Pathogenicity of the Novel A/H7N9 Influenza Virus in Mice

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ABSTRACT A novel avian-origin influenza A/H7N9 virus infecting humans was first identified in March 2013 and, as of 30 May 2013, has caused 132 human infections leading to 33 deaths. Phylogenetic studies suggest that this virus is a reassortant, with the surface hemagglutinin (HA) and neuraminidase (NA) genes being derived from duck and wild-bird viruses, respectively, while the six “internal gene segments” were derived from poultry H9N2 viruses. Here we determine the pathogenicity of a human A/Shanghai/2/2013 (Sh2/H7N9) virus in healthy adult mice in comparison with that of A/chicken/Hong Kong/HH8/2010 (ck/H9N2) virus, highly pathogenic avian influenza (HPAI) A/Hong Kong/483/1997 (483/H5N1) virus, and a duck influenza A H7N9 virus of different genetic derivation, A/duck/Jiangxi/3286/2009 (dk/H7N9). Intranasal infection of mice with Sh2/H7N9 virus doses of 10^3, 10^4, and 10^5 PFU led to significant weight loss without fatality. This virus was more pathogenic than dk/H7N9 and ck/H9N2 virus, which has six internal gene segments that are genetically similar to Sh2/H7N9. Sh2/H7N9 replicated well in the nasal cavity and lung, but there was no evidence of virus dissemination beyond the respiratory tract. Mice infected with Sh2/H7N9 produced higher levels of proinflammatory cytokines in the lung and serum than did ck/H9N2 and dk/H7N9 but lower levels than 483/H5N1. Cytokine induction was positively correlated with virus load in the lung at early stages of infection. Our results suggest that Sh2/H7N9 virus is able to replicate and cause disease in mice without prior adaptation but is less pathogenic than 483/H5N1 virus.

IMPORTANCE An H7N9 virus isolate causing fatal human disease was found to be more pathogenic for mice than other avian H9N2 or H7N9 viruses but less pathogenic than the highly pathogenic avian influenza virus (HPAI) H5N1. Similarly, the ability of Sh2/H7N9 to elicit proinflammatory cytokines in the lung and serum of mice was intermediate to ck/H9N2 and dk/H7N9 on the one hand and HPAI H5N1 on the other. These findings accord with the observed epidemiology in humans, in whom, as with seasonal influenza viruses, H7N9 viruses cause severe disease predominantly in older persons while HPAI H5N1 can cause severe respiratory disease and death in children and young adults.
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### RESULTS

Pathogenesis of the H7N9 and H9N2 viruses in mice. To examine the pathogenicity of human H7N9 viruses in an in vivo model, we intranasally infected 6- to 8-week-old healthy female BALB/c mice (six mice in each group) with doses ranging from $1 \times 10^2$ to $1 \times 10^3$ PFU of the A/Shanghai/2/2013 (H7N9) (Sh2/H7N9) virus isolated from a human patient with fatal infection (1). As controls, A/duck/Jiangxi/3286/2009 (H9N2) (dk/H7N9), a virus with genetic makeup different from that of Sh2/H7N9, A/chicken/Hong Kong/H188/2010 (H9N2) (ck/H9N2), which has an “internal gene” constellation genetically similar to that of Sh2/H7N9, and A/chicken/Hong Kong/483/1997 (H5N1) (483/H5N1) were infected in parallel. The differences in genetic sequence at some of the key amino acid positions between the viruses used in this study are shown in Table 1. Mice inoculated with the viruses were monitored for 14 days for signs of illness, weight loss, and mortality. We found that the mice infected by the Sh2/H7N9 virus showed signs of illness and weight loss starting from 2 days postinfection (Fig. 1). The mice showed around a 15% loss of weight, while mice of illness and weight loss starting from 2 days postinfection found that the mice infected by the Sh2/H7N9 virus showed signs of illness, weight loss, and mortality. We carried out experimental infections of mice with an isolate from a human patient with fatal disease, A/Shanghai/2/2013. Mortality, weight loss, virus titers, and cytokine induction in the lungs and evidence of virus dissemination were determined. Other viruses were used for comparison.

#### Table 1: Comparison of the selected amino acids with the A/Shanghai/2/2013 (Sh2/H7N9) virus

| Virus (abbreviation) | Amino acid(s) at each position | PB2(627) | HA(138) | HA(160) | HA(226) | NS1(42) | NS (PDZ domain) |
|----------------------|--------------------------------|----------|---------|---------|---------|--------|-----------------|
| A/Shanghai/2/2013    | K A T Q S EPEV                  |          |         |         |         |        |                 |
| (Sh2/H7N9)           |                                |          |         |         |         |        |                 |
| A/Dk/TX/3286/2009    | E A A Q S ESEV                 |          |         |         |         |        |                 |
| (dk/H7N9)            |                                |          |         |         |         |        |                 |
| A/Ck/HK/HH8/10       | E S A L S Deletion             |          |         |         |         |        |                 |
| (ck/H9N2)            |                                |          |         |         |         |        |                 |
| A/A/HK/483/97 H5N1   | E A A Q S ESEV                 |          |         |         |         |        |                 |
| (483/H5N1)           |                                |          |         |         |         |        |                 |
| A/Shanghai/2/2013    | K A T Q S EPEV                  |          |         |         |         |        |                 |
| (Sh2/H7N9)           |                                |          |         |         |         |        |                 |
| A/Dk/TX/3286/2009    | E A A Q S ESEV                 |          |         |         |         |        |                 |
| (dk/H7N9)            |                                |          |         |         |         |        |                 |
| A/Ck/HK/HH8/10       | E S A L S Deletion             |          |         |         |         |        |                 |
| (ck/H9N2)            |                                |          |         |         |         |        |                 |
| A/A/HK/483/97 H5N1   | E A A Q S ESEV                 |          |         |         |         |        |                 |
| (483/H5N1)           |                                |          |         |         |         |        |                 |

a H3 numbering.

Viral replication of H7N9, H9N2, and H5N1 viruses in mice. Sh2/H7N9, ck/H9N2, dk/H7N9, and HPAI A/HK/483/97 (483/H5N1) viruses were compared. Mice were infected intranasally with a dose of $10^5$ PFU, with the exception of H5N1 virus, for which a dose of two 50% lethal doses (LD$_{50}$) (around $10^3.7$ PFU) was used because this virus is rapidly lethal for mice. Sh2/H7N9 virus replicated to higher titers in the lung than did ck/H9N2 virus at both day 3 and day 5 postinfection and to higher titers than dk/H7N9 at day 3 postinfection. However, the virus titers were lower than those for 483/H5N1 (Table 2). Interestingly, although dk/H7N9 did not cause dramatic weight loss in the mice, it still replicated well in the nasal cavity and lung of the mice, but with a slower replication kinetic in the lung. Among the tested virus strains, ck/H9N2 replicated the least well, although it possesses 6 internal gene segments of similar genetic derivation to those of Sh2/H7N9 virus. With the exception of 483/H5N1, we did not observe any systemic spread of virus from the mice infected by either human or avian H7N9 virus or the ck/H9N2 virus. No virus titers were detected at 8 days postinfection in either H7N9 or H9N2 virus, suggesting that mice can clear the virus effectively.

To further investigate the replication efficiency among the viruses we used in this study, MDCK cells were infected by Sh2/H7N9, dk/H7N9, and ck/H9N2 at a multiplicity of infection (MOI) of 0.01 and infectious virus titer was measured by 50% tissue culture infective dose (TCID$_{50}$) titrations at 24, 48, and 72 h postinfection. The virus titer of Sh2/H7N9 was higher than those of the other two virus strains at all time points (see Fig. S1 in the supplemental material).

Cytokine induction by H7N9, H9N2, and H5N1 viruses in experimentally infected mice. It has been shown that cytokine dysregulation is associated with the pathogenicity of H5N1 virus, as shown by clinical, in vivo (mice and ferret), and in vitro models. To better understand the cytokine responses of mice infected by Sh2/H7N9 virus, we analyzed 10 proinflammatory cytokines in the lungs and serum from the mice infected by different viruses. Compared to dk/H7N9 and ck/H9N2 viruses, lungs from the mice infected by Sh2/H7N9 exhibited higher levels of the proinflammatory cytokines tumor necrosis factor alpha (TNF-α), macrophage inflammatory protein 1α/β (MIP-1α/β), RANTES, monocyte chemotactic protein 1 (MCP-1), MCP-3, alpha interferon (IFN-α), gamma interferon (IFN-γ)-induced protein 10 (IP-10), and...
FIG 1  Weight change of the mice infected with A/Shanghai/2/2013 (H7N9) compared with other avian H9N2 and duck H7N9 viruses. Female BALB/c mice were infected intranasally with $10^5$ PFU (A), $10^4$ PFU (B), or $10^3$ PFU (C) of the Sh2/H7N9, dk/H7N9, or ck/H9N2 virus. The virus-infected mice were monitored for 14 days, and the weight was determined daily. Results from each group and each time point are expressed as means ± standard deviations (SD) of six infected mice. *, $P < 0.05$. 

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KC at days 3 and 5 postinfection (Fig. 3 and Fig 4). The 483/H5N1 virus, which is lethal to mice, induced higher KC, MCP-3 and MCP-1, RANTES, and TNF-α levels than Sh2/H7N9, even though the infecting dose was lower.

We next investigated whether the higher cytokine induction by Sh2/H7N9 than by dk/H7N9 and ck/H9N2 is related to its increased replication competence in the lung of mice. Viral TCID<sub>50</sub> titers were positively correlated with cytokine protein levels (IP-10, TNF-α, and MIP-1β) at day 3 postinfection (Fig. 3). However, there was no significant correlation between replication and cytokine levels at day 5 postinfection (data not shown).

The levels of cytokine in the serum were also investigated. A high level of IP-10, which is comparable to those in mice infected by the H5N1 virus, was detected in Sh2/H7N9-infected mice. Significantly higher levels of MCP-1, MCP-3, KC, and IFN-α were detected in the serum of H5N1-infected mice than in that of mice infected with the Sh2/H7N9, dk/H7N9, and ck/H9N2 viruses (Fig. 5).

FIG 2  Histology and immunohistochemistry of the mice infected with Sh2/H7N9 compared with dk/H7N9 virus. (A and B) Histology of lung sections stained by hematoxylin and eosin from mice infected with Sh2/H7N9 (A) or dk/H7N9 (B) at 5 days postinfection. (C and D) Immunohistochemical detection of virus nucleoprotein in lungs from mice infected with Sh2/H7N9 (C) or dk/H7N9 (D) at 5 days postinfection. Arrows indicate positively stained lung alveolar epithelial cells. Magnification, ×10.

DISCUSSION
In this study, we have shown that the human Sh2/H7N9 virus is more pathogenic in mice than dk/H7N9 and ck/H9N2 viruses but less pathogenic than HPAI 483/H5N1. None of these viruses were lethal to mice, in contrast to infection with 483/H5N1 virus, which was uniformly fatal to mice even when administered at a lower dose (dose of 10<sup>2.7</sup> PFU). Both the Sh2/H7N9 and dk/H7N9 viruses replicate effectively in the upper (nasal) and lower (lung) respiratory tract of mice, infecting alveolar epithelial cells, but only Sh2/H7N9 appears to cause significant weight loss. Higher levels of proinflammatory cytokines were detected from the lung and serum of the human Sh2/H7N9-infected mice than from those of mice infected with dk/H7N9 and ck/H9N2 viruses. Cytokine induction by 483/H5N1 in the lung and in serum was higher than that induced by Sh2/H7N9, even though the infecting dose of H5N1 virus was lower than that used for Sh2/H7N9 virus. Overall, the differences in cytokine induction may be correlated...
with efficiency of virus replication, at least at day 3 postinfection (Fig. 3).

The viral determinants of human pathogenicity of H7N9 virus remain to be clarified. It is likely that PB2-E627K contributes to mammalian host adaptation and pathogenicity of Sh2/H7N9. An E627K substitution in the PB2 gene has been identified in many human virus isolates, and a number of studies have shown that this mutation is associated with increased virulence of avian influenza viruses for mammals (6–11). Although some of the recent H7N9 human isolates still retain 627E, the occurrence of the PB2-E627K mutation in these viruses may compensate as the mammalian host adaptation mutation in these viruses (12, 13). The D701N mutation in these viruses may compensate as the mammalian host adaptation mutation in these viruses (12, 13). The mutation at HA-Q226L of the virus may also contribute to pathogenicity in humans, as this change increases the affinity of the receptor is the dominant sialic acid species in the respiratory tract.

Human H7N9 virus originated from multiple reassortment events (16), with the HA and NA genes originating from duck and migratory bird viruses, respectively, and the six internal genes originating from two different groups of H9N2 avian strains. We found that the purely avian H7N9 virus (dk/H7N9) can replicate effectively in mice, although with minimal symptoms. Thus, our results with dk/H7N9 virus suggest that the transmission of the duck H7 precursor virus is unlikely to be pathogenic in mammals. The lack of PB2-K627 may contribute to this difference in phenotype. Moreover, we have shown that an H9N2 virus which possesses an internal gene segment constellation similar to that of Sh2/H7N9 also failed to exhibit a pathogenic phenotype comparable to that of Sh2/H7N9 in mice. Whether the surface proteins of human H7N9 contribute to the pathogenicity still needs to be further investigated.

Previous data suggested that cytokine dysregulation is a contributory factor in H5N1-related disease pathogenesis (17–20). One study has shown high serum IL-10 levels in a patient severely ill with H7N9 disease (2). Our results showed that mice infected with Sh2/H7N9 induced a range of proinflammatory cytokines in the lung and serum, although their clinical relevance remains unclear. We found that the level of cytokines induced at an early stage of infection (day 3 postinfection) is positively correlated to virus replication, implying that the higher cytokine levels with Sh2/H7N9 may be a reflection of more-efficient virus replication. 483/H5N1 virus appeared even more potent in eliciting proinflammatory cytokine responses than Sh2/H7N9 virus, even though the infecting dose was 1.3 log10 lower. Studies on H5N1 virus have demonstrated that blocking of key proinflammatory cytokines, such as TNF-α or IP-10, or its signaling pathways reduces the severity of the virus-infected mammalian hosts (21–23).

In view of its apparent lethality in humans, it was surprising that Sh2/H7N9 virus was not lethal to mice, even at the highest challenge dose (106 PFU), and there was no dissemination of virus beyond the respiratory tract, in contrast to 483/H5N1 virus. The epidemiological differences in the age distributions of H7N9 and H5N1 disease in humans, with H7N9 being lethal predominantly for older adults rather than young adults, may be a reflection of the lower virulence of H7N9 than H5N1 virus. The median age of H7N9-infected patients is around 63 years, and 46% of 82 cases occurred in persons 65 years of age or older (3). The lower virulence in mice may suggest that H7N9 infection may lead to mild self-limited disease in children and young adults. Sh2/H7N9 infection of older mice may be instructive in this regard, as will human-population-based seroepidemiological studies, to ascertain whether mild H7N9 infections in children and young adults are common in affected areas.

### MATERIALS AND METHODS

**Viruses and cells.** A/Shanghai/2/2013 (H7N9) (Sh2/H7N9) virus was initially isolated from a patient with a fatal disease. The virus isolate had two passages in Madin-Darby canine kidney (MDCK) cells and one subsequent passage in the allantoic cavity of embryonated eggs and was the virus used in the experiments. The sequences of the virus were confirmed by resequencing of the entire genome using a Genome Sequencer Junior (Roche) to be identical to the initially published sequence (1). The control viruses A/duck/IX/3286/2009 (H7N9) (dk/H7N9) and ck/HK/H8/10 (H9N2) (ck/H9N2) were also passaged in the allantoic cavity of embryonated eggs twice, while A/HK/483/97 H5N1 was passaged in Madin-Darby canine kidney (MDCK) cells as previously described (17). All procedures involving live H7N9 viruses were carried out in a biosafety level 3 facility at the University of Hong Kong.
Experimental infection of mice. Specific-pathogen-free female BALB/c mice (6 to 8 weeks old) were infected with 1 × 10^5 PFU of each virus (except that a dose of 1 × 10^3 PFU was used for the H5N1 virus) in a volume of 25 μl intranasally and monitored daily for weight loss. In addition, a dose titration experiment of Sh2/H7N9 in mice was done with infectious doses of 1 × 10^4, 1 × 10^3, and 1 × 10^2 PFU virus. The mice were sacrificed at the indicated days postinfection for virological and cytokine assays. The lung, brain, and liver were homogenized in 1 ml phosphate-
FIG 4 Cytokine responses in the lungs of mice infected with A/Shanghai/2/2013 (H7N9) virus or other avian H9N2, duck H7N9, and highly pathogenic avian influenza H5N1 viruses. Cytokine levels of MCP-1 (A), MCP-3 (B), KC (C), IFN-α (D), MIP-1α (E), IFN-γ (F), and RANTES (G) from virus-infected lungs (n = 3 mice per virus group; days 3, 5, and 8 postinoculation) were measured individually by the FlowCytomix system. Results from each time point are expressed as means ± SD of three infected mice. *, P < 0.05.
FIG 5  Serum cytokine responses of A/Shanghai/2/2013 (H7N9) virus compared with those of avian H9N2, duck H7N9, and highly pathogenic avian influenza H5N1 viruses. Cytokine levels of IP-10 (A), TNF-α (B), MCP-1 (C), MCP-3 (D), IFN-γ (E), MIP-1β (F), KC (G), and IFN-α (H) from virus-infected lungs (n = 3 mice per virus group; days 3 and 5 postinoculation) were measured individually by the FlowCytomix system. Results from each time point are expressed as means ± SD of three infected mice. *, P < 0.05.
buffered saline (PBS). A total of 200 μl of PBS was used for the nasal wash. All animal procedures were carried out under institutionally approved protocols.

**Quantitative analysis of cytokines.** Expression levels of IFN-α, TNF-α, MIP-1α, MIP-1β, MCP-1, MCP-3, RANTES, IFN-γ, KC, and IP-10 in the lung homogenates and blood serum were quantitatively determined by flow cytometry-based immunoassay (FlowCytomix Multiplex; Bender MedSystems). In brief, both lung homogenates and blood serum were collected at day 3, day 5, and day 8 postinfecion. A total of 25 μl of each sample was processed according to the manufacturer’s protocol. The amount of cytokine (pg/ml) in the samples was acquired on a BD LSRII (BD BioScience) and was calculated by FlowCytomix Pro 2.3 software (Bender MedSystems).

**Virus titration.** The virus stock used for infection of mice was titered in a plaque-forming assay on MDCK cells. The quantification of virus in the homogenates of mouse organs was titered on MDCK cells, and the titers were reported as tissue culture infectious dose units per 100 μl (TCID₅₀/100 μl).

**Collection of serum.** Virus-infected or mock-infected mice were deeply anesthetized with ketamine (80 mg/kg of body weight) and xylazine (15 mg/kg), and 0.7 ml of blood was collected through intracardiac puncture, followed by subsequent euthanization. The blood was allowed to clot, and the serum was separated by centrifugation and stored at −80°C.

**Histology and immunohistochemistry of mouse lung.** Lung tissues from virus-infected mice were fixed in 10% neutral buffered formalin for at least 24 h before processing. The tissues were embedded in paraffin by standard tissue processing procedures, and sections were cut at 4 μm and affixed on glass slides. Standard hematoxylin and eosin staining was carried out. Immunohistochemical staining of NP antigens in the lungs was performed by using antibody HB65 (European Veterinary Laboratories) according to our previously published protocol (24).

**Statistical analysis.** Statistical significance of differences between experimental groups was determined by using Student’s t test, assuming a normal distribution of the underlying data. P values of <0.05 were considered significant. Spearman’s correlation coefficient (r) was calculated between certain cytokine levels and viral load.

### SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl/doi:10.1128/mBio.00362-13/-/DCSupplemental.

Figure S1, TIF file, 0.5 MB.

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