HK TS HH. Contributed reagents/materials/analysis tools: HK NN MT AA MT YA SH MW TS. Wrote the paper: MK.

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Characterization of H5N1 highly pathogenic avian influenza virus strains isolated from migratory waterfowl in Mongolia on the way back from the southern Asia to their northern territory

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ABSTRACT

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

Introduction

H5N1 highly pathogenic avian influenza (HPAI) virus infections have spread in poultry in more than 60 countries in Eurasia and Africa since 1996, when the first outbreak occurred at a goose farm in Guangdong province in China (Smith et al., 2006; Xu et al., 1999). H5N1 virus infections have become endemic at poultry farms in some countries and cause accidental transmissions to humans, so H5N1 viruses are recognized as the most likely candidate for the next pandemic (Li et al., 2004; Peiris et al., 2007). The widespread presence of H5N1 HPAI viruses in poultry, especially in domestic ducks reared in free range, has inevitably resulted in the transmission of viruses to wild bird populations. Domestic ducks and geese infected with HPAI virus shed progeny viruses in feces at the ponds in the farms, where migratory waterfowl visit. Thus, water-borne transmission easily occurs from domestic waterfowl to migratory waterfowl. In the past, such infections had been restricted to wild birds found dead in the vicinity of infected poultry farms, but there are concerns that infections of wild birds in which HPAI virus has caused mild or no clinical signs (e.g., ducks) could result in spread of the virus over large areas and long distances (Kim et al., 2009). Infections with HPAI viruses in many wild bird species at 2 waterfowl parks in Hong Kong were recorded in 2002 (Ellis et al., 2004) and further, more significant outbreaks in wild waterfowl were found at Lake Qinghai in Western China (Chen et al., 2005). H5N1 HPAI virus infections in poultry and wild birds now spread in Asia, Europe, and Africa, and it has been suggested that the H5N1 virus could spread by migratory waterfowl to the west and south, since genetically closely related H5N1 viruses (clade 2.2) were isolated in several countries from 2005 to 2006 (Monne et al., 2008; Salzberg et al., 2007; Starick et al., 2008). From intensive surveillance in China, 2 antigenically distinct virus groups, clade 2.3.2 and clade 2.3.4, were characterized as the dominant isolates in wild birds (Kou et al., 2009; Smith et al., 2009).

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

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Table 1
Identification of H5N1 isolates in Mongolia.

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

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

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

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

Results

Isolation and identification of H5N1 HPAI viruses from dead waterfowl

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

Phylogenetic analysis of H5N1 isolates

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

Pathogenicity of H5N1 influenza viruses in pigs

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

Pathogenicity of H5N1 influenza viruses in ducks

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

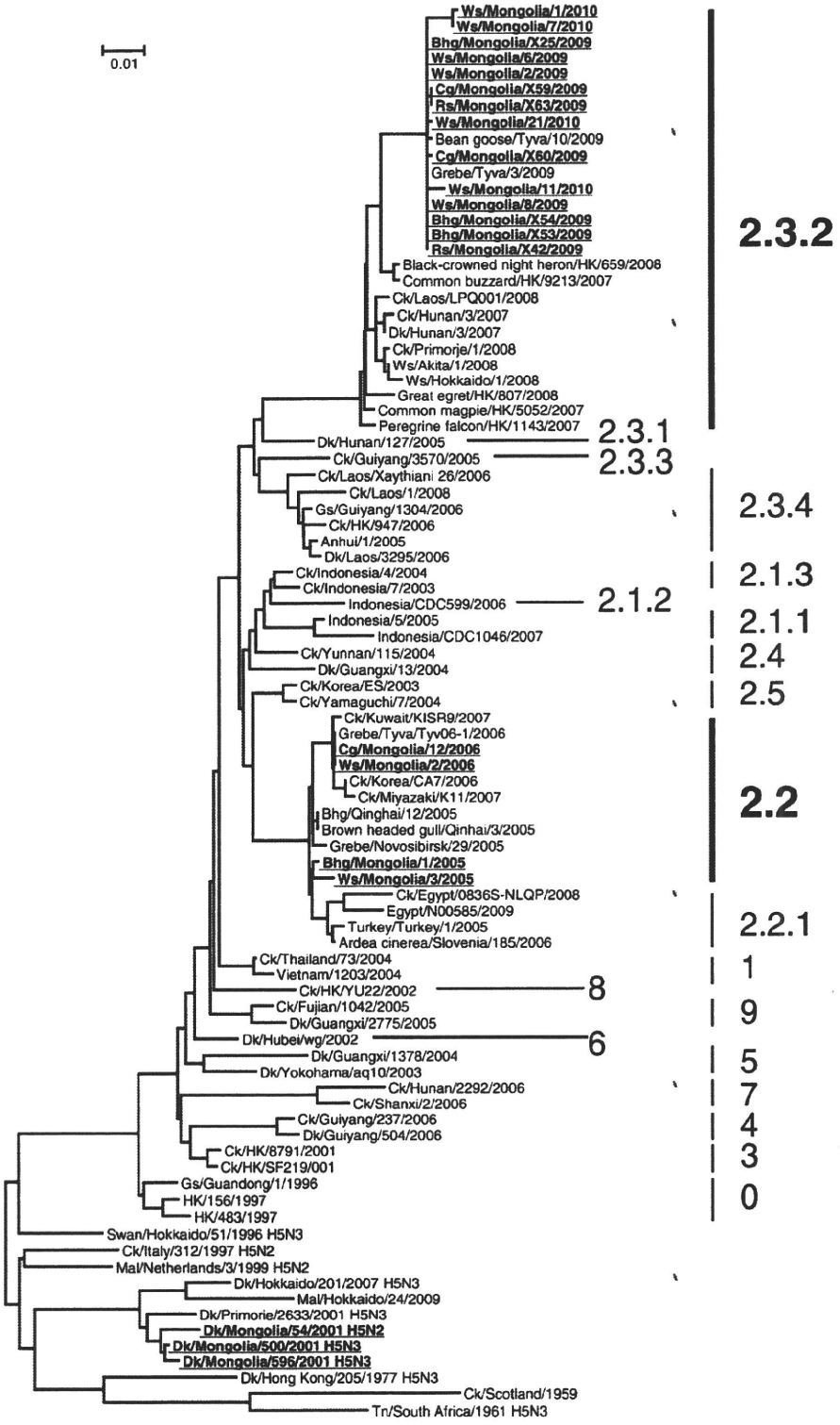

Fig. 1. Phylogenetic trees of the HA genes of H5 influenza viruses. Nucleotide sequences (976 bp) of the HA genes of H5 avian influenza viruses isolated in Mongolia (shown in bold and underlined) and the sequence information of other related viruses were cited from the public database for phylogenetic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

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

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

[a] –: <0.5 log EID50/ml.

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

Materials and methods

Isolation and identification of viruses

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

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

Sequencing and phylogenetic analysis

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

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

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

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

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

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

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

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AIK-C measles vaccine expressing fusion protein of respiratory syncytial virus induces protective antibodies in cotton rats

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ABSTRACT

Respiratory syncytial virus (RSV) is the most common cause of respiratory infection in infants, and no vaccine is available. In this report, recombinant AIK-C measles vaccines, expressing the RSV G or F protein of subgroup A (MVAIK/RSV/G or F), were investigated as a RSV vaccine candidate. MVAIK/RSV/G or F had the original *ts* phenotype and expressed RSV/G or F protein. Cross-reactive neutralizing antibodies against RSV subgroups A and B were detected in cotton rats immunized intramuscularly with MVAIK/RSV/F but not MVAIK/RSV/G. In cotton rats infected with RSV, RSV was recovered and lung histopathological finding was compatible with interstitial pneumonia, demonstrating thickening of alveolar walls and infiltration of mononuclear cells. When cotton rats immunized with MVAIK/RSV/F were challenged with homologous RSV subgroup A, no infectious RSV was recovered and very mild inflammation was noted without RSV antigen expression. When they were challenged with subgroup B, protective efficacy decreased. When cotton rats immunized with MVAIK/RSV/G were challenged with RSV subgroup A, low levels of infectious virus were recovered from lung. When challenged with subgroup B, no protective effects was demonstrated, demonstrating large amounts of RSV antigen in bronchial-epithelial cells. MVAIK/RSV/F is promising candidate and protective effects should be confirmed in monkey model.

1. Introduction

Human respiratory syncytial virus (RSV) is a member of the family *Paramyxoviridae* in the order *Mononegavirales*. The *Paramyxoviridae* consist of two subfamilies, *Paramyxovirinae* and *Pneumovirinae* [1]. Classified into the genus *Pneumovirus*, RSV is characterized by a non-segmented, negative sense, single-stranded RNA genome, and has approximately 15,200 nucleotides. All members of the paramyxovirus family are similar in structure and characteristics [2]. Viral particles of RSV are surrounded by a lipid bilayer with two viral glycoproteins, G and F [1], involved in the attachment to, fusion with, and entry into cells during infection. G protein is not always required for infection and cell fusion and the expression of F protein alone leads to cell fusion [3]. RSV was first isolated in 1956 and two antigenically different subgroups, A and B, co-circulate [4]. RSV is the most common cause of lower respiratory infections in infants and young children worldwide, and is responsible for a variety of illnesses, including 20–25% of pneumonia cases and 45–50% of bronchiolitis cases among hospitalized children [5]. The peak of serious RSV infections is at 2–6 months of age and most children experience an RSV infection by two years of age [6]. The infection causes serious illnesses especially in babies born prematurely and having chronic lung diseases, or congenital heart diseases. RSV also causes lower respiratory tract infections in the elderly, and in immunocompromised hosts [7]. The global annual morbidity and mortality for RSV are estimated to be 64 million and 160,000 deaths, respectively [8].

A recent study of the immune response to RSV showed the importance of innate immunity in regulating adaptive immune responses [9]. Adaptive immunity is generally considered effective due to neutralizing antibodies (NT) and cellular immune responses for the clearance of viruses are influenced by innate inflammatory responses. Secretory and NT antibodies were generated after repeated infections with RSV, although the responses were weak in young infants [10]. The presence of IgG antibodies in the lung has been shown to reduce viral load [11]. Even a natural infection did not provide long-term protective immunity against reinfection in young infants, and a humanized monoclonal antibody against the F protein is available as a prophylaxis against RSV, or for reducing serious diseases in high-risk infants during epidemics [12]. However, the high medical costs for monthly administration mean that there is a great need to develop an RSV vaccine [13]. There are several obstacles to developing a RSV vaccine. An aluminium-precipitated formalin-inactivated RSV vaccine (FI-RSV) was developed in the 1960's, but did not prevent infections [14]. In fact, symptoms were exacerbated among recipients subsequently

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infected with RSV. FI-RSV generated only binding antibodies without neutralizing activity because of the denatured F protein, and did not induce cytotoxic T cell lymphocytes (CTL) activity [15]. Several strategies have been adopted to develop subunit vaccines, live attenuated vaccines through conventional methods of cloning or selecting *ts* mutants, genetically modified-strain by reverse genetics, and vaccinia virus vector-based recombinant vaccines [16–18].

Recently, a method for direct manipulation of the genomic RNA of *Mononegavirales* has been established, known as the infectious cDNA clone system [19]. The transcription and replication of minigenome RNA are driven by viral proteins, which are co-expressed by plasmids or helper viruses. Using this system, the infectious recombinant viruses can be retrieved from the authentic full-size genome cDNA [20,21]. These "reverse genetics" techniques are powerful tools not only for basic research into viral properties, such as the characteristics of viral proteins, and mechanisms of replication, transcription and pathogenesis, but also for practical purposes, such as the development of new vaccines and viral vectors. As vector-based recombinant vaccines, human parainfluenza virus type III (HPIV III) vector-based, or Sendai virus vector-based vaccines have been evaluated [22,23].

Current measles vaccines used throughout the world were attenuated from the Edmonston strain, classified as genotype A [24]. The AIK-C strain of the measles vaccine was developed in 1976 in Japan from the Edmonston strain, by plaque cloning through passages in sheep kidney cells and chicken embryonic cells at 33 °C [25]. It shows optimal growth at 33 °C and little or no growth at 39 °C [21]. The safety and immunogenicity of the AIK-C measles vaccine were established through clinical trials [26–29]. Reverse genetics of the AIK-C live attenuated vaccine was performed and in this study, recombinant AIK-C MV vaccine strains encoding the RSV G or F protein were constructed, and immunogenicity and protective effects against RSV were investigated in cotton rats immunized with recombinant measles vaccines, expressing RSV G or F protein.

2. Materials and methods

2.1. Viral strains and cell cultures

The AIK-C seed strain for vaccine production was used. Wild-type strains of RSV subgroups A and B were isolated in HEp-2 cells from patients. Long and wild-type strains were used for the neutralization test (NT) against RSV subgroups A and B. 293T and HEp-2 cells were maintained in Eagle's MEM (Sigma–Aldrich, Dorset, UK) supplemented with 10% fetal bovine serum (FBS). Vero cells were maintained in Eagle's MEM supplemented with 5% FBS. B95a cells are marmoset B cell line, and maintained in RPMI-1640 medium (Sigma–Aldrich, Dorset, UK) supplemented with 10% FBS [30]. These media were supplemented with 4 mM L-glutamine, 10,000 IU/ml penicillin, and 10,000 μg/ml streptomycin.

2.2. Cloning of the RSV G and F genes

Genomic RNA was extracted from a clinical isolate of subgroup A and B, and the RSV genome was amplified by RT-PCR. The viral RNA was first converted to cDNA using a cDNA primer: 5′-ACACGATTTGCAATCAAACC-3′. The RSV G gene was amplified with 5′-GTTTCCATGGCCAAAACCAAGGACCAA-3′ and 5′-CCAAGCGGCCGCTAGTTTGTTGTGTTGGATGGAGA-3′, which amplified 894 bp. The RSV F gene was amplified with 5′-GTTGCCATGGAGTTGCCAATCCTCAA-3′ and 5′-TGTGGCGGCCGCTAACTAAATGCAATATTATTTT-3′, which amplified 1722 bp. The F and G genes were cloned into pMV/20-77 using two restriction enzymes, Nco I and Not I (underlined sequences).

2.3. Construction of recombinant AIK-C

A schematic diagram of the strategy used for the construction of the recombinant cDNA plasmid is shown in Fig. 1. The full length plasmid was divided from two parts as previously reported. The first half contained the N, P, M and F genes from the leader sequence to the Pac I site at nucleotide position 7238 of the AIK-C genome. The second half contained the H and L regions from the Pac I site from position 7238 of the AIK-C genome to the trailer sequence. The full-length cDNA, pMVAIK, was constructed using these two plasmids [31].

The cloning vector for the RSV genome, pMVAIK/20-77, was constructed from positions 2040 (Sac II) to 7761 (EcoT22 I). The RSV G or F PCR product was digested with Nco I and Not I and ligated into pMVAIK/20-77, resulting in pMVAIK/20-77/RSV/G and pMVAIK/20-77/RSV/F, respectively. The pMVAIK/20-77/RSV/G or pMVAIK/20-77/RSV/F was digested with Sac II and Pac I and ligated into pMVAIK. Then, full-length infectious cDNA clones, pMVAIK/RSV/G and pMVAIK/RSV/F, were constructed.

2.4. Rescue of the infectious recombinant virus from cloned cDNA

Monolayers of 293T cells in 6-well plates were infected with the vaccinia virus MVAT7 pol, expressing T7 RNA polymerase. MVAT7 pol was derived from a highly attenuated and host range-restricted vaccinia virus, the Ankara strain [32]. Open reading frames of the N, P, and L genes were cloned downstream of the T7 promoter of pBluescript SK, and the expression plasmids pCIAN01, pCIAP01, and pCIAL01 were constructed [19,21]. After 1 h of adsorption, the cells were washed with Opti-MEM (GIBCO, Grand Island, NY, US) and transfected with 0.5 μg of pCIAN01, 0.25 μg of pCIAP01, 0.1 μg of pCIAL01, and 1.5 μg of pMVAIK/RSV with TransIT-LT1 Reagent (Mirus Bio Corporation, US). After incubation at 33 °C for 3 h, the medium containing the transfection reagent/plasmid complex was replaced with fresh MEM supplied with 5% FBS. The transfected cells were incubated at 33 °C in 5% CO_2 for 3 days. After 3 days, 293T cells were detached and co-cultured with B95a cells. When a demonstrable cytopathic effect (CPE) was observed, the supernatant and cell lysate were harvested and stocked.

2.5. Virus growth

To examine viral growth, B95a cells were infected with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F (m.o.i. = 0.02) and the plates were placed at temperatures of 33, 35, 37 and 39 °C. The culture fluids were obtained on days 1, 3, 5, and 7 of culture and infective titers were examined and expressed as $TCID_{50}$/ml in B95a cells.

2.6. Indirect immuno-staining and Western blotting

B95a cells were infected with MVAIK, MVAIK/RSV/G or MVAIK/RSV/F at m.o.i. of 0.01 in 24-well plates and cultured for two days at 33 °C. B95a cells were collected and subjected to indirect immuno-staining without fixation to detect the surface expression. Polyclonal antibodies against RSV raised in goat (Abcam, Cambridge, UK) were used and the cells incubated for 1 h at 37 °C. The cells were washed extensively with phosphate-buffered saline with 0.05% Tween 20 (PBST), and stained with second antibodies against goat IgG conjugated with FITC, raised in rabbit (Vector Laboratories, Burlingame, CA, US), and thereafter, mouse monoclonal antibody against MV HA protein (kindly supplied by Dr. Sato, National Institute of Infectious Diseases, Japan) was used and followed by second antibodies against mouse IgG conjugated with rhodamine raised in goat (Rockland Immunochemicals, Gilbertsville, PA, US).

Vero cells were infected with MVAIK, MVAIK/RSVF, and MVAIK/RSV/G and HEp-2 were infected with RSV subgroup A, Long

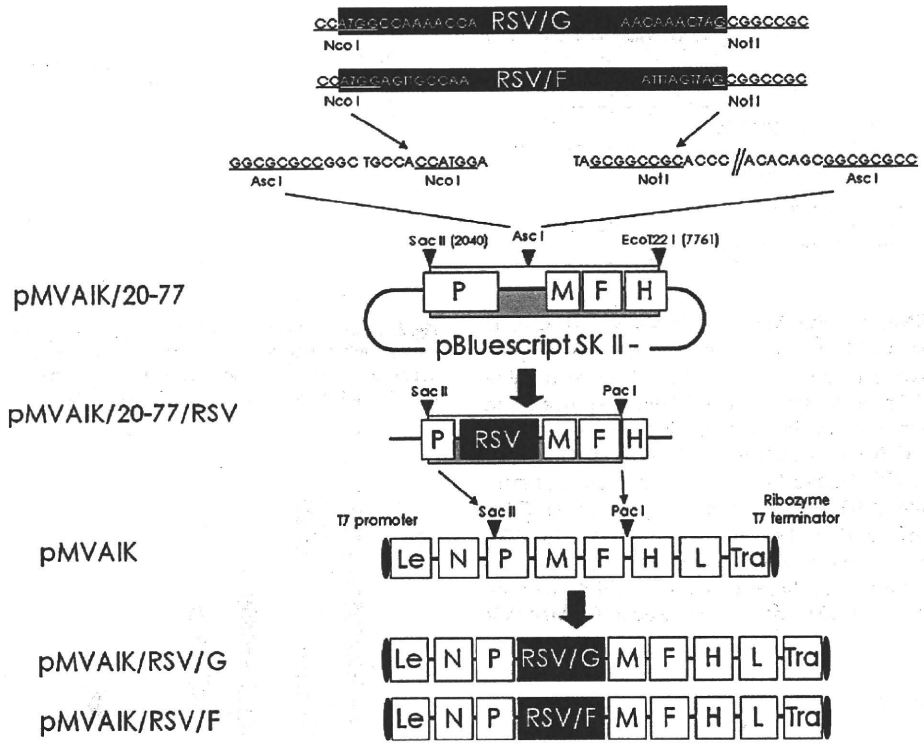

Fig. 1. Strategy for the construction of the recombinant AIK-C genome cDNAs having RSV protein genes. The recombinant AIK-C viral cDNAs expressing RSV G or F protein were constructed based on AIK-C cDNA (pMVAIK). pMVAIK/20-77 was constructed for the cloning of foreign genes. The Asc I site was introduced by adding GGCGCG after position 3432 of AIK-C and R1 and R2 sequences were added. The Nco I–Not I fragment of RSV G or F was cloned into pMVAIK/20-77, designed as pMVAIK/20-77/RSV. pMVAIK/20-77/RSV had unique restriction enzyme sites, Sac II and Pac I sites, located in the P gene and between the F and H gene. The DNA fragments between the Sac II and Pac I sites of pMVAIK/20-77/RSV/G and pMVAIK/20-77/RSV/F were inserted into pMVAIK using Sac II and Pac I sites. The recombinant plasmid constructs were designated pMVAIK/RSV/G and pMVAIK/RSV/F, respectively.

strain in a 24-well plate. Culture supernatants were collected and cells were freeze-thawed and total protein of 4 μg of supernatants and cell lysate was applied. Samples were subjected to Western blotting. Briefly, after SDS-PAGE, proteins were transferred to membrane (Immobilon; Millipore, Danvers, MA, US). Membranes were washed with PBST, incubated with an RSV polyclonal antibody raised in goats, washed again, and incubated with a donkey anti-goat IgG (H+L) conjugated with horse radish peroxidase (HRP). The final reaction was performed with a DAB SUBSTRATE KIT FOR PEROXIDASE (Vector Laboratories, Burlingame, CA, US) used as recommended by the manufacturer.

Culture medium of Vero cells infected with MVAIK/RSV/G or F was collected and fractionated through sucrose discontinuous gradient ultra-centrifugation. Fraction 1 was obtained at the top of the gradient, 30% sucrose, Fraction 2 between 30% and 45% sucrose, and Fraction 3 between 45% and 60% sucrose. Each fraction was electrophoresed and analyzed by Western blotting, using RSV polyclonal antibodies and monoclonal antibodies against MV N protein.

2.7. Immunogenicity in experimental animals

Six-week-old cotton rats were purchased from Harlan (Indianapolis, IN, US) and Charles River (USA). Five cotton rats for each group were immunized intramuscularly with 1×10^6 $TCID_{50}$ of MVAIK, MVAIK/RSV/G or MVAIK/RSV/F. Serum samples were obtained immediately before and 1, 3, 5, 8, 12 and 16 weeks after immunization. Cotton rats immunized with MVAIK/RSV/G or F were boosted with the same dose after 16 weeks, and serum samples were collected one week after re-immunization (17 weeks).

2.8. Serology

Neutralization tests (NTs) against RSV were performed with the 50% plaque reduction assay, using Long strain and wild-type isolate of subgroup B. Briefly, serum samples were serially diluted by1:4, starting from a 1:10 dilution, and mixed with an equal volume of RSV (100 PFU) in MEM for 1 h at room temperature. The mixtures were inoculated on monolayers of HEp-2 cells in 24-well plates. Plates were incubated for 1 h at 37 °C in 5% CO_2 and then overlaid with MEM supplemented with glutamine, antibiotics, 5% fetal bovine serum and 0.5% agar. After incubation for six days at 37 °C in 5% CO_2, cells were fixed with 1% formalin. Agar was removed and cells were stained with neutral red. Plaque numbers were counted and NT antibody titers were calculated as the reciprocal of the serum dilutions that showed a 50% reduction of the plaque number.

For the particles agglutination (PA) test, gelatin particles were coated with purified measles virus antigen (Serodia®-Measles, Fuji Rebio, Tokyo, Japan). Sera were serially diluted two-fold, starting from a 1:10 dilution, and each serum dilution was mixed with an equal volume of gelatin particles to detect agglutination, according to the recommendations of the manufacturer. The PA antibody titers were expressed as the reciprocal of the serum dilution which induced particle agglutination.

2.9. Detection of the MV genome

Cotton rats were sacrificed 10 days after immunization with MVAIK/RSV/G and F, and samples of liver, kidney, spleen, lung, thymus, and nasal turbinate were obtained to detect the MV genome. The tissues were homogenized, and total RNA was

Table 1
Primer and probe sequences for the detection of the MVAIK N gene and RSV N gene by TaqMan real-time PCR.

Primers	Sequences (5′–3′)	Genomic position
RSV-Long-N-(+)	aatgctaaaagaaatgggagagg	
Probe	gctccaga	411–470
RSV-Long-N-(−)	ccacaatcaggagaatcatgc	
MV-AIK-C-N-(+)	caagatcagtagagcggttgg	
Probe	agcccaag	1212–1274
MV-AIK-C-N-(−)	ctttgatcaccgtgtagaaatga	

extracted using an RNeasy® Plus Mini Kit (QIAGEN, MD, US), as recommended. TaqMan PCR was performed in the MV N gene region. Reverse-transcribed real-time PCR was performed using a FastStart TaqMan® Probe Master (Roche Meylan, France), and LightCycler®480 System II (Roche Meylan, France) using 1 μg of extracted mRNA. cDNA was synthesized using an One Step PrimeScript® RT-PCR Kit (TaKaRa Bio, Otsu, Japan). The parameters used were 1 cycle of 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s, and 1 cycle of 40 °C for 30 s. Reactions were performed in triplicate and genome copy numbers were determined by referring to the results of serial dilution of the corresponding plasmid, pCIAN01. The primers used in TaqMan PCR are shown in Table 1.

2.10. Protection against RSV

Seven week-old cotton rats were immunized intramuscularly with MVAIK/RSV/F or MVAIK/RSV/G and, five weeks later, challenged with 10^6 PFU/0.5 ml of RSV subgroups A and B. They were sacrificed four days after the challenge and nasal wash, BAL, nasal turbinate, and lung tissues were obtained. Lung samples were divided into two portions, one for pathological examination, and another for recovering the infective particles and RSV genome.

Tissues were homogenized and 0.1 ml volumes of serial 10-fold dilutions of homogenized samples were placed on HEp-2 cells and overlaid with MEM 5% FBS and 0.5% agar. Plaque numbers were counted after incubation for six days at 37 °C and infectivity was expressed as the number of plaques. RNA was extracted from nasal wash, BAL, nasal turbinate and lung homogenate. cDNA was synthesized and reverse-transcribed real-time PCR was done at position 1212–1274 of the RSV N genome, using the primers and TaqMan probe listed in Table 1. The RSV genome copy number was calculated by referring to a linear regression assay of serial dilutions of the corresponding plasmid.

Lungs were inflated to their normal volumes with 4% formalin and submerged in formalin for overnight fixation. The fixed tissue was embedded in paraffin, sectioned, and stained with hematoxylin–eosin, and immuno-staining was performed using four clone blend monoclonal antibodies against RSV P, F, and N proteins (AdB Serotec, UK), and anti-mouse IgG conjugated with HRP.

3. Results

3.1. Characteristics of recombinant viruses

MVAIK/RSV/G and MVAIK/RSV/F were recovered from full-length recombinant cDNA and MVAIK from vector cDNA. B95a cells were infected with MVAIK, MVAIK/RSV/G and MVAIK/RSV/F at a m.o.i. of 0.02. The culture medium was harvested on days 1, 3, 5, and 7 at 33 °C and the results are shown in Fig. 2. Infectivity showed a peak titer of 10^5 $TCID_{50}$/ml 5 days after infection. MVAIK/RSV/G and MVAIK/RSV/F grew as well as MVAIK in B95a cells. AIK-C has temperature-sensitivity (*ts*), showing efficient virus

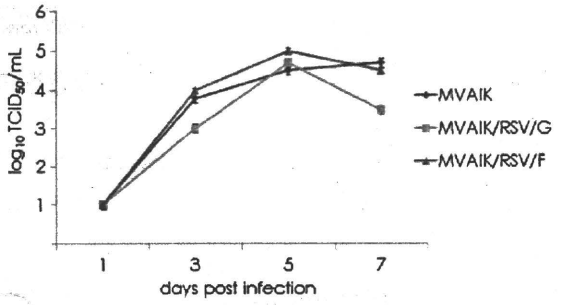

Fig. 2. Growth of MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. B95a cells were infected with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F at m.o.i. of 0.02. Culture fluid was obtained on days 1, 3, 5, and 7 of culture at 33 °C. Infectivity is shown as mean titers of $TCID_{50}$/ml assayed in B95a cells. Error bars show 1.0 S.D.

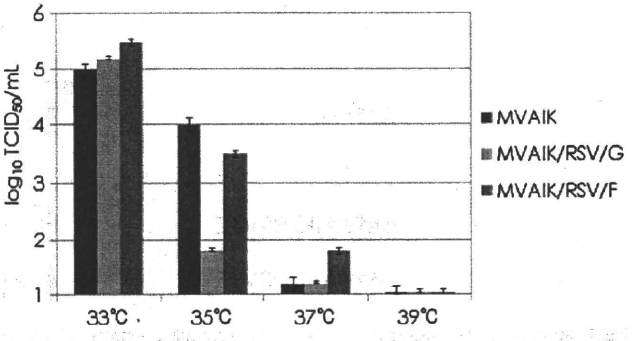

Fig. 3. Temperature sensitivity of MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. B95a cells were infected with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F at a m.o.i. of 0.02. Culture fluid was obtained on day 5 and the infectivity at 33 °C, 35 °C, 37 °C, and 39 °C is shown as mean infectious titer ($TCID_{50}$/ml). Error bars show 1.0 S.D.

growth at 33 °C, but extremely poor at 39 °C, less than 10^{-4} in comparison with the result at 33 °C. MVAIK/RSV/G and MVAIK/RSV/F were examined for virus growth at 33, 35, 37 and 39 °C. The culture supernatants were harvested on day 7 of the culture and infectivity was examined. Both MVAIK/RSV/G and MVAIK/RSV/F showed 10^5 $TCID_{50}$/ml at 33 °C, and MVAIK/RSV/F grew little at 37 °C. But, however, no infectious virus was detected at 39 °C, and the *ts* phenotype was maintained (Fig. 3).

3.2. Detection of RSV G or F protein

B95a cells were infected with MVAIK/RSV/F, MVAIK/RSV/G, and MVAIK at a m.o.i. of 0.01. Live cells were stained with monoclonal antibodies against measles HA and polyclonal antibodies against RSV and visualized with second antibodies conjugated with rhodamine or FITC, as shown in Fig. 4. RSV F and MV HA proteins were observed diffusely on the surface of B95a cells infected with MVAIK/RSV/F. RSV G protein was detected in speckled pattern together with MV HA protein on the surface of B95a cells infected with MVAIK/RSV/G.

Culture medium and cell lysate were examined for the expression of RSV G and F by Western blotting and the results are shown in Fig. 5. Live vector virus MVAIK and RSV were used for the negative and positive controls. RSV G and F proteins were detected in both supernatant and cell lysate infected with MVAIK/RSVF, and MVAIK/RSV/G, similar to those infected with RSV (Fig. 5, Panel A).

Culture fluid was collected and fractionated through sucrose discontinuous gradient ultra-centrifugation. Fraction 1 was obtained at the top of the gradient, 30% sucrose, Fraction 2 between 30% and 45% sucrose, and Fraction 3 between 45% and 60% sucrose. Each fraction was electrophoresed and analyzed by Western blotting, using RSV polyclonal antibodies and a monoclonal antibody

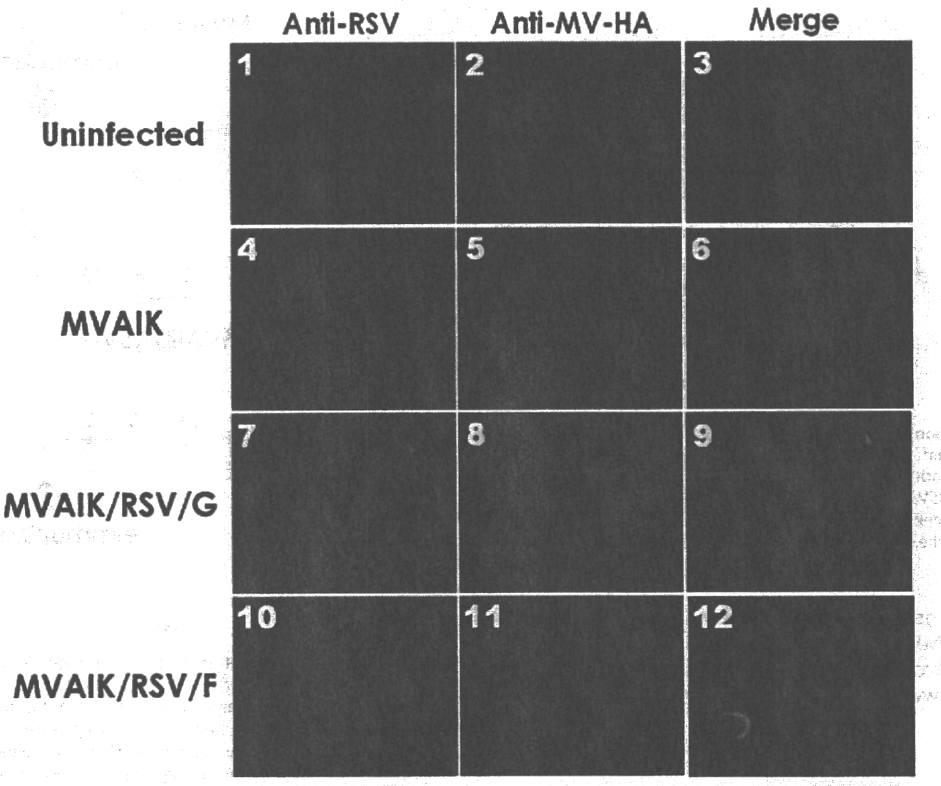

Fig. 4. Expression of MV HA and RSV G or F protein. B95a cells were infected with MVAIK (panels 4 and 5), MVAIK/RSV/G (panels 7 and 8) or MVAIK/RSV/F (panels 10 and 11) at a m.o.i. of 0.01 in 24-well plate and cultured for two days at 33 °C. Uninfected B95a cells are shown in panels 1 and 2. B95a cells were collected and subjected to live cell staining without fixation to detect the surface expression. The expression of RSV (panels 1, 4, 7, and 10) and MV HA protein (panels 2, 5, 8, and 11) are shown. Panels 3, 6, 9 and 12 are merged images.

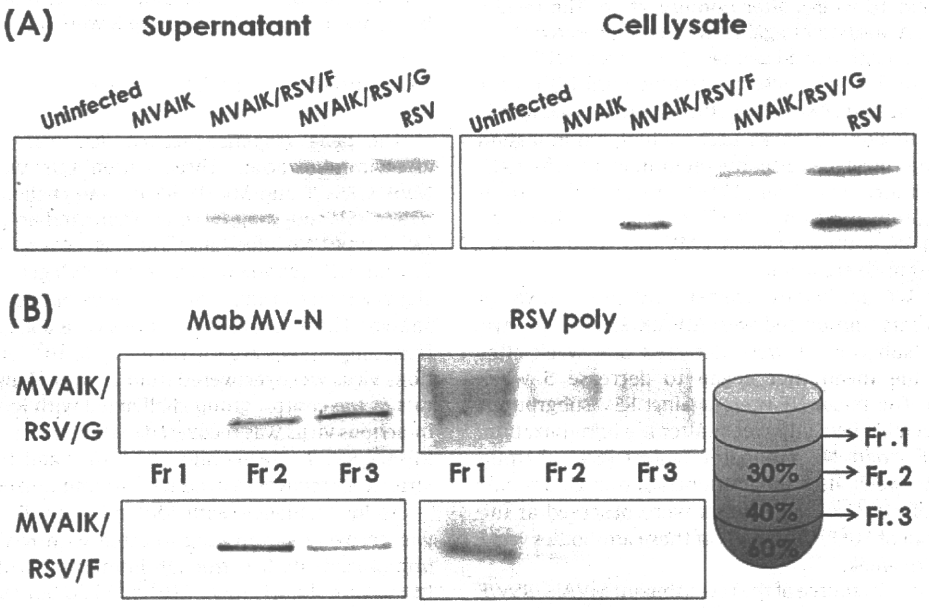

Fig. 5. Results of Western blotting of culture supernatant, cell lysate, and purified recombinant measles viral particles. (A) Vero cells were infected with MVAIK, MVAIK/RSVF, and MVAIK/RSV/G and HEp-2 cells were infected with RSV subgroup A, Long strain, and were cultured in 1 ml in a 24-well plate. Just before the appearance of CPE, culture media was replaced with serum free medium (VP-SFM). 1 ml of culture medium was harvested and 100 μl of PBS was added in plate. Cells were freeze-thawed and cell lysate was clarified. As for the Western blotting, 1/30 of initial supernatants and 1/100 of cell lysate were subjected for experiments. They were stained with polyclonal antibodies against RSV. (B) Infectious particles were obtained through sucrose discontinuous gradient ultra-centrifugation. Fraction 1 was obtained at the top of the gradient of 30% sucrose, Fraction 2 between 30% and 45% sucrose, and Fraction 3 between 45% and 60% sucrose. Each fraction was analyzed by Western blotting, using RSV polyclonal antibodies and monoclonal antibodies against MV N protein.

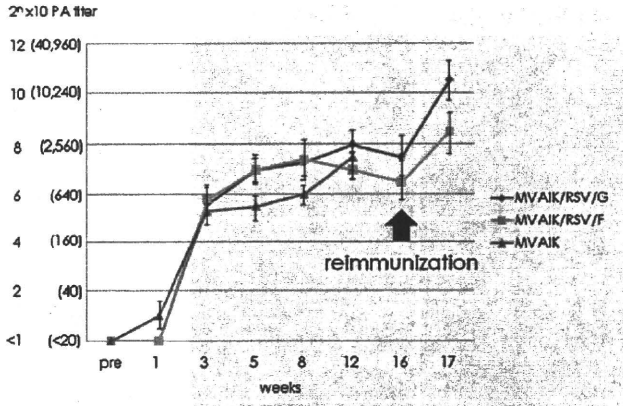

Fig. 6. Serological response of PA antibodies against MV. PA antibody titers were examined, using Serodia®-Measles. PA titers are expressed as $2^n \times 10$. Sera were collected before immunization, and 1, 3, 5, 8, 12, 16, and 17 weeks after immunization with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. Five cotton rats were immunized and followed for 12 weeks. Mean PA titers ± 1.0 S.D. are shown. Two rats for each were reimmunized at the 16th week.

against the MV N protein. RSV G or F was detected in Fraction 1, and, whereas the MV N protein was detected in Fractions 2 and 3 (Fig. 5, Panel B). Accordingly, RSV G or F protein translated from the inserted gene was considered not to be incorporated into MV particles.

3.3. Immunogenicity of recombinant measles viruses

The recombinant viruses, MVAIK/RSV/G and MVAIK/RSV/F, were inoculated into cotton rats to confirm the immunogenicity intramuscularly of the inserted RSV G or F protein. Five cotton rats were immunized with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F for each study group and serum samples were obtained before and 1, 3, 5, 8, 12, and 16 weeks after immunization. The results are shown in Fig. 6. PA antibodies against MV were detected three weeks after the immunization in all animals. High levels of PA antibody, $2^{6-8} \times 10$ (1:640–1:2560), were maintained until 16th week in those immunized with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F. Two rats were reimmunized 16 weeks after the first immunization and sera were obtained one week after the reimmunization for each group. PA antibodies increased from $2^{7.5 \pm 1.5}$ to $2^{10.5 \pm 1.5} \times 10$ in the MVAIK/RSV/G group, and from $2^{6.5 \pm 1.5}$ to $2^{8.5 \pm 1.5} \times 10$ in the MVAIK/RSV/F group. PA antibodies against MV increased after the reimmunization by four to eight-fold.

The results for NT antibodies against RSV are shown in Fig. 7. In the cotton rats immunized with MVAIK/RSV/G, NT antibodies against RSV subgroup A were detected one week after immunization but the mean titer began to decrease 5 week after immunization. The mean NT titers against RSV subgroup A decreased to undetectable levels 12 weeks after the immunization. In the MVAIK/RSV/F group, NT antibodies against RSV subgroup A were detected one week after the immunization in all animals with a mean titer of $10^{2.0 \pm 0.7}$. High titers were observed at the 5th week with a mean of $10^{2.6 \pm 1.0}$. Levels of these antibodies were maintained until 16th week.

In this experiment, RSV source of the recombinant MVAIK/RSV/F or MVAIK/RSV/G was derived from the RSV subgroup A wild type. Cross immunity against RSV subgroup B was further investigated. In cotton rats immunized with MVAIK/RSV/F, NT antibodies against RSV subgroup B were detected at the 3rd week with a mean titer of 150 ($10^{2.1}$) and maintained for 16 weeks. However, cross-reactive antibodies against RSV subgroup B were not detected in the cotton rats immunized with MVAIK/RSV/G.

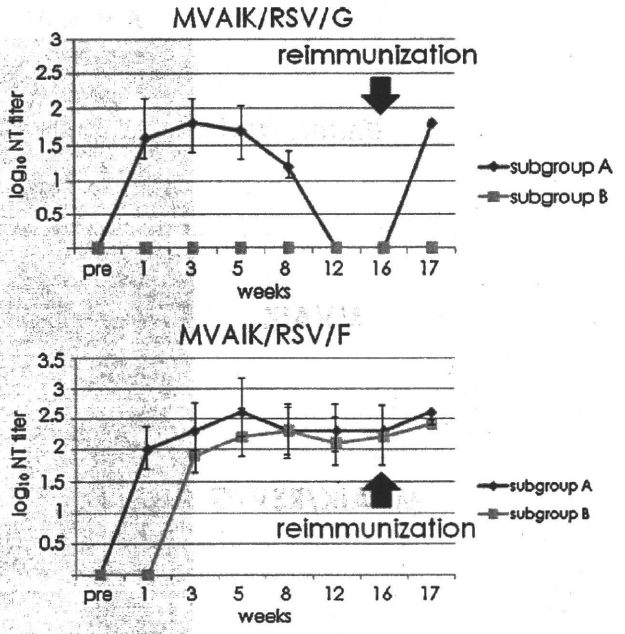

Fig. 7. Development of NT antibodies against RSV. NT antibodies were examined using the RSV Long strain (Subgroup A) and wild-type RSV subgroup B strain. Sera were collected before immunization, and 1, 3, 5, 8, 12, 16, and 17 weeks after immunization. 50% plaque reduction NT titers are expressed 10^n and are shown as mean NT titers with 1.0 S.D. The upper panel shows the immune response after immunization with MVAIK/RSV/G. The lower panel shows the results after immunization with MVAIK/RSV/F.

In the cotton rats immunized with MVAIK/RSV/F, NT antibodies against RSV subgroups A and B increased after reimmunization by two fold, but not significantly. As for the rats immunized with MVAIK/RSV/G, NT antibodies against RSV subgroup A were boosted from an undetectable level before the reimmunization to $10^{1.8 \pm 0.1}$, but those against RSV subgroup B were not detected.

3.4. Protection against RSV challenge

The peak response against RSV was observed five weeks after immunization. Three cotton rats were immunized with MVAIK/RSV/F and MVAIK/RSV/G and challenged with the homologous RSV subgroup A (Long strain) and heterologous subgroup B (wild-type). No infectious virus was recovered from nasal wash and BAL but RSV genome was detected. RSN genome copy number was slightly lower in immunized groups but not significant (data not shown). The recovery of infectious virus and genome copy numbers from lung tissues are shown in Fig. 8. $10^{5.4}$ and $10^{4.5}$ PFU of infectious virus were recovered from 20 mg of lung tissue in two cotton rats of the control group challenged with RSV subgroup A, but no infectious virus was recovered in three cotton rats immunized with MVAIK/RSV/F. Meanwhile, $10^{4.5}$, $10^{2.8}$ and $10^{3.3}$ PFU of infectious virus were recovered in cotton rats immunized with MVAIK/RSV/G.

As for challenge with RSV subgroup B, $10^{5.0-5.8}$ PFU of RSV was recovered from lung infected with RSV subgroup B in non-immunized rats. In cotton rats immunized with MVAIK/RSV/F, virus titers were slightly lower, $10^{4.4-4.5}$ PFU but $10^{5.0-5.3}$ PFU from their lung tissues in the cotton rats immunized with MVAIK/RSV/G. There was no significant reduction in RSV N gene copy number.

For histopathological examinations, lung tissues were obtained four days after the challenge with RSV subgroups A and B and the results of HE staining and immuno-staining against RSV antigens are shown in Fig. 9. The non-immunized rat challenged with RSV subgroup A showed prominent interstitial pneumonia

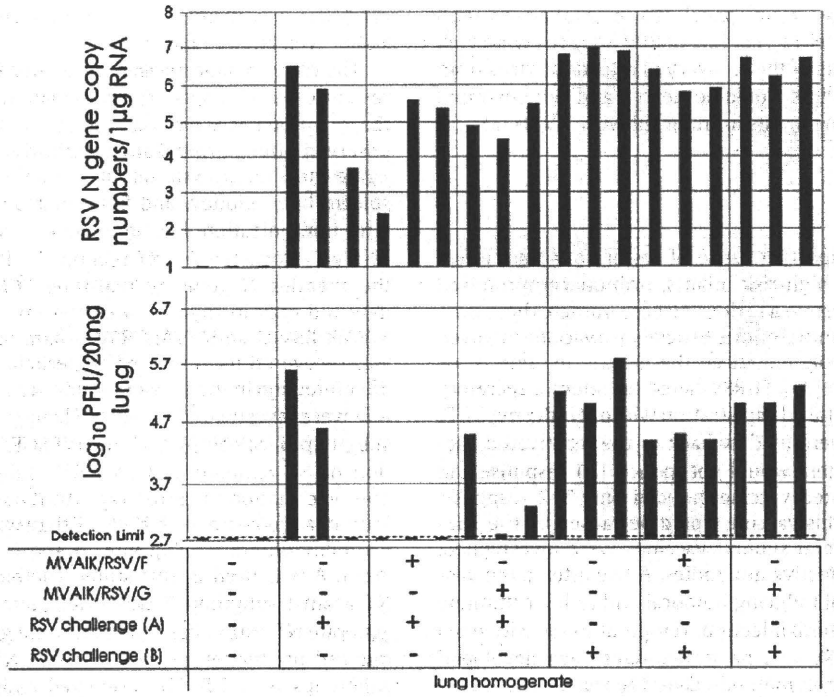

Fig. 8. Recovery of RSV infectious virus and genome copy numbers after challenge with RSV subgroups A and B. Three cotton rats were investigated in the normal control group, non-immunized group, and group immunized with MVAIK/RSV/F or MVAIK/RSV/G. Animals non-immunized group, and group immunized with MVAIK/RSV/F or MVAIK/RSV/G were challenged with 1.0×10^6 PFU of the homologous RSV Long strain and wild-type subgroup B five weeks later. Virus infectivity was monitored in lung homogenate, and RSV infectivity is shown as PFU in 20 mg of lung tissue. And 1 μg of total RNA of lung tissue was used for real-time PCR, and each column represents individual result.

(panel 2; thickening of alveolar wall, and infiltration of inflammatory mononuclear cells) with RSV antigens in bronchial epithelial cells (panel 6). In cotton rat immunized with MVAIK/RSV/F showed very mild inflammation (panel 3), though most sections were normal, without RSV antigen in bronchial tissue after RSV challenge with subgroup A (panel 7). In cotton rat immunized with MVAIK/RSV/G, moderate interstitial pneumonia was observed with a small amount of RSV antigen (panels 4 and 8).

As for the challenge with RSV subgroup B, histological findings in non-immunized rat challenged with subgroup B were similar to the results challenged with RSV subgroup A. The results of immunostaining are shown. Large amounts of RSV antigen were detected in non-immunized rat (panel 9). Small amounts of RSV antigens were

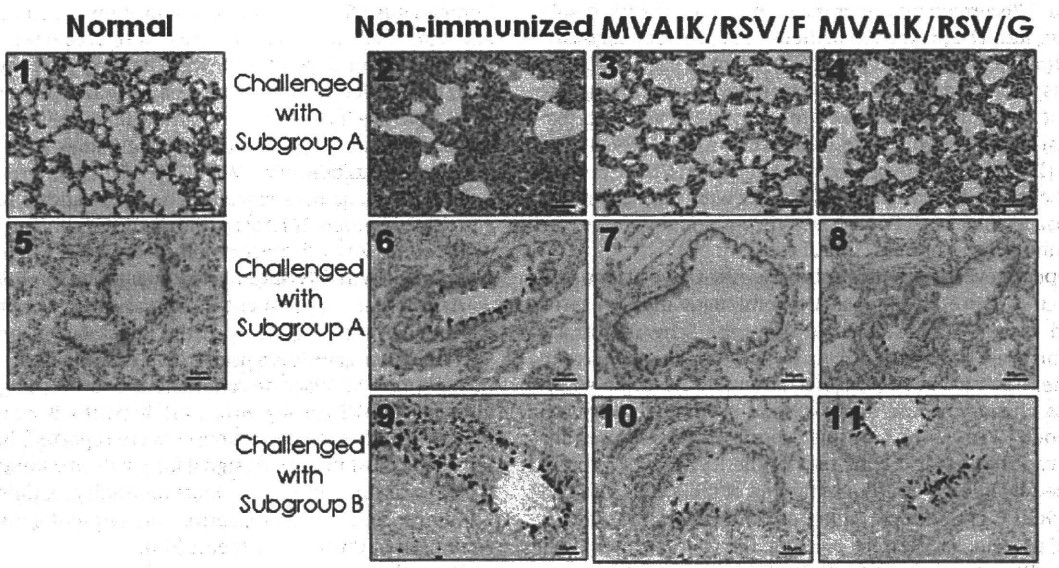

Fig. 9. Pulmonary histopathology in cotton rats challenged with RSV subgroups A and B. Cotton rats were immunized intramuscularly with MVAIK/RSV/F (panels 3, 7, and 10) or MVAIK/RSV/G (panels 4, 8, and 11) and then challenged five weeks later, with RSV subgroup A (panels 2, 3, 4, 6, 7, and 8) and subgroups B (panels 9, 10 and 11). They were sacrificed four days after the challenge. Histological examination was performed by HE staining of lung tissues (panels 1, 2, 3, and 4) and the results of immuno-staining of bronchiolar regions are shown in panels 5, 6, 7, 8, 9, 10, and 11. Immuno-staining was performed using four clone blend monoclonal antibodies against RSV P, F, and N proteins and anti-mouse IgG conjugated with HRP. HE staining and immuno-staining of normal control are shown in panels 1 and 5.

detected in MVAIK/RSV/F group (panel 10) in comparison with MVAIK/RSV/G group (panel 11). Finding of RSV antigens were well correlated with the results of the recovery of infectious virus from lung tissues. Inoculated virus would be cleared and demonstrated a mild pathological finding in rats immunized with MVAIK/RSV/F.

4. Discussion

RSV is a clinically important cause of respiratory tract infections, especially among high-risk infants, immunocompromised hosts, and the elderly. Despite a serious disease burden, there is no licensed vaccine for RSV. Initial efforts to develop a vaccine involved FI-RSV which unexpectedly enhanced the disease in clinical trials in RSV-naive children [33]. FI-RSV failed to induce a secretory IgA response after pareteral administration without inducing a CTL response, which was a serious drawback of the inactivated vaccine. The defeated F protein would not induce Th1 response and the aluminium-precipitated vaccine induced only Th2 response. The allergic reaction to this vaccine would be caused by the Th2-prone reaction [34]. Several subunit vaccines were investigated, but failed to generate effective antibodies. A live attenuated vaccine has the advantage of inducing humoral and cellular immune responses similar to a natural infection. Temperature-sensitive (*ts*) and cold-adapted (*ca*) RSV vaccine strains have been developed by conventional attenuation methods. Over the last 40 years, cautious and deliberate progress has been made toward developing a RSV vaccine using various experimental approaches, including live attenuated strains and vector-based and viral protein subunit vaccine candidates. But the balance between the safety and immunogenicity is a key issue to the development of a live attenuated vaccine, and the (*ts*) RSV vaccine candidate resulted in insufficient attenuation, causing similar respiratory illness [35]. Based on a vaccine candidate having the *ts* phenotype, several recombinant vaccine candidates were developed by deletion of the SH gene or NS1 gene or mutation by reverse genetics. These recombinant RSV vaccines induced sufficient immune response in chimpanzees [36]. Another approach involved the application of reverse genetics to express RSV protein in a recombinant vector-based vaccine. The first vector-based candidate was evaluated using vaccinia virus. Recombinants expressing RSV F or G was highly immunogenic, induced protection in mouse but provided inconsistent protection in chimpanzees [37]. MVA strain of vaccinia-based recombinants expressing RSV G and F protein were immunogenic in rodent but not in rhesus monkey model [38]. Several vector-based live vaccine platforms were established using HPIV-III and Sendai virus [23,39]. Through preceding experiments, the F protein is known to be more effective than G. But there were no experiences for clinical usage and the HPIV-III-based recombinant vaccine was poorly immunogenic in human clinical trials.

In this report, reverse genetics using the AIK-C live attenuated measles vaccines were developed. A recombinant measles virus vector-based vaccine was established using the Schwartz strain, expressing the West Nile virus [40]. As well as the Schwartz strain, the AIK-C measles vaccine is a further attenuated vaccine strain having the *ts* phenotype, and its safety and immunogenicity has been confirmed [26,29]. Thus, in this report, AIK-C was used for a live virus vaccine-vector. Expression of the RSV G or F protein was confirmed by indirect immuno-staining of B95a cells infected with MVAIK/RSV/G or F with polyclonal and monoclonal antibodies against the F protein. By Western blotting, the G or F protein was detected in culture medium and cell lysate of B95a cells infected with MVAIK/RSV/G or F. The RSV G and F proteins were considered not to be incorporated into MV particles because theoretically they had no binding site for the MV M protein. MV envelop proteins bound the M protein [41]. The genetic stability of the vaccine candidate was examined and inserted genes for RSV G and F were stable even after 15 passages.

The recombinant measles virus (MVAIK) triggered an immune response three weeks after vaccination in cotton rats. Levels of these antibodies were maintained for 16 weeks. The same was observed after immunization with MVAIK/RSV/G or F. To investigate the viral growth, samples of nasal turbinate, lung, thymus, spleen, liver, kidney, and bone marrow were obtained 10 days after immunization, but no infectious virus was recovered. Total RNA was extracted and RT-real time PCR was performed to detect the measles N gene by real-time PCR. The MV genome was detected only in thymus in cotton rats immunized with MVAIK, MVAIK/RSV/G, and MVAIK/RSV/F (data not shown). Infectious virus was recovered from inguinal superficial lymph nodes three days after infection in the previous study [42]. NT antibody titers against RSV were investigated, using RSV Long (subgroup A) and wild-type subgroup B. MVAIK/RSV/G or MVAIK/RSV/F induced the production of NT antibodies against RSV subgroup A from one week after vaccination in cotton rats. Antibody titers were higher after immunization with MVAIK/RSV/F than with MVAIK/RSV/G. RSV has distinctly different subgroups, A and B. The G or F gene of subgroup A was used in this study. Therefore, the cross reaction of NT against subgroup B was investigated. MVAIK/RSV/G did not generate NT antibodies against RSV subgroup B, but MVAIK/RSV/F induced production of cross-reactive NT antibodies against RSV subgroups A and B. The predicted amino acid sequence of the RSV F protein used in this study exhibited 98.6% homology among F proteins of subgroup A strains and 90.8% in comparison with those of subgroup B strains. The predicted amino acid sequence of RSV G protein has 86.9% homology among subgroup A strains but 49.7% in comparison with subgroup B. Thus, F protein was relatively conserved between subgroups A and B but the G protein of RSV was variable and thought not suitable as a vaccine antigen. Recently, a humanized monoclonal antibody against the RSV F protein was used for prevention of serious RSV infections in young infants having cardiac and pulmonary disorders, with a low birth weight, or born prematurely. In this study, recombinant MVAIK/RSV/G or F was administered intramuscularly and induced sufficient NT antibodies. Secretory IgA antibodies and CTL response were not examined but it protected against the challenge with homologous RSV subgroup A. In non-immunized cotton rats, $10^{5.4}$ and $10^{4.5}$ PFU of infectious virus were recovered from 20 mg of lung tissue four days after the RSV challenge. But those immunized with MVAIK/RSV/F were protected, without recovery of infectious virus from the lung tissues. And they did not demonstrate interstitial pneumonia. Cross reactive NT antibodies were demonstrated after immunization with MVAIK/RSV/F but its protective effect is not sufficient against subgroup B, demonstrating slightly lower levels (approximately 1/10 of non-immunized control) of the recovery of infectious virus. Protective effects of MVAIK/RSV/G were poor in comparison with MVAIK/RSV/F similar to the serological responses.

As for the experimental animal models, transgenic mice expressing human CD46 with the knock out of type I interferon (IFN) receptor gene were used to evaluate the immunogenicity of a recombinant MV vaccine candidate produced using the West Nile virus [40], SARS corona virus [43], hepatitis B virus [44] and HIV [45]. Efficient immune responses were reported, but the IFN system is the most important signal for innate immunity. In the case of the RSV vaccine candidate, innate immunity modified the adaptive immunity, and, therefore, cotton rats without gene manipulation were used in these experiments [46].

Recombinant MV vaccine-based vectors have practical limitation for timing of immunization. In young infants, maternal conferred immunity would interfere with vaccine effects. In field trials, AIK-C gave efficient sero-conversion and induction of cell-mediated immunity even when the vaccine was given at the age

of six months [26,27]. They demonstrated more than 80% seroconversion rate to overcome maternal conferred immunity and the safety was similarly confirmed, suggesting no evidence of immunesuppression. RSV infection was observed even after six months of age, and, therefore, MVAIK/RSV/F would be applicable for six months to provide protective immunity both against RSV and measles especially for developing countries.

As for the effective protection against RSV infection, intranasal administration is desired. But we have no experience of intranasal administration of AIK-C vaccine, and, in our previous experiments, the recombinant MVAIK did not induce serum NT against MV through intranasal administration because of the strict *ts* phenotype in cotton rat model, having high body temperature [21,42]. Therefore, the comparative studies are planning to investigate the immunogenicity and challenge tests in monkeys immunized with MVAIK/RSV/F.

In conclusion, a new MV vaccine-strain-based RSV vaccine candidate was demonstrated to confer protection against RSV in cotton rats. The xenogeneic recombinant might induce simultaneously protective immunity against backbone-MV and inserted-RSV infections. Recombinant MVAIK expressing RSV F protein is a promising candidate and protective effects should be confirmed in monkey model, considering the immunization routes.

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ORIGINAL ARTICLE

Incidence of bacterial coinfection with respiratory syncytial virus bronchopulmonary infection in pediatric inpatients

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Abstract Bacterial coinfection occurs in pediatric bronchopulmonary infections caused by respiratory syncytial virus (RSV), but the incidence is uncertain. Our subjects are 188 pediatric inpatients having RSV bronchopulmonary infection in two hospitals in Chiba Prefecture between 2005 and 2007. On admission, antigen detection kits using nasopharyngeal aspirate were performed to detect RSV infection and washed sputum bacterial culture was performed to detect bacterial infection. Of the 188 pediatric inpatients with RSV bronchopulmonary infection, 95 (50.5%) patients were aged less than 1 year, 57 (30.3%) were aged 1–2 years, and 36 (19.1%) were aged 2 years or more. Thirty-six (19.1%) patients were associated with bronchial asthma attacks. Pathogenic bacteria were predominantly isolated from 43.6% of the patients. The three most frequently isolated bacteria were *Haemophilus influenzae* (43.9%), *Streptococcus pneumoniae* (36.6%), and *Moraxella catarrhalis* (29.3%). We found that 38.9% of *H. influenzae* strains were β-lactamase-nonproducing ampicillin-resistant strains. All *S. pneumoniae* strains were penicillin G (PcG) sensitive. However, 21.9% of *S. pneumoniae* strains showed PcG minimum inhibitory concentration values of 2 μg/ml. RSV bronchopulmonary infections in hospitalized children are often associated with antimicrobial-resistant bacterial infection in their lower airways. These results indicate that we should be aware of bacterial coinfections in the management of pediatric inpatients with RSV bronchopulmonary infection.

Keywords Respiratory syncytial virus · *Haemophilus influenzae* · *Streptococcus pneumoniae* · Bronchopulmonary infection

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Introduction

Respiratory syncytial virus (RSV) is one of the major pathogens in upper and lower respiratory tract infection in children. RSV infections are often associated with bacterial coinfections, which increase the severity of the RSV infection [1–3]. Several papers that have examined the occurrence of bacterial coinfection in children with severe RSV infection requiring pediatric intensive care unit (PICU) admission have found the incidence to be between 17.5 and 44% [4–6]. However, both the incidence of bacterial coinfection with RSV bronchopulmonary infection in pediatric inpatients not requiring PICU admission and the prevalence of antimicrobial-resistant pathogenic bacteria isolated from these patients are poorly understood.

Most pediatric patients with RSV bronchopulmonary infections exhibit wheezing and productive cough. Therefore, sputum can be obtained easily from children with RSV infection, irrespective of age. Unfortunately, the diagnostic yields of sputum culture are limited because of potential contamination from the upper respiratory tract. For this reason, previous studies have considered expectorated sputum washing as a useful means for improving sputum for microbiological examination [7–11].

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