

Short Communication

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Recent H5N1 avian *Influenza A virus* increases rapidly in virulence to mice after a single passage in mice

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To evaluate the potential pathogenicity to mammals of the recent H5N1 avian *Influenza A virus*, viruses recovered from dead mice infected with A/chicken/Yamaguchi/7/2004 isolated in Japan were examined. All recovered viruses from the brains of dead mice infected with this strain (without any prior adaptation to mice) had substituted the amino acid at position 627 of the PB2 protein from glutamic acid to lysine. Their mouse lethality had increased by approximately 5×10^4 times over that of the original virus. Histopathological analysis reinforced the finding that these variants caused more rapid and severe damage to mice than the original virus. This revealed that it might be useful to characterize the recovered virus to assess its potential pathogenicity to mammals.

Beginning in 2003, the highly pathogenic H5N1 avian influenza virus has caused great economic losses in the poultry industry throughout east Asia, including Japan (Chen *et al.*, 2004, 2005; Li *et al.*, 2004; Liu *et al.*, 2005; Mase *et al.*, 2005b). Incidents of the causative H5N1 virus being transmitted directly from birds to humans occurred in 1997 and 2003 in Hong Kong (Peiris *et al.*, 2004; Subbarao *et al.*, 1998; Yuen *et al.*, 1998). From 2004 to 2006, these viruses were transmitted to humans in Azerbaijan, Cambodia, China, Egypt, Indonesia, Iraq, Thailand, Turkey and Vietnam, and this transmission resulted in over 100 deaths (WHO, 2006). Therefore, an epidemic of H5N1 avian influenza still poses a serious threat to public health.

Mice have been shown to be a good mammalian model for the human H1N1, H2N2 and H3N2 influenza viruses. Therefore, mice were used for the H5N1 influenza viruses as well, and it was revealed that recent isolates of the H5N1 influenza virus replicated well in mice without prior adaptation (Gao *et al.*, 1999; Guan *et al.*, 2002, 2004; Lipatov *et al.*, 2003; Lu *et al.*, 1999; Nishimura *et al.*, 2000; Shortridge *et al.*, 1998).

In Japan, an outbreak of highly pathogenic H5N1 avian influenza in chickens was confirmed in 2004 for the first time (Mase *et al.*, 2005b). The causative H5N1 viruses in Japan were genetically close to A/chicken/Shantou/4231/2003, termed genotype V, which belongs to a genotype different from that of the dominant epidemic viruses in South-East Asian countries, such as Indonesia, Thailand and Vietnam (i.e. genotype Z) (Li *et al.*, 2004). This suggested that multiple H5N1 virus genotypes have been circulating and are associated with the occurrence of the serious outbreaks in poultry in Asian countries.

The H5N1 virus isolated in Japan during the 2003–2004 outbreaks was able to replicate in mice without prior adaptation, but the isolate was less virulent than the Hong Kong 1997 H5N1 viruses isolated from humans (Gao *et al.*, 1999; Mase *et al.*, 2005b). However, it was previously revealed experimentally that highly pathogenic H5N1 variants could be selected rapidly in mice after a single passage (Lipatov *et al.*, 2003). Here, we describe the biological and pathological characterization of recovered viruses with rapidly increasing virulence to mice by only one amino acid substitution in the complete genome after a single passage in mice.

The Japanese H5N1 virus A/chicken/Yamaguchi/7/2004 (Ck/Yama/7/04), isolated from chickens (Mase *et al.*, 2005b), was used in this study. To prepare the original virus stock for this study, virus was propagated once in the allantoic cavity of embryonated eggs at 37 °C for 1–2 days and then stored at –80 °C until use. All experiments using the live H5N1 virus were performed in biosafety level 3 facilities under the recommended procedures.

First, we examined the pathogenicity to mice of the original Ck/Yama/7/04 strain. Six-week-old female BALB/c mice ($n=18$; SLC Japan) were used in all experiments. The mice were anaesthetized by pentobarbital injection and 50 µl infectious virus [10^6 50% egg infectious dose (EID₅₀)] diluted in PBS was inoculated intranasally (i.n.). The mice were checked daily for clinical signs and mortality for 14 days post-infection (p.i.). Reisolation of the virus from the brain, lung, liver, spleen and kidney of the dead mice was conducted immediately after the animals' deaths, as described previously (Mase *et al.*, 2005a).

Fourteen mice in total died during the observation period, and the virus was reisolated from the brains of all dead mice. The mean time to death of the mice infected with the original Ck/Yama/7/04 strain was 8.3 days. To examine the extent of mutations, we determined the nucleotide sequences of isolates from the mouse brains. First, partial nucleotide sequences of the PB2 gene, which was related to the virulence to mice of the Hong Kong H5N1/97 viruses, of all isolates from the mouse brains were determined. Reverse transcription, PCR amplification and sequencing were performed as described previously (Mase *et al.*, 2005b).

All viruses recovered from the brains of dead mice inoculated with the Ck/Yama/7/04 strain had an amino acid substitution from glutamic acid (Glu) to lysine (Lys) at position 627 of the PB2 protein, as shown by partial nucleotide sequencing of the PB2 gene. Next, the complete nucleotide sequences of all segments of five chosen isolates (termed mouse brain variants; MBVs) recovered from five respective mice were determined as described previously (Mase *et al.*, 2005a). Interestingly, as for the results of the complete nucleotide sequencing of all segments of the five chosen recovered isolates, four viruses have only one amino acid substitution at position 627 of the PB2 protein from Glu to Lys. From these viruses, one isolate was selected and designated mouse brain variant A (MBV-A). The remaining isolate has an additional amino acid substitution, methionine (Met) to isoleucine (Ile), at position 531 of the haemagglutinin (HA), and was designated MBV-B. In the following tests, the MBV-A and -B strains were used as mouse variants derived from the original Ck/Yama/7/04 strain.

Next, the pathogenicity to mice of the original virus and MBVs were compared. Six-week-old female BALB/c mice (SLC Japan) were used. Fifty per cent mouse infectious dose (MID_{50}) and 50 % mouse lethal dose (MLD_{50}) titres were determined by inoculating groups of eight mice i.n. with serial 10-fold dilutions of virus according to the method described by Lu *et al.* (1999). Three days later, four mice from each group were euthanized and the lungs were collected and homogenized. The homogenates were frozen at -80°C and later thawed for ease of handling. Solid debris was pelleted by centrifugation and the supernatants were titrated for virus infectivity in eggs. The four remaining mice in each group were checked daily for disease signs and death for 14 days p.i. The MID_{50} and MLD_{50} titres were calculated by the method of Reed & Muench (1938).

The scores of MID_{50} and MLD_{50} of the two MBVs were 5 EID_{50} and 8.9 EID_{50} , respectively. However, the scores of MID_{50} and MLD_{50} of the original Ck/Yama/7/04 strain were 5×10^3 EID_{50} and 5×10^5 EID_{50} , respectively. Comparing these scores, the MLD_{50} of two MBVs were increased by 5×10^4 times over that of the original Ck/Yama/7/04 strain.

Next, a histopathological examination of the mice infected with the original and MBV-A viruses was performed. Six-week-old female BALB/c mice ($n=18$ for each of the

original and MBV-A viruses) were inoculated i.n. with 50 µl virus (10^6 EID_{50}). A total of three sacrificed or dead mice were examined on day 3 and another three on day 6 p.i. The major organs were removed and fixed in 10 % neutral phosphate-buffered formalin, then processed routinely and stained with haematoxylin and eosin (HE) for histopathological examination. A section mounted on silane-coated slide glass was heated by microwave as described previously (Tanimura *et al.*, 2004) and goat anti-influenza A virus polyclonal antibody (Chemicon) was applied. The primary antibody was allowed to incubate for 30 min at room temperature and then detected by the application of horseradish peroxidase anti-goat Ig conjugate (Histofine Simple Stain; Nichirei Inc.). Diaminobenzidine (Nichirei Inc.) served as the substrate chromogen and haematoxylin was used as a counterstain. For the negative control, the primary antibody was applied to tissues of a normal mouse. In addition, the primary antibody was substituted with normal goat serum (DakoCytomation). There was no non-specific staining in these negative controls, except for the mast cells in the connective tissue, which showed endogenous peroxidase activity in their cytoplasmic granules. The viral titres of the brain, lung, liver, spleen and kidney of these mice were determined as described previously (Mase *et al.*, 2005b).

The results of the histopathological and immunohistochemical analyses are summarized in Table 1. In the respiratory organs, such as the lungs and nasal turbinates, severe damage and viral antigens were detected earlier in the MBV-A-infected mice than in those infected with the original strain (Table 1b; Fig. 1a–d). Viral antigen-positive alveolar cells appeared to be alveolar macrophages or type II alveolar epithelial cells. In the central nervous system (CNS), whereas viral antigens were detected in the MBV-A-infected mice at day 3 p.i., they were not detected in the original Ck/Yama/7/04-infected mice until day 6 p.i. (Table 1b; Fig. 1e). Lesions and viral antigens were only observed in the olfactory bulbs of the MBV-A strain-infected mice (Fig. 1f).

Viruses were recovered from all organs collected from the MBV-A strain-infected mice at day 3, whereas viruses were recovered only from the lung and spleen collected from the original virus-infected mice (Table 2). The virus titres of the variant virus-infected mice were higher than those of the original virus-infected mice. At day 6 p.i., viruses were recovered from all examined organs except the kidneys collected from MBV-A strain-infected mice.

The outbreaks beginning in 2003 of the highly pathogenic H5N1 avian influenza virus in many Asian countries, including Japan, have posed a very serious threat to public health. Ever since H5N1 viruses killed humans in 1997 for the first time in Hong Kong, this virus has often been transmitted from poultry to humans and has recently caused human deaths again in several countries (WHO, 2006). The molecular basis of the lethal virulence of the recent epidemic H5N1 viruses to humans in these countries is not understood completely, but in the case of Hong Kong H5N1 in

Table 1. Histopathological changes of and distribution of viral antigen-positive cells in sacrificed or dead mice at days 3 and 6 post-infection

(a) Lesions: –, no significant lesion; ±, minimum; +, mild; ++, intermediate; +++, severe. (b) No. viral antigen-positive cells: –, negative; ±, minimum; +, small; ++, intermediate; +++, large.

Tissue	Lesions (a)/antigen-positive cells (b)	Time post-infection (days)			
		3		6	
		Original	MBV-A	Original	MBV-A
(a) Histopathological changes					
Nasal turbinate	Rhinitis	—	+	—	+ ~ + +
Middle ear	Tympanitis	—	+	+	+
Lung	Bronchointerstitial pneumonia	± ~ + +	++	+ ~ + + +	+ + ~ + + +
Olfactory bulb	Degeneration/necrosis of neurons and glial cells	—	+	—	+
Cerebrum	Degeneration/necrosis of neurons and glial cells	—	+	—	+ ~ + +
Brainstem	Degeneration/necrosis of neurons and glial cells	—	—	+	+ ~ + +
Cervical spinal cord	Degeneration/necrosis of neurons and glial cells	—	—	+	+
Truncus sympathicus ganglion	Necrotic ganglionitis	—	—	+ ~ + +	+ + ~ + + +
Thoracic spinal cord	Degeneration/necrosis of neurons and glial cells	—	—	+	+ ~ + +
Lumbar spinal cord	Degeneration/necrosis of neurons and glial cells	—	—	—	+
Liver	Focal necrosis of hepatocytes	—	+	—	+
Spleen	Focal necrosis, hyperplasia of reticulum cells	+	+	—	—
(b) Distribution of viral antigen-positive cells					
Respiratory area of nasal cavity	Epithelium	± ~ +	++	± ~ +	++
Olfactory area of nasal cavity	Olfactory cells	± ~ +	++	± ~ +	+
Middle ear	Epithelium of tympanic cavity	—	+	++	++
Lung	Epithelium of bronchiolus, alveolar cells	+ ~ + +	+ + ~ + + +	+ ~ + +	++
Olfactory bulb	Neurons	—	+	—	+ ~ + +
Cerebrum	Neurons, glial cells, ependymal cells	—	+	—	+ ~ + +
Brainstem	Neurons, glial cells	—	—	+ ~ + + +	+ ~ + +
Cervical spinal cord	Neurons, glial cells, ependymal cells	—	+	+ ~ + +	+
Truncus sympathicus ganglion	Ganglion cells, satellite cells	—	+	++	++ +
Thoracic spinal cord	Neurons, glial cells, ependymal cells	—	±	++	+ ~ + + +
Lumbar spinal cord	Neurons, glial cells, ependymal cells	—	—	—	+
Liver	Hepatocytes	—	+	—	+
Adipose (fat) tissue	Fat cells	—	+	±	+

1997, the molecular basis of the high virulence was studied in the mouse model (Hatta *et al.*, 2001). By using reverse genetics, a Lys at residue 627 in the PB2 protein and polybasic cleavage site in HA were found to be crucial for the highly virulent and systematic replication of the A/Hong Kong/483/97 (H5N1) virus in mice. In particular, the presence of Lys leads to more aggressive virus replication, overwhelming the host's defence mechanisms and resulting in high mortality rates in mice (Gabriel *et al.*, 2005; Massin *et al.*, 2001; Naffakh *et al.*, 2000; Shinya *et al.*, 2004; Subbarao *et al.*, 1993). Interestingly, the Dutch H7N7 virus isolated in 2003 from humans had Lys at position 627 of the PB2 protein, whereas H7N7 in 2003 isolated from chickens maintained Glu at this position (Fouchier *et al.*, 2004). The PB2s of most human H1N1, H2N2 and H3N2 influenza viruses examined thus far possess Lys at position 627, whereas those of their progenitor avian viruses examined thus far all contain Glu at this position (Wright & Webster, 2001). Taken together, it was suggested that the substitution

of Glu to Lys at this position arises easily in transmission from birds to mammals.

Here, we demonstrated experimentally that the Japanese H5N1 viruses isolated in 2004 substituted their amino acid at position 627 of the PB2 protein from Glu to Lys after a single passage in mice, with increasing virulence. A variant virus containing Lys at position 627 replicated more rapidly than the original virus containing Glu at this position. This substitution may be a first step for adaptation to mammals, as human H1N1, H2N2 and H3N2 influenza viruses have Lys at position 627 of the PB2 protein (Wright & Webster, 2001). Comparing the MID₅₀ and MLD₅₀ of both the MBV-A and MBV-B viruses, the amino acid substitution at position 531 of the HA seemed not to be critical for high virulence. By pathological analysis of the MBV-A-infected mice, severe pneumonia was observed in the earlier phase (3 days p.i.) and encephalomyelitis was also observed at the later phase (6 days p.i.). Viruses were recovered from the internal organs of the MBV-A-infected mice, but the virus

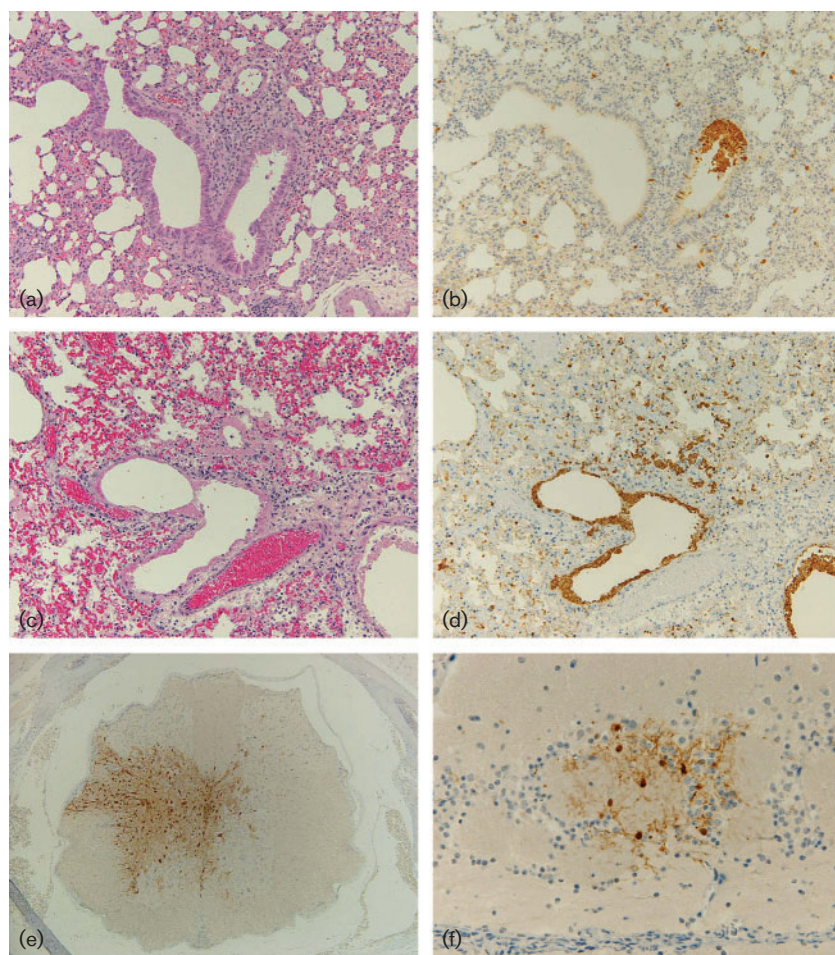


Fig. 1. Histological and immunohistochemical analyses of lung (a–d), thoracic spinal cord (e) and olfactory bulb (f) obtained from mice. (a) Lung; original Ck/Yama/7/04 strain-infected mouse at day 6 p.i. Mild bronchiointerstitial pneumonia is present. HE stain. (b) Lung; original Ck/Yama/7/04 strain-infected mouse at day 6 p.i. A small amount of viral antigen is present in the epithelium of the bronchiolus and alveolar cells. Immunoperoxidase labelling, haematoxylin counterstain. (c) Lung; MBV-A strain-infected mouse at day 6 p.i. Severe necrotizing bronchiolitis and diffuse alveolar damage can be seen. HE stain. (d) Lung; MBV-A strain-infected mouse at day 6 p.i. Abundant viral antigen is present in the epithelium of the bronchiolus and alveolar cells. Immunoperoxidase labelling, haematoxylin counterstain. (e) Thoracic spinal cord; original Ck/Yama/7/04 strain-infected mouse at day 6 p.i. Viral antigen is present in the neurons, glial cells and central canal ependymal cells. Immunoperoxidase labelling, haematoxylin counterstain. (f) Olfactory bulb; MBV-A strain-infected mouse at day 3 p.i. Viral antigen is present in the neurons. Immunoperoxidase labelling, haematoxylin counterstain. Magnification, $\times 20$ (a–d); $\times 10$ (e); $\times 40$ (f).

titres of the tissues at 6 days p.i. were lower than those at 3 days p.i., except in the brain. Moreover, histopathological analysis suggested that the viruses isolated from the kidney might be derived from adipose tissues around this organ. However, the appearance of mild lesions and the detection of viral antigens in the olfactory bulb in MBV-A-infected mice suggested rapid replication and spreading in this tissue. The routes of invasion of the HK483 strain into the CNS were suggested previously to be through afferent fibres of the olfactory, vagal, trigeminal and sympathetic nerves following replication in the respiratory mucosa (Park *et al.*, 2002).

The invasion of the MBV-A strain into the CNS in mice seemed to be facilitated strongly by these three routes compared with the original strain.

Although the Japanese H5N1 viruses were different in H5N1 genotype (genotype V) from the South-East Asian isolates of H5N1, including those from Thailand and Vietnam (genotype Z), the PB2 protein of Japanese H5N1 (Ck/Yama/7/04) was very similar (about 99.5%) to that of Thailand H5N1 (A/goose/Thailand/79/2004) at the amino acid level. In fact, many recent H5N1 isolates (genotype Z)

Table 2. Growth of original Ck/Yama/7/04 and MBV-A strains in mice

Virus titres in the organs shown were determined. Mean \pm SD is shown for the number of virus-positive mice that died or were sacrificed.

Time post-infection (days)	Virus	Virus titre [$\log_{10}(\text{EID}_{50} \text{ g}^{-1})$]				
		Brain	Lungs	Spleen	Liver	Kidneys
3	Original	0/3	3/3 (5.4 ± 1.0)	3/3 (4.3 ± 1.0)	0/3	0/3
	MBV-A	3/3 (4.4 ± 0.1)	3/3 (8.2 ± 0.6)	3/3 (4.8 ± 0.5)	3/3 (3.7 ± 1.2)	3/3 (4.7 ± 0.3)
6	Original	2/3 (4.0, 3.3)	3/3 (5.2 ± 1.9)	1/3 (3.3)	0/3	0/3
	MBV-A	3/3 (5.0 ± 0.9)	3/3 (6.4 ± 1.3)	3/3 (2.7 ± 0.3)	3/3 (2.4 ± 0.4)	0/3

from mammals have this substitution at position 627 of the PB2 protein (Govorkova *et al.*, 2005; Keawcharoen *et al.*, 2004; Puthavathana *et al.*, 2005). Urgent measures to deal with a possible pandemic, such as the development and application of effective vaccines and the stockpiling of anti-influenza drugs, are needed.

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