Histopathology and growth kinetics of influenza viruses (H1N1 and H3N2) in the upper and lower airways of guinea pigs

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Recent investigations have shown that guinea pigs are important for the study of influenza A virus (IAV) transmission. However, very little is known about IAV replication and histopathology in the guinea pig respiratory tract. Here, we describe viral growth kinetics, target cells and histopathology in the naso-sinus, trachea and lungs of IAV-infected guinea pigs. We found that guinea pigs infected with either A/Puerto Rico/8/34 (H1N1) or A/Hong Kong/8/68 (H3N2) developed a predominantly upper airway infection with high nasal viral titres. IAV grew to moderate titres in the lungs but induced marked inflammatory responses, resulting in severe bronchopneumonia and alveolitis. Although non-lethal at the high dose of 2×10⁵ p.f.u., infections with these IAV strains were associated with reduced weight gain. IAV infection in guinea pigs is characterized by extensive viral replication in the ciliated nasal epithelial cells followed by heavy nasal mucus secretion.

Both inbred and out-bred mice are by far the most commonly used animal models for the study of influenza A virus (IAV) pathogenesis, and for the evaluation of vaccine and antiviral drugs (Gubareva et al., 1998). However, the use of mice has several drawbacks because mice are not natural hosts for the influenza virus infection. Furthermore, a recent report showed that mice lack 2,6-linked sialic acid, which is the preferred receptor for human IAV (Ibricevic et al., 2006). However, other investigators have shown that the presence of a major 2,6 sialic acid on N-linked glycoproteins is not essential for IAV infection in mice (Glaser et al., 2007). More importantly, mice are not suitable for the study of viral transmission because infected mice failed to transmit virus to other mice housed even within the same cages (Lowen et al., 2006). Unlike mice, ferrets are suitable for transmission and pathogenesis studies but they are not as readily available and their housing requirements are beyond the resource of many investigators (Zitzow et al., 2002). More recently, cotton rats are shown to be promising for IAV studies but it is not known if these animals are susceptible to transmission (Ottolini et al., 2005).

Guinea pigs (Cavia porcellus), which are not rodents based on phylogenetic analysis of amino acid sequences (Graur et al., 1991; D’Erchia et al., 1996), have recently been shown to be an excellent model for the study of IAV transmission (Lowen et al., 2006). Earlier studies in guinea pigs were limited to the analysis of IAV-induced lung changes by histology and electron microscopy (Azoulay-Dupuis et al., 1984) and immune responses such as delayed-type hypersensitivity (Wetherbee, 1973; Phair et al., 1979). However, despite the new found importance of guinea pigs for the study of environmental factors that affect IAV transmission (Lowen et al., 2007, 2008), very little is known about viral replication and histopathology in the respiratory tissues of this species. Here, we describe viral growth kinetics, target cells and histopathology in upper and lower airways of guinea pigs infected with the IAV strain A/Puerto Rico/8/34 (H1N1), designated PR8 (ATCC VR-95) and A/Hong Kong/8/68 (H3N2), designated HK/68 (ATCC VR-544). These IAV strains were grown in embryonated eggs and titrated in Madin-Darby canine kidney (MDCK) cells as previously reported (Chong et al., 2008). For these studies, we used juvenile out-bred Hartley guinea pigs of mixed sex at 190–220 g body weight (Charles River Laboratories Inc., Wilmington, Massachusetts, USA) that were housed individually in cages with food and water provided ad libitum. These animals were maintained and handled in accordance with the guidelines set by the Institutional Animal Care and Use Committee.

We initially inoculated non-anaesthetized guinea pigs by dosing them with 10 μl HK/68 virus per nostril to take advantage of the reported high susceptibility of the animals to droplet transmission. Animals infected with 2×10⁵ p.f.u. showed little respiratory symptoms and there was no significant difference in body weight compared to mock-infected animals during the duration of infection. For the determination of virus titre, we collected tissue specimens...
from nasosinus mucosa, trachea and whole lungs of guinea pigs as previously reported (Chong et al., 2008). As shown in Fig. 1(a), infected animals showed a large increase in nasal titre between 4 and 8 h post-infection (p.i.) that led to a mean peak nasal titre of $\sim 3 \times 10^5$ p.f.u. (g tissue)$^{-1}$ at 1 day p.i. Following peak infection, there was a 1 log reduction in nasal viral levels by day 5 p.i. and a further 1 log reduction by day 7 p.i. Infected animals developed relatively low levels of virus in the lungs with mean peak titres of $\sim 10^5$ p.f.u. (g tissue)$^{-1}$ on day 3 p.i., which subsequently declined to undetectable levels (Fig. 1a). Viral growth in the trachea was significantly lower than in the nasal mucosa, but was more persistent than in the lungs.

To produce more severe infections, we infected guinea pigs at 10-fold higher dose levels in all subsequent experiments. Animals were infected at $2 \times 10^6$ p.f.u. (100 μl per nostril) under anaesthesia as previously reported (Lowen et al., 2006). With this higher dose of virus, infected animals displayed ruffled fur and were noticeably less active, but no animal died during the experimental duration. Tissue dissection showed macroscopic gross pathology, including excessive mucus in the nasosinus tract and varying levels of lung congestion and inflammation on day 3 p.i. Most infected animals also showed areas of lung lobes that appeared normal and this may account for the mild disease symptoms in these animals. Unlike the lower dose group, animals infected with the higher dose were slower in gaining weight compared with mock-infected control animals on days 1 and 3 p.i., but this reduction in body weight was not statistically significant ($P > 0.05$ Student’s $t$-test).

Similar to the low dose infection group, these animals also showed a rapid increase (~100-fold) in titre within the nasal tract between 4 and 8 h p.i. (Fig. 1b), but reached a much higher titre in the nasal tract with mean peak titre of $\sim 2 \times 10^7$ p.f.u. (g tissue)$^{-1}$ on day 1 after infection. Nasal titre decreased rapidly to $\sim 3 \times 10^4$ p.f.u. g$^{-1}$ by day 3 p.i. It was interesting to note that in the high dose infection groups animals displayed rapidly decreasing nasal titres, suggesting that infection was limited by the availability of susceptible epithelial cells. Moreover, the higher dose infection did not produce greater infection in the trachea or the lungs with lung titres peaking at $\sim 10^3$ p.f.u. g$^{-1}$ by day 3 p.i. and declining rapidly so that virus titres were below the assay limit of detection by day 5 p.i. In separate experiments using the PR8 strain of H1N1 virus, we noted that PR8 showed similar growth kinetics to the HK/68 strain except that titres were lower in all airway tissues, especially in nasal mucosa that showed a mean peak nasal titre of $\sim 2 \times 10^6$ p.f.u. g$^{-1}$ (Fig. 1c). Unlike HK/68 strain, the PR8 virus that we used in this study may have been...

Fig. 1. Time course of virus replication in guinea pigs inoculated intranasally with $2 \times 10^5$ (a) or $2 \times 10^6$ (b) p.f.u. of HK/68 (H3N2) and $2 \times 10^6$ p.f.u. (c) of PR8 (H1N1). Data point represents virus titre (mean±SD) of the log$_{10}$ p.f.u. (g tissue)$^{-1}$ from individual animals (three animals per group) at 4 and 8 h, and 1, 3, 5 and 7 days p.i. Lungs titres for days 5 and 7 p.i. (data not shown) were below the detection threshold of 50 p.f.u. (g tissue)$^{-1}$. 

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mouse-adapted and this would account for its poor replication in guinea pigs.

To correlate viral growth kinetics with histopathological analysis, we infected separate groups of guinea pigs with either HK/68 or PR8 strains by the high dose infection procedure as described above. Tissue specimens from infected animals on days 1, 3 and 7 p.i. were fixed in buffered formalin and processed for routine histology. In HK/68-infected animals, the conducting airway tissues including trachea, nasal, paranasal sinuses, bronchus and bronchioles showed varying levels of epithelial cell desquamation and inflammation beginning on day 1 p.i. Infected nasal mucosa showed a moderate increase in the number of goblet cells on days 1 (Fig. 2a and b) and 3 p.i. (Fig. 2d and e) and disorganized epithelial cells most likely due to epithelial tissue remodelling induced by viral cytopathic effect. To localize IAV antigen to target cells, we performed immunostaining on tissue sections that were treated for antigen retrieval by immersion in 0.01 M sodium citrate buffer (pH 6.0) and steam heated for 15 min. Subsequently, sections were stained using antibody preparations against influenza virions as previously reported (Chong et al., 2008). The staining results showed widespread viral antigens in nasal mucosa predominantly in ciliated epithelial cells on day 1 p.i. (Fig. 2c), and to a smaller extent on day 3 p.i. (Fig. 2f). Thereafter, the occurrence of virus-positive cells declined sharply so that only occasional foci of infection in the olfactory epithilia were demonstrable by day 7 p.i. (Fig. 2i). In contrast, excessive mucus secretion within the nasal tract was still evident by day 7 (Fig. 2g and h). For PR8-infected animals, we noted similar nasal tract histological changes (data not shown).

As early as 1 day after infection with either HK/68 or PR8 virus, localized areas of individual lung lobes showed varying levels of lung inflammatory changes and consolidation. In histological analysis, HK/68-infected lungs showed evidence of acute bronchiolitis, bronchointerstitial pneumonia and alveolitis. Early lesions following either PR8 (data not shown) or HK/68 infection consisted of massive immune cell infiltrates predominantly of neutrophils and mononuclear cells but also some eosinophils (Fig. 3a and b). By day 3 p.i., infected lungs showed severe bronchiolitis and alveolitis that were characterized by the presence of inflammatory cells within alveolar spaces (Fig. 3d and e). Viral antigens were readily demonstrable in the airway epithelial cells, including the bronchioles, alveolar epithelial cells and macrophages (Fig. 3c and f). Lung lesions persisted for 7 days p.i. as indicated by prominent alveolitis with extravasated red blood cells and oedema (Fig. 3g and h), but virus antigens were not detected in infected lung sections (Fig. 3i).

In our studies, guinea pigs were readily infected with PR8 or HK/68, which are well-characterized IAV strains. However, we were surprised that given at $2 \times 10^5$ p.f.u. in 10 μl volume, HK/68 virus produced only a relatively mild nasal infection with mean titres on days 1 and 3 p.i. of $\approx 2 \times 10^5$ and $\approx 5 \times 10^4$ p.f.u. (g tissue)$^{-1}$, respectively. In contrast, it was reported that guinea pigs inoculated with $10^6$ p.f.u. led to nasal wash titres on days 1 and 3 p.i. of $\approx 3 \times 10^5$ and $\approx 3 \times 10^6$ p.f.u. ml$^{-1}$, respectively (Lowen et al., 2006). One interpretation of this is that the nasopharyngeal washing procedure yields more virus than could be obtained in microdissected nasosinus mucosa. However, this is not likely to be a major factor since our animals also showed $> 1.5 \log$ lower lung titres. A more important difference between the earlier transmission report and our findings is the nature of virus strains and their propagation methods. Whereas we have used an older non-mouse-adapted HK/68 isolate that was propagated in chick embryos, Lowen et al. (2006) had used a newer H3N2 virus human isolate (A/Panama/2007/99) that was cultured in MDCK cells. However, it is not known how virus strain differences can lead to differences in guinea pig susceptibility, since there have been very few studies of IAV infection in this animal species. Our findings suggest that it would be worthwhile to investigate if HK/68 and PR8 infections are also transmissible in guinea pigs, but we have not performed any transmission study.

Our results showed that pathological lesions that developed in the upper and lower airways of guinea pigs corresponded with the time course of acute IAV infection. Since both HK/68 and PR8 have been widely used in animal model studies, our findings allow comparison of IAV-induced airway disease in guinea pigs with published results on airway lesions in mice and ferrets (Mbawuike et al., 2006). For these IAV strains, infected guinea pigs showed somewhat more severe nasal and lung disease than have been previously reported for mice (Iwasaki et al., 1999; Chong et al., 2008). For instance, guinea pigs produced excessive amounts of mucus in the nasosinus tract by day 3 p.i., which were much greater than we previously observed in infected mice (Chong et al., 2008). This suggests that infection in guinea pigs produced acute airway symptoms more similar to upper airway infection in humans. This is perhaps not surprising since guinea pigs are the preferred species for the study of upper airway responses such as antigen-induced rhinitis that involved the measurement of nasal secretion and sneezing responses (Fujita et al., 1999). As early as 1 day after infection, we noted widespread localization of IAV in nasal mucosa, which suggested that ciliated nasal epithelial cells in guinea pigs are highly susceptible to IAV infection. Therefore, the ease with which nasal epithelial cells supported IAV growth along with the excessive nasal mucus secretions might contribute to the susceptibility of guinea pigs to droplet spread.

Our results suggested that IAV-inoculated guinea pigs produced a predominantly upper airway infection with localized infection foci in the lungs. Infection in the lungs induced marked lung inflammatory responses, resulting in severe bronchopneumonia and alveolitis. Unlike IAV infection in mice, infected guinea pigs developed
pronounced nasal tract mucus secretion, which resembled rhinitis in IAV-infected persons. In addition, elevated tissue eosinophils were suggestive of the induction of airway hypersensitivity.

Therefore, in addition to their use for viral transmission studies, guinea pigs should be further explored for their potential contributions in the understanding of virus–host interactions in IAV infection and airway allergic responses.

Fig. 2. Representative histological changes in haematoxylin and eosin stained nasal tissues from guinea pigs at days 1, 3 and 7 following infection with $2 \times 10^6$ p.f.u. with HK/68. Sections shown are infected nasal mucosa at 1 (a), 3 (d) and 7 (g) days p.i. and mock-infected control (j). More detailed histology from these sections is shown at higher magnifications in (b), (e), (h) and (k) along with IAV antigen-positive cells as depicted by brown immunostaining (c, f and i). Tissue sections from the mock-infected control were negative for IAV antigen (l). Only very small foci of virus-positive cells were detectable on day 7 p.i. [arrow in panel (i)], which is shown at higher magnification in the insert. Excessive mucus secretion within the nasal tract is indicated by an asterisk symbol (*) in (g), (h) and (i). Bars, 50 μm.
such as asthma. In this respect, the guinea pig is unique because its airway innervation is very similar to that of humans (Wang et al., 2005). Moreover, guinea pigs are widely used in pulmonary pharmacology because their responses to various mediators and drugs are more similar to that of the human airway (Ressmeyer et al., 2006). For instance, leukotrienes readily cause bronchoconstriction in humans and guinea pigs, but not in mice or rats (Dahlen et al., 1980; Drazen et al., 1980; Hedqvist et al., 1980; Held et al., 1999).

Fig. 3. Representative histological changes in haematoxylin and eosin stained lungs from guinea pigs at days 1, 3 and 7 following infection with $2 \times 10^6$ p.f.u. with HK/68. Sections shown are infected lungs depicting bronchioles and varying degrees of cellular infiltrations at 1 (a), 3 (d) and 7 (g) days p.i., and mock-infected control (j). More detailed histology is shown at higher magnifications (b), (e), (h) and (k) along with IAV antigen-positive cells depicted by brown immunostaining in macrophages, bronchial and alveolar epithelial cells (c and f). Tissue sections at day 7 p.i. (i) and from mock-infected control (l) were negative for IAV antigen. An arrow in (b) points to the presence of eosinophils, which are shown at higher magnification in the insert. Bars, 50 μm.
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References


