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Differential host gene responses in mice infected with two highly pathogenic avian influenza viruses of subtype H5N1 isolated from wild birds in Thailand

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ABSTRACT

In Thailand, highly pathogenic avian influenza (HPAI) viruses of subtype H5N1 had been isolated from various wild birds during the HPAI outbreak in poultries. In this study, we examined the pathogenicity of two wild bird isolates (A/Pigeon/Thailand/VSMU-7-NPT/2004; Pigeon04 and A/Tree sparrow/Ratchaburi/VSMU-16-RBR/2005; T.sparrow05) in mice. They showed similar replication in several organs and lethal outcome. However, on day 3 post-infection, Pigeon04 induced mRNA expression of proinflammatory cytokines (IL6 and TNFα) and MIP-2, neutrophil chemoattractant, in the lungs, resulting in severe pneumonia that was accompanied by neutrophil infiltration. In contrast, on day 7 post-infection, T.sparrow05 induced the expression of several cytokines to a greater extent than Pigeon04; it also potently induced mRNA expression of several cytokines in brains of the infected mice that triggered frequent inflammatory events. In sum, our study demonstrated that two HPAI viruses induced different host responses, despite having similar replications, resulting in lethal outcome in mice.

Introduction

In Thailand, there were three major outbreaks of highly pathogenic avian influenza (HPAI) subtype H5N1 in poultry during 2004–2005, followed by 4 sporadic ones up to 2007. During these outbreaks, H5N1 HPAI virus was isolated not only from poultry, but also from wild birds such as pigeon, tree sparrow and the open-bill stork (Siengsanan et al., 2009; Uchida et al., 2008). Wild bird surveillances of HPAI viruses during 2004–2007 in Thailand revealed that the HPAI viruses were detected in wild birds during the outbreaks and even after the goal of the eradication program in poultry had been accomplished in the area (Siengsanan et al., 2009).

Fatal HPAI viral infections have also been reported in mammalian species such as tigers, leopards, dogs, and cats (Amonsin et al., 2006; Keawcharoen et al., 2004; Songserm et al., 2006a; Songserm et al., 2006b). Such infections are thought to have occurred through ingestion of poultry meat or wild bird carcasses infected with the HPAI viruses (Amonsin et al., 2006; Keawcharoen et al., 2004; Songserm et al., 2006a,b). Human infections of the H5N1 HPAI virus in Thailand have also been reported (Chotpitayasunondh et al., 2005; Tiensin et al., 2005). In total, 25 human cases, 17 of which were fatal, have been reported during 2004–2006. Chotpitayasunondh et al. (2005) showed that most patients with HPAI had been exposed to infected poultry. These findings demonstrate sporadic interspecies transmission of the H5N1 HPAI viruses and imply that routine monitoring of not only poultries but also wild birds and domestic mammals is necessary for early detection as well as for preventing resurgence of the viruses.

Previous studies have shown that many patients infected with the H5N1 HPAI virus developed acute respiratory distress syndrome (ARDS), which is characterized by diffuse alveolar damage (DAD), lymphopenia, and multiple organ failure (MOF) (The Writing Committee of the World Health Organization Consultation on Human Influenza, 2005). Moreover, a typical finding in H5N1 infected patients is an aberrant level of proinflammatory cytokines and chemokines in the serum (de Jong et al., 2006; Ka-Fai et al., 2001). de Jong et al. (2006) reported that levels of proinflammatory

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cytokines and chemokines, such as IP-10, MIG, IL-8, IL-10, IL-6 and IFNγ in the serum of H5N1 infected patients increased significantly compared to those of H3N2 or H1N1 human influenza patients or healthy controls. It is noteworthy that serum levels of most of these cytokines in the fatal cases of H5N1 were significantly higher than those in the survivors; these phenomena correlated with the high viral load in pharyngeal specimens (de Jong et al., 2006). A high level of TNFα expression in the pneumocyte of an H5N1-infected patient compared with a patient who died from a non-infective illness was reported (Peiris et al., 2004). Apoptosis was detected in alveolar epithelial cells or leukocytes in lung specimens of H5N1-infected patients (Uiprasertkul et al., 2007). In animal models, virulent H5N1 HPAI viral infection caused depletion of lymphocytes in the blood, lungs, and lymphoid tissues as well as apoptosis of lymphocytes in the lungs (Tumpey et al., 2000). Thus, apoptosis is thought to contribute to lymphopenia that is typically observed in H5N1 HPAI-infected patients (Korteweg and Gu, 2008; Tumpey et al., 2000).

Pathogenicity studies in animal models are useful tools for better understanding of the diseases caused by influenza viruses in humans. Previous studies have demonstrated that ARDS, high production of cytokine and apoptosis are found in lung tissue of mouse infected with the HPAI virus, suggesting that mouse is a suitable model for evaluating the pathogenesis of HPAI virus in humans (Perrone et al., 2008; Tumpey et al., 2000; Xu et al., 2006).

Wild waterfowls are thought to act as a natural reservoir of influenza A viruses, and these viruses do not usually cause fatalities in wild birds. However, in China, unexpected H5N1 HPAI outbreaks in wild migratory waterfowls occurred at Qinghai Lake in 2005, and thousands of migratory birds were found dead due to the viral infection, suggesting that H5N1 HPAI viruses could cause fatal infections in wild birds (Chen et al., 2005). Very importantly, Gilsdorf et al. (2006) suggested that HPAI viruses were transmitted to humans following close contact or the process of de-feathering infected wild swans. There is report that these wild bird isolates were pathogenic to not only chickens but also mice (Chen et al., 2006; Zhou et al., 2006). Songserm et al. (2006a) showed that a domestic cat was infected with an H5N1 HPAI virus due to eating a pigeon carcass infected with HPAI viruses in Thailand. HPAI viruses in wild birds have been suggested as possible potential threats not only to poultry but also to the public health of humans (Gilsdorf et al., 2006).

In this study, we examined the pathogenic characters of two H5N1 HPAI viruses isolated from wild birds during HPAI outbreaks among poultries in Thailand. One is a HPAI virus isolated from dead pigeon (Pigeon04), and the other is a HPAI virus isolated from live tree sparrow (T.sparrow05). Interestingly, these two HPAI viruses exhibited different levels of virulence *in vivo* in chickens and different growth properties *in vitro* in MDCK cells. In contrast, they showed similar replications *in vivo* in the lungs and brain of the infected mice, resulting in fatal outcome. Thus, to determine whether the differences of the replication profiles of two HPAI viruses between *in vitro* and *in vivo* were related to alterations of host gene responses in the lungs and brain infected with these two viruses, we compared host responses, particularly in host gene responses in the lungs and brain of mice infected with the two HPAI viruses.

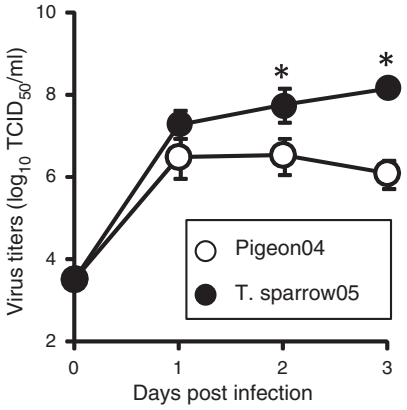

Fig. 1. Growth properties of two H5N1 HPAI viruses in MDCK cells. MDCK cells were infected with the HPAI viruses at a MOI of 0.01 $TCID_{50}$ (3.7 log_{10} $TCID_{50}$/ml). Supernatants were collected on days 1, 2 and 3 post-infection, and virus titers were determined on MDCK cells by $TCID_{50}$ assay. The data were indicated as mean virus titers ± standard deviations of three independent experiments. Asterisks indicate statistically significant differences (*$p < 0.05$ by Student t test).

Results

Characteristics of two H5N1 HPAI viruses isolated from wild birds

We isolated two HPAI viruses from wild birds during routine surveillance during the outbreak of HPAI viruses in Thailand in 2004–2005. A/Pigeon/Thailand/VSMU-7-NPT/2004 (Pigeon04) was isolated from a dead pigeon in Nakhon Pathom province in 2004, and A/Tree sparrow/Ratchaburi/VSMU-16-RBR/2005 (T.sparrow05) was from a clinically healthy tree sparrow in Ratchaburi province in 2005. We determined nucleotide sequences of protein-coding regions of all eight gene segments of Pigeon04 (AB576199-AB576206). All eight gene segments of T.sparrow05 were also sequenced in this study to find a mix population (A/G) at position 415 in NS1 compared to the sequenced done by Uchida et al. This was submitted to the Genbank (AB576207). Pigeon04 and T.sparrow05 had more than 99% and 97% identities in nucleotide and amino acid sequences, respectively. Phylogenetic analysis of the HA gene showed that these two viruses belong to clade 1 of the classification system (WHO/OIE/FAO H5N1 Evolution Working Group 2007) (Table 1, data not shown). The two viruses had similarly high infectivity titers in embryonated eggs and MDCK cells with titers, ranging from 7.6 to 7.9 $log_{10}EID_{50}$/mL and 7.1 to 7.2 log_{10}PFU/mL, respectively (Table 1). Interestingly, the plaque size of the two HPAI viruses varied. Pigeon04 produced mostly pinpoint plaques, whereas T.sparrow05 produced medium to large plaques, indicating different growth properties in the cultured cells *in vitro* between the two viruses (Table 1). Therefore, to assess the growth properties of two viruses, we examined viral growth kinetics of these viruses in MDCK cells. As shown in Fig. 1, T.sparrow05 robustly replicated in MDCK cells on days 2 and 3 post-infection, and the mean virus titers were 7.8 and 8.2 $log_{10}TCID_{50}$/mL, respectively. In

Table 1
Characteristics of highly pathogenic avian viruses used in this study.

Virus	Abbreviation	Date of specimen collection	Province	Status of host	H5 HA gene clade	Log_{10} EID_{50}/mL	Log_{10} PFU/ml in MDCK cells	Plaque size in MDCK cells[a]	MLD_{50} (log_{10} EID_{50})
A/Pigeon/Thailand/VSMU-7-NPT/2004	Pigeon04	13/2/2004	Nakornpatom	Dead	1	7.6	7.2	Pinpoint (76%) Medium (24%)	0.8
A/Tree sparrow/Ratchaburi/VSMU-16-RBR/2005	T.sparrow05	3/5/2005	Ratchaburi	Live	1	7.9	7.1	Pinpoint (6%) Medium (72%) Large (22%)	2.2

Pinpoint plaque, <1 mm in diameter; medium plaque, 1–2 mm in diameter; large plaque, >2 mm in diameter.
[a] Plaque size was measured in MDCK cells infected with HPAI viruses at 3 day post-infection.