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Avian influenza virus isolates from wild birds replicate and cause disease in a mouse model of infection

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ABSTRACT

The direct transmission of highly pathogenic avian influenza (HPAI) viruses to humans in Eurasia and subsequent disease has sparked research efforts leading to better understanding of HPAI virus transmission and pathogenicity in mammals. There has been minimal focus on examining the capacity of circulating low pathogenic wild bird avian influenza viruses to infect mammals. We have utilized a mouse model for influenza virus infection to examine 28 North American wild bird avian influenza virus isolates that include the hemagglutinin subtypes H2, H3, H4, H6, H7, and H11. We demonstrate that many wild bird avian influenza viruses of several different hemagglutinin types replicate in this mouse model without adaptation and induce histopathologic lesions similar to other influenza virus infections but cause minimal morbidity. These findings demonstrate the potential of wild avian influenza viruses to directly infect mice without prior adaptation and support their potential role in emergence of pandemic influenza.

Introduction

The 1997 outbreak of highly pathogenic H5N1 avian influenza (HPAI) virus in humans marked the beginning of intense investigation into the pathogenesis of human infections with avian influenza viruses (AIV) (Subbarao et al., 1998). These "novel" AIVs are of concern not only because of the severity of disease observed but also because they have a pandemic potential (Li et al., 2004; Maines et al., 2005). Since the 1997 H5N1 outbreak, many other examples of human infections with AIVs have occurred. These outbreaks have also demonstrated that other hemagglutinin subtypes of AIVs are capable of direct human infection, such as H7 and H9, with varying morbidity and mortality (Belser et al., 2009; Butt et al., 2005; Fouchier et al., 2004; Guo, Li, and Cheng, 1999; Koopmans et al., 2004). In cases of H5N1 infections, disease in humans has been geographically and temporally associated with a HPAI outbreak in poultry (Beigel et al., 2005). Human infections with H7 and H9 AIV subtypes have also most frequently been attributed to transmission from poultry (Belser et al., 2009; Butt et al., 2005). The mechanism of how AIVs are capable of directly infecting humans and other mammals is multifactorial, including differences in hemagglutinin receptor specificity and polymerase activity, and is still being elucidated (Gabriel et al., 2007; Gambaryan et al., 2006; Labadie et al., 2007; Li et al., 2005; Rogers and Paulson, 1983; Thompson et al., 2006; van Riel et al., 2007). These studies have primarily focused on H5N1 virus isolates, and there is minimal information available on the abundant other AIVs that exist in nature.

Direct transmission of AIV from birds is not unique to humans but also observed in other mammals not traditionally considered susceptible to influenza, such as domestic cats and dogs, wild cats, and seals (Geraci et al., 1982; Hinshaw et al., 1984; Keawcharoen et al., 2004; Klingeborn et al., 1985; Klopfleisch et al., 2007; Song et al., 2008; Songserm et al., 2006a, 2006b). Again, a range of avian influenza hemagglutinin subtypes have been demonstrated to infect other mammals, including H4, H5, H6, H7, H9 and H10, by natural or experimental infection. Investigators are finding increasingly more avian influenza subtypes that can productively infect mammals resulting in varied morbidity (Belser et al., 2007; Dybing et al., 2000; Gillim-Ross et al., 2008; Hinshaw et al., 1981; Joseph et al., 2007; Rigoni et al., 2007; Wan et al., 2008). The mouse model for influenza infection has been widely used in studies of human and avian influenza to elucidate virulence and pathogenesis of these viruses (Fislova et al., 2009; Lu et al., 1999; Tumpey et al., 2007; Ward, 1997). Pathogenicity studies have primarily focused on HPAI isolates that have been involved in human infections, with only a small number of studies examining low pathogenic avian influenza (LPAI) isolates from poultry and even fewer isolates examined from wild birds (Gillim-Ross et al., 2008; Henzler et al., 2003; Joseph et al., 2007; Wan et al., 2008). Wild birds are the reservoirs for all influenza subtypes and recently have been implicated in the spread of HPAI H5N1 (Cattoli and Capua, 2007; Keawcharoen et al., 2008; Li et al., 2004; Olsen et al., 2006; Stallknecht and Brown, 2007). Examining the

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behavior of a large variety of wild bird avian influenza isolates in the mouse model is a step towards deeper understanding of the risks and mechanisms of avian influenza infections in mammals.

All pandemic influenza viruses since 1918, including the 2009 H1N1 virus have at least an AIV component (Glaser et al., 2005; Matrosovich et al., 2000; Stevens et al., 2006). There is a possibility that the next pandemic influenza precursor is circulating in the wild bird population. In this study, the mouse model is utilized for influenza to examine a large variety of AIVs isolated from wild birds from influenza surveillance in the United States. Results demonstrate that many of these isolates can replicate in the lung of mice and induce pulmonary lesions with minimal morbidity. For some isolates, virus was localized with immunohistochemistry and demonstrated robust replication in respiratory epithelial tissues. Serology after infection showed that immunogenicity was variable and unrelated to replication or pathology. These data support the idea that humans and other mammals may be directly infected with wild bird AIVs, in some cases subclinically and without seroconversion, providing a potential avenue for emergence of influenza viruses with pandemic potential via mutation and/or reassortment.

Results

Wild bird avian influenza viruses replicate robustly in mice

As a primary in vitro screen, more than 400 wild bird avian influenza virus isolates were screened for replication in MDCK cells by plaque assay. As plaquing in cell culture in the absence of exogenous trypsin has been shown to indicate the potential for increased pathogenicity for avian influenza viruses (Rimmelzwaan et al., 2006; Zitzow et al., 2002) and the 1918 influenza virus was shown to plaque to a high titer in culture without addition of trypsin (Tumpey et al., 2005), wild bird isolates were tested for replication in MDCK cells in the presence and absence of trypsin. While this phenotype is not associated with infectivity in mice, it provided a measure of infectivity in a mammalian culture system and a means to select for potentially pathogenic viruses. The MDCK plaque assay identified 114 viruses that replicated to high titer in MDCK cells and most isolates lost less than 20 fold of the titer the absence of exogenous trypsin (data not shown). Twenty-eight of these viruses were selected for in vivo screening.

Wild bird isolates selected for *in vivo* replication analysis covered six hemagglutinin (HA) subtypes and four known avian species (Table 1). Replication of each isolate in mice was categorized as: "No replication" (no virus was detected by $TCID_{50}$ assay from lung samples), "poor replication" (virus detected from lung samples was low titer and/or only present in mice inoculated with a high concentration inoculum), and "efficient replication" (virus detected from lung samples was high titer and present in mice inoculated with both concentrations of virus). Many of the isolates (71%) were able to replicate in the mouse lung when assessed on day 4 post-inoculation (p.i.) by $TCID_{50}$ assay, where 29% of these isolates showed efficient replication (Table 1). Lung titers in individual mice from isolates that demonstrated efficient replication are shown in Fig. 1. Inoculum concentration was not standardized as viruses only grew to a moderate titer and repeated passage of virus in embryonated chicken eggs (ECE) to derive a high-titer stock was specifically avoided. Despite this lack of standardization, the wide range of inoculum concentrations (as low as 5.5E+02 PFU/mouse and as high as 3.5E+05 PFU/mouse) between isolates that had efficient replication in the mouse lung did not appear to be associated with the magnitude of $TCID_{50}$ virus titer from the lung on day 4 p.i. (Fig. 1). Virus was not detected in mock infected mice via $TCID_{50}$ assay.

Three virus isolates that exhibited efficient replication in the mouse lung were further selected for detailed pathogenesis studies (viruses RT/645, RT/625, and RK/470). These particular isolates were selected to diversify the HA subtypes examined. A 50% mouse infectious dose (MID_{50}) was elucidated for each of these three selected isolates. The MID_{50} was similar for RT/645 (2.5E+03 PFU/mouse) and RT/625 (1.2E+03 PFU/mouse) but much lower for RK/470 (3.0E+01 PFU/mouse).

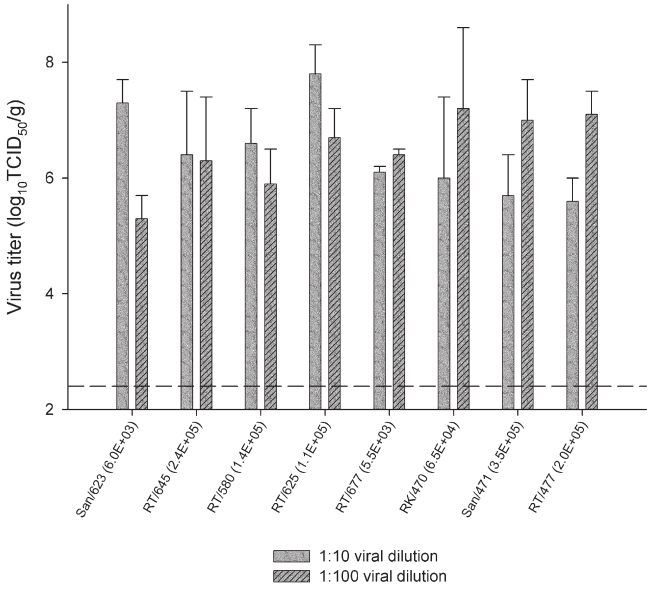

Fig. 1. Wild aquatic bird influenza virus isolates replicate to high titer in mice without adaptation. Groups of two bars represent a single isolate that replicated efficiently in mice (from Table 1) inoculated i.n. at two dilutions (1:10 and 1:100) with lung collected and titrated at day 4 p.i. in MDCK cells. The concentration of the inoculum for the 1:10 dilution is listed after the isolate name (expressed in PFU/mouse). Each bar represents the average lung titer for a group of four mice. The dashed line is the limit of detection (2.4 $log_{10}TCID_{50}/g$).

Wild bird avian influenza virus infection exhibits low pathogenicity in mice despite robust replication in mouse lung

Clinical signs in mice inoculated with $20MID_{50}$ of RT/645, RT/625, or RK/470 were mild and included slightly ruffled fur on days 1 and 3 post-inoculation. Similar clinical signs were observed on day 1 p.i. in mock infected mice; these clinical signs were attributed to anesthetic recovery. Clinical signs in X31 inoculated mice included ruffled fur and lethargy on days 3 and 5 post-inoculation. Mice infected with RT/645 and RK/470 exhibited more weight loss than mice infected with RT/625 and mock infected control mice on day 2 p.i. and weight gain remained poor in mice infected with RK/470 on day 3 p.i. (Fig. 2). Despite these differences, mice infected with RT/625, RT/645 and RK/470 exhibited overall minimal weight loss early p.i. that was not significantly different from mock infected control mice by a Student's *t* test. In comparison, mice infected intranasally with X31 and monitored for weight loss for other studies in our laboratory exhibit approximately 15–20% peak weight loss on day 5 post-inoculation. One PBS inoculated mouse was found to be an outlier by Dixon's *Q* test based on weight loss data and dropped from the group.

Virus replication in the mouse lung was further examined over time by infecting mice with $20MID_{50}$ of each of the selected isolates (RT/645, RT/625, and RK/470) and euthanizing mice to determine virus titer in the lung at multiple time points (Fig. 3). All viruses reached a similar peak titer in mouse lungs (2.4E +07 to 7.7E+07 $TCID_{50}/g$). Mice infected with RT/625 and RK/470 both had a pattern of peak virus in the lung at day 5 p.i. but RT/645 had a less clear time point of peak virus, with highest titers around days 1 and 3 post-inoculation. Virus was present in mouse lung up to day 7 p.i. for all three viruses and cleared by day 14 p.i.

The spleen, liver, and kidney of mice infected with RT/625 and RT/645 (day 5 p.i.) and the brain of mice infected with RT/625 (day 5 p.i.)

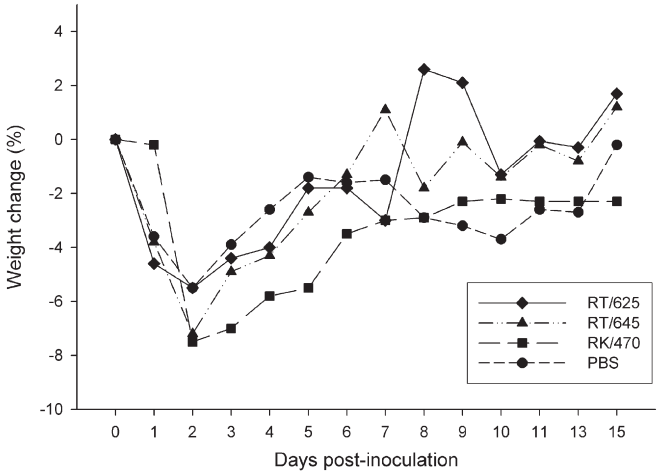

Fig. 2. Weight loss in mice infected with selected wild aquatic bird influenza virus isolates. Groups of five mice were inoculated with 20MID$_{50}$ of RT/625 (H6N4), RT/645 (H2N9), RK/470 (H7N3), or mock-infected with PBS only. Weights were tracked daily and percent weight lost compared to starting weight calculated. Daily weight loss was not statistically significant by Student's *t* test between virus inoculated mice and mock infected mice.

was examined for presence of virus via titration on MDCK cells. A subset of mice from both RT/625 and RT/645 had evidence of low titers of virus in the spleen and liver and these samples were further evaluated via virus isolation in ECE. Virus isolation in eggs indicated that a single mouse infected with RT/645 had influenza virus present in the liver, which was confirmed by real-time PCR for influenza (data not shown).

Wild bird avian influenza virus infection induces pulmonary lesions in mice

The lungs of mice infected with RT/645, RT/625, and RK/470 exhibited similar histopathologic lesions as well as a similar resolution of lesions; however, development of lesions differed with RT/625 lagging slightly behind RT/645 and RK/470. No significant histopathologic lesions were present in RT/645, RT/625, or RK/470 infected mice day 1 post-inoculation. By day 3 p.i., mice infected with RT/645 and RK/470 had a necrotizing bronchiolitis in many bronchioles and peribronchiolar inflammation, but mice infected with RT/625 exhibited only rare scattered areas of peribronchiolar inflammation (Fig. 4). A similar average percentage of lung was affected by inflammation on day 3 p.i. for all viruses (1.5% for RT/625, 2.7% for RT/645, and 2.0% for RK/470). Peribronchiolar inflammation present in mice infected with RT/625 at day 3 p.i. was primarily lymphocytes, with fewer macrophages and neutrophils present in adjacent alveoli, where in RT/645 and RK/470 infected mice the composition of inflammatory cells was more neutrophilic. Mild tracheal inflammation, characterized by small numbers of lymphocytes within the tracheal submucosa, was present in some RT/625 and RK/470 infected mice. Many apoptotic tracheal epithelial cells were observed only in RK/470 infected mice at day 3 post-inoculation. In X31 infected mice on day 3 p.i., there was a striking necrotizing bronchitis and bronchiolitis with loss of epithelium and peribronchiolar cell debris with some inflammatory cells.

Histopathology in mice on day 5 p.i. again showed that lesions in mice infected with RT/645 and RK/470 were ahead of mice infected with RT/625. Mice infected with RT/645 and RK/470 had continued peribronchiolar pneumonia and necrotizing bronchiolitis; however, the necrotizing bronchiolitis was resolving by this point in time and the bronchiolar epithelium was beginning to become hyperplastic (Fig. 4). Conversely, mice infected with RT/625 had continued peribronchiolar pneumonia that increased in severity and also a necrotizing bronchitis and bronchiolitis appeared at this time point. Mice infected with RT/625 exhibited prominent lymphocytic perivascular cuffing. The degree of inflammation present in the lung was slightly higher on day 5 p.i. compared to day 3 p.i., but similar between all viruses examined (8.6% for RT/625, 5.7% for RT/645, and 3.5% for RK/470). No lesions were present in the lungs of mock infected mice (Fig. 4). Mice infected with X31 had continued lesions of necrotizing bronchitis and bronchiolitis with peribronchiolar

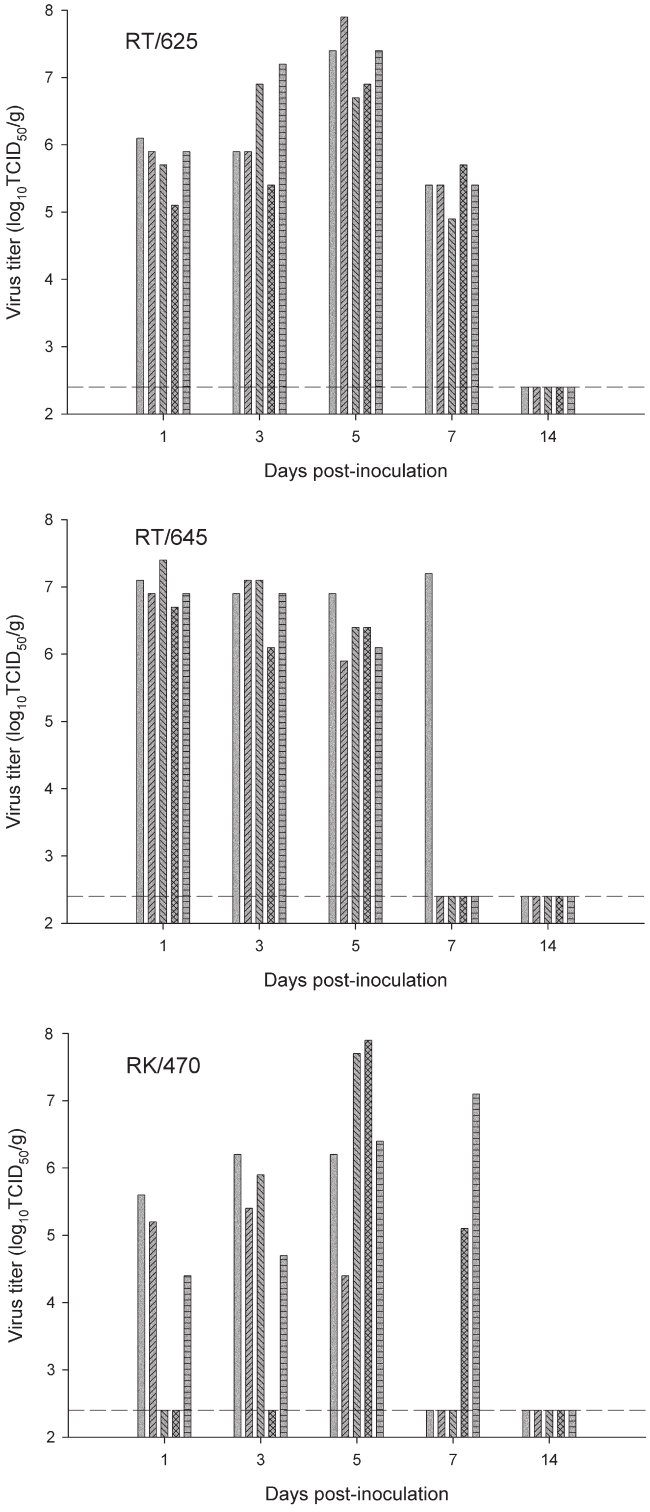

Fig. 3. Kinetics of viral infection of RT/625, RT/645, and RK/470 in the lung of infected mice. Each bar represents the viral titer in the lung of an individual mouse infected with 20MID50 of virus for viruses RT/625 (H6N4), RT/645 (H2N9), and RK/470 (H7N3). The dashed line is the limit of detection (2.4 log$_{10}$TCID$_{50}$/g).

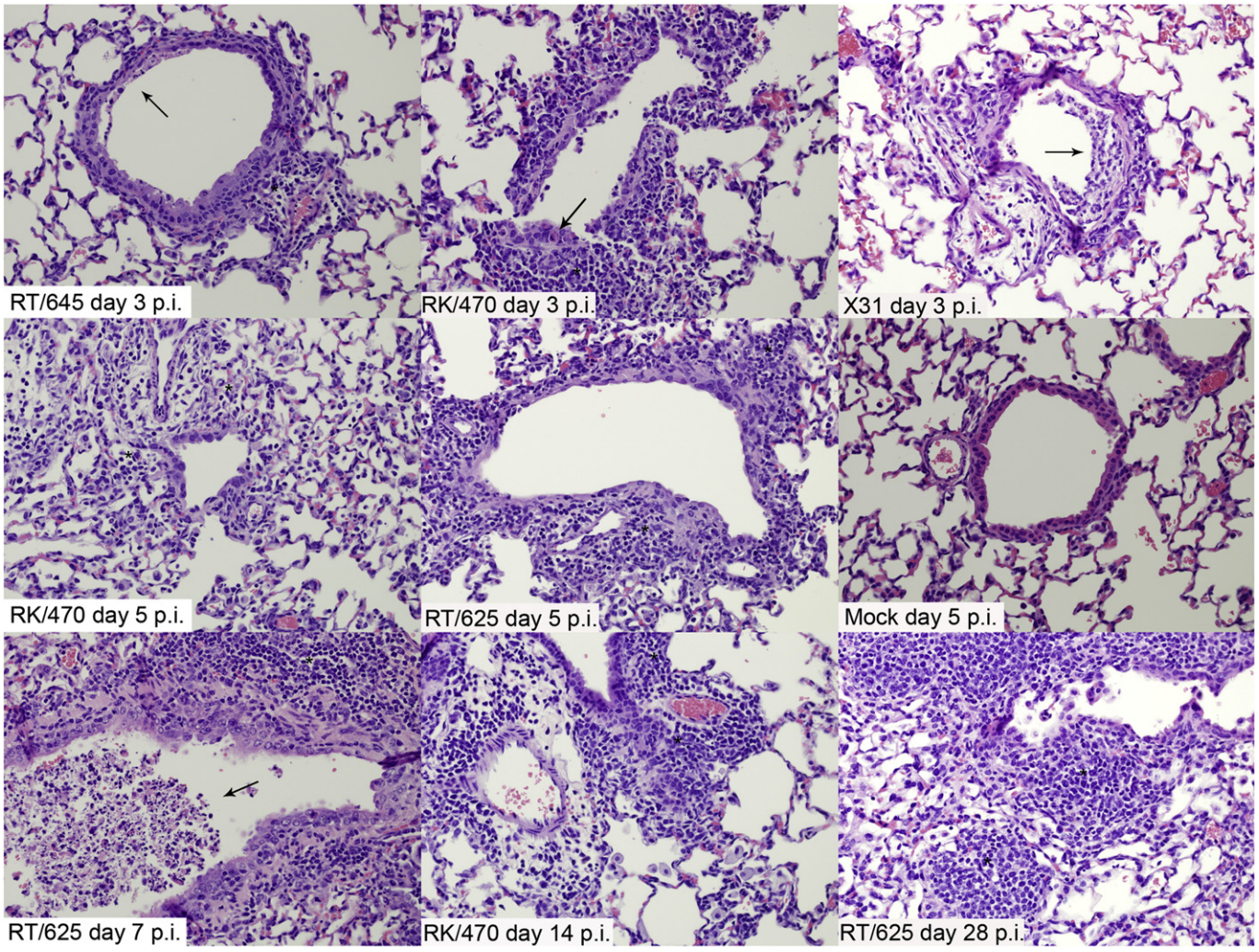

Fig. 4. Histopathology in lung of AIV infected mice on days 3 through 28 post-inoculation (p.i.). Mice infected with RT/625 (H6N4), RT/645 (H2N9), or RK/470 (H7N3) have bronchiolar necrosis (arrow) and peribronchiolar inflammation (asterisk). Inflammation was most severe in RT/625 (H6N4) infected mice on day 7 post-inoculation. Peribronchiolar inflammation was resolving as dense nodular collections of lymphocytes on days 14 and 28 post-inoculation. Positive control infected mice (X31 day 3 p.i.) have similar bronchiolar necrosis as AIV inoculated mice. Mock infected mice have no lesions on histopathology.

inflammation day 5 p.i., but many areas of bronchiolar epithelium were hyperplastic at this time point.

Mice infected with RT/625 had continued peribronchiolar pneumonia and necrotizing bronchiolitis at day 7 p.i.; however, the pneumonia was more widespread (Fig. 4). Mice infected with RT/645 and RK/470 had similar peribronchiolar pneumonia day 7 p.i. as described for day 5 p.i.; however, the degree of inflammation was much less in severity than RT/625 (24.9% lung affected for RT/625, 3.6% for RT/645, and 5.6% for RK/470) and there was complete resolution of bronchiolar necrosis. Additionally, there was prominent type II pneumocyte hyperplasia observed in some mice infected with RT/645 beginning at day 7 postinoculation.

Examination of lungs in infected mice on days 14 and 28 p.i. revealed similar resolution of mice infected with RT/625, RT/645, and RK/470. Peribronchiolar inflammation in all of these mice was primarily consolidated areas of lymphocytes and there were many peribronchiolar areas of type II pneumocyte hyperplasia. Lesions progressed to scattered nodular collections of peribronchiolar lymphocytes by day 28 p.i. in all virus infected mice. Mice infected with X31 had similar histopathologic changes of lymphocytic inflammation and type II pnuemocyte hyperplasia on day 14 as the three AIVs that were examined.

Immunohistochemistry (IHC) for the nucleoprotein of influenza A on the lungs of mice infected with RT/625, RT/645, and RK/470 all exhibited strong positive intranuclear staining of tracheal, bronchiolar, and/or alveolar epithelium in areas of histopathologic lesions between days 3 and 5 p.i. (Fig. 5). Mice infected with RT/625 had very minimal positive staining on day 3 p.i. but staining became more prominent by day 5 p.i., versus RT/645 and RK/470, which both had more prominent positive staining on day 3 p.i. and magnitude of positive staining was lessened by day 5 post-inoculation. These observations are in concord with histopathology that indicates earlier productive infection in mice infected with RT/645 and RK/470 versus mice infected with RT/625. Additionally, no positive staining of tracheal epithelium was observed in mice infected with RT/625, but there was good staining of the tracheal epithelium on day 3 p.i. for mice infected with RT/645 and RK/470. There was no positive staining in the lung of mock infected mice examined on day 5 post-inoculation.

Mice infected with RT/625 and RT/645 did not have any histopathologic lesions in the nasal turbinates at any time point (days 1, 3, 5, 7, and 14 p.i.). Two mice that were infected with RK/470 (day 5 p.i.) and one mouse that was infected with RK/470 (day 7 p.i.) had a single area in the nasal cavity that contained mixed mucus and neutrophils, but there were no lesions in the nasal epithelium in any of the RK/470 infected mice. Although epithelial lesions were not observed, there was positive intranuclear staining in very rare ciliated epithelial cells of the paranasal sinuses in mice infected with RT/645 and in mice infected with RK/470. There was no positive staining for

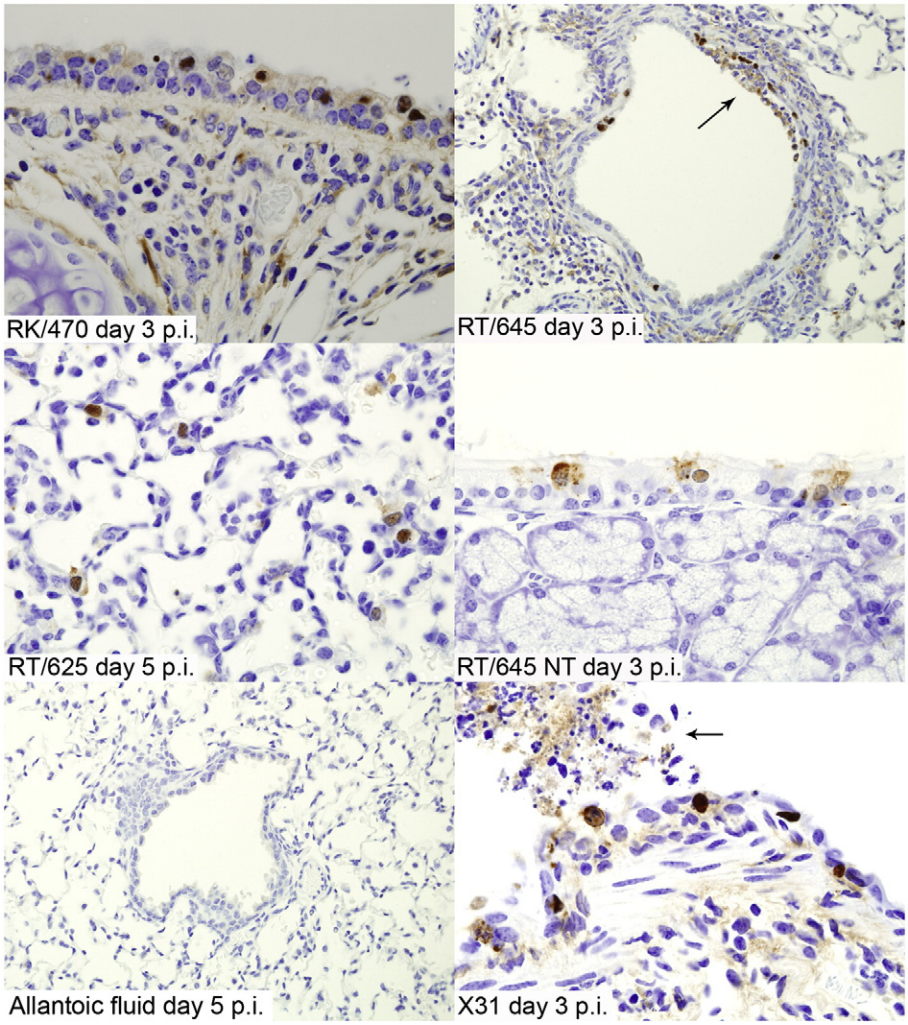

Fig. 5. Viral antigen in the lung and nasal turbinates of AIV infected mice on days 3 and 5 post-inoculation. By immunohistochemistry, mice infected with RT/625 (H6N4), RT/645 (H2N9), or RK/470 (H7N3) have strong positive intranuclear staining for the nucleoprotein of influenza A in tracheal epithelium (RK/470 day 3 p.i.) , bronchiolar epithelium (RT/645 day 3 p.i.), and alveolar epithelium (RT/625 day 5 p.i.) for all three AIV isolates in infected mice. There is also strong positive intranuclear staining of the nasal turbinate epithelium (RT/645 NT day 3 p.i.) in some AIV infected mice. Mock infected mice have no staining for viral antigen (Allantoic fluid day 5 p.i.). Positive control infected mice (X31 day 3 p.i.) have similar strong positive intranuclear staining of bronchiolar epithelium as AIV inoculated mice. Cellular necrosis is evident by luminal debris in the airways (arrow).

influenza in other organs examined on day 5 p.i. for any of the three viruses.

There was only a single extrapulmonary lesion observed in mice infected with the three AIVs, this was thymic atrophy with lymphocyte depletion present in a mouse infected with RT/645 on day 3 post-inoculation. Thymic atrophy has been associated with influenza infection in mice; however, we did not consistently observe this lesion in mice infected with this virus and observation of thymic atrophy in a different study occurred much later in infection (Fislova et al., 2009). No other lesions were observed in any of the organs examined for mice infected with RT/625, RT/645 or RK/470 on days 1, 3, 5, 7, or 14 p.i.

Wild bird avian influenza viruses exhibit differences in immunogenicity in mice

A serum ELISA was performed on sera collected from mice infected with RT/625, RT/645, or RK/470 on day 23 p.i. to assess seroconversion after AIV infection. All five mice infected with RT/645 or RK/470 had a robust IgG response to the specific AIV with a prominent signal from serum dilutions up to 1:320 (Fig. 6). In contrast, mice infected with RT/625 had a poor IgG response to the viral infection, with little difference in signal from serum dilutions when compared to mock infected mice (Fig. 6).

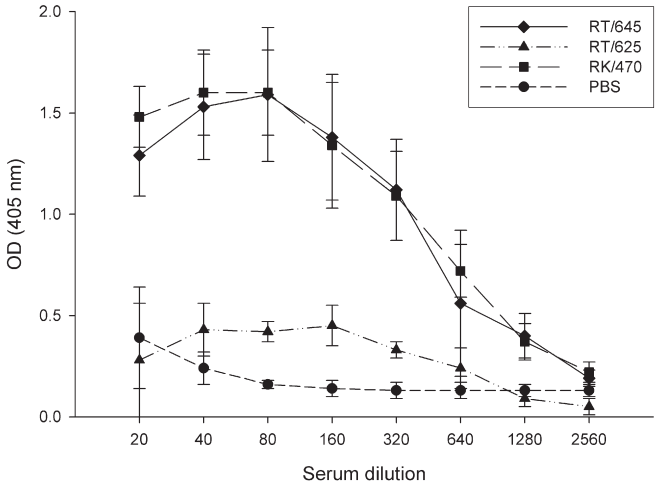

Fig. 6. Serum antibody response in mice after wild AIV infection. Groups of 5 mice were inoculated with $20MID_{50}$ of RT/625 (H6N4), RT/645 (H2N9), or RK/470 (H7N3) and sera were collected day 23 post-inoculation. Sera from individual mice were tested against whole virus and allantoic fluid, and the difference was averaged and plotted. Sera from PBS inoculated mice were tested against allantoic fluid.

Discussion

The perspectives regarding the host range and mechanisms of influenza infections are ever broadening to a view of a more promiscuous virus than previously thought. Through natural and experimental infections, the list of mammals that are susceptible to AIV infection is rapidly increasing along with the scope of organ tropism of these viruses. These findings apply not only to highly pathogenic AIVs, but also to low pathogenic viruses from poultry (Belser et al., 2007; Gillim-Ross et al., 2008; Joseph et al., 2007; Wan et al., 2008). These evolving viewpoints are further supported with these data that includes a large scope of avian influenza isolates from wild birds, demonstrating that many AIVs from wild birds can replicate in the BALB/c mouse model without adaptation and that hemagglutinin subtype was not a barrier to infection (Table 1). Methodology in screening the isolates for replication in the mouse lung was limited by the titer of the virus stock, as the virus isolates were minimally passaged to avoid mutations associated with egg adaptation. For the purpose of these studies it was critical to keep these isolates as close as possible to the original sample from the wild bird, to better assess replication of the original isolate. This resulted in many isolates that were only of moderate titer. The inoculum concentration for the primary screening experiment could not be standardized, as lowering the inoculum concentration for some isolates to normalize the concentration could have resulted in missing some isolates that were able to replicate. This method enabled identification of the maximum number of isolates that replicated in the mice and did not have any effect on peak viral titer for isolates that exhibited efficient replication (Fig. 2), although some concentration effect was observed on isolates that exhibited poor replication (data not shown).

Mice did not require a high concentration inoculum for infection with RT/625, RT/645, or RK/470, as evident in the MID_{50} for each virus. Studies with LPAI H7 viruses have also shown that low concentrations of virus are adequate for infection of mice (Belser et al., 2007). RK/470 had a very low MID_{50} in mice, however, when mice were inoculated with $20MID_{50}$ for pathogenicity studies; several mice did not become infected. Therefore, it is possible that a higher concentration of inoculum was required for consistent infection of all these mice. Despite these observations, it is also interesting to note that all H7 subtype viruses examined in this screen replicated to a high titer despite a low inoculum concentration, suggesting there is a lower threshold for infection in the H7 subtypes (Fig. 1). Additionally, although there was not an equal number of hemagglutinin subtypes represented, all viruses of the H7 subtype exhibited efficient replication compared to other subtypes that exhibited varied ability to replicate in mouse lung (Table 1). The H7 subtype of avian influenza is of particular concern in human infections (Belser et al., 2009; Gillim-Ross and Subbarao, 2006). The data shown here is suggestive of an increased ability of the H7 viruses to replicate in the mouse model and further investigation to examine more isolates of this subtype may provide insight to mechanisms of mammalian infection. While previous studies focused on LPAI and HPAI H7 influenza viruses isolated from humans and poultry, the viruses tested here were exclusively isolates from North American waterfowl and shore birds. This presents additional opportunities for transmission; whether through contact with infected wild birds or environmental exposure in the birds' habitats (Rohani et al., 2009).

Infections in these mice were very similar to infections with human or other LPAIVs in BALB/c mice regarding pathogenesis and lesion development (Buchweitz, Karmaus, and Harkema, 2007). Peak viral titers in the lung for other LPAIVs are generally at day 4 but can occur as early as day 2 with virus present in the lung through day 7 (Gillim-Ross et al., 2008; Joseph et al., 2007). In this study, there is a similar peak of infection based on virus titers from the lung as RT/625 and RT/470 had peak titers in most mice on day 5 p.i. and RT/645 had peak titers in most mice at days 1 and 3 p.i. (Fig. 3). Previous immunohistochemistry studies on influenza infected mice also demonstrate similar strong intranuclear staining of bronchiolar epithelium (Fislova et al., 2009; Rigoni et al., 2007). Interestingly, all of the viruses we examined demonstrated positive viral antigen staining of not only bronchiolar epithelial cells but also alveolar epithelial cells, but in the X31 infected mice viral antigen staining was restricted to bronchiolar epithelium. Similar findings were present in a study of mouse adapted influenza viruses, where IHC staining for influenza was only observed in bronchiolar epithelial cells (Fislova et al., 2009). Moreover, there was prominent type II pneumocyte hyperplasia present in mice infected with viruses RT/625, RT/645, and RK/470 by day 14 p.i., also supporting that there was significant alveolar epithelial damage. HA receptor specificity is thought to be a large contributor to host range, as avian influenza strains preferentially bind to cell glycoproteins/glycolipids that have terminal sialyl-galactosyl (SA) residues with a 2–3 linkage [Neu5Ac(α2-3)Gal] and human influenza strains preferentially bind to terminal 2–6 linked glycoproteins/glycolipids [Neu5Ac(α2-6)Gal] (Connor et al., 1994; Mansfield, 2007). Additionally, the distribution of these receptors on cells of the respiratory tract is demonstrated to vary between mammalian species and mice have been shown to have primarily the 2–3 linked SA receptor in ciliated epithelial cells in large airways and in type II pneumocytes (Ibricevic et al., 2006; van Riel et al., 2007). Indeed, many studies of HPAI H5N1 have demonstrated enhanced alveolar damage in humans and mice, suggesting increased affinity for binding of these cells compared to human strains of influenza (Abdel-Ghafar et al., 2008; Dybing et al., 2000). This affinity for binding cells of the lower respiratory tract was also demonstrated with some LPAI isolates (van Riel et al., 2007). Our results are also supportive that these wild bird AIVs have an increased binding affinity for receptors present on alveolar epithelial cells compared to human strains. In mice infected with RT/645 and RK/470 there was minimal presence of viral antigen in the nasal cavity via IHC with no antigen present in RT/625. Other influenza isolates, particularly human origin, typically exhibit replication in this area of the upper respiratory tract. The minimal presence of antigen in the nasal turbinates despite efficient replication and lesions in the lung supports that these isolates, similar to other avian isolates, have a preference for replication in the lower respiratory tract. There is recent evidence that the regional replication preferences of avian influenza in mammals are in part due to mutations in PB2 that change temperature and cell type replication preferences in the virus (Hatta et al., 2007). Further investigation into the molecular aspects of these wild type avian viruses may provide insight into common molecular mechanisms of host range and pathogenicity in mammals.

It has been shown that many HPAI viruses have extrapulmonary spread, with virus presence and lesions most frequently in the brain and spleen of infected mice (Belser et al., 2007; Joseph et al., 2007; Maines et al., 2005; Rigoni et al., 2007). Examination of LPAIVs has demonstrated that some of these viruses that exhibit pulmonary replication also have the capacity for extrapulmonary spread, while others do not (Gillim-Ross et al., 2008; Joseph et al., 2007). Interestingly, mouse adapted influenza viruses have also been shown to have extrapulmonary spread to numerous organs with possible viremia, regardless of virulence (Fislova et al., 2009). Additionally, a case study of a human infection with HPAI H5N1 demonstrated viral antigen in extrapulmonary sites without lesions in these sites (Zhang et al., 2009). We also have evidence of inconsistent extrapulmonary spread of one of the viruses examined (RT/645) without significant morbidity or extrapulmonary lesions in these mice. This may be further supportive evidence that viremia and extrapulmonary spread is not as uncommon as previously believed and conclusions of virulence of an influenza isolate must be evaluated not only in context of viral presence, but also with consideration of morbidity and severity of lesions in the mouse model.

In depth studies of isolates RT/625, RT/645, and RK/470 revealed very similar peak titers of virus, pulmonary lesions, and location of viral antigen. However, slight differences were present between these viruses, most notably in RT/625. This isolate exhibited slower progression of viral infection, characterized by a later peak of pulmonary lesions and viral antigen compared to RT/645 and RK/470 (Fig. 4). Additionally, the amount of peribronchiolar inflammation in RT/625 was greater in magnitude than RT/645 and RK/470 on day 5 post-inoculation. Viral titer in the lungs corresponded with the progression of lesions in RT/625 (peak virus at day 5) and RT/645 (peak virus at days 1 and 3) but not as well with RK/470 (apparent peak virus at day 5) regardless of inclusion or exclusion of the several mice of RK/470 that did not become infected (Fig. 3). Interestingly, there was a good antibody response in mice infected with RT/645 and RK/470 but a poor response in mice infected with RT/625. In addition, RT/625 infection caused marked lymphocytic infiltrates early in the lung, whereas RT/645 and RK/470 infection resulted in primarily neutrophilic infiltrates and followed a more typical inflammatory pattern (Buchweitz, Karmaus, and Harkema, 2007). It is possible that the differences in inflammation with RT/625 infection may be related to an altered immune response or poor specific immune response to the virus. Virus was present in all RT/625 infected mice through day 7 p.i.; additional sampling days between day 7 and day 14 p.i., in addition to analysis of the cytokine and chemokines expressed during infection would be useful to determine if there was an altered immune response influencing viral clearance from the lung and seroconversion compared to RT/645 and RK/470 infected mice.

Many inbred mouse strains including BALB/c are more susceptible to influenza infection because of defective alleles in the Mx1 gene (Staeheli et al., 1988). Studies have shown that mice with a wild-type Mx1 gene are resistant to infection with LPAI and have lower viral replication with HPAI H5N1 infections than BALB/c mice (Henzler et al., 2003; Tumpey et al., 2007). Therefore, interpretation of the potential of mammalian and human infection with these wild bird AIVs must be carefully evaluated in additional models. Nonetheless, the evaluation of these wild AIVs in the BALB/c mouse model are invaluable for selection of isolates for additional molecular and *in vivo* studies using other animal models. Many of the isolates in this study should be further explored to better understand mechanisms of AIV infection in mammals. From this study, we conclude that numerous wild bird AIVs replicate without adaptation and to high titer in the mouse model. While all viruses induced lung pathology, the extent, type and time course varied. Moreover, virus titer and lung pathology were not necessarily associated with seroconversion. Deductions about the behavior of influenza in BALB/c mice and mammals in general should not simply be based on magnitude of replication of the virus or seroconversion, but also correlated with clinical signs, evidence of morbidity, and severity of lesions. Avian influenza viruses from wild bird populations could be crossing species barriers and causing sub-clinical disease with limited seroconversion; providing unknown opportunities for mutation and emergence of novel influenza viruses.

Methods

Viruses

Avian influenza viruses used were cloacal swab isolates from wild birds in the United States acquired from Southeastern Cooperative Wildlife Disease Study at the University of Georgia collected between 1998 and 2006. Viruses used in this study are shown in Table 1. Isolates used for in vitro screening were isolated from cloacal swabs in 9-day-old ECE at 37 °C for 72 h and then minimally passaged (3 or fewer passages) in ECEs. Approximately 400 influenza virus isolates

Table 1
Study viruses and their ability to replicate in mouse lung.

Virus[a]	Name in this study	Subtype	Replication in mouse lung[b]
A/Ruddy Turnstone/NJ/650624/02	RT/624	H2N4	Poor
A/Sanderling/DE/650623/02	San/623	H2N4	Efficient
A/Ruddy Turnstone/DE/650621/02	RT/621	H2N9	No
A/Ruddy Turnstone/NJ/650638/02	RT/638	H2N9	Poor
A/Ruddy Turnstone/DE/650645/02[c]	RT/645	H2N9	Efficient
A/Ruddy Turnstone/DE/650580/02	RT/580	H2N9	Efficient
A/Ruddy Turnstone/NJ/650627	RT/627	H2N9	No
A/Mallard/MN/199105/99	Ma/105	H3N4	Poor
A/Mallard/MN/355808/00	Ma/808	H3N4/8	Poor
A/Ruddy Turnstone/NJ/1321394/05	RT/394	H3N6	No
A/Ruddy Turnstone/NJ/1321398/05	RT/398	H3N6	No
A/RuddyTurnstone/NJ/1321397/05	RT/397	H3N6	Poor
A/RuddyTurnstone/NJ/1321396/05	RT/396	H3N6	No
A/Ruddy Turnstone/NJ/1321395/05	RT/395	H3N8	Poor
A/Mallard/MN/199084/99	Ma/084	H3N8	Poor
A/Ruddy Turnstone/NJ/1321399/05	RT/399	H3N9	No
A/Mallard/MN/355807/00	Ma/807	H4N6	Poor
A/Ruddy Turnstone/DE/650625/02[c]	RT/625	H6N4	Efficient
A/RuddyTurnstone/NJ/650677/02	RT/677	H6N4	Efficient
A/Sanderling/DE/650680/02	San/680	H6N4	Poor
A/feces/DE/650574/02	Fe/574	H6N8	Poor
A/Red Knot/NJ/1523470/06[c]	RK/470	H7N3	Efficient
A/Sanderling/NJ/1523471/06	San/471	H7N3	Efficient
A/Ruddy Turnstone/1523477/06	RT/477	H7N3	Efficient
A/Ruddy Turnstone/NJ/650615/02	RT/615	H11N2	No
A/Ruddy Turnstone/NJ/650626/02	RT/626	H11N9	No
A/Mallard/MN/199116/99	Ma/116	H11N9	Poor
A/feces/DE/650619/02	Fe/619	H11N9	Poor
X31	X31	H3N2	Efficient

[a] Each of the 28 avian virus isolates listed were surveyed for ability to infect lungs of mice by infecting groups of three to four mice with two serial tenfold dilutions of the virus.
[b] Isolates were scored as: "No" which represents absence of virus in infected mice lung for either inoculum dilution on $TCID_{50}$ assay, "Poor" which represents virus presence in infected mice lung only at the higher inoculum dilution and/or low titer virus at either inoculum dilution on $TCID_{50}$ assay, and "Efficient" which represents high titer virus presence in infected mice lung at both inoculum dilutions on $TCID_{50}$ assay. The isolate X31 was used as a positive control.
[c] Indicates isolates that were selected for in depth pathogenicity studies.

were examined in vitro. Viruses that exhibited plaquing on Madin-Darby canine kidney (MDCK) cells without the addition of trypsin were selected for additional in vivo selection in mice, as this phenotype is suggestive of enhanced pathogenicity of influenza viruses. The original low passage isolates, once selected by in vitro screening methods, were grown once more in 9- to 10-day-old ECE to generate a stock of the virus. Allantoic fluid pooled from eggs was frozen in aliquots at −80 °C. Virus stocks were plaqued on MDCK cells with trypsin to elucidate a PFU/mL titer.

Mouse experiments

Female 6- to 8-week-old BALB/c mice (Harlan Laboratories, Indianapolis, IN) were anesthetized with intraperitoneal injection of 2,2,2-tribromoethanol in *tert*-amyl alcohol and inoculated intranasally with 50 μL of diluted virus in sterile phosphate buffered saline (PBS). A screen to determine the replication capacity of selected AIV isolates in mice was performed by harvesting lungs of mice inoculated with a 1:10 dilution of virus stock (3–4 mice per virus) or a 1:100 dilution of virus stock (3–4 mice per virus) on day 4 post-inoculation. A group of mice was mock infected with PBS and another group was infected with X31 to serve as controls. Mouse experiments were performed in enhanced BSL2 facilities in HEPA filtered isolators. Studies were conducted under guidelines approved by the Animal Care and Use Committee of the University of Georgia. Clarified lung homogenate and fixed lung tissue from selected influenza inoculated mice (RT/625) exhibiting histopathologic lesions were negative for *Mycoplasma pulmonis* via PCR.

The MID_{50} was determined as previously described (Cottey, Rowe, and Bender, 2001). Briefly, mice were infected with 10-fold dilutions of each virus. Five mice per group were euthanized on day 4 p.i. and clarified lung homogenate was serially titrated in MDCK cells to determine the MID_{50} calculated by the method of Reed and Muench (1938). The pathogenesis of RT/625, RT/645, and RK/470 was determined by inoculation of groups of mice with $20MID_{50}$ of virus and harvesting lungs on days 1, 3, 5, 7, and 14 p.i. and harvesting spleen, liver, kidney and brain on day 5 p.i (5 mice per virus per day). Tissues were homogenized in 1 mL PBS, clarified by centrifugation, and frozen at −80 °C for later titration. Clarified lung homogenate was titrated in MDCK cells starting at a 1:10 dilution with a limit of detection at $10^{2.4}$ $TCID_{50}$/gram. Clarified organ homogenate (spleen, liver, kidney, and brain) was initially titrated in MDCK cells starting at a 1:10 dilution. Samples were further selected for virus isolation in 9- to 10-day-old ECE. Real time RT-PCR was performed on hemagglutination assay positive samples from virus isolation. Briefly, viral RNA was extracted from allantoic fluid by using RNeasy mini kit (Qiagen, Valencia, CA) and the Qiagen one-step RT-PCR kit was used for RRT-PCR with a Stratagene MX300P/3005P thermocyler and Mx Pro QPCR software (La Jolla, CA). Reaction mixture and PCR cycling protocol is available upon request. An influenza virus matrix gene specific primer and probe set were used as follows: primer M+25, sequence AGA TGA GTC TTC TAA CCG AGG TCG; primer M-124, sequence TGC AAA AAC ATC TTC AAG TCT CTG; and probe M+64, sequence FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA (Biosearch Technologies, Novato, CA).

For morbidity and seroconversion studies, five mice per group for viruses RT/625, RT/645, RK/470, and a mock infected PBS group were inoculated with $20MID_{50}$ of virus and weighed daily for 11 days and then every other day for an additional 4 days. Serum was collected from these mice on day 23 p.i. to assay serum antibody response to the AIVs. Statistical significance of weight loss between groups of mice was determined using Student's *t* test.

Histopathology and immunohistochemistry

Tissues from infected and control mice were examined by histopathology and immunohistochemistry. Mice were inoculated with $20MID_{50}$ virus and lung, trachea, thymus, thyroid, esophagus, heart, spleen, liver, stomach, intestine, pancreas, kidneys, adrenal gland, ovary, uterus, bladder, brain, and nasal turbinates were collected on days 1, 3, 5, 7, 14, and 28 p.i. (three mice per virus per day). The lungs were inflated with 10% neutral buffered formalin and all tissues were preserved in 10% neutral buffered formalin. Additional groups of mice were inoculated with allantoic fluid in sterile PBS (3 mice) or with X31 (9 mice) to serve as controls and organs were collected on day 5 p.i (PBS) or days 3, 5, and 14 p.i. (X31). Five equal transverse sections were made through the entire lungs. Mouse skulls were decalcified and four transverse sections were made through the nasal cavity to examine the nasal turbinates. Tissues were routinely processed, embedded and stained with hematoxylin and eosin. The severity of inflammation in lungs of infected mice was calculated by finding the average percent area of lung affected by inflammation per day p.i. for each virus using Image Pro Plus software vs. 4.5.1 (MediaCybernetics, Bethesda, MD).

Immunohistochemical staining was performed on lung tissue (days 1, 3, 5, and 7 p.i. for 3 mice per day) or on nasal turbinates (days 3 and 5 p.i. for 2 mice per day) and all other organs (day 5 p.i. for 2 mice per day) in mice infected with RT/625, RT/645, and RK/470. Immunohistochemistry on mouse lung tissue was performed using a commercially available mouse monoclonal antibody to the nucleoprotein of influenza A virus at a 1:200 dilution (Biodesign International, Sako, Maine) or on mouse nasal turbinates and organs other than lung using a commercially available goat polyclonal antibody to the nucleoprotein of influenza A virus at a 1:10,000 dilution (Biodesign International, Sako, Maine), as excessive background staining was observed in nasal turbinates and organs using the mouse monoclonal. Tissues were deparaffinized and blocked with a commercial protein blocking agent (Dako Cytomation, Carpinteria, CA) and a linked strepavidin-biotin immunoperoxidase system was used for immunolabeling. The reaction was visualized with 3,3′-diaminobenzidine substrate (Dako Cytomation, Carpinteria, CA).

Serum ELISA assay

Sera from individual mice were assayed via ELISA against each whole influenza virus (RT/625, RT/645, or RK/470) inoculated in the individual mouse. Additionally, the same sera were assayed against allantoic fluid to account for any antibodies generated against components other than the virus. Virus or allantoic fluid was coated 100 μL per well on 96 well Immulon 2HB microtiter plates and incubated 24 h at 4 °C. Two-fold dilutions of sera were applied to pre-absorbed plates, starting with a dilution of 1:20 and virus specific antibodies were measured using alkaline phosphatase labeled goat anti mouse IgG(H+L) (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The p-nitrophenyl phosphate substrate (Kiregaard and Perry Laboratories, Gaithersburg, MD) was added and absorbance measured at 405 nm on a 96-well format plate reader (BioTek, Winooski, VT). Absorbance readings for sera against allantoic fluid were subtracted from absorbance readings for sera against virus and plotted.

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