

## **Human and avian influenza viruses target different cell types in cultures of human airway epithelium**

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### **Abstract**

The recent human infections caused by H5N1, H9N2, and H7N7 avian influenza viruses highlighted the continuous threat of new pathogenic influenza viruses emerging from a natural reservoir in birds. It is generally believed that replication of avian influenza viruses in humans is restricted by a poor fit of these viruses to cellular receptors and extracellular inhibitors in the human respiratory tract. However, detailed mechanisms of this restriction remain obscure. Here, using cultures of differentiated human airway epithelial cells, we demonstrated that influenza viruses enter the airway epithelium through specific target cells and that there were striking differences in this respect between human and avian viruses. During the course of a single-cycle infection, human viruses preferentially infected nonciliated cells, whereas avian viruses as well as the egg-adapted human virus variant with an avian virus-like receptor specificity mainly infected ciliated cells. This pattern correlated with the predominant localization of receptors for human viruses (2-6-linked sialic acids) on nonciliated cells and of receptors for avian viruses (2-3-linked sialic acids) on ciliated cells. These findings suggest that although avian influenza viruses can infect human airway epithelium, their replication may be limited by a nonoptimal cellular tropism. Our data throw light on the mechanisms of generation of pandemic viruses from their avian progenitors and open avenues for cell level-oriented studies on the replication and pathogenicity of influenza virus in humans.

Influenza pandemics originate from birds which provide a reservoir for a large variety of influenza A viruses containing at least 15 hemagglutinin (HA) and nine neuraminidase (NA) subtypes. Fortunately, both initial human infections by avian viruses and the emergence of pandemic viruses are restricted by a limited fitness of avian viruses in humans. In the case of virus genes other than the HA gene, this restriction can be overcome by gene reassortment between avian and human viruses similar to that which occurred in the 1957 and 1968 pandemic viruses. However, any pandemic virus must carry the HA from a nonhuman virus. Therefore, one of the key themes in the study of pandemic influenza is to understand the mechanisms of HA-mediated restriction and how avian viruses manage to breach it.

Avian influenza viruses bind to cell-surface glycoproteins or glycolipids containing terminal sialyl-galactosyl residues linked by 2-3-linkage [Neu5Ac(2-3)Gal], whereas human viruses, including the earliest available isolates from the 1957 and 1968 pandemics, bind to receptors that contain terminal 2-6-linked sialyl-galactosyl moieties [Neu5Ac(2-6)Gal]. The 1918 influenza pandemic viruses presumably also had a human-virus-like receptor specificity. By contrast, H5N1 chicken viruses that caused the influenza outbreak in humans in Hong Kong in 1997 had an avian virus-like receptor specificity (12) and were unable to transmit efficiently from human to human (13). It is

believed, therefore, that an alteration of the receptor specificity is essential for the emergence of pandemic viruses from avian progenitors, but the mechanism of this phenomenon is not clear.

In humans, influenza viruses replicate in the ciliated epithelium of conducting airways, which consists of several distinct cell types with different functions. Observations made late in the infectious process in humans and monkeys show that influenza viruses infect many different types of airway epithelial cells. However, neither initial targets of the virus attack nor specific cell types that are essential for virus replication have been defined. Our knowledge on the receptor equipment of human airway epithelium is also limited. Predominant expression of 2-6-linked sialic acids on the apical surface of human tracheal epithelial cells has been reported, but little is known about the presence of 2-3-linked sialic acids on such cells or about variations in sialic acid expression on different cell types.

To address these questions and to understand how avian-virus-like receptor specificity limits efficiency of virus replication in humans, we studied cellular tropism of human and avian influenza viruses and distribution of virus receptors in differentiated cultures of human tracheobronchial epithelium (HTBE). These cultures are pseudostratified and polarized, contain ciliated, secretory, and basal cells, and they resemble human airway epithelium *in vivo* both morphologically and functionally. Here we show that different receptor-binding specificity of human and avian viruses determines their preferential tropism to nonciliated cells and ciliated cells, respectively. This finding suggests that infection of nonciliated epithelial cells in human airways is essential for the efficient replication and/or transmission of influenza viruses and that fitness of avian viruses in humans may be compromised by their nonoptimal cellular tropism.

#### Materials and Methods:

**Airway Epithelial Culture:** Primary human epithelial cells from tracheal/bronchial and nasal tissues were purchased from Clonetics and Promo Cell, Heidelberg, respectively. Cells of passage 2 were grown on membrane supports (12-mm Transwell-Clear, Corning) at the air-liquid interface in serum-free and hormone- and growth factor-supplemented medium as described. Fully differentiated 4- to 8-wk-old cultures were used for all experiments.

**Viruses and Ab:** Human influenza A/Bayern/7/95-like virus (H1N1), A/Sydney/5/97-like virus (H3N2), and B/Sichuan/379/99-like virus were isolated from clinical material and passaged in Madin-Darby canine kidney (MDCK) cells. Sheep antisera against human viruses were provided by Alexander Klimov at the Centers for Disease Control and Prevention, Atlanta. Human clinical isolates A/Memphis/14/96-M (H1N1), A/Memphis/5/98 (H3N2), and B/Memphis/25/99 in MDCK cells, avian influenza viruses, and rabbit antisera against avian viruses were kindly provided by Robert Webster of St. Jude Children's Research Hospital, Memphis, TN.

**Virus Infection:** The apical surfaces of the cultures were washed 10 times to remove accumulated mucins before inoculation with 0.2 ml of virus dilutions in the complete growth medium. The inoculum was removed after 1 h of incubation at 35°C; the cultures were incubated either for 6 h or for 23 h at the air-liquid interface and fixed with 4% paraformaldehyde for 1 h at 20°C. No trypsin was added to the cultures because previous studies in similar cultures demonstrated efficient proteolytic activation of influenza viruses by endogenous proteases.

**Double Immunostaining and Light Microscopy:** Fixed cultures were permeabilized with 0.2% Triton X-100 and immunostained by sequential incubations with reagents from the apical side. Cilia were stained by using the  $\alpha$ -tubulin-specific mAb (Sigma), horseradish peroxidase (HRP)-labeled secondary Abs, and Vector SG substrate (Vector Laboratories). Virus-infected cells were stained by using antiviral Abs, followed by corresponding HRP-labeled secondary Abs (Dianova, Hamburg, Germany) and aminoethylcarbazole substrate (Sigma). Some cultures were additionally stained by Alcian blue (pH 3) and periodic acid-Schiff reagent (Sigma) to identify secretory cells. Immunostained cultures were mounted by using Crystal Mount (Biomed, Foster City, CA) and photographed en face by using a Nikon Optiphot-2 microscope equipped with a charge-coupled device camera. For cell counting, the cultures were observed en face with oil-immersion at x1,000 magnification. In microscopic fields containing between 5% and 25% ciliated cells with respect to the total amount of superficial cells, each infected cell was classified as ciliated (based on the presence of cilia), nonciliated, or undefined. Percentages of ciliated and nonciliated infected cells with respect to the total amount of infected cells were calculated. For each sample, 25-35 fields were analyzed and the results were averaged.

**Fluorescent Confocal Microscopy:** Infection and immunostaining were performed as described above, using FITC-labeled and Texas red-labeled secondary Abs (Dianova) to detect cilia and the virus antigen, respectively. The cultures were mounted and photographed by using an LSM 510 laser scanning microscope (Zeiss).

**Receptor-Binding Activity:** Monospecific HRP-labeled fetuin containing either 2-6-linked or 2-3-linked sialic acid was prepared, and its binding to the viruses was determined by using a solid-phase binding assay as described.

**Detection of 2-6- and 2-3-Linked Sialic Acid:** HTBE cultures were washed 10 times with growth medium to remove overlying mucus and incubated for 1 h on ice with digoxigenin-labeled lectins Sambucus nigra agglutinin (SNA; 4  $\mu$ g/ml) or Maackia amurensis agglutinin (MAA; 20  $\mu$ g/ml) (Roche Molecular Biochemicals) in Tris-buffered saline (pH 7.2) containing 1% BSA and 1 mM Mg<sup>2+</sup>, Ca<sup>2+</sup>, and Mn<sup>2+</sup>. After washing and fixation with 4% paraformaldehyde, the cultures were incubated for 1 h at 20°C with HRP-labeled anti-digoxigenin Abs (Roche Molecular Biochemicals) in 1% BSA followed by incubation with aminoethylcarbazole substrate. The cells were permeabilized, and cilia were immunostained as described above. Control cultures were treated with lectin solutions that had been preincubated for 1 h with 10 mg/ml bovine fetuin. They showed no significant staining.