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Replication and pathogenesis of avian influenza A (H5N1) virus infection in polarised human bronchial and alveolar epithelium

Key Messages
1. In vitro models of polarised human respiratory epithelial cells were established to investigate the tropism and innate host responses of influenza A (H5N1 and H1N1) viruses.
2. Both viruses efficiently infected alveolar epithelial cells of both the apical and basolateral surfaces of the epithelium, whereas release of newly formed virus was mainly from the apical surface of the epithelium.
3. H5N1 virus was a more potent inducer of cytokines and chemokines in alveolar epithelial cells than H1N1 virus. Such chemokines were secreted onto both the apical and basolateral surfaces of the polarised alveolar epithelium.
4. In bronchial epithelium, the H5N1 virus replicated more efficiently and induced a stronger type I interferon response in the undifferentiated NHBE cells than did H1N1 virus. In contrast, in well-differentiated cultures, H5N1 virus replication was less efficient and elicited a lower interferon-beta response than did H1N1 virus.
5. Reombinant virus with vRNPs of a mammalian PB2 and an avian PB1 had the strongest polymerase activities, and replicated better in human cell cultures, especially at a high incubation temperature. These viruses were potent inducers of cytokines and chemokines in primary human alveolar epithelial cells.

Introduction
Avian influenza A (subtype H5N1) transmitted from poultry to humans in Hong Kong in 1997 and caused a potentially fatal human respiratory disease. So far, human-to-human transmission of the H5N1 subtype influenza virus appears to be inefficient, although it can cause exceptionally severe disease in 60% of humans it infects. Most such patients have a primary viral pneumonia complicated by acute respiratory distress and multiple organ dysfunction, and notably there may be lymphopaenia and haemophagocytosis. These haematological abnormalities have been ascribed to cytokine dysregulation.

The respiratory epithelial cells are the primary targets for influenza A virus replication. In primates experimentally infected with H5N1/97 virus, types I and II pneumocytes and alveolar macrophages were found to contain viral antigen. Virus infection of alveolar pneumocytes was also demonstrated in the lung of a patient with fatal H5N1 disease. Human alveolar epithelium is vital for the maintenance of lung function and the pulmonary air-blood barrier. Human respiratory epithelial cells respond to viral infections by mounting a cytokine response that contributes to innate and adaptive host defences. In our previous studies, we investigated the proinflammatory cytokine profile associated with influenza A (H5N1) virus infection of primary monocyte-derived macrophages and non-polarised respiratory epithelial cells. We hypothesised that the hyper-induction of cytokines may contribute to the unusual severity of human H5N1 disease.

However, the physiological relevance of findings from non-polarised and undifferentiated cells, as well as transformed cells lines is uncertain. It is therefore important to study the influenza A (H5N1) virus–cell interactions using polarised and differentiated respiratory epithelial cells. We aimed to (1) establish a well-differentiated and polarised primary human respiratory epithelial cell culture model in vitro to study the replication and pathogenesis of avian influenza A (H5N1) virus disease in humans, (2) identify differences in the virus replication kinetics of H5N1 and H1N1 viruses in polarised human alveolar and bronchial epithelial cells in vitro, (3) define the contribution of different viral genes to the viral replication kinetics and polarisation using recombinant influenza viruses containing different gene combinations between human H1N1 and avian H5N1 viruses, and (4) define the contribution of different viral genes to the cytokine induction profile of H1N1 and H5N1 viruses.

Methods
This study was conducted from March 2007 to December 2009. Primary human in vitro well-differentiated alveolar and bronchial epithelial cells were infected with influenza A (H5N1 and H1N1) viruses in vitro. Virus replication was monitored by measuring the levels of the influenza matrix gene, by immune-fluorescence detection of the influenza matrix and nucleoprotein, and by titration of the infectious virus in MDCK cells. Using reverse genetics derived
Fig 1. Replication kinetics and innate host response of influenza A virus–infected polarised alveolar epithelial cells
(a) Virus titres of the A/HK/54/98 (H1N1) and A/HK/483/97 (H5N1) viruses are determined after apical and basolateral infections of the type I-like alveolar epithelial cells at 3 and 24 hours. At 24 hours following basolateral infection, the titre of influenza H5N1 virus at the apical surface of the cells is significantly more than that of H1N1 infected cells. (b) The cytokine (IFN-β and IL-6) and chemokine (RANTES and IP-10) gene expression from type I-like alveolar epithelial cells as well as release of RANTES and IP-10 proteins from alveolar epithelial cells after 24 hours of apical and basolateral infections of H1N1 and H5N1 viruses. The means and the standard errors from three representative experiments are shown. The dotted line represents the lowest detection limit of the TCID₅₀ assay.
Fig 2. Replication kinetics and innate host response of influenza A virus–infected well-differentiated bronchial epithelial cells
(a) Virus titres of the H1N1 and H5N1 viruses are determined after infection in the ud- and wd-NHBE cells for 1 to 48 hours at MOI of 2. At 24 hours post infection, viral replication kinetics of the 2 viruses are compared. (b) The IFN-β, RANTES, and IP-10 gene expression of the ud- and wd-NHBE cells at 6 and 24 hours post infection of H1N1 and H5N1 viruses. The means and standard errors from three independent experiments are shown. The dotted line represents the detection limit of the TCID₅₀ assay.
recombinant viruses, the role of viral PB1 and PB2 segments of H5N1 virus on viral replication and cytokine induction in the human respiratory tract was demonstrated. Gene and protein expression profiles (cytokine and chemokine) of respiratory epithelial cells infected with various influenza A viruses and recombinant viruses were compared by using real-time quantitative reverse transcriptase PCR.

**Results**

Both influenza H1N1 and H5N1 viruses efficiently infected alveolar epithelial cells of both the apical and basolateral surfaces of the epithelium, whereas release of newly formed virus was mainly from the apical surface of the epithelium (Fig 1a). H5N1 virus was a more potent inducer of cytokines and chemokines in alveolar epithelial cells than H1N1 virus. Such chemokines were secreted onto both the apical and basolateral surfaces of the polarised alveolar epithelium (Fig 1b). The H5N1 virus replicated more efficiently and induced a stronger type I interferon response in the undifferentiated NHBE cells than did H1N1 virus (Fig 2a). For other cytokines (eg RANTES and IP-10), H5N1 virus led to significantly higher induction of IF-10 and RANTES mRNA expression in undifferentiated NHBE cells than did H1N1 or mock infection (Fig 2b). In contrast, in well-differentiated cultures, H5N1 virus replication was less efficient and elicited a lower interferon-beta response than did H1N1 virus. Recombinant vRNPs with a mammalian PB2 and an avian PB1 had the strongest polymerase activities in human cells, and replicated better in cell cultures, especially at a high incubation temperature (Fig 3a). These viruses were potent inducers of cytokines and chemokines (eg RANTES and IP-10) in primary human alveolar epithelial cells (Fig 3b).

**Discussion**

Influenza viral infections are major causes of economic loss and of morbidity and mortality worldwide, and threaten economies and social stability as well as human health. These in vitro models of human respiratory epithelial cells enables better understanding of the transmission, prevention, and control of influenza A virus infections (especially the highly pathogenic H5N1 virus) and other emerging respiratory infections (such as SARS-CoV), as well as the underlying mechanism of inter-species transmission of animal pathogens to humans. These models have also been used to investigate the tropism and host responses of the swine-origin pandemic (H1N1pdm) virus. This is especially important when a good small animal model is lacking and in adherence to the 3R principles pertaining to the use of animals in research. Nonetheless, further investigations with relevant animal models of influenza virus infection and the potential use of immunomodulators in addition to antivirals may be considered. Cytokine dysregulation may play an important role in severe human influenza disease. The use of immunomodulators in combination with antivirals may help in reducing the severity of the disease.

**Fig 3. Characterisation of viral replication and cytokine and chemokine responses of recombinant viruses with chimeric polymerase complexes**

The origins of PB2, PB1, PA, and NP in each recombinant virus are shown (A=avian, M=mammalian). (a) Growth properties of the WSN (MAMM) and recombinant viruses in MDCK cells: the number of infectious progeny viral particles generated from MDCK cells infected with the corresponding virus at a MOI of 0.01 is determined by standard plaque assay. Mutant AMMM and AMMA are significantly attenuated. At 8 hours post infection, the amounts of infectious progeny of MAAA and MAMM are significantly higher than the wild type controls. (b) Cytokine and chemokine (RANTES and IP-10) gene expression profiles from primary human alveolar epithelial cells: total RNA from cells infected at a MOI of 2 is harvested at the indicated time points and tested by the corresponding quantitative RT-PCR. The means of triplicate assays and the recombinant viruses used in the experiments are shown.
with antivirals may have clinical benefits. Our results may provide a biological basis for the observed therapeutic impact of immunomodulators for treating severe human influenza disease.

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References