A Critical Role of Cell Tropism for the Pathogenesis of Influenza

Debby van Riel

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A Critical Role of Cell Tropism for the Pathogenesis of Influenza

Een kritieke rol van celtropisme in de pathogenese van influenza

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Voor papa en mama

Table of contents


influenza
virus
HPAIV
viruses
cells
H5N1
species
humans
disease
infection
Influenza
avian
human
birds
alveolar
respiratory
transmission
epithelial
caused
pandemic
host
also
tract
viral
replication
population
poultry
type
cell
infected
mammalian
outbreak
among
cases
pneumocytes
relatively
wild
severe
may
different
seasonal
pigs
HA
attachment
evidence
several
estimated
infections
cause
mortality
pathogenesis
replicates
chickens
spread
transmitted
direct
antigen
detected
site
two
barrier
clear
H7
NA
swine
pandemics
nucleus
important
damage
occur
high
Although
contrast
flu
varied
able
case
alveolus
addition
types
proteins
million
compartments
primary
seals
surface
alveoli
circulating
segments
lumen
differs
RNA
primarily
often
columnar
studies
outbreaks
observed
fatality
result
fatal
human-to-human
harbor
dogs
first
mild
intestinal
considered
rate
pneumocyte
subtypes
generally
cats
tissues
largely
mammals
subtype
fluid
low
cytoplasm
bird
H1N1
endothelial
Since
lung
H3N2
LPAIV
efficiently
time
including
However
ciliated
nasal
Furthermore
nuclear
people
became
example
macrophages
recognize
horses
another
domestic
ribonucleoproteins
lined
H7N7
capacity

Chapter 1

General Introduction

Influenza A viruses

Influenza A virus, together with Influenza B virus, Influenza C virus, Isavirus and Thogotovirus, are the five genera forming the family *Orthomyxoviridae*. *Orthomyxoviridae* are enveloped, negative-stranded RNA viruses with a segmented genome. Influenza A viruses can be further categorized into subtypes based on two surface glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA). To date, 16 different HAs and 9 different NAs have been found and may occur in virtually all possible combinations.[1] Free-ranging waterbirds are considered to be the natural reservoir of all influenza A viruses.[1;2] Even though some mammalian species—humans, pigs, horses and dogs—harbor their own influenza A viruses, there is strong evidence that these mammalian viruses originate from avian influenza A viruses.[3] In addition, avian influenza A viruses may cross the species barrier to other mammals without developing into a stable lineage in the new host species. An example is the outbreak of avian influenza virus of the subtype H7N7 in harbor seals in 1979.[4] Interestingly, the primary replication site for influenza A virus differs between birds and mammals. In birds, the virus replicates primarily in the intestinal tract, whereas in mammals, it replicates primarily in the respiratory tract.

An important feature of avian influenza viruses is their capacity to mutate into forms that cause high mortality in poultry. Such mutations only have been recorded for the subtypes H5 and H7 and occur after transmission of these viruses to poultry. Based on their capacity to cause morbidity and mortality in chickens, avian influenza viruses are classified as low pathogenic avian influenza virus (LPAIV) or high pathogenic avian influenza virus (HPAIV).[5]

Human respiratory tract

The human respiratory tract has many different anatomical compartments. The laryngo-tracheal transition is often considered as the border between the upper (URT) and lower respiratory tract (LRT). The URT consists of nasal vestibule, nasal septum, nasal concha, nasopharynx, oropharynx, paranasal sinuses and larynx. Depending on the site, these tissues are lined by different cell types: non-ciliated squamous epithelial cells, ciliated columnar epithelial cells, goblet cells, and olfactory epithelial cells.[6] Below the epithelial lining, submucosal glands may be found throughout the URT. These glands produce mucus, which trap pathogens, inhibit their replication, and clear them from airways, so forming an important line of defence against respiratory infections.[7]

The human LRT consists of trachea, bronchi, bronchioles, and alveoli (Figure 1). The trachea and bronchi are lined by ciliated columnar epithelial cells and goblet cells. The bronchioles are lined by ciliated columnar epithelial cells, goblet cells and—in the smallest divisions—by non-ciliated cuboidal cells, or Clara cells. The alveoli are

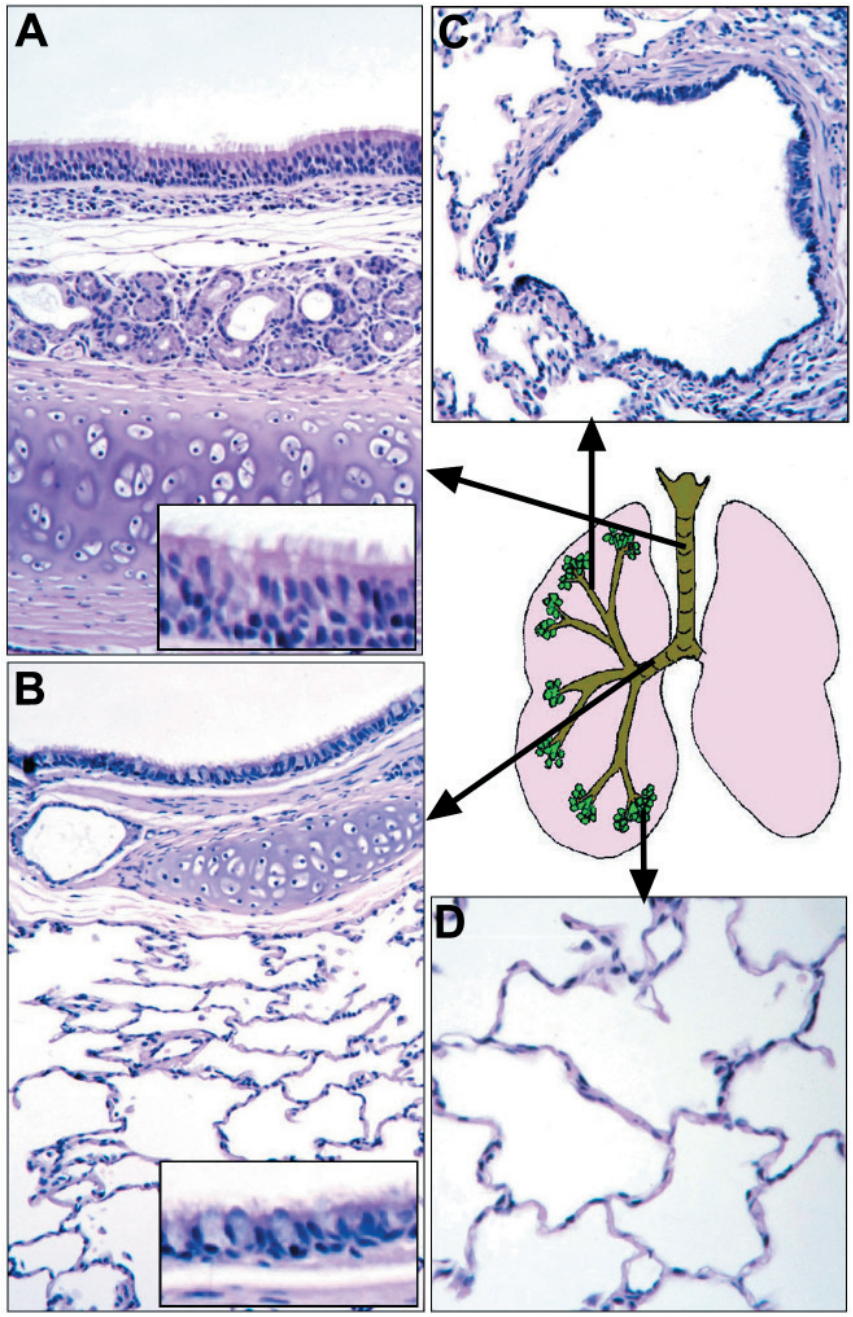


Figure 1: *A schematic overview of the mammalian respiratory tract. (A) The trachea, which is lined by ciliated epithelial cells and a few goblet cells (insert). (B) The bronchus, which is lined by ciliated epithelial cells and goblet cells (insert). (C) A bronchiole, which is lined by ciliated epithelial cells and non-ciliated cuboidal epithelial cells. (D) The alveoli, which are lined by type I and type II pneumocytes.*

lined by type I and type II pneumocytes. The alveoli are the part of the lung where gas exchange occurs between alveolar lumen and lung capillaries. The alveoli are also the site where the main pathological changes occur in influenza pneumonia and are therefore discussed in more detail below.

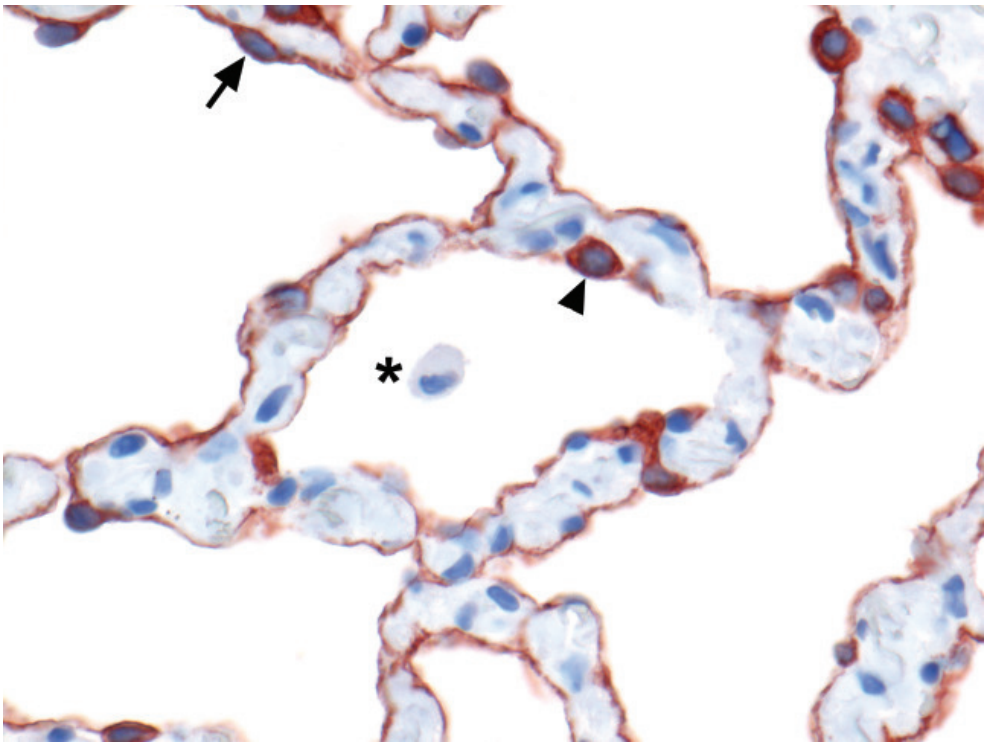


Figure 2: *Alveoli in human lung stained by immunohistochemistry for pancytokeratin (epithelial cells). The alveolar wall is lined by flat type I pneumocytes (arrow) and more cuboidal type II pneumocytes (arrowhead). The alveolar macrophage (asterix) resides in the alveolar lumen.*

The pulmonary alveolus forms a unique micro-environment. Important cell types are the type I pneumocyte, type II pneumocyte, and alveolar macrophage, each with its own function (Figure 2). Type I pneumocytes are flat cells that comprise 40% of the alveolar epithelial cells in number, yet cover more than 90% of the surface of the alveolar wall.[8] Because they are so flat, they presumably facilitate gas exchange between air in the alveolar lumen and blood in the lung capillaries.[9] Type I pneumocytes are metabolically rather inactive. Type II pneumocytes are cuboidal cells that comprise 60% of the alveolar epithelial cells.[8] Their functions include re-epithelization after alveolar damage, they are the progenitor cells of type I pneumocytes, ion transport, fluid resorption and surfactant production. They are metabolically highly active. Alveolar macrophages are pleomorphic cells that reside in the alveolar lumen and are in constant contact with the epithelial lining. Each alveolus is estimated to contain one alveolar macrophage on average.[10] Alveolar

macrophages protect the alveolus by phagocytosis of pathogens and apoptotic cells. They also tightly regulate immune responses by constant interaction with alveolar epithelial cells, and so prevent excessive inflammation.[11;12] The pulmonary alveolus has several other cell types, which are not discussed here; these include fibroblasts, endothelial cells, mast cells, dendritic cells, and interstitial macrophages

Replication cycle of influenza viruses

There are several stages in the replication cycle of influenza viruses: (1) attachment, (2) entry, (3) fusion and uncoating, (4) nuclear import of ribonucleoproteins, (5) virus RNA synthesis, (6) transcription and replication, (7) nuclear export of ribonucleoproteins and (8) virus assembly and release.[13] During the first step, attachment, the HA of the influenza virus attaches to sialic acids (SA) on the surface of the host cell. The ability of a specific HA to recognize and interact with the SA depends in part on the type of linkage of the SA to the underlying galactose. For example, the HA of human influenza viruses generally recognize SA with an α-2,6 linkage, whereas the HA of avian influenza viruses generally recognize SA with a α-2,3 linkage.[14] However, the attachment of an influenza virus to its host cell also depends on other factors, such as the type of the SA, and glycosylation and sialylation of the HA close to the receptor binding site.[15] Limited studies have shown that the distribution of receptors for influenza virus differs largely among species. This indicates that virus attachment is an important limiting factor in the host range of influenza viruses. However, the character and the distribution of these receptors in humans and other host species have so far not been studied extensively.

After attachment, influenza viruses are internalized by endocytic compartments. In these compartments, the low pH activates fusion of the viral membrane with the endosome and releases the viral contents into the cytoplasm. The eight viral RNA segments are imported into the nucleus, where the viral RNA synthesis is dependent on host cell nuclear functions. The recognition of viral proteins by host importins, which are required for nuclear entry, are another important factor in host restriction of influenza viruses.[16] Viral RNA is synthesized in the nucleus, while viral proteins are translated from mRNA, after it has been transported to the cytoplasm. Viral ribonucleoproteins are assembled in the nucleus, from where they are actively exported to the cytoplasm. The viral envelope proteins, HA, NA and matrix protein 2 (M2) accumulate at the cell surface. Matrix protein 1 (M1) accumulates just underneath the lipid envelop, which facilitates the incorporation of the eight viral RNA segments. M1 initiates budding and the separation of the viral envelope from the cell membrane. Finally, the enzymatic activity of the NA cleaves the sialic acid from the HA and thereby releases the virus from the host cell.[17;18] Influenza virus infection is a lytic infection. Therefore, infection of the host cell with an influenza virus will always result in death of the infected cell.

Influenza virus pathogenesis in birds

LPAIV

Although LPAIV infections have not been studied extensively in their natural hosts, free-ranging waterbirds, recent studies in mallard ducks (*Anas platyrhynchos*) indicate that LPAIV infections cause little if any disease.[19;20] Virus can be isolated from cloacal swabs and—to a lesser extent—from pharyngeal swabs of infected birds, indicating that the virus replicates in intestinal and respiratory tracts. We confirmed replication in the intestinal tract of mallards recently by the detection of influenza virus antigen in the epithelium of bursa of Fabricius and intestine by immunohistochemistry (Daoust, in preparation). Remarkably, the presence of virus antigen, indicative for active virus replication, was not associated with any histological lesions in these birds.

HPAIV

HPAIV, which arise from LPAIV subtypes H5 and H7, cause severe disease with up to 100% mortality in poultry. We and others have shown that HPAIV viruses infect endothelial cells systemically in chickens, although the degree of viral tropism for endothelial cells may vary among tissues.[21] Replication of influenza virus in endothelial cells causes damage to the blood vessels, which leads to edema and hemorrhage. In addition to endothelial cells, HPAIV are able to replicate in cells of many parenchymal organs such as the brain, liver and spleen, resulting in widespread inflammation and necrosis.[5;21]

Until 2005, HPAIV infection in wild birds was considered to be rare, and transmission of HPAIV among wild birds even rarer if not non-existent. Until then, the only reported outbreak of HPAIV in wild birds was H5N3 virus in common terns (*Sterna hirundo*) along the coast of South Africa in 1961.[22] In 2005, however, HPAIV H5N1 caused a massive die-off among several wild waterbird species at Qinghai Lake, China.[23;24] After this outbreak, HPAIV H5N1 spread westwards to India, Europe and Africa. Since then, there is substantial evidence that HPAIV H5N1 occurs in wild waterbird species, but it is not clear whether HPAIV H5N1, like LPAIVs, is able to maintain itself in wild waterbirds.[25] Experimental studies have revealed that HPAIV H5N1 productively infects different duck species, but that the severity of disease ranges from subclinical infection to fatal systemic disease. In contrast to chickens with HPAIV H5N1 infection, Eurasian pochards (*Aythya ferina*) and tufted ducks (*Aythya fuligula*), in which HPAIV H5N1 replicates systemically, do not show virus replication in endothelial cells.[26] This indicates that the pathogenesis of HPAIV H5N1 infection not only varies among duck species, but also differs largely from the pathogenesis of this infection in chickens.

Influenza virus pathogenesis in humans

Seasonal influenza viruses typically infect the upper respiratory tract and larger airways in humans, causing mild disease. Signs and symptoms of uncomplicated influenza virus infections are both local (nasal obstruction, cough, sore throat) and systemic (headache, fever, chills anorexia and myalgia).[17] The primary complication of seasonal influenza is pneumonia. Furthermore, in rare cases there is evidence for extra-respiratory spread of influenza virus, which may result in central nervous system disease, myocarditis and myopathy.[27]

Infection of humans by HPAIV H5N1 differs considerably from that by seasonal influenza viruses. HPAIV H5N1 infection is sporadic and probably requires a high virus dose, e.g., by close contact with infected poultry. In contrast with seasonal influenza virus infection, HPAIV H5N1 infection primarily causes severe pneumonia, with a case fatality rate of about 60%. The primary lesion observed in human HPAIV H5N1 fatal cases is diffuse alveolar damage. The alveolar lumina contain edema fluid, fibrin, hyaline membranes, macrophages and desquamated epithelial cells. There is type II pneumocyte hyperplasia and infiltration of lymphocytes and, in some cases, neutrophils. An additional histologic feature is bronchiolitis.[27;28] By immunohistochemistry, influenza virus has been detected in type II pneumocytes and tracheal epithelial cells.[29;30] In addition to replication in the respiratory tract, HPAIV H5N1 may also replicate in extra-respiratory tract tissues. By immunohistochemistry and in situ hybridisation, influenza virus has been detected in neuronal cells in the brain, epithelial cells and mononuclear cells in the intestinal tract, Kupffer cells in the liver, lymphocytes in the lymph nodes, and Hofbauer cells and cytotrophoblasts in the placenta. In a fetus, influenza virus antigen has been detected in the lung. Furthermore, HPAIV H5N1 has been isolated from cerebrospinal fluid in a child with central nervous system disease without any signs of respiratory tract disease.[31] There only have been a few suspected cases of human-to-human transmission, indicating that transmission of HPAIV H5N1 between humans is inefficient.[32]

Influenza virus pathogenesis in other mammals

Not only humans, but also pigs, horses and dogs harbor their own influenza viruses. These viruses are able to cause respiratory disease in their host, and are transmitted efficiently in the host population. The severity of disease caused by these viruses varies; normally disease is relatively mild with recovery within two weeks, but more severe respiratory disease also may be observed.

Cross-species transmissions of influenza virus has been observed in several mammalian species, including ferrets, cats, horses, pigs, harbor seals (*Phoca vitulina*) and mink.[4] Spread of infection in the recipient host species ranged from solitary cases to large outbreaks. The resulting disease caused by these infections

also varied greatly. For example, avian H7N7 virus infection in harbor seals caused severe respiratory disease with an estimated mortality of 25% of the affected population.[33] In contrast, pandemic human H3N2 virus infection in cats was subclinical and infection was confirmed by serology only.[34]

HPAIV H5N1 also has proven successful in crossing the species barrier to a wide range of mammalian species. The first report of a non-human HPAIV H5N1 infection in mammals dates from December 2003, when two captive tigers (*Panthera tigris*) and two leopards (*Panthera pardus*) died after feeding on HPAIV-H5N1-infected chicken carcasses.[35;36] Since then, HPAIV H5N1 has also transmitted to numerous other mammalian species, including domestic cats, domestic dogs, Owston's palm civets (*Chrotogale owstoni*), a stone marten (*Mustela foina*), an American mink (*Mustela vison*) and domestic pigs. As in avian species, the severity of disease caused by HPAIV H5N1 varied greatly among mammalian species; domestic pigs had a relatively mild disease,[37-39] while the other mammalian species suffered severe respiratory disease, often associated with extra-respiratory spread of the virus.[4]

Disease burden of influenza in humans

Influenza A viruses in their different forms—seasonal, zoonotic, and pandemic—inflict a large burden of disease on humans. Seasonal influenza viruses circulate in the human population and are responsible for annually recurring epidemics. In the moderate climate zones, these are restricted to the fall and winter periods. It is estimated that, worldwide, between 250,000 and 500,000 people die from seasonal influenza each year.[40]

Zoonotic influenza virus infections in humans are the result of direct transmission of an influenza virus from another host species. Most of these transmission events involve birds or pigs. In the past, direct transmission of avian influenza virus from birds to humans was not thought to occur at all, as it was generally believed that humans did not have the appropriate receptor for these viruses. Therefore, it was postulated that avian influenza viruses only could cross the species barrier to humans after infection of pigs (or possibly another mammal) as an intermediate host.[41;42]

However, in 1997 a HPAIV of the H5N1 subtype, which was causing HPAIV outbreaks in China in poultry at that time, was transmitted in Hong Kong to 18 individuals, six of whom died. Most of these people had recently been to live bird markets in Hong Kong, where infected birds, imported from mainland China, had been present.[43] From 2003 onwards, HPAIV of the H5N1 subtype caused outbreaks in poultry in Vietnam, Indonesia and Thailand, and subsequently spread to larger parts of Asia, the Middle East, Europe and western Africa. Besides poultry flocks, wild bird populations also were affected from time to time during this unprecedented

spread.[23;24] Furthermore, in areas where these HPAIV of the H5N1 subtype were present in wild bird populations or mass mortality in poultry occurred, several mammalian species—predominantly carnivores—became infected with often fatal consequences.[4] Since 2003, there have been recurrent reports of sporadic HPAIV H5N1 infections in humans. These are all single cases or small clusters, without any evidence of sustained human-to-human transmission. So far, about 500 severe human cases have been identified with a case fatality rate of about 60% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_06_08/en/index.html).

Zoonotic influenza also occurred during an outbreak in poultry caused by HPAIV H7N7 in 2003 in the Netherlands. Although this outbreak was controlled within three months, it was the largest outbreak of a HPAIV of the H7 subtype ever recorded. In total, 255 flocks became infected and over 30 million birds were culled. An unusual aspect of this outbreak was the frequent transmission of the H7N7 virus to humans. In total, 89 people became infected with clinical symptoms, including one person who died. In addition, there was evidence of human-to-human transmission in three cases.[44]

Outbreaks of HPAIV as described not only have major consequences for animal health and animal welfare but obviously also for local food production and economies. Although the direct impact on human health is relatively limited, it should be realized that this is largely due to the absent or limited human-to-human transmission of these viruses. However, once such an avian influenza virus acquires the capacity to spread efficiently among humans—either directly or after passage through another mammal like the pig—it may result in a pandemic.

Influenza pandemics occur when a new influenza virus from the animal world is introduced into the human population and is subsequently transmitted efficiently among humans, resulting in attack rates of more than 20% of the world population. The burden of disease of the previous influenza pandemics varied largely. The most severe influenza pandemic reported was caused by an H1N1 virus that was introduced in the human population in 1918 (Spanish flu). It is estimated that this pandemic resulted in more than 40 million excess deaths worldwide.[45] In 2005, this virus was recovered from archived formalin-fixed lung autopsy materials and from frozen, unfixed lung tissues from an Alaskan influenza victim who was buried in permafrost. Studies on these materials revealed that this virus was most likely the result of the direct transmission of an avian influenza virus from birds to humans.[46] The Asian flu pandemic in 1957 (H2N2 virus), and the Hongkong flu pandemic in 1968 (H3N2 virus) originated from the reassortment between human and avian viruses. These pandemics were less severe and each caused an estimated 1-4 million excess deaths.[17] In 1977, H1N1 viruses re-emerged after having disappeared from the human population after the introduction of the H2N2 pandemic virus. These viruses were closely related to the H1N1 viruses circulating in the 1950s, and it is

therefore believed that the re-introduction was the result of an accidental release from a laboratory. Unlike the previous pandemics, this re-emergence of H1N1 virus did not replace the circulating H3N2 viruses, and since then both viruses have co-circulated in the human population.[17]

The currently ongoing “swine flu” or “Mexican flu” pandemic is caused by an H1N1 (pH1N1) virus that is genetically and antigenically distinct from circulating seasonal H1N1 influenza viruses. This pH1N1 virus apparently is a virus of swine origin that contains gene segments from both North American and Eurasian swine virus lineages.[47] It had started spreading among humans in Mexico before April 2009 when it was first recognized in California (USA) in a hospitalized child that came from Mexico. To date, the impact of this pandemic is not yet clear as it is still ongoing. Although the disease was relatively mild and the case fatality rate relatively low, recent data indicate that, due to the relatively young age of the fatal cases, the number of years lost in the U.S. population alone is between 330000 and 2000000.[48]

Outline of this thesis

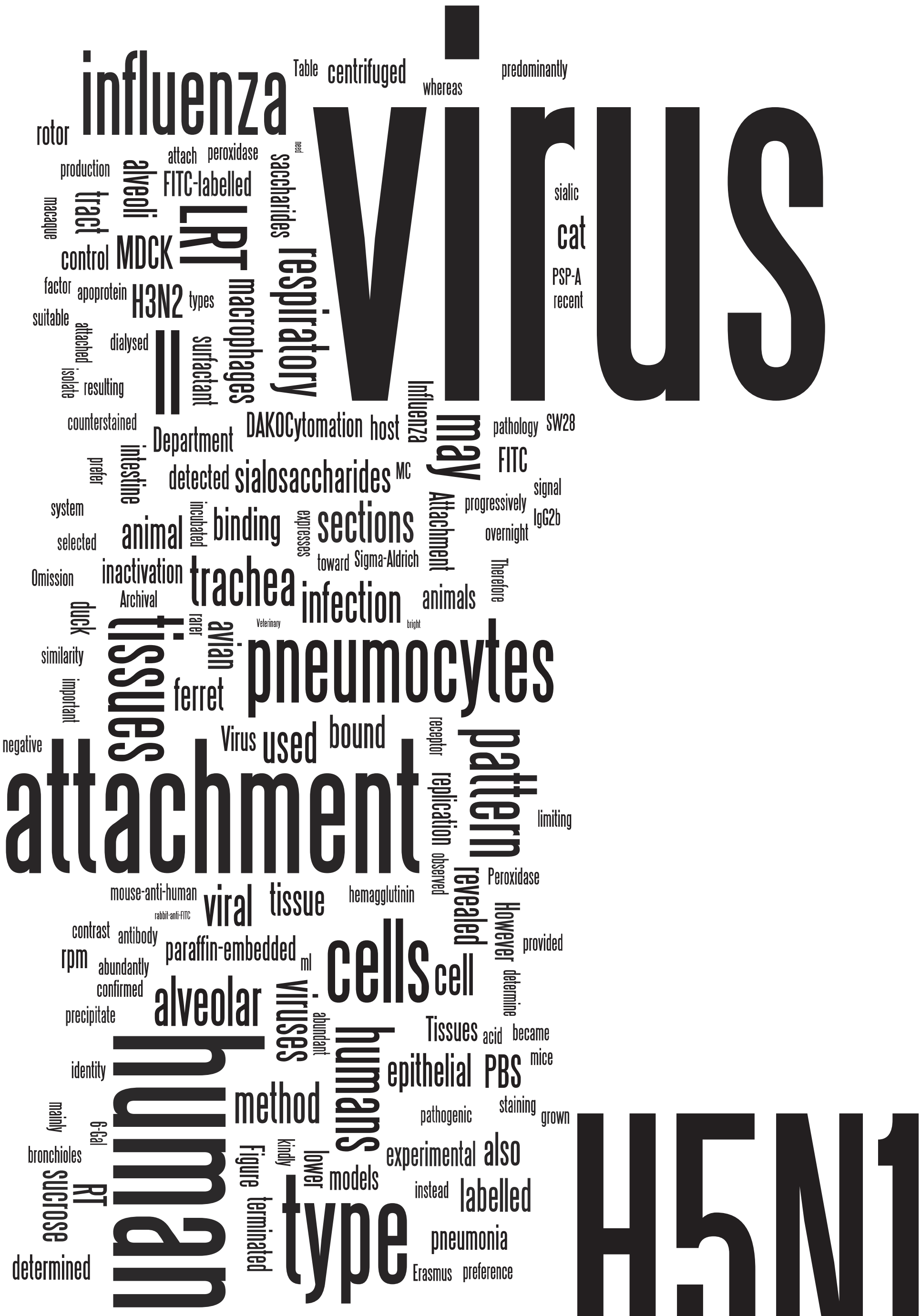
To better understand the pathogenesis of influenza A viruses in humans and other mammals we first determined the attachment pattern of different influenza viruses in the respiratory tract of humans and laboratory mammals which are commonly used to study influenza virus infections. **Chapter 2** and **3** focus on the lower respiratory tract, and it is shown that seasonal human and avian influenza viruses attach to different sites and cell types in the respiratory tract of humans. Furthermore, it is shown that there is a large difference in the pattern of attachment between different influenza A viruses in different animal species. **Chapter 4** focuses on the upper respiratory tract, and examines the attachment of seasonal human influenza viruses, pH1N1 virus, and avian influenza viruses, including HPAIV H5N1.

In **chapter 5** we studied mutant H5N1 viruses, of which the HA contained mutations that could increase the tropism to the human URT. By virus histochemistry, the influence of the mutations on the attachment to the human URT is studied and thereby the influence on the pandemic potential of these viruses.

Chapter 6 addresses the question whether seasonal influenza virus, pH1N1 virus or HPAIV H5N1 infects human alveolar macrophages productively and whether this leads to excessive cytokine production. In addition, we examine whether alveolar macrophages and macrophages cultured from monocytes interact differently after influenza virus infections.

Based on the attachment studies presented in chapter 3, **chapter 7** and **8** focus on the pathogenesis of HPAIV infections in cats, as this may be a good model for studying the pathogenesis of these infections in humans. Special attention is paid to differences between HPAIV H5N1 and HPAIV H7N7 infections, with an emphasis on severity of pneumonia, extra-respiratory dissemination of the respective viruses and the nature of lesions in extra-respiratory organs.

Chapter 9 consists out of a discussion on the implications of the data generated for our understanding of the role that viral tropism plays in the pathogenesis of influenza.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Chapter 2

H5N1 Virus Attachment to Lower Respiratory Tract

Based on:
Debby van Riel, Vincent J. Munster, Emmie de Wit, Guus F. Rimmelzwaan, Ron A.M. Fouchier, Ab D. M. E. Osterhaus and Thijs Kuiken.

And: Supporting online Material

Science. 2006; 312: 399

Abstract

Highly pathogenic avian influenza virus (H5N1) may cause severe lower respiratory tract (LRT) disease in humans. However, the LRT cells to which the virus attaches are unknown for both humans and other mammals. We show here that H5N1 virus attached predominantly to type II pneumocytes, alveolar macrophages, and non-ciliated bronchiolar cells in the human LRT, and this pattern was most closely mirrored in cat and ferret tissues. These findings may explain, at least in part, the localization and severity of H5N1 viral pneumonia in humans. They also identify the cat and the ferret as suitable experimental animals based on this criterion.

Introduction

Highly pathogenic avian influenza virus of the subtype H5N1 may cause infection of the lower respiratory tract (LRT) and severe pneumonia in humans.[49] However, the cell types in the LRT to which the virus attaches are unknown for both humans and experimental animals. Although attachment is not the only factor required for virus replication, it is the first step in the replication cycle. Therefore, this information is important both to better understand the pathogenesis of H5N1 influenza and to assess the suitability of animal models.

Influenza viruses attach to host cells by binding of the hemagglutinin to sialosaccharides on the host cell surface. Human influenza viruses prefer sialic acid (SA)-α-2,6-Gal terminated saccharides, whereas avian influenza viruses prefer those terminating in SA-α-2,3-Gal.[15] Lectins that specifically detect α-2,6– and α-2,3–linked sialosaccharides are an indirect measure of influenza virus attachment to host tissues, and lectins do not account for other variables that influence the binding avidity, such as type of SA, and glycosylation and sialylation of the hemagglutinin close to the receptor binding site.[15] Therefore, we compared the pattern of H5N1 virus attachment to the LRT of humans and four animal species commonly used for influenza virus research with a method that directly visualised the attachment of an FITC-labelled H5N1 virus. This method is a more direct method, which was modified from a previously used technique.[50]

Materials and Methods

Experimental design

To determine the cell types to which H5N1 virus binds in the lower respiratory tract of humans, we determined the attachment pattern of a recent highly pathogenic H5N1 virus isolate from Asia in human tissues. We determined whether attachment occurred to epithelial cells of the trachea, bronchi, bronchioles, and alveoli. The binding pattern of this H5N1 virus was also determined in mammals that are used for experimental influenza virus infections. Tissues from the lower respiratory tract of cynomolgus macaque, domestic cat, ferret, and mouse were included. As a control for the validity of the method, we examined the attachment of H5N1 virus and a currently circulating human H3N2 virus on human and avian tissues with known receptor identity.

Viruses

Influenza virus A/Vietnam/1194/04 (H5N1) was isolated from a fatal human case. The virus was kindly provided by Dr. W. Lim, Queen Mary Hospital, Hong Kong and propagated once in Madin-Darby canine kidney (MDCK) cells. Influenza virus A/Netherlands/213/03 (H3N2) is a recent human isolate grown on MDCK cells, and was kindly provided by the National influenza Center, The Netherlands.

Virus preparation, inactivation and labelling

The H5N1 virus was grown on MDCK cells. The supernatant was harvested and cleared by low speed centrifugation. Cleared supernatants were subsequently centrifuged for 2 h at 27000 rpm in a SW28 rotor at 4 °C on a 0.5 ml layer of 60% sucrose. The lowermost 2.5 ml virus on sucrose cushion was transferred to a 60-20 % sucrose gradient and centrifuged overnight at 39000 rpm in a SW41 rotor at 4 °C. To deplete the sucrose from the virus fraction, it was diluted in PBS and centrifuged for 2 h at 27000 rpm in a SW28 rotor at 4 °C, and the virus pellet was resuspended in PBS. H5N1 virus was inactivated by dialysing against 0.1 % formalin for 3 days at RT. After inactivation, the virus solution was dialysed against PBS and complete inactivation was confirmed by passaging on MDCK cells. Virus was labelled by mixing with an equal volume of 0.1 mg/ml of fluorescein isothiocyanate (FITC) (Sigma-Aldrich, Saint Louis, MO) in 0.5 M bicarbonate buffer (pH 9.5) for 1 h at RT while constantly stirring. To lose all unbound FITC, labelled virus was dialysed against PBS.

Tissues from humans and animals

Archival paraffin-embedded human tissue sections were from the Department of Pathology, Erasmus MC. Archival paraffin-embedded animal tissue sections were from the Department of Virology, Erasmus MC, or from the Department of Pathobiology, Faculty of Veterinary Medicine, University of Utrecht. All tissues selected were histologically normal by microscopic examination of hematoxylin-and-eosin-stained sections and were obtained from humans or animals that had no evidence of respiratory infection.

Virus histochemistry on tissue sections

Formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and hydrated with alcohol. FITC-labelled influenza virus was incubated overnight at 4°C at a titre of 50-100 HAU. The FITC label was detected with a peroxidase labelled rabbit-anti-FITC (DAKOCytomation, Glostrup, Denmark). The signal was amplified with a tyramide signal amplification system (Perkin Elmer, Boston, MA) according to the instructions of the manufacturer. Peroxidase was revealed with 3-amino-9-ethyl-carbazole (Sigma-Aldrich) resulting in a bright red precipitate. Tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck, Whitehouse Station, NJ). Omission of the FITC-labelled virus was used as a negative control. To validate the method, we incubated labelled H5N1 virus and H3N2 virus with human trachea and mallard duck intestine. The pattern of attachment of both viruses to these tissues was as expected. H5N1 virus, which has retained a preference for sialic acid (SA)-α-2,3-Gal terminated saccharides,[51] bound abundantly to epithelial cells in duck intestine, which expresses mainly sialosaccharides with α-2,3 linkage,[42] and bound rarely to human trachea, which expresses mainly sialosaccharides with α-2,6 linkage.[50] In contrast, H3N2 virus, which has a preference for SA-α-2,6-Gal terminated saccharides,[51] bound abundantly to ciliated epithelial cells in human trachea, and bound poorly to duck intestine.

Double staining for H5N1 virus attachment and identification of type II pneumocytes

Attachment of H5N1 virus in selected sections of human lung was detected as above except peroxidase was not yet revealed. Type II pneumocytes were detected by incubation of a monoclonal mouse-anti-human surfactant apoprotein A (PSP-A) antibody (DAKOCytomation) for 1 h at RT, followed by an alkaline phosphatase-labelled goat-anti-mouse IgG2b (Southern Biotechnology Associates Inc., Birmingham, AL) for 1 h at RT. Peroxidase was revealed as described above. Alkaline phosphatase was revealed with BCIP/NBT substrate system (DAKOCytomation), resulting in a dark blue precipitate. Slides were not counterstained. Omission of H5N1 virus or an IgG2b isotype control (instead of mouse-anti-human PSP-A) were used as negative controls.

Results

In the human LRT, H5N1 virus attached predominantly to type II pneumocytes, alveolar macrophages, and nonciliated cuboidal epithelial cells in terminal bronchioles. Attachment became progressively rarer toward the trachea (Figure 1 and Table 1). The identity of type II pneumocytes was confirmed by double staining with antibody to human surfactant apoprotein A (Figure 2).

Table 1: *H5N1 virus attachment in respiratory tract of humans and four animal species. The mean abundance of cells binding H5N1 virus was scored as follows: -, no attachment; +/-, attachment to rare or few cells; +, attachment to a moderate number or many cells. When H5N1 virus attached only to rare or few cells, the predominant cell type usually could not be analyzed (n.a.).*

	Tissue							
	Trachea		Bronchus		Bronchiolus		Alveolus	
Species	Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type
Human	- *	n.a.	+ *	ciliated	+	non-ciliated	+ †	type II
Mouse	+ *	ciliated‡	+	ciliated	+	non-ciliated	+/-	n.a.
Ferret	-	n.a	-	n.a.	+/-	n.a.	+/- §	type II
Macaque	-	n.a	+/-	n.a.	+	non-ciliated	+	type I
Cat	-	n.a.	-	n.a.	+	non-ciliated	+ †	type II

† H5N1 virus also attaches to alveolar macrophages
‡ H5N1 virus also attaches to many non-ciliated cells
§ The number of cells binding H5N1 virus varied strongly per animal
* H5N1 virus also attaches to mucus and submucosal glands

The pattern of H5N1 virus attachment to cat LRT and, to a lesser extent, ferret LRT most closely resembled that in human tissues (Figure 1 and Table 1). In macaque alveoli, H5N1 virus attached predominantly to type I pneumocytes instead of type II pneumocytes, as in human tissues. In mice, H5N1 virus attachment to cells was most abundant in the trachea and became progressively rarer toward the alveoli, whereas the opposite trend was observed in human tissues.

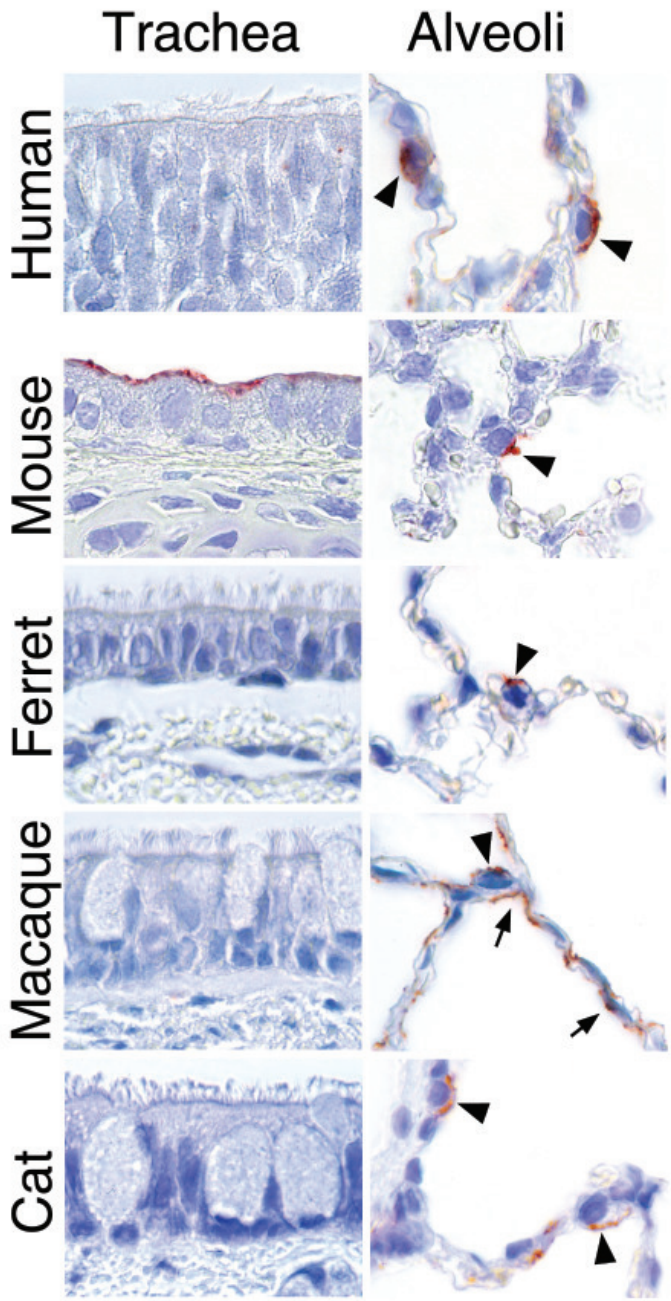


Figure 1: *Attachment of H5N1 virus to respiratory tissues of humans and four animal species. In the trachea, H5N1 virus—visible as red staining— attached only to epithelial cells of mice. In the alveoli, H5N1 virus attached predominantly to type II pneumocytes (arrowheads) in humans and all animal species except the macaque, where attachment was predominantly to type I pneumocytes (arrows).*

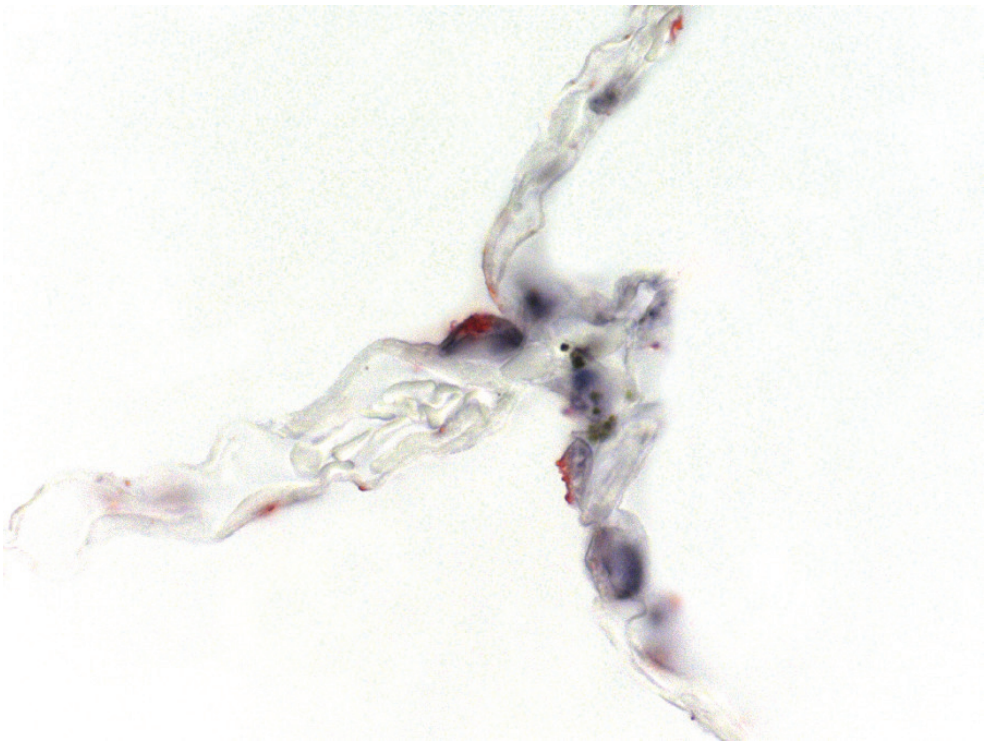


Figure 2: *Double staining of H5N1 virus and type II pneumocyte (arrowhead) in human alveolar tissue. The H5N1 virus is visible as red staining. Type II pneumocytes are identified by the expression of surfactant, visible as dark blue staining.*

Discussion

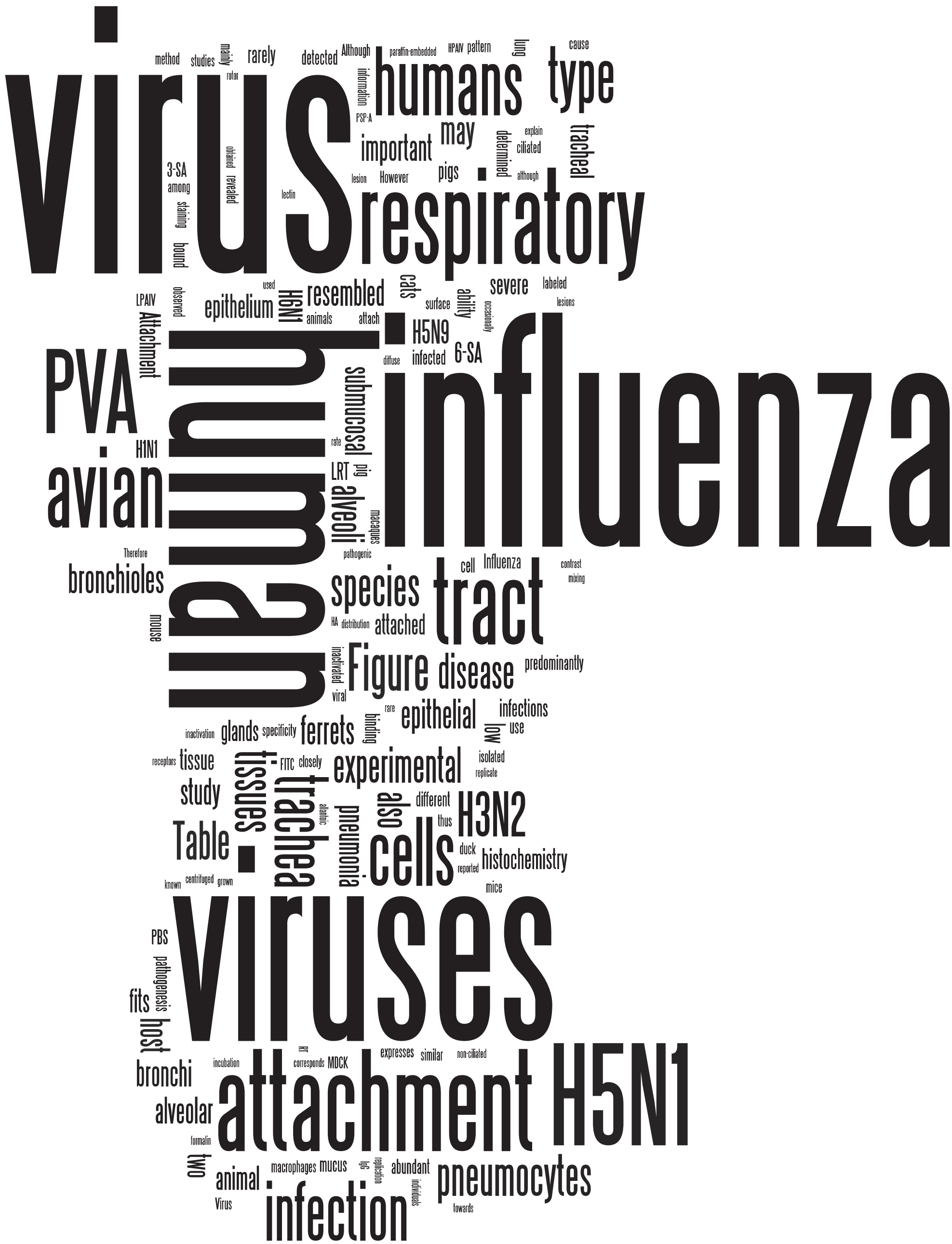
Attachment of H5N1 virus to type II pneumocytes and alveolar macrophages fits with the limited pathology data for H5N1 virus infection in humans, which show diffuse alveolar damage[49] and the presence of H5N1 virus antigen in type II pneumocytes.[29] However, they contrast with the idea that avian influenza viruses generally have little affinity for human respiratory tissues.[15] The predilection of H5N1 virus for type II pneumocytes and alveolar macrophages may contribute to the severity of the pulmonary lesion. Because type II pneumocytes are metabolically active and are the most numerous cell type lining the alveoli, targeting of this cell type may lead to abundant virus production. Damage to type II pneumocytes may impair their functions, including re-epithelialization after alveolar damage, ion transport, and surfactant production, and so may inhibit tissue repair. Targeting of alveolar macrophages may be important because of their role in limiting viral replication and the immune response to viral infection.

Based on virus attachment, the cat and the ferret would be the most suitable models for H5N1 viral pneumonia in humans. However, other factors also need to be considered, such as the availability of reagents and immunologic similarity. Overall the observed pattern of H5N1 virus attachment to the LRT is consistent with the respective pathology and immunohistochemistry results of experimental H5N1 virus infection in mice,[52] ferrets,[53] macaques,[54] and cats.[55]

This study demonstrates the attachment of H5N1 virus to the human LRT in a pattern that corresponds with autopsy findings. It also identifies cat and ferret as the most suitable animal models for human H5N1 viral pneumonia, on the basis of the similarity of viral attachment pattern. This technique also could be applied to further determine H5N1 virus attachment to the upper respiratory tract. Failure to attach to this site may be a limiting factor in human-to-human transmissibility of H5N1 virus.

Acknowledgements

We thank W. Lim for providing the H5N1 virus isolate; M. den Bakker, H. Sharma, M. Vermeij, and J. van den Brand for providing tissues; and F. van der Panne for technical assistance.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replication
IgG
RT

Chapter 3

Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals

Based on:

Debby van Riel, Vincent J. Munster, Emmie de Wit, Guus F. Rimmelzwaan, Ron A. M. Fouchier, Albert D.M.E. Osterhaus and Thijs Kuiken

American Journal of Pathology. 2007;171:1215-1223

Abstract

Viral attachment to the host cell is critical for tissue and species specificity of virus infections. Recently, pattern of viral attachment (PVA) in human respiratory tract was determined for highly pathogenic avian influenza virus of subtype H5N1. However, PVA of human influenza viruses and other avian influenza viruses in either humans or experimental animals is unknown. Therefore, we compared PVA of two human influenza viruses (H1N1 and H3N2) and two low pathogenic avian influenza viruses (H5N9 and H6N1) to that of H5N1 virus in respiratory tract tissues of humans, mice, ferrets, cynomolgus macaques, cats, and pigs by virus histochemistry. We found that human influenza viruses attached more strongly to human trachea and bronchi than H5N1 virus, and attached to different cell types than H5N1 virus. These differences correspond to primary diagnoses of tracheobronchitis for human influenza viruses and diffuse alveolar damage for H5N1 virus. The PVA of low pathogenic avian influenza viruses in human respiratory tract resembled that of H5N1 virus, demonstrating that other properties determine its pathogenicity for humans. The PVA in human respiratory tract was most closely mirrored by that in ferrets and pigs for human influenza viruses and in ferrets, pigs, and cats, for avian influenza viruses.

Introduction

Infections with human influenza A viruses of the subtypes H1N1 and H3N2 are an important cause of respiratory tract disease. The most common lesion in immunocompetent individuals is a tracheobronchitis.[56] Uncommonly, human influenza A virus infection causes severe pneumonia, which requires hospitalization and may be fatal. This pattern of disease contrasts with the ongoing outbreak of highly pathogenic avian influenza A virus (HPAIV) infection of the subtype H5N1. In this outbreak, severe pneumonia is the most common lesion in the >300 patients with confirmed H5N1 virus infection, and the case fatality rate is over 50% (World Health Organisation http://www.who.int/csr/disease/avian_influenza/country/cases_table_2007_06_04/en/index.html). Until now, there is no evidence that this avian virus has become efficiently transmissible among humans, which could result in a new pandemic.[57]

The increased interest in H5N1 virus infection has highlighted large gaps in our knowledge of the pathogenesis of influenza A virus infections in humans. An important factor in this pathogenesis is tissue tropism, which depends largely on the ability of the virus to attach to the host cell.[15] Influenza A viruses attach to host cells by binding of the hemagglutinin (HA) protein to sialosaccharides on the host cell surface. The HAs of influenza A viruses from different host species differ in their specificity of binding. For example, HAs of human influenza A viruses preferentially recognize sialic acid (SA)-α-2,6-Gal terminated saccharides (α-2,6-SA), whereas HAs of avian influenza viruses preferentially recognize SA-α-2,3-Gal terminated saccharides (α-2,3-SA).[14;40;58] These differences generally correspond with the variation in the type of SAs expressed at important sites for influenza A virus replication in the respective host species. For example, human tracheal epithelium expresses mainly α-2,6-SA,[50] while duck intestinal epithelium expresses mainly α-2,3-SA.[42] Therefore, the type and distribution of SA is considered to be an important factor in the susceptibility of different host species to influenza A viruses.[59] The SA recognized by influenza A virus is not only important in the host species range, but also in its transmissibility. The latter was demonstrated in experimental infections of ferrets with the 1918 pandemic influenza virus. In this study, horizontal transmission was abolished by two amino acid mutations in the HA that caused a switch in binding preference from human α-2,6-SA to avian α-2,3-SA.[60]

A traditional method for studying the tissue tropism of different influenza A viruses is to measure the distribution of SAs by use of lectin histochemistry. Lectin histochemistry makes use of the property of a wide variety of lectins to specifically bind to SAs.[61] Studies on receptors for influenza virus have made use of the plant lectins *Sambucus nigra* agglutinin, which has a major specificity for α-2,6-SA, and *Maackia amurensis* agglutinin, which has a major specificity for α-2,3-SA.[62;63] Although useful for determining the distribution of SAs in tissues, these lectin histochemistry techniques are only an indirect measure of influenza A virus attachment to host tissues. They

do not account for other variables that influence the binding specificity. For HA, these include glycosylation and sialylation close to the receptor binding site;[40] for the receptor, these include type of SA, alternative linkages,[64] and sulfation and fucosylation of the saccharide residues.[65]

To circumvent these problems, we made use of virus histochemistry to study the pattern of virus attachment (PVA) in respiratory tissues. This method, modified from Couceiro and colleagues[50] directly displays the attachment of influenza virus to tissues. By use of this method, we recently determined that H5N1 virus attachment in the human respiratory tract is progressively more abundant towards the alveoli, where the virus attaches predominantly to type II pneumocytes and alveolar macrophages.[66] This attachment pattern fits with the limited pathology data on H5N1 virus infection in humans, which show diffuse alveolar damage as the primary lesion.[29;67;68] Our results on H5N1 virus were supported by recent experimental studies in human *ex vivo* lung cultures, which demonstrated H5N1 virus replication in the lower respiratory tract (LRT).[63;69]

Although limited studies have been done on the human trachea,[50] the PVA for human influenza A viruses in the LRT is not known.[66] This information is important to better understand the pathogenesis of influenza pneumonia, which is centred on the LRT. Also, it is not clear whether the PVA of H5N1 virus that we observed is unique among avian influenza viruses and therefore may in part explain its ability to cause respiratory disease in humans. Finally, the PVA of human influenza A virus in experimental animals is not known. This information is important to help select the most appropriate animal model for influenza pneumonia. Of particular interest is the domestic pig, which is permissive for both human and avian influenza A virus infections, and may thus act as a "mixing vessel" for the generation of reassortant viruses.[2] Therefore, we here describe the PVA of two currently circulating subtypes of human influenza A virus (H3N2 and H1N1) and low pathogenic avian influenza viruses (LPAIV) (H5N9 and H6N1) to compare these with the PVA of HPAIV H5N1 in human. Furthermore, we determined the PVA of these human and avian influenza A viruses in respiratory tract of known experimental animals.

Materials and methods

Experimental design

To determine the PVA of human and avian influenza A viruses in the trachea and LRT of humans, we used two LPAIV (H5N9 and H6N1), a HPAIV H5N1 isolate and two recently circulating human influenza viruses (H3N2 and H1N1). We determined whether attachment occurred to epithelial cells in the trachea or LRT (including

bronchi, bronchioles, and alveoli) and to alveolar macrophages. The PVA of all of the above viruses was also determined in mammalian species, which are used for experimental influenza A virus infections. Animals included were cynomolgus macaque (*Macaca fascicularis*), European shorthair cat, ferret, Yorkshire-Landrace pig and C57/BL6 mouse.

Viruses

Influenza virus A/Netherlands/35/05 (H1N1) and A/Netherlands/213/03 (H3N2) are recent human isolates grown on Madin-Darby canine kidney (MDCK) cells, and were kindly provided by the National Influenza Center, The Netherlands. Influenza virus A/Mallard/Sweden/79/02 (H5N9) and A/Mallard/Sweden/81/02 (H6N1) were obtained from cloacal swabs of migratory mallard ducks (*Anas platyrhynchos*) during ongoing influenza virus surveillance of wild birds and were subsequently passaged twice in embryonated hens' eggs.[70] Influenza virus A/Vietnam/1194/04 (H5N1) was isolated from a fatal human case. The virus was kindly provided by Dr. W. Lim, Queen Mary Hospital, Hong Kong, and propagated once in MDCK cells.

Virus preparation, inactivation and labeling

The H1N1, H3N2 and H5N1 viruses, isolated from humans, were grown in MDCK cells. The supernatant was harvested and cleared by low speed centrifugation. The H5N9 and H6N1 viruses, isolated from mallards, were grown in the allantoic cavity of 11-day-old embryonated hens' eggs. The allantoic fluid was harvested after 2 days and cleared by low speed centrifugation. Cleared supernatants and allantoic fluid samples were subsequently centrifuged 2 h at 85000 x *g* in a SW28 rotor at 4 °C. The virus pellet was resuspended in 2 ml phosphate-buffered saline (PBS) and loaded on a 20-60% sucrose (w/w) gradient and centrifuged overnight at 300000 x *g* in a SW41 rotor at 4 °C. To deplete the sucrose, the viruses were additionally centrifuged 2 h at 85000 x *g* in a SW28 rotor at 4 °C, and the virus was resuspended in PBS.

H5N1 virus was inactivated by dialysing against 0.1% formalin for 3 days. All other viruses were inactivated by incubation with 1:1 v/v 10% formalin for 1 h at RT. After inactivation, virus suspensions were dialysed against PBS. Inactivation was confirmed by failure to passage on MDCK cells.

Viruses were labeled by mixing concentrated, inactivated viruses, suspended in PBS, with an equal volume of 0.1 mg/ml of fluorescein isothiocyanate (FITC) (Sigma) in 0.5 M bicarbonate buffer (pH 9.5) for 1 h while constantly stirring. To lose all unbound FITC, labeled viruses were dialysed against PBS.

To check for the continued capacity for hemagglutination by the inactivated viruses, the hemagglutination titre of the viruses was determined after formalin inactivation and FITC labeling.[71]

Respiratory tract tissues from humans and animals

Archival paraffin-embedded human tissue sections were obtained from the Department of Pathology, Erasmus MC. Archival paraffin-embedded animal tissue sections were obtained from the Department of Virology, Erasmus MC, or from the Department of Pathobiology, Faculty of Veterinary Medicine, University of Utrecht. All tissues selected were from individuals without histological lesions or evidence of respiratory tract infection at the time of death. Three individuals per species were analyzed.

Virus histochemistry on tissue sections

Formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and hydrated using graded alcohols. FITC-labeled influenza viruses were incubated overnight at 4 °C at a titre of 50-100 HAU/50 μl. For the visualization by light miscroscopy, FITC label was detected with a peroxidase-labeled rabbit-anti-FITC (DAKO). The signal was amplified with a tyramide signal amplification system (Perkin Elmer) according to the instructions of the manufacturer. Peroxidase was revealed with 3-amino-9-ethyl-carbazole (Sigma) resulting in a bright red precipitate. Tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck). Attachment of influenza virus to tissues was visible as granular to diffuse red staining on the apical surface of epithelial cells. Cytoplasmic staining in epithelial cells and staining of other cell types was seen occasionally. For each tissue tested, in each run, an omission control was included to check for non-specific amplification.

To validate the method, we incubated labeled H5N1 virus and H3N2 virus with human trachea and mallard duck intestine. The pattern of attachment of both viruses to these tissues was as expected. H5N1 virus, which has retained a preference for α-2,3-SA,[65] bound abundantly to duck intestinal epithelium, which expresses mainly α-2,3-SA,[42] and bound rarely to human tracheal epithelium, which expresses mainly α-2,6-SA.[50] In contrast, H3N2 virus, which has a preference α-2,6-SA, bound abundantly to human tracheal epithelium, and bound poorly to duck intestinal epithelium.

Double staining for H5N1 virus attachment and type II pneumocytes

H5N1 virus attachment in human lung was detected as described above, but peroxidase was not yet revealed. Type II pneumocytes were detected by incubation with a monoclonal mouse anti-human surfactant apoprotein A (PSP-A) antibody (DAKO) for 1 h at RT, followed by incubation with an alkaline phosphatase (AP)-labeled goat-anti-mouse IgG2b (Southern biotechnology Associates Inc.) for 1 h at RT. Peroxidase was revealed as described above and AP was revealed with BCIP/NBT substrate system (DAKO), resulting in a dark blue precipitate. Sections were not counterstained. Omission of H5N1 virus or an IgG2b isotype control (instead of mouse anti-human PSP-A) were included as negative controls in each run.

Table 1: *Virus attachment in respiratory tract of humans and five animal species. The mean abundance of cells where virus attached to was scored as follows: -, no attachment; +/- attachment to rare or few cells; + attachment to a moderate number of cells; ++ attachment to many cells. Where possible, the predominant cell type to which virus attached is indicated: ciliated cells (cil), non-ciliated cuboidal cell (non-cil) type I pneumocytes (type I) or type II pneumocytes (type II).*

Virus	Species	Trachea		Bronchus		Bronchiolus		Alveolus	
		Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type
H3N2	**Human**	++ †	cil	++ †	cil	+	cil	+	type I
	Mice	-		-		-		+/-	
	Ferret	++ *	cil	+/- *		+/-		+	type I
	Cyno	-		-		-		-	
	Pig	++ †	cil	++ †	cil	++	cil	+	type I
	Cat	-		-		-		-	
H1N1	**Human**	++ †	cil	++ †	cil	+	cil	+	type I
	Mice	-		-		-		+/-	
	Ferret	+ *	cil	+ *	cil	+/-		+	type I
	Cyno	-		-		-		-	
	Pig	++ †	cil	++ †	cil	++	cil	+	type I
	Cat	-		-		-		-	
H5N1	**Human**	- *		+ *	+ cil	+	non-cil	+ ‡	type II
	Mice	++	both	+	non-cil	+	non-cil	+	type II
	Ferret	-		-		+/-		+	type II
	Cyno	-		+/-		+/-		+	type I
	Pig	-		-		-		+	type II
	Cat	-		-		+	non-cil	+ ‡	type II
H5N9	**Human**	- *		+ *	cil	+	non-cil	+ ‡	type II
	Mice	++	both	++	non-cil	++	non-cil	+	type II
	Ferret	-		-		-		+/-	
	Cyno	-		-		+/-		+/-	
	Pig	-		-		+/-		+/-	
	Cat	-		-		+/-		+ ‡	type II
H6N1	**Human**	+/- *		+/- *		+	non-cil	+ ‡	type II
	Mice	++	both	+	non-cil	+	non-cil	+	?
	Ferret	-		-		-		+/-	type II
	Cyno	-		+/-		+/-		+	type I
	Pig	-		-		+/-		+	type II
	Cat	-		-		+/-	non-cil	+ ‡	type II

* (sub)mucosal glands positive
† goblet cells occasionally positive
‡ macrophages positive

Results

Attachment of human influenza A viruses to human respiratory tract

The human influenza A viruses H3N2 and H1N1 had a similar PVA to the human respiratory tract (Table 1, Figure 1). Virus attachment in the trachea and bronchi was more abundant than in the bronchioles. In trachea, bronchi, and bronchioles, virus attached predominantly to the surface of ciliated epithelial cells, occasionally to goblet cells and rarely to bronchiolar non-ciliated cuboidal cells. In alveoli, virus attached more to type I than to type II pneumocytes, and very rarely to alveolar macrophages.

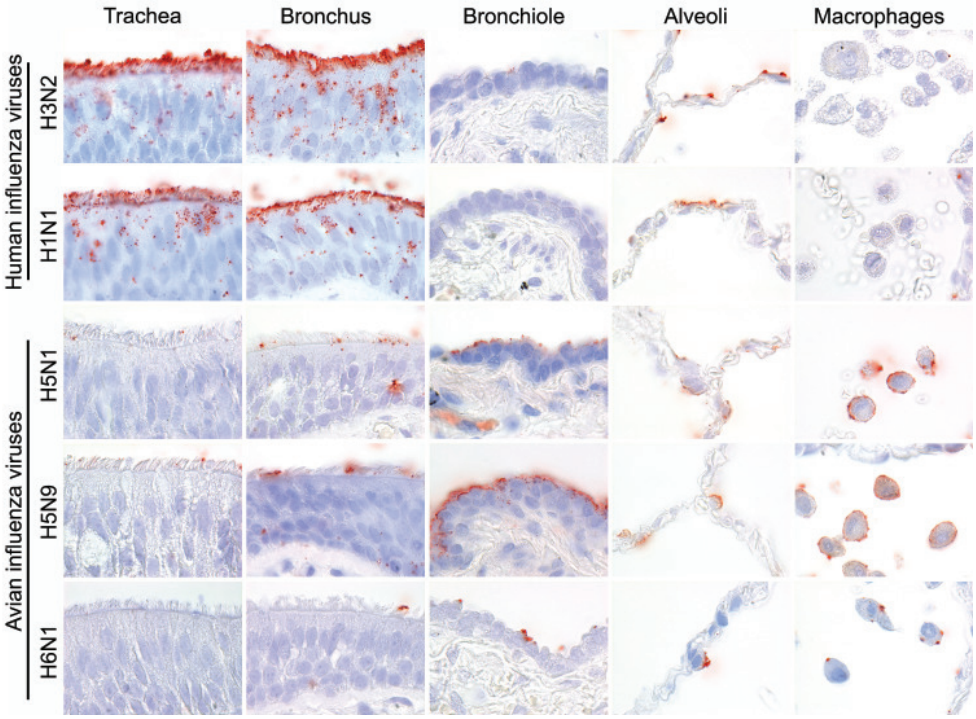

Figure 1*: Attachment of human (H3N2 and H1N1) and avian (highly pathogenic H5N1, low pathogenic H5N9 and H6N1) influenza viruses in human trachea, lower respiratory tract (bronchus, bronchiole and alveoli) and alveolar macrophages.*

Attachment of avian influenza viruses to human respiratory tract

The avian influenza viruses H5N9 and H6N1 showed a similar PVA to tissues of the human respiratory tract, that also resembled the PVA of H5N1 virus (Table 1, Figure 1). Attachment to the apical cell membrane was usually granular for H6N1 virus and more diffuse for H5N9 virus. In contrast to the human influenza A viruses, attachment of the avian influenza viruses was rare in the trachea and increased progressively towards the bronchioles. The avian influenza viruses also attached preferentially to different cell types than the human influenza A viruses: acinar cells of the tracheal and bronchial submucosal glands (Figure 2) as well as to mucus at these sites, non-ciliated cuboidal cells in the bronchioles, and type II pneumocytes and alveolar macrophages in the alveoli. To confirm that avian influenza viruses attached to type II pneumocytes, human lung tissue was double stained with H5N1 virus and PSP-A, which is a surfactant produced specifically by type II pneumocytes (Figure 3).

Attachment of human influenza A viruses to experimental animal respiratory tract

The PVA of human influenza A viruses to respiratory tract tissues of some experimental animal species resembled that of humans more than others (Table 1,

Figure 2, 4 and 5). The PVA of ferrets and pigs resembled that of humans most closely, because they were the only two species in which the viruses attached to the surface of ciliated epithelial cells in the airways, and to type I pneumocytes in alveoli. In ferrets, there also was virus attachment to submucosal glands and mucus (Figure 2). In mice, there was no attachment to trachea, bronchi and bronchioles and only occasional attachment to alveolar epithelial cells of indeterminate type. In macaques and cats, virus attachment was not or rarely observed at any level of the respiratory tract.

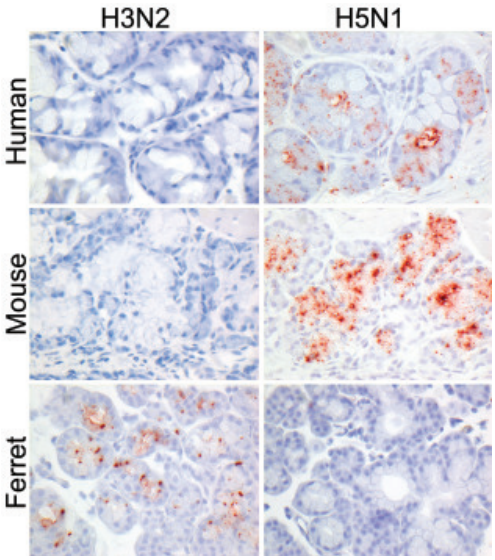

Figure 2*: Attachment of H3N2 virus and H5N1 virus to the submucosal glands in human, mouse and ferret trachea.*

Attachment of avian influenza viruses to experimental animal respiratory tract

The PVA of H5N9 and H6N1 viruses to respiratory tract tissues resembled that of H5N1 virus in each of the five animal species (Table 1). As for human influenza A viruses, the PVA of avian influenza viruses to respiratory tract tissues of some animal species resembled that of humans more than others. The PVA of cats, ferrets, and pigs (Figure 5) resembled that of humans most closely, with rare virus attachment in the trachea and bronchi, rare to occasional attachment to non-ciliated cuboidal cells in the bronchioles, and predominant attachment to type II pneumocytes in the alveoli. In cats, there also was virus attachment to alveolar macrophages, as in humans. The PVA of macaques also resembled that of humans, except that virus attachment was predominantly to type I instead of type II pneumocytes, although attachment to type II pneumocytes was also observed. In mice, virus attachment was most abundant in the trachea and became progressively weaker towards the alveoli, which was opposite to the PVA in humans. However, virus attachment to submucosal glands in mice mirrored that in humans (Figure 2).

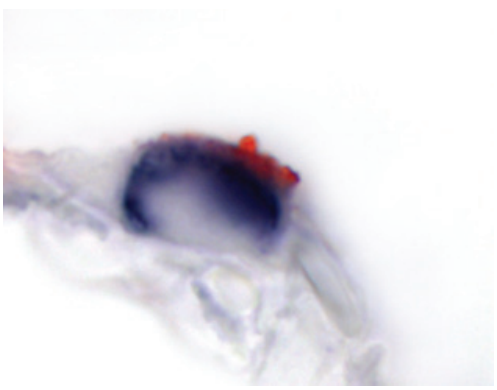

Figure 3*: Confirmation of H5N1 virus attachment to type II pneumocytes in human alveoli by staining for human surfactant apoprotein A (PSP-A). H5N1 virus attachment is visible as red staining on the apical cell surface, while PSP-A expression, characteristic for type II pneumocytes, is visible as diffuse dark blue staining in the cytoplasm*

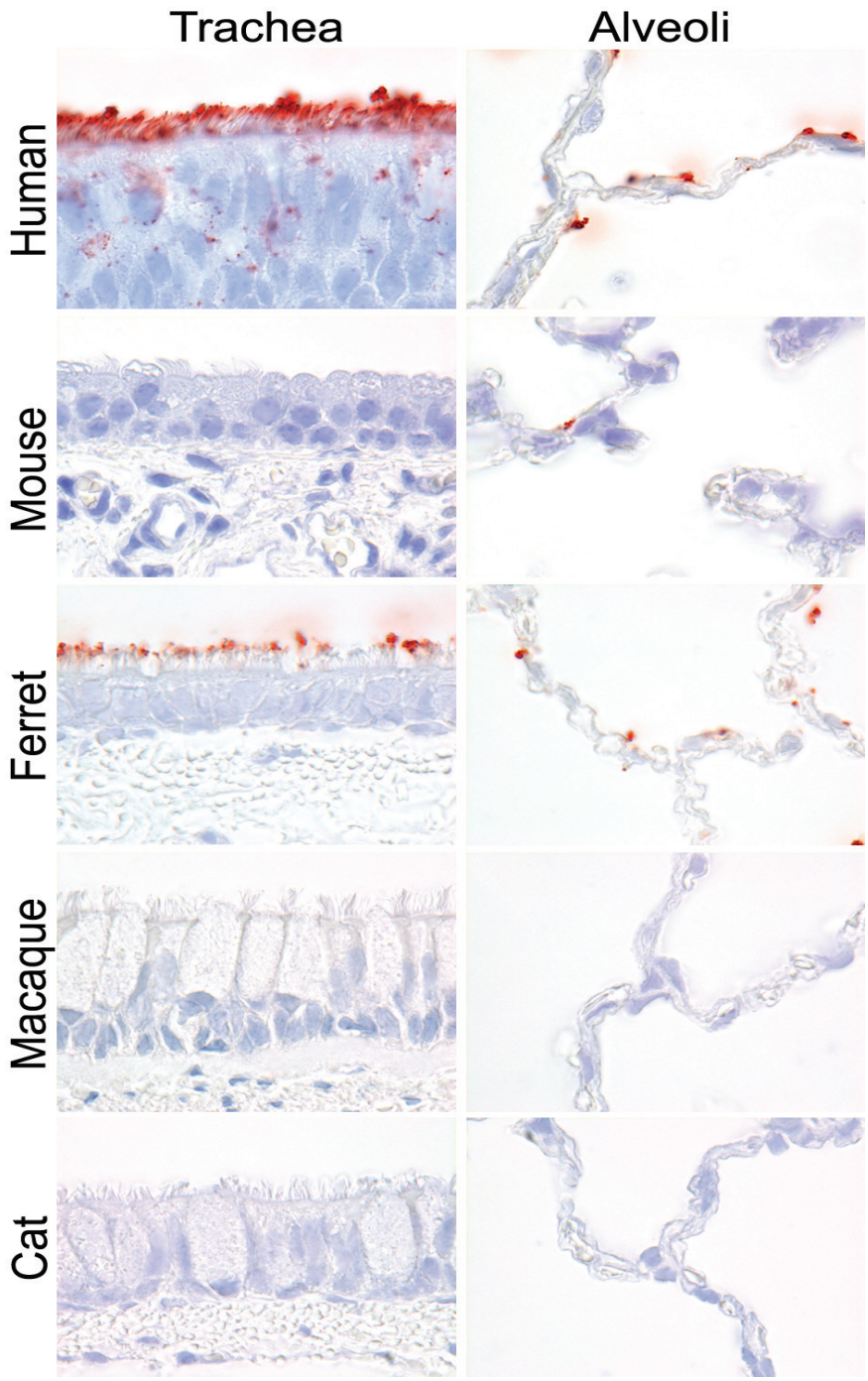

Figure 4*: Attachment of H3N2 virus to the trachea and alveoli of human, mouse, ferret, macaque and cat.*

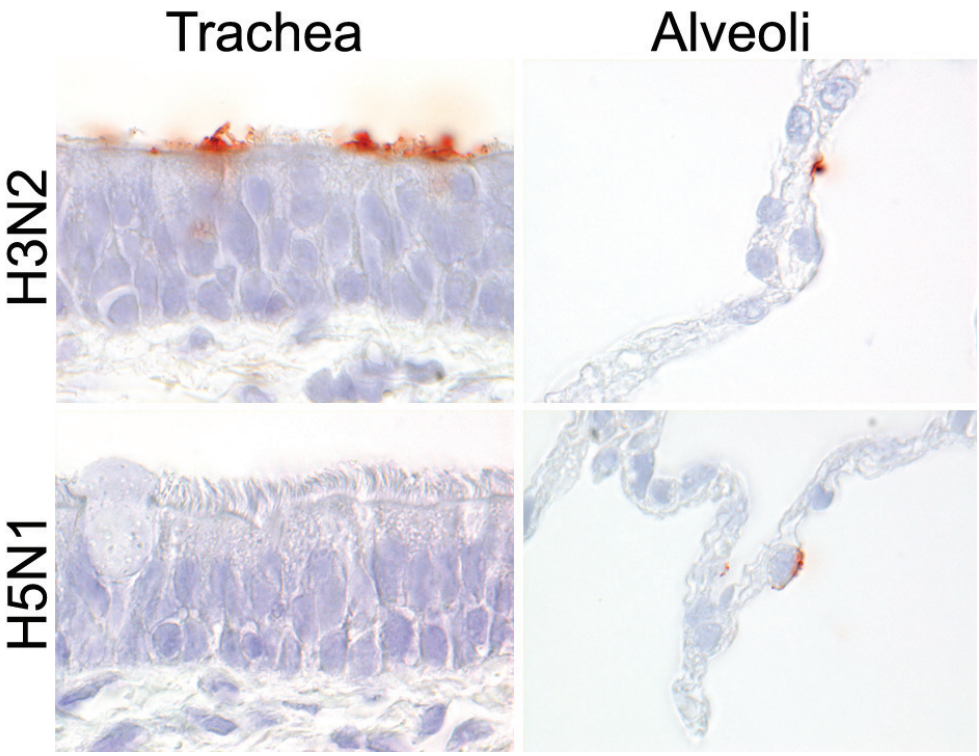

Figure 5: *Attachment of H3N2 virus and H5N1 virus to pig trachea and alveoli.*

Discussion

This study shows for the first time the PVA of human influenza A viruses in the human respiratory tract, from the trachea down to the alveoli. This information is important to better understand the pathogenesis of influenza pneumonia. The PVA of these viruses differs markedly from that of a H5N1 strain.[66] These differences may explain, at least in part, the contrasts in localization and severity of respiratory disease between these virus infections in humans.

The common presentation of human influenza A virus infection is tracheobronchitis, which fits with the abundant attachment of these viruses to tracheal and bronchial epithelium (Table 1, Figure 1). Although rarely, human influenza A viruses can cause severe pneumonia. This fits with the ability of human influenza A viruses to attach to the human LRT. This difference in disease outcome between human influenza A viruses and H5N1 virus infection, where the primary lesion is severe pneumonia,[49] fits with differences in virus attachment in the alveoli. Human influenza A viruses attached primarily to type I pneumocytes (Table 1, Figure 1), which are less numerous than type II pneumocytes (40% vs. 60% of alveolar epithelial cells)[8] and have low

metabolic activity.[72] We therefore speculate that if attachment leads to infection, virus production by type I pneumocytes is relatively low and can more easily be controlled by the host innate immune response. In addition, a large proportion of the human population has serum neutralizing IgG antibodies against human influenza A viruses due to prior exposure. While IgA is important for the protection of the respiratory tract from nose to bronchi, IgG is the main antibody involved in protecting the lung from pneumonia. These IgG antibodies are known to protect the richly vascularized lung parenchyma from infection and thus from development of severe pneumonia.[73-75]

The two LPAIV H5N9 and H6N1 had a similar PVA to human respiratory tract to that of H5N1 virus (Table 1, Figure 1), even though LPAIV rarely infect or cause disease in humans.[3;76] This means that the ability of H5N1 virus to attach to human respiratory tract cells—although necessary—is not sufficient to explain the ability of this virus to induce severe disease in humans. Alternative explanations for the uniqueness of H5N1 virus among avian influenza viruses may be its ability to replicate efficiently in human respiratory tract cells,[69;76;77] to induce pro-inflammatory cytokine production,[77-80] or to inhibit the host's innate immune response.[81]

The respiratory tract of ferrets and pigs most closely resembled that of humans with regard to PVA of human influenza A viruses (Table 1, Figure 4 and 5). This corresponds with the permissiveness of pig[82;83] and ferret[84] for infection with non-adapted isolates of human influenza A viruses. As in humans, ferrets infected with human H3N2 virus usually develop upper respiratory tract disease and occasionally bronchitis and pneumonia,[84,85] with similar histological changes as in human disease and expression of influenza virus antigen in ciliated epithelial cells of bronchi and bronchioles.[86] Attachment of human influenza A virus to ciliated epithelial cells in the ferret trachea has been observed before.[87] The PVA of human influenza A viruses in respiratory tracts of mouse, cynomolgus macaque and cat differed strongly from that in human respiratory tract, with no attachment to trachea, bronchus and bronchioles (Table 1, Figure 4). This fits with the results of experimental infections. Mice are not permissive for non-adapted human influenza A viruses.[88] Cynomolgus macaques could be infected experimentally with human H3N2 virus, but no clinical signs were observed.[89] In a recent study, we were unable to show experimental infection in domestic cats with a human H3N2 virus, although the same protocol resulted in productive infection of H5N1 virus.[90]

The respiratory tract of cats, ferrets, and pigs most closely resembled that of humans with regards to PVA of avian influenza viruses (Table 1). For cats and ferrets, the PVA of H5N1 virus to respiratory tract tissues has been reported previously.[66] However, a species not reported upon previously is the pig. Although both α-2,3-SA (the preferred receptors of avian influenza viruses) and α-2,6-SA (the preferred receptors of human A influenza viruses) have been detected in pig trachea by lectin histochemistry,[42] only human influenza A viruses attached to pig trachea in our study (Figure 5 and Table 1). Pigs can be infected by both human and avian influenza A viruses, and

reassortant viruses have been isolated from pigs.[91] Therefore, pigs have traditionally been considered as potential mixing vessel for avian and human influenza A viruses, from which pandemic influenza viruses may develop.[2] However, re-assortment of two influenza A viruses requires infection of the same host cell. Because our study indicates that such a reassortant is unlikely to occur in tracheal epithelium, it raises the question where in porcine respiratory tract such reassortments can occur.

The PVA of avian influenza viruses, including H5N1 virus, in experimental animals corresponds overall to the localization of respiratory tract disease reported for experimental H5N1 virus infection in these species. In cats,[90] ferrets,[53] macaques,[92] and pigs,[93] the lesions are most severe in alveoli and bronchioles, whereas bronchi and trachea are less or not affected. This fits with the PVA of H5N1 virus in these species, which is predominantly in alveoli and bronchioles (Table 1). In contrast, mice infected with H5N1 virus show substantial necrotizing lesions in trachea and bronchi in addition to alveolar and bronchiolar involvement.[52;94] This corresponds to the moderate to abundant attachment of H5N1 virus to mouse trachea and bronchus (Table 1).

Tracheo-bronchial submucosal glands play two potentially opposite roles in influenza virus infection. On the one hand, they produce mucus that can inactivate virus and thus inhibit viral infection. On the other hand, the glands themselves may become infected with influenza virus, thus enhancing viral infection. In humans, tracheal submucosal glands produce a different sialosaccharide (α-2,3-SA) from that predominantly expressed by tracheal epithelial cells (α-2,6-SA).[95] This fits with our attachment study, where avian influenza viruses bound to human submucosal gland cells and their mucus, but human influenza viruses did not (Figure 2). This mucus could trap these avian viruses and result in their expulsion by the mucociliary pathway before they can reach the LRT.[95] This fits with the low transmission rate of avian influenza (both low and highly pathogenic) viruses from birds to humans,[76] and of H5N1 virus among humans.[49] However, it is inconsistent with the observation that human H3N2 virus can replicate in human submucosal glands.[96] The only species in which we detected attachment of human H3N2 influenza virus to tracheo-bronchial submucosal glands was the ferret (Figure 2), in which human H3N2 virus also has been shown to replicate in these glands.[97] A possible explanation is that attachment of H3N2 virus to tracheo-bronchial submucosal gland is below the detection limit of our test for humans and above the limit for ferrets.

Together, the results of this virus attachment study improve our understanding of the pathogenesis of human respiratory tract disease from both human and avian influenza A virus infection. This information on virus attachment needs to be combined with studies on other factors that may contribute to pathogenicity of influenza A virus infection, such as replication rate,[32,98] escape mechanisms from innate immune response,[99] and induction of cytokine production.[77] Virus attachment studies also may help in the choice of animal model to study disease mechanisms

and to test preventive and therapeutic strategies against both human and avian influenza. The viral histochemistry technique used here for influenza A virus also may be useful in the research of other viral diseases.

Acknowledgements

We thank W. Lim for providing the H5N1 virus isolate; F. Van der Panne for technical assistance; and M. den Bakker, H. Sharma, M. Vermeij, and J. van den Brand for providing tissues.


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Chapter 4

Seasonal and pandemic human influenza viruses attach better to human upper respiratory tract epithelium than avian influenza viruses

Based on:
Debby van Riel, Michael den Bakker, Lonneke Leijten, Salin Chutinimitkul, Vincent Munster, Emmie de Wit, Guus Rimmelzwaan, Ron Fouchier, Ab Osterhaus and Thijs Kuiken

American Journal of Pathology. 2010; 176:1614-1618

Abstract

Influenza viruses vary markedly in the efficiency of human-to-human transmission. This variation has been speculated to be determined in part by the tropism of influenza virus for the human upper respiratory tract. To study this tropism, we determined the pattern of virus attachment by virus histochemistry of three human and three avian influenza viruses in human nasal septum, conchae, nasopharynx, paranasal sinuses and larynx. We found that the human influenza viruses—two seasonal influenza viruses and pandemic H1N1 virus—attached abundantly to ciliated epithelial cells and goblet cells throughout the upper respiratory tract. In contrast, the avian influenza viruses, including the highly pathogenic H5N1 virus, attached only rarely to epithelial cells or goblet cells. Both human and avian viruses attached occasionally to cells of the submucosal glands. The pattern of virus attachment was similar among the different sites of the human upper respiratory tract for each virus tested. We conclude that influenza viruses that are transmitted efficiently among humans attach abundantly to human upper respiratory tract, while inefficiently transmitted influenza viruses attach rarely. These results suggest that the ability of an influenza virus to attach to human upper respiratory tract is a critical factor for efficient transmission in the human population.

Introduction

Influenza is an important cause of morbidity and mortality in humans during seasonal, pandemic, and zoonotic outbreaks. Seasonal influenza is estimated to cause 250 000 to 500 000 deaths per year worldwide. Pandemic influenza viruses of the previous century resulted in an estimated 1 to 4 million deaths for the 1957 H2N2 (Asian flu) and the 1968 H3N2 (Hong Kong flu) influenza pandemics, and 20 to 50 million deaths for the 1918 H1N1 (Spanish flu) influenza pandemic.[45;100] The first influenza pandemic of the 21st century, the currently ongoing new H1N1 virus outbreak (Mexican flu), has caused at least 3486 deaths as of 13 September 2009 (http://www.who.int/csr/don/2009_09_18/en/index.html). The zoonotic highly pathogenic avian influenza virus (HPAIV) H5N1, which is causing an ongoing outbreak in poultry, only occasionally infects humans, but has a high mortality rate, with 262 deaths out of 400+ confirmed infections as of August 2009 (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2009_08_11/en/index.html).

The pandemic potential of an influenza virus depends largely on its efficiency of human-to-human transmission. Human influenza viruses, including seasonal H1N1 and H3N2 viruses, and the pandemic H1N1 virus, are transmitted efficiently.[101] In contrast, the zoonotic HPAIV H5N1 is only rarely transmitted from human to human.[102] However, the factors determining efficient virus transmission among humans are poorly understood.

Tropism of influenza virus for the human upper respiratory tract (URT) has been speculated to be an important determinant for the efficiency of virus transmission, based both on receptor distribution and virus replication studies.[63;66] Based on lectin histochemistry, the human URT has abundant receptors for human influenza viruses, which are efficiently transmitted.[63;69] This fits with the ability for human influenza viruses to replicate in human URT tissues based on *in vivo*,[103] *ex vivo*,[69] and *in vitro* studies.[104-106] In contrast, the human URT has only limited receptors for avian influenza viruses.[63;69] This fits with the absence or rarity of HPAIV H5N1 transmission among humans.[102] However, it is discordant with a study of Nicholls and others, who showed that HPAIV H5N1 can replicate in URT tissues. They explained this discordance by suggesting that HPAIV H5N1 attached to receptors not detected by the lectins used. Therefore, there is currently no consensus on the tropism of HPAIV H5N1 for the human URT. In addition, the studies to date have not studied the human URT systematically, and it is not known what the tropism of the new H1N1 virus is for the human URT.

To address the question whether URT tropism of influenza viruses is linked to efficient transmission, we determined the pattern of attachment of selected influenza viruses in the human URT: human influenza viruses, including seasonal H1N1 and H3N2 viruses and pandemic H1N1 virus, which are transmitted efficiently, and avian

influenza viruses, including a HPAIV H5N1, isolated from a fatal human case, which is not transmitted efficiently among humans. We measured the pattern of virus attachment by use of virus histochemistry instead of lectin histochemistry.[66] Virus histochemistry measures the attachment of influenza virus to its host cell directly. Therefore, any receptors other than SA-alpha-2,3-Gal terminated saccharides and SA-alpha-2,6-Gal terminated saccharides also would be detected by virus histochemistry. We have used this technique previously to show that the pattern of attachment in the human lower respiratory tract is different for human and avian influenza viruses, and correlates with differences in primary disease.[107]

Materials and Methods

The pattern of attachment was determined by virus histochemistry of the seasonal H1N1 virus (A/Netherlands/35/05), seasonal H3N2 virus (A/Netherlands/213/03), HPAIV H5N1 (A /Vietnam/1194/04), low pathogenic avian influenza virus (LPAIV) H5N9 (A/Mallard/Sweden/79/02) and LPAIV H7N7 (A/Mallard/Sweden/100/02). To study the pattern of attachment of the pandemic H1N1 virus, we used a reassortant virus rather than the pandemic H1N1 virus itself to obtain sufficiently high titers in cell culture for the virus histochemical assay. This reassortant virus consisted of 6 gene segments of A/PR/8/34 and the HA and NA of pandemic H1N1 virus (A/NL/602/09). Because the surface glycoproteins of this reassortant virus were those of the pandemic H1N1 virus, attachment of the reassortant virus was expected to be the same as that of the pandemic H1N1 virus and is referred to as such in the rest of the text. The pandemic H1N1 virus, the two seasonal human influenza viruses and HPAIV H5N1 were grown on MDCK cells and the two LPAIV were grown in the allantoic cavity of 11-day-old embryonated hens' eggs. Viruses were purified, concentrated, inactivated and labeled with FITC as described previously.[66;107]

Histologically normal, archival, formalin-fixed, paraffin-embedded, human URT tissues from the nasal septum (n=2), nasal inferior concha (n=5), medial concha (n=3), nasopharynx (n=5), paranasal sinuses (n=3) and larynx (n=6) were included. In total, tissues originated from 20 different individuals. Tissue sections were incubated with FITC-labeled influenza viruses, as described before.[66;107] Briefly, binding of FITC-labeled influenza virus was detected with a peroxidase-labeled rabbit-anti-FITC antibody (DAKO, Glostrup, Denmark). The signal was amplified with a tyramide signal amplification system (Perkin Elmer, Boston, MA). Peroxidase was revealed with 3-amino-9-ethyl-carbozole (Sigma, St Louis, MO) resulting as a granular to diffuse red precipitate. For each tissue tested, in each run, an omission control was included to check for non-specific staining. Visualization by light microscopy provides a better overview of the tissues than is possible by fluorescence microscopy. For the precise localization of submucosal glands and goblet cells, serial sections were stained by HE or periodic acid Schiff stain.

Table 1*: Attachment of human and avian influenza viruses to different parts of the human upper respiratory tract. Scores are median scores from individual tissues. Attachment of influenza viruses to the apical site of ciliated epithelial cells (- no attachment; +/- < 10% cells positive; + < 50% cells positive; ++ ≥ 50% cells positive) was scored, and to the cytoplasm of goblet cells (- no attachment; + intracellular attachment). All viruses attached occasionally to epithelial cells of the submucosal glands.*

		Human						Avian					
		Seasonal H3N2		Seasonal H1N1		Pandemic H1N1		HPAIV H5N1		LPAIV H5N9		LPAIV H7N7	
	n	cilia	goblet	cilia	goblet	cilia	goblet	cilia	goblet	cilia	goblet	cilia	goblet
Nasal septum	2	++	+	++	+	++	+	-	-	+/-	-	+/-	-
Concha inferior	5	++	+	++	+	++	+	-	-	-	-	+/-	-
Concha media	3	++	+	++	+	+	+	-	-	+/-	-	+/-	-
Nasopharynx	5	++	+	++	+	++	+	+/-	-	+	-	+/-	-
Paranasal sinuses	2	++	+	++	+	++	+	+/-	-	+/-	-	+/-	-
Larynx	6	++	+	++	+	+/-	+	-	-	-	-	-	-

Results

Attachment of the studied influenza viruses to the mucociliary epithelium showed two distinct patterns (Table 1; Figure 1 and 2). All human influenza viruses (seasonal H1N1 and H3N2 viruses, pandemic H1N1 virus) attached abundantly to the apical site of ciliated epithelial cells. Furthermore, all human influenza viruses attached to goblet cells, both to the apical site and intracellularly to the mucus. In contrast, the avian influenza viruses (HPAIV H5N1 and LPAIV H5N9 and H7N7) attach only rarely to ciliated epithelial cells and not to goblet cells. Attachment to the submucosal gland

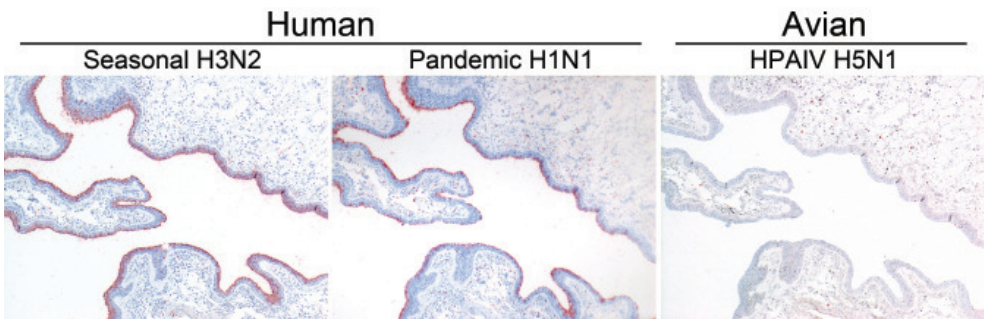


Figure 1: *Overview of attachment of seasonal H3N2, pandemic H1N1 and HPAIV H5N1 to the inferior concha.*

epithelium was similar for all viruses tested (Figure 2). All human and avian influenza viruses attached occasionally to the apical side and cytoplasm of submucosal gland epithelial cells.

In general, the pattern of virus attachment was similar among the different sites of the human URT for each virus tested (Table 1). Exceptions were less attachment of pandemic H1N1 virus to ciliated epithelial cells of the larynx and medial concha.

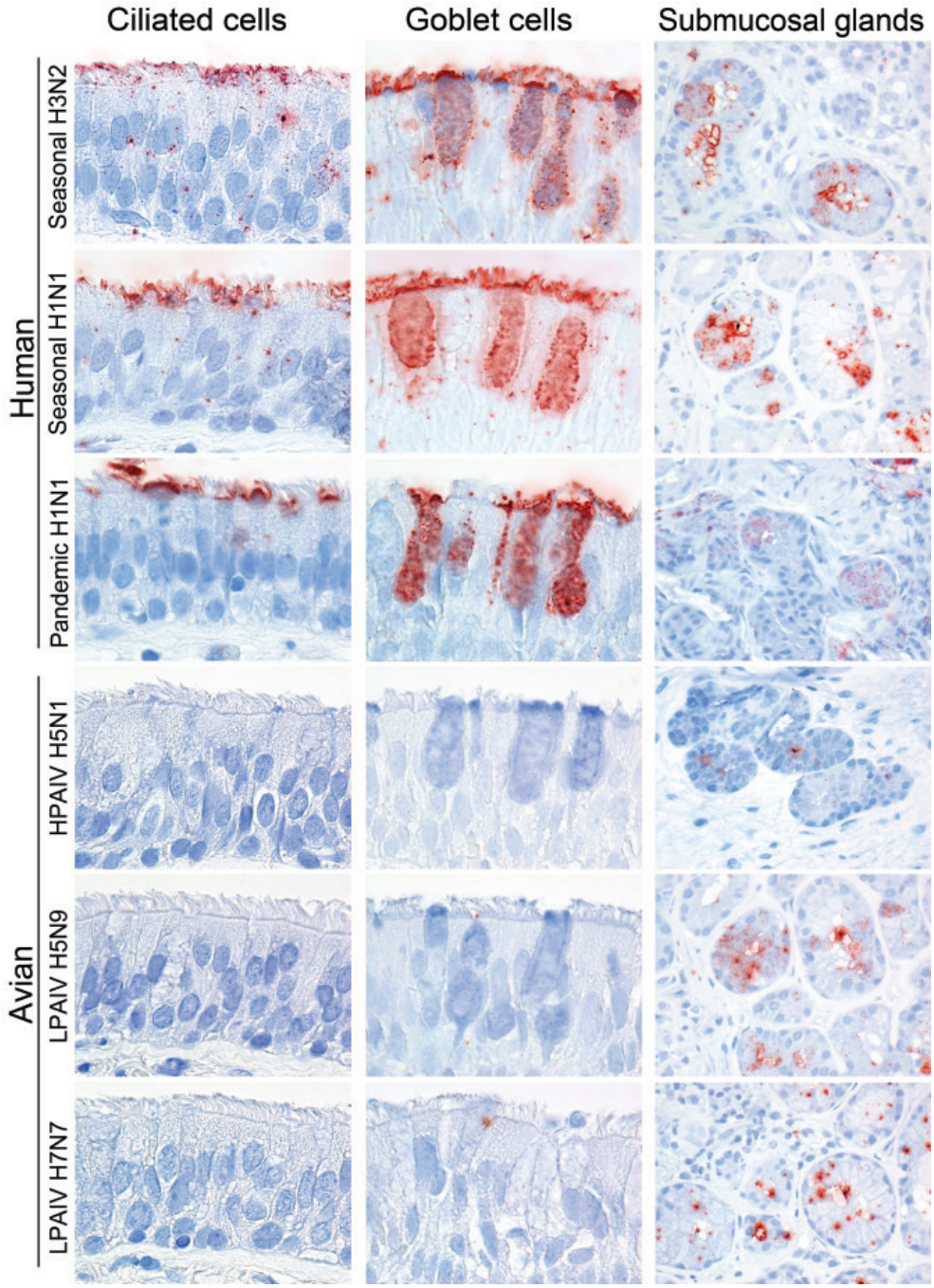


Figure 2: *Attachment of human and avian viruses to ciliated epithelial cells, goblet cells and submucosal glands in the human URT.*

Furthermore, attachment of avian influenza viruses to different sites of the human URT varied slightly.

Discussion

We here show that seasonal H1N1 and H3N2 viruses and pandemic H1N1 virus—that are transmitted efficiently among humans—attach abundantly to ciliated epithelial cells in the human upper respiratory tract. In contrast HPAIV H5N1—that is inefficiently transmitted among humans—attach rarely. These results indicate that the ability of an influenza virus to bind to human URT epithelium is a critical factor for efficient transmission in the human population.

Although there is no proof for the causal link between attachment and infection, our virus histochemistry data on human influenza viruses correspond with data from lectin histochemistry and from *in vitro*, *ex vivo*, and *in vivo* infections.[63;69;103-106] They also correspond with clinical data on human influenza virus infections, which commonly cause URT disease, specifically rhinitis, paranasal sinusitis, pharyngitis, and laryngitis (or croup).[108-117] The similarity that we found between pandemic H1N1 virus and seasonal influenza viruses for the attachment to the URT corresponds to the similarity between these viruses for attachment to the trachea.[118]

Our virus histochemistry data on HPAIV H5N1 and other avian influenza viruses correspond with some previous studies, but not with others. They correspond with lectin histochemistry studies,[63;69] which showed poor binding of MAA2, which is considered to specifically recognize SA-alpha-2,3-Gal —the preferred receptor of avian influenza viruses— to URT epithelium. They also correspond with clinical data on H5N1 virus infection, where URT symptoms are only present in a minority of hospitalized patients.[49] However, it contrasts with productive replication of HPAIV H5N1 in *ex vivo* cultures of human URT epithelium.[69] Although attachment does not necessarily lead to infection or attachment could be below detection limit, our virus histochemistry results suggests that infection of epithelial cells in the URT by H5N1 virus is possible, but likely not very widespread.

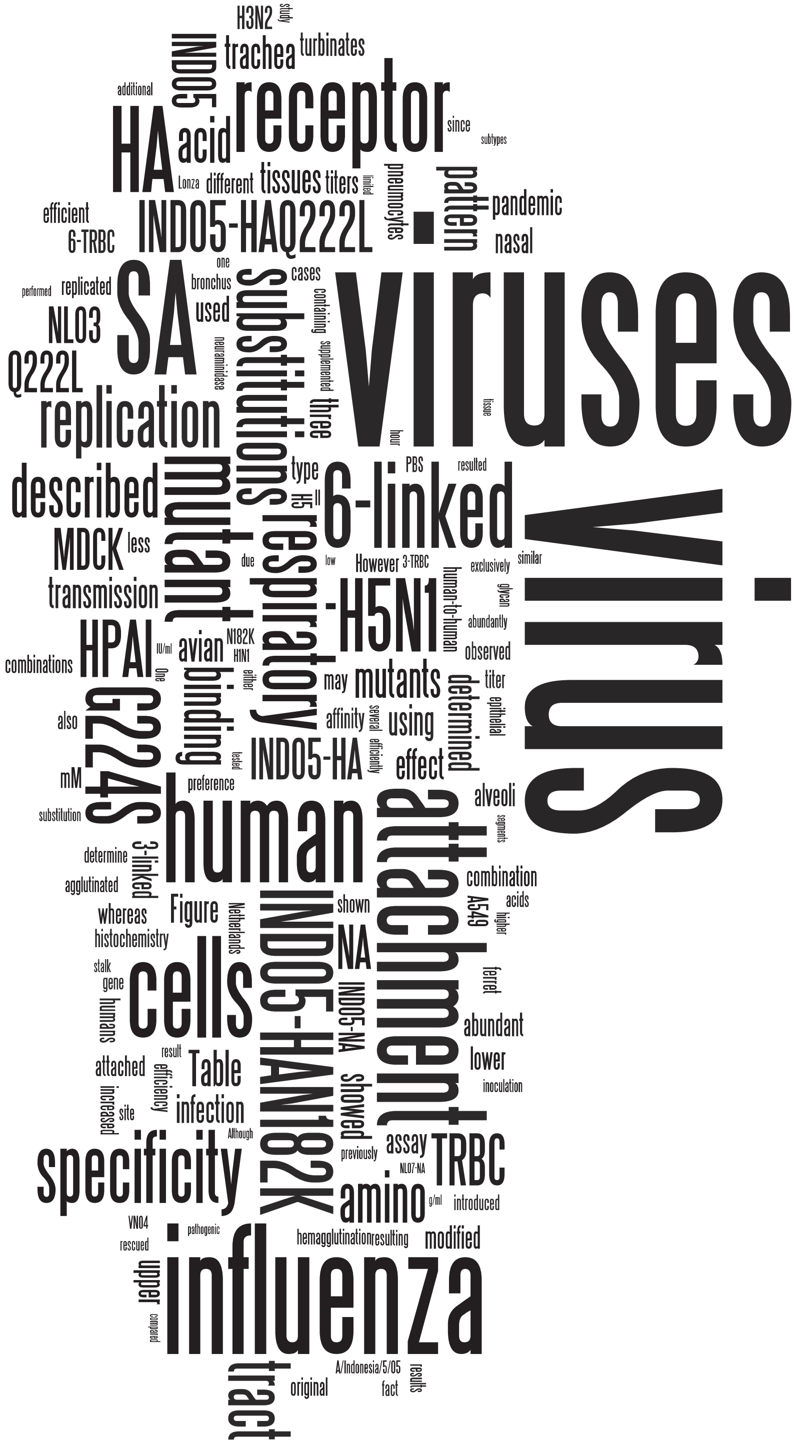
The significance of influenza virus attachment to submucosal glands and goblet cells is not clear. Our attachment results correspond with *in vivo* data showing infection of human submucosal glands by human seasonal influenza viruses.[96] Infection of submucosal glands and goblet cells could not only lead to the production of progeny virus, but also decrease their production of mucus, which is known to inhibit virus infection.[97]

In conjunction with our previous study[66;107] we now have a systemic overview of the pattern of attachment of influenza viruses at different levels of both upper and lower respiratory tract. However, attachment is only the first step in the replication cycle of

influenza virus in its host cell. Therefore, the next challenge will be to systematically investigate how influenza viruses replicate in tissues at different levels of the human respiratory tract. Together, these studies will help us to understand how respiratory tract tropism of influenza viruses affect both their pathogenicity and their transmission in the human host.

Acknowledgements

We thank W.Lim for providing the H5N1 virus isolate; F. van der Panne and P. van Run for technical assistance.



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Chapter 5

In vitro assessment of attachment pattern and replication efficiency of H5N1 influenza A viruses with altered receptor specificity

Based on:
Salin Chutinimitkul, Debby van Riel, Vincent J. Munster, Judith M.A. van den Brand, Guus F. Rimmelzwaan, Thijs Kuiken, Albert D.M.E. Osterhaus, Ron A.M. Fouchier and Emmie de Wit

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Abstract

The continuous circulation of the highly pathogenic avian influenza (HPAI) H5N1 virus has been a cause of great concern. The possibility of this virus acquiring specificity for the human influenza A virus receptor, α2,6-linked sialic acids (SA), and being able to transmit efficiently among humans is a constant threat to human health. Different studies have described amino acid substitutions in hemagglutinin (HA) of clinical HPAI H5N1 isolates or that were introduced experimentally, that resulted in an increased, but not exclusive, binding of these virus strains to α2,6-linked SA. Here, we have introduced all previously described amino acid substitutions and combinations thereof into a single genetic background, influenza virus A/Indonesia/5/05 HA and tested the receptor specificity of these 27 mutant viruses. The attachment pattern to ferret and human tissues of the upper and lower respiratory tract of viruses with α2,6-linked SA receptor preference was then determined and compared to the attachment pattern of a human influenza A virus (H3N2). At least three mutant viruses showed an attachment pattern to the human respiratory tract similar to that of the human H3N2 virus. Next, the replication efficiency of these mutant viruses and the effect of three different neuraminidases on virus replication were determined. These data show that influenza virus A/Indonesia/5/05 potentially requires only a single amino acid substitution to acquire human receptor specificity, while at the same time remaining replication competent, thus suggesting that the pandemic threat posed by HPAI H5N1 is far from diminished.

Introduction

Influenza A virus is a negative strand RNA virus with a segmented genome within the family of *Orthomyxoviridae*. Influenza A viruses are divided into subtypes based on the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Currently, 16 subtypes of HA and 9 subtypes of NA have been identified in the natural reservoir of all influenza A viruses, wild aquatic birds.[13] Occasionally, viruses from this reservoir cross the species barrier into mammals, including humans. When animal influenza viruses are introduced in humans, the spread of the virus is generally limited, but may on occasion result in sustained human-to-human transmission. Three influenza A virus subtypes originating from the wild bird reservoir, H1, H2 and H3, have formed stable lineages in humans, starting off with a pandemic and subsequently causing yearly influenza epidemics. In the 20th century, three such pandemics have occurred, in 1918 (H1N1), 1957 (H2N2) and 1968 (H3N2). In 2009, the swine-origin H1N1 virus caused the first influenza pandemic of the 21st century.[119]

Efficient human-to-human transmission is a prerequisite for any influenza A virus to become pandemic. Currently, the determinants of efficient human-to-human transmission are not completely understood. However, it is believed that a switch of receptor specificity from α2,3-linked sialic acids (SA), used by avian influenza A viruses, to α2,6-linked SA, used by human influenza viruses, is essential.[14;60;120] It has been shown that the difference in receptor use between avian and human influenza A viruses combined with the distribution of the avian and human virus receptors in the human respiratory tract results in a different localization of virus attachment.[63;66;107;121] Human viruses attach more abundantly to the upper respiratory tract and trachea whereas avian viruses predominantly attach to the lower respiratory tract.[66;107;121;122] Theoretically, the increased presence of virus in the upper respiratory tract, due to the specificity of human influenza A viruses for α2,6-linked SA, could facilitate efficient transmission.

Since 1997, highly pathogenic avian influenza (HPAI) H5N1 virus has been circulating in South-East Asia and has spread westwards to Europe, the Middle East and Africa, resulting in outbreaks of HPAI H5N1 virus in poultry and wild birds, and sporadic human cases of infection in 15 different countries (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_03_16/en/index.html). The widespread, continuous circulation of the HPAI H5N1 strain has spiked fears that it may acquire specificity for α2,6-linked SA, potentially resulting in a pandemic. Given the currently high case-fatality rate of HPAI H5N1 virus infection in humans of approximately 60%, the effect of such a pandemic on the human population could be devastating. Over the past years, several amino acid substitutions in HA of HPAI H5N1 viruses have been described, either in virus isolates from patients or introduced experimentally, that increased the binding of the HPAI H5N1 HA to α2,6-linked SA.[65;123-129] However, none of the described substitutions conferred a full switch of receptor specificity from α2,3-linked SA to α2,6-linked SA and the substitutions were described in virus

strains of different geographical origins. Furthermore, it is unknown whether these substitutions led to increased attachment of the virus to cells of the upper respiratory tract, the primary site of replication of human influenza A viruses.

Here, we have introduced all the 21 previously described amino acid substitutions or combinations thereof that changed the receptor specificity of HPAI H5N1 virus strains and six additional combinations not previously described, into HA of influenza virus A/Indonesia/5/05 (IND05). Indonesia is the country that has the highest cumulative number of human cases of HPAI H5N1 virus infection (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_03_16/en/index.html).

The receptor specificity of 27 mutant H5N1 viruses was determined and the attachment pattern of a subset of these viruses to tissues of the respiratory tract of ferret and human was determined and compared to the attachment pattern of human influenza A virus (H3N2). Subsequently, the role of NA in efficient replication of these mutant viruses was investigated. The data presented here show that receptor specificity of HA of the IND05 virus can be changed by introducing a single amino acid substitution in the receptor binding domain, resulting in replication competent viruses that attach abundantly to the human upper respiratory tract.

Materials and methods

Cells

Madin-Darby Canine kidney (MDCK) cells were cultured in EMEM (Lonza, Breda, The Netherlands) supplemented with 10% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml Sodium Bicarbonate, 10 mM Hepes and non-essential amino acids. 293T cells were cultured in DMEM (Lonza) supplemented with 10 % FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1 mM Sodium Pyruvate and non-essential amino acids. A549 cells were cultured in HAM F12 medium (Lonza) supplemented with 10% FCS (Hyclone), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine.

Viruses

Seasonal influenza viruses A/Netherlands/213/03 (NL03; H3N2) and A/Netherlands/26/07 (NL07; H1N1) were isolated from human cases of influenza and subsequently passaged in MDCK cells. Influenza virus A/Indonesia/5/05 (IND05; H5N1) and A/Vietnam/1194/04 (VN04; H5N1) were isolated from human cases of HPAI virus infection and passaged once in embryonated chicken eggs and once in MDCK cells. Reassortant and mutant viruses consisting of six or seven gene segments of influenza virus A/PR/8/34 (PR8) and HA and/or NA of influenza virus A/Indonesia/5/05 were produced using reverse genetics techniques as described previously.[130] The genotypes of recombinant viruses were confirmed by sequencing prior to use.

Plasmids

HA and/or NA of influenza viruses NL03, NL07, VN04 and IND05 were amplified by RT-PCR and cloned in the BsmBI site of a modified version of plasmid pHW2000.[130] Twelve nucleotides encoding four amino acid residues (underlined) making up the multibasic cleavage site PQRER/SRRKKRG in the HA gene of influenza virus VN04 and IND05 were removed as described previously,[131] so viruses containing these HA genes, were no longer considered highly pathogenic. For the construction of 27 plasmids containing HA of influenza virus IND05 with one or several nucleotide substitutions, a QuickChange Multi Site-Directed Mutagenesis kit (Qiagen, Venlo, The Netherlands) was used according to instructions of the manufacturer.

For insertion of 20 amino acids in the stalk region of NA of influenza virus IND05, a 60 nucleotide fragment encoding the NA stalk of HPAI H5N1 virus A/HongKong/213/03 (CNQSIITYENNTWVNQTYVN) was amplified and inserted at nucleotide position 165 of IND05 NA. All plasmids were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and a 3130XL Genetic Analyser (Applied Biosystems), according to the instructions of the manufacturer. All primer sequences are available upon request.

Transfection and infection of cells

Transfection of 293T cells and production of recombinant viruses was performed by transient calcium phosphate-mediated transfection as previously described.[130] The supernatant of the transfected cells was harvested 48h after transfection and was used to inoculate MDCK cells. One hour after inoculation, MDCK cells were washed once with PBS and cultured in infection media consisting of EMEM (Lonza) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 1.5 mg/ml Sodium Bicarbonate, 10 mM Hepes, non-essential amino acids and 17.5 µg/ml trypsin (Lonza).

Modified turkey red blood cell hemagglutination assay

Modified turkey red blood cells (TRBC) were prepared with modifications from.[132] Briefly, all α2,3- α2,6- α2,8- and α2,9-linked SA were removed from the surface of TRBC, by incubating 62.5 µl of 20% TRBC in PBS with 50 mU *Vibrio cholerae* Neuraminidase (VCNA; Roche, Almere, The Netherlands) in 8 mM calcium chloride at 37°C for 1 hour. Removal of sialic acids was confirmed by complete loss of hemagglutination of the TRBC by control influenza A viruses. Subsequently, resialylation was performed using 0.5 mU α2,3-(N)-sialyltransferase (Calbiochem, CA, USA) or 2 mU α2,6-(N)-sialyltransferase (Japan Tobacco Inc., Shizuoka, Japan) and 1.5 mM CMP-sialic acid (Sigma-Aldrich, Zwijndrecht, the Netherlands) at 37°C in 75 µl for 2 hours to produce α2,3-TRBC and α2,6-TRBC respectively. After washing the TRBC were resuspended in PBS containing 1% BSA to a final concentration of 0.5% TRBC. Resialylation was confirmed by hemagglutination of viruses with known receptor specificity. Receptor specificity of mutant viruses was tested by performing a standard HA assay with the modified TRBC. In brief, serial 2-fold dilutions of virus

in PBS were made in a 50 µl volume; 50 µl of 0.5% TRBC was added and incubated for 1 hour at 4°C before reading the hemagglutination titer.

Virus purification and labeling

Viruses were purified and labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich) as described previously.[107] Briefly, virus stocks prepared in MDCK cells were concentrated and purified using sucrose gradients, inactivated by dialysis against 0.1% formalin and labeled with an equal volume of 0.1 mg/ml of FITC.

Respiratory tract tissues

The paraffin-embedded human respiratory tract tissue sections were obtained from the Department of Pathology, Erasmus Medical Center. The paraffin-embedded ferret respiratory tract tissue sections were obtained from the Department of Virology, Erasmus Medical Center. All of the selected tissues were without histological lesions or evidence of respiratory tract infection. Tissues from three individuals of each species were analyzed.

Virus histochemistry on tissue sections

Virus histochemistry was performed as described previously.[50;107] Briefly, formalin-fixed paraffin-embedded tissues were deparaffinized with xylene and rehydrated with graded alcohol. FITC-labeled influenza viruses (50-100 hemagglutinating units) were incubated with tissues overnight at 4˚C. The FITC label was detected with a peroxidase-labeled rabbit anti-FITC antibody (Dako, Heverlee, Belgium) and the signal was amplified with a Tyramide Signal Amplification System (Perkin Elmer, Groningen, The Netherlands) according to the manufacturers instructions. Peroxidase was revealed with 3-amino-9-ethyl-carbazole (Sigma-Aldrich) and tissues were counterstained with hematoxylin and embedded in glycerol-gelatin (Merck, Darmstadt, Germany). Attachment of influenza virus to tissues was visible as granular to diffuse red staining on the apical surface of epithelial cells.

Virus titrations

Viruses were titrated by end-point dilution in MDCK cells as described previously.[130] Briefly, MDCK cells were inoculated with tenfold serial dilutions of culture supernatants. One hour after inoculation, cells were washed and supplemented with infection media. Three days after inoculation, the supernatants were tested for agglutination activity using TRBCs as an indicator of infection of the cells. Infectious titers were calculated from 5 replicates by the method of Spearman-Karber.

Replication kinetics

Multi step replication kinetics were determined by inoculating MDCK or A549 cells at a multiplicity of infection (moi) of 0.01 TCID50 per cell. One hour after inoculation, at time point 0, cells were washed once with PBS and fresh infection media was added. Supernatants were sampled at 6, 12, 24 and 48 hours after inoculation and virus titers in these supernatants were determined.

Table 1: *Overview of the 27 mutant A/Indonesia/5/05 HA molecules used in this study. Amino acid mutations introduced and source of these substitutions are indicated.*

Amino acid substitution[a]	Amino acid position by H3 numbering	Virus rescue	References
S129V[b]	133	+	(123)
A134V	138	+	(123)
S133A[c]	137	+	(129)
G139R	143	+	(128)
N154S[c]	158	+	(125)
N182K	186	+	(128)
E186D[c]	190	+	(127)
T188I[c]	192	+	(129)
Q192R	196	+	(128)
N193K	197	+	(128)
K218E[c]	222	+	(126)
G221D[c]	225	+	(127)
Q222L[c]	226	+	(127)
S223N[c]	227	+	(65, 128)
G224S[c]	228	+	(127)
E186D G221D[c]		+[d]	(127)
Q192R S223N		+	(128)
S133A T188I[c]		+	(129)
G139R N182K		+	(128)
S129V[b] A134V		+	(123)
Q222L G224S[c]		+	(127)
N182K Q222L G224S		+	This study
Q192R Q222L G224S		+	This study
Q222L S223N G224S		+	This study
N182K Q222L S223N G224S		+	This study
Q192R Q222L S223N G224S		+	This study
N182K Q192R Q222L S223N G224S		+	This study

[a] Amino acid position by H5 numbering
[b] L129V in (123)
[c] H3 amino acid numbering used in reference
[d] Virus rescue positive but hemagglutination titer too low for further analysis

Results

Production of mutant H5 viruses

Over the past years, there have been several reports of HPAI H5N1 viruses with increased affinity for the human influenza A virus receptor, α2,6-linked SA, in combination with residual affinity for α2,3-linked SA.[65;123;124;126-129] These amino acid substitutions resulting in increased affinity for α2,6-linked SA were described in different HPAI H5N1 viruses and using different experimental assays. The 21 described amino acid substitutions or combinations thereof and 6 additional combinations not previously described (Table 1) were introduced in the HA gene of influenza virus A/Indonesia/5/05 (IND05) from which the multibasic cleavage site was deleted, so that viruses containing these HAs were no longer highly pathogenic. Using reverse genetics, rescue of these mutant HAs in combination with 7 gene segments encoding influenza virus A/PR/8/34 was attempted. All of the 27 mutant viruses could be rescued (Table 1). The virus containing IND05-$HA_{E190D,G225D}$ was excluded from further experiments because it had a low HA titer using a standard HA assay and its receptor specificity could not be determined.

Receptor specificity of the mutant H5 viruses

A hemagglutination assay using modified TRBC that contained either α2,3-linked SA (α2,3-TRBC) or α2,6-linked SA (α2,6-TRBC) exclusively on their cell surface, was used to determine the receptor specificity of the viruses with mutant HAs. NL03 (H3N2) and VN04 (H5N1) were used as a prototype human and avian influenza A virus respectively, since their attachment pattern to the human respiratory tract was characterized previously.[66;107;121] As shown in table 2, NL03 exclusively showed attachment to α2,6-linked SA, whereas VN04 exclusively bound α2,3-linked SA. These control viruses also showed that modified TRBC contained amounts of SA that yielded equivalent HA titers to the titers obtained with regular TRBC. Next, the receptor specificity of the 26 viruses with mutant HAs was tested. Based on the results, the viruses were divided into four groups (Table 2). Group I contained viruses that agglutinated unmodified TRBC, but not the modified TRBC containing either α2,3- or α2,6-linked SA. The 10 mutant viruses in group II agglutinated α2,3-TRBC, but not α2,6-TRBC, despite the introduced amino acid substitutions. In group III, the mutants IND05-HA_{N182K}, IND05-HA_{Q222L}, IND05-HA_{S223N}, IND05-HA_{G224S}, IND05-$HA_{Q192R,S223N}$, and IND05-$HA_{Q222L,G224S}$ agglutinated only α2,6-TRBC but not α2,3-TRBC, despite the fact that these amino acid substitutions or combinations thereof were described to have dual receptor binding in their original background.[127;128] This prompted us to create additional combinations of mutations, resulting in six additional mutants (Table 1). Of these additional mutants, only the IND05-$HA_{N182K,Q222L,G224S}$ virus agglutinated α2,6-TRBC as well as the single and double mutants, whereas the other combinations resulted in lower affinity for α2,6-TRBC (Table 2). In fact, the IND05-$HA_{N182K,Q192R,Q222L,S223N,G224S}$ virus agglutinated α2,6-TRBC, but also retained affinity for α2,3-TRBC (Table 2) placing them in group IV together with two other viruses.

Table 2: *Receptor specificity of the different viruses as determined by a modified TRBC hemagglutination assay. All amino acid substitutions are indicated by H5 numbering.*

Group	Mutant	HA titer (HAU/50µl)		
		TRBC	α2,3-TRBC	α2,6-TRBC
I	A134V	16	0	0
	E186D	16	0	0
II	VN04 (H5N1)	128	128	0
	IND05	64	64	0
	S129V	64	64	0
	S133A	64	64	0
	G139R	64	64	0
	T188I	64	64	0
	Q192R	64	64	0
	N193K	64	32	0
	K218E	16	1	0
	G221D	64	4	0
	S129V A134V	8	1	0
	S133A T188I	128	128	0
III	NL03 (H3N2)	128	0	128
	N182K	64	0	16
	Q222L	32	0	8
	S223N	64	0	2
	G224S	64	0	32
	Q192R S223N	64	0	8
	Q222L G224S	32	0	32
	N182KQ222L G224S	64	0	64
	Q192R Q222L G224S	64	0	4
	Q222L S223N G224S	64	0	2
	N182K Q222L S223N G224S	64	0	32
	Q192R Q222L S223N G224S	64	0	1
IV	N154S	32	32	1
	G139R N182K	64	1	2
	N182K Q192R Q222L S223N G224S	64	2	64

Attachment of selected H5 mutants to the ferret respiratory tract

Next, the attachment pattern of virus with IND05-HA and the IND05-HA_{N182K}, IND05-HA_{E186D}, IND05-HA_{Q222L}, IND05-HA_{G224S}, IND05-$HA_{G139R,N182K}$, IND05-$HA_{Q222L,G224S}$ and IND05-$HA_{N182K,Q222L,G224S}$ mutants to fixed tissues of the upper and lower respiratory tract, including nasal turbinates, trachea, bronchus, bronchiole and alveoli, was determined using virus histochemistry. Because of the limited availability of human tissues for such studies, the initial screening was performed with ferret tissues.

Table 3: *Attachment of selected mutant virus to upper and lower respiratory tract tissues of ferrets and human origin. The mean abundance of cells to which virus attached was scored as follows: -, no attachment; +/-, attachment to rare or few cells; +, attachment to a moderate number of cells; + +, attachment to many cells. Scores are median scores of three individual tissues. Where possible, the predominant cell type to which virus attached is indicated: ciliated cells (cil), non-ciliated cuboidal cell (non-cil), type I pneumocytes (type I), or type II pneumocytes (type II). ND: not determined.*

Virus	Tissue origin	Nasal turbinates		Trachea		Bronchus		Bronchiole		Alveoli	
		Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type	Score	Predominant cell type
NL03	**Ferret**	++[a,b]	cil	++[a]	cil	+[a,b]	cil	+/-		++	Type I
	Human	++[a,b]	cil	++[a,b]	cil	++[a,b]	cil	++	cil	++	Type I
IND05	**Ferret**	-		-		-		+/-		++	Type II
	Human	-		-		-		+	non-cil	+	Type II
IND05-HA$_{E186D}$	**Ferret**	-		-		-		-		+	Type II
	Human	ND		ND		ND		ND		ND	
IND05-HA$_{G139R,N182K}$	**Ferret**	-[a]		-		-		-		++	Type II
	Human	ND		ND		ND		ND		ND	
IND05-HA$_{N182K}$	**Ferret**	++[a,b]	cil	+[a]	cil	+[a,b]	cil	-		+/-	
	Human	++[a,b]	cil	+[a,b]	cil	++[a,b]	cil	+[b]	cil	+	Type I
IND05-HA$_{Q222L}$	**Ferret**	+	cil	-		+/-[a]		+	non-cil	+	Type II
	Human	ND		ND		ND		ND		ND	
IND05-HA$_{G224S}$	**Ferret**	+/-		-		-		-		+	Type II
	Human	ND		ND		ND		ND		ND	
IND05-HA$_{Q222L,G224S}$	**Ferret**	++[a,b]	cil	+/-		+/-[a,b]		+	non-cil/cil	+	Type I/II
	Human	++[a,b]	cil	+[a,b]	cil	++[a,b]	cil	++	cil	++	Type I
IND05-HA$_{N182K,Q222L,G224S}$	**Ferret**	++[a,b]	cil	+/-[a]		+[a]	cil	+/-		+/-	
	Human	++[a,b]	cil	+/-[a]		++[a,b]	cil	+	cil	++	Type I

[a] Submucosal glands positive
[b] Goblet cells occasionally positive

Ferrets are a widely accepted animal model for influenza A virus infections and attachment of avian and human influenza A viruses to the ferret lung was shown to be comparable to the attachment pattern to human respiratory tract.[107;133] The IND05-HA_{E186D} and IND05-$HA_{G139R,N182K}$ viruses were selected because of their ability to agglutinate unmodified TRBC, but not, or to a much lesser extent, α2,3- or α2,6-TRBC. Both viruses showed an attachment pattern similar to that of IND05, with some small differences (Table 3). Neither of the viruses attached to the nasal turbinates, trachea or bronchus. In the bronchioles IND05-HA virus attached rarely, but the IND05-HA_{E186D} and IND05-$HA_{G139R,N182K}$ viruses did not; in the alveoli all three viruses attached to type II pneumocytes.

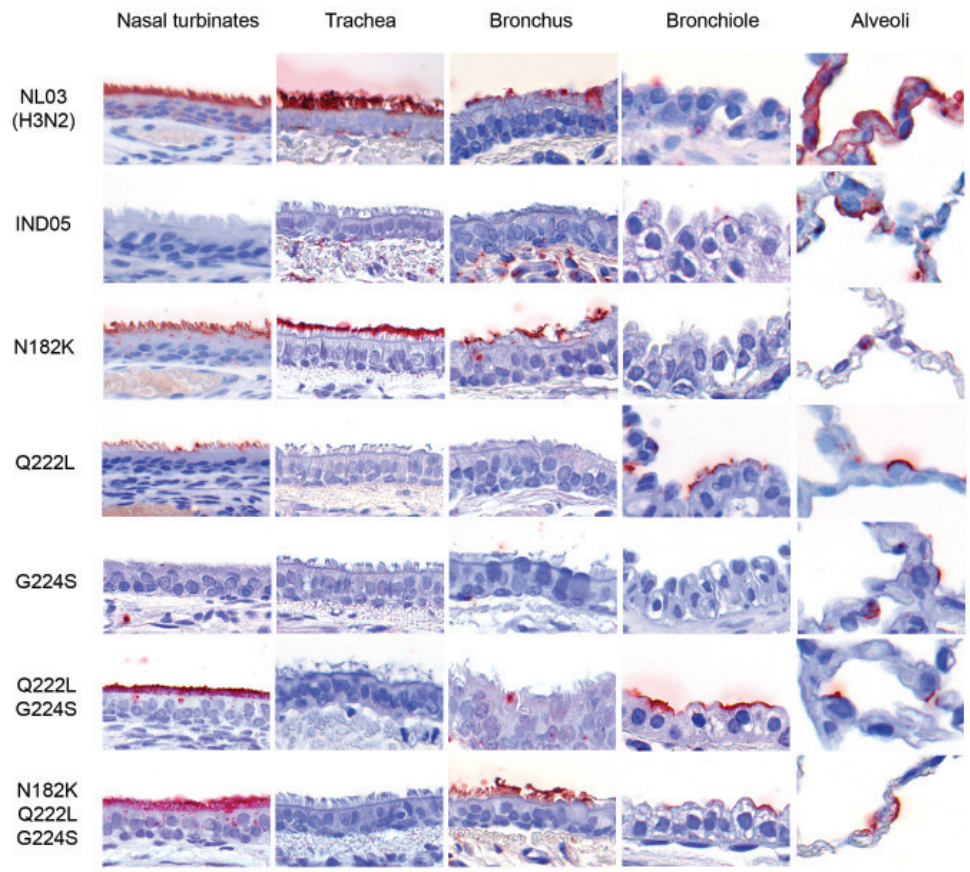

Figure 1: *Attachment of viruses with altered receptor specificity to tissues of the ferret upper and lower respiratory tract. The attachment pattern of viruses NL03 (H3N2) and IND05 (H5N1) and the five mutant viruses IND05-HA_{N182K}, IND05-HA_{Q222L}, IND05-HA_{G224S}, IND05-$HA_{Q222L,G224S}$ and IND05-$HA_{N182K,Q222L,G224S}$ to ferret nasal turbinates, trachea, bronchus, bronchiole and alveoli was determined. Virus attachment is shown in red. The panels were chosen to reflect the attachment pattern in the whole tissue section as much as possible, but small differences between the single panels and overall view may exist.*

The remaining mutants were from group III and thus agglutinated α2,6-TRBC exclusively. In order to determine which of these mutants exhibited an attachment pattern similar to that of a human influenza A virus, the attachment pattern of the mutant viruses was compared to that of the human influenza virus NL03 (H3N2) and to IND05 as a control. The IND05 virus predominantly attached to type II pneumocytes in the alveoli, whereas the NL03 virus attached abundantly to ciliated epithelial cells in the nasal turbinates, trachea and bronchi and to type I pneumocytes in the alveoli (Table 3, Figure 1). The attachment pattern of the IND05-HA_{G224S} was least comparable to NL03, with rare attachment in the nasal cavity, no demonstrable attachment in trachea, bronchus or bronchioles and moderate attachment to type II pneumocytes in the alveoli (Table 3). The attachment pattern of the IND05-HA_{Q222L} mutant was also dissimilar from that of NL03, with moderate attachment in nasal turbinates, no demonstrable attachment in the trachea, rare attachment in the bronchus and moderate attachment in the bronchiole to non-ciliated epithelial cells and to type II pneumocytes in the alveoli (Table 3). The remaining mutants, IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$ and IND05-$HA_{N182K,Q222L,G224S}$ all showed abundant attachment to ciliated epithelial cells in the nasal turbinates, like the human NL03 virus (Table 3, Figure 1), but did not show similarly abundant attachment to the trachea or type I pneumocytes in the alveoli.

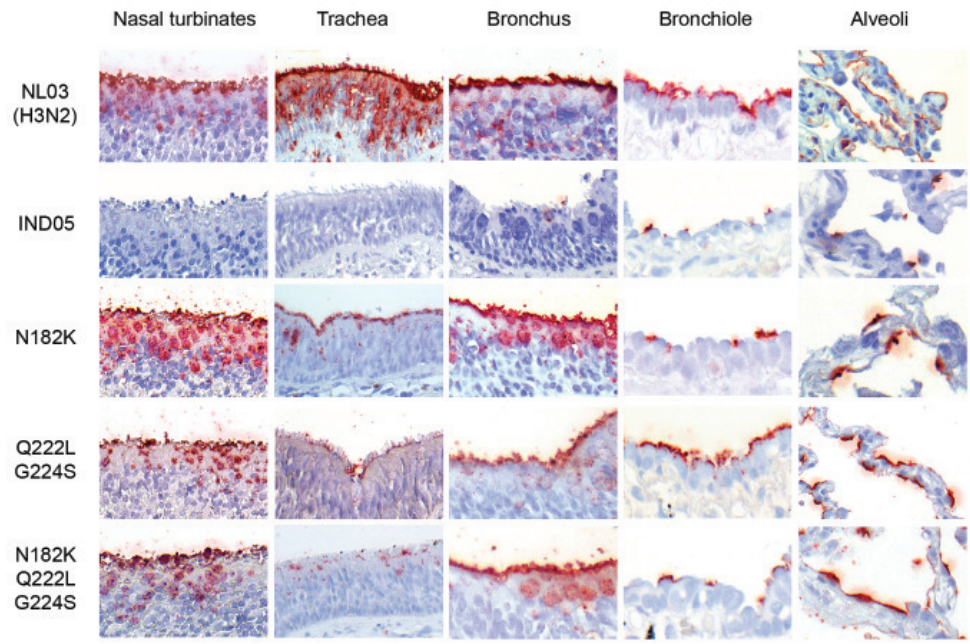

Figure 2: *Attachment of viruses with altered receptor specificity to tissues of the human upper and lower respiratory tract. The attachment pattern of viruses NL03 (H3N2) and IND05 (H5N1) and three mutant viruses IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$ and IND05-$HA_{N182K,Q222L,G224S}$ to human nasal turbinates, trachea, bronchus, bronchiole and alveoli was determined. Virus attachment is shown in red. The panels were chosen to reflect the attachment pattern in the whole tissue section as much as possible, but small differences between the single panels and overall view may exist.*

Attachment of three mutant H5 viruses to the human respiratory tract

Since the IND05-HA$_{N182K}$, IND05-HA$_{Q222L,G224S}$ and IND05-HA$_{N182K,Q222L,G224S}$ viruses had an attachment pattern to ferret respiratory tract most similar to that of the human NL03 virus, the attachment pattern of these viruses to tissues of the human upper and lower respiratory tract, including the nasal turbinates, trachea, bronchus, bronchiole and alveoli, was determined. Like the human NL03 virus, all three mutant viruses showed abundant attachment to the ciliated epithelial cells of the nasal turbinates and bronchus, but attachment to the trachea and lower respiratory tract differed (Table 3, Figure 2). The mutant viruses attached less abundantly than the NL03 virus to the ciliated epithelial cells of the trachea, with attachment of the IND05-HA$_{N182K,Q222L,G224S}$ mutant being only rare. In the bronchiole, the IND05-HA$_{Q222L,G224S}$ mutant attached as abundantly as the NL03 virus, but attachment of the other two mutants to this tissue was less abundant. In the alveoli, all three mutant viruses attached to type I pneumocytes, in agreement with attachment of the NL03 virus and in contrast with the attachment of the IND05 virus to type II pneumocytes (Table 3, Figure 2).

H5 viruses with altered receptor specificity are replication competent

Besides the receptor specificity and attachment pattern of the mutant H5 viruses, that showed similarity to that of a human influenza A virus, we determined the replication efficiency of these viruses, since efficient replication is a prerequisite for the emergence of a mutant strain. The functionality of the mutant HAs was investigated by determining the replication kinetics of viruses consisting of IND05-HA, IND05-HA$_{N182K}$, IND05-HA$_{Q222L,G224S}$ or IND05-HA$_{N182K,Q222L,G224S}$ and seven PR8 gene segments in MDCK and A549 cells. Although all viruses replicated to low titers in A549 cells, the three viruses with the mutant HAs replicated to virus titers similar or slightly higher than those of the virus with IND05-HA in both MDCK and A549 cells (Figure 3A and E), indicating that the amino acid substitutions in HA did not have a negative effect on replication kinetics in vitro.

Effect of neuraminidase on replication efficiency of mutant H5

The receptor binding properties of HA have to be balanced with the neuraminidase activity of NA.[134;135] Since the viruses with IND05-HA$_{N182K}$, IND05-HA$_{Q222L,G224S}$ and IND05-HA$_{N182K,Q222L,G224S}$ substitutions had an altered receptor specificity, this raised the question whether viruses with these HA molecules could still replicate efficiently in combination with IND05-NA. Thus, recombinant viruses consisting of six gene segments of influenza virus PR8, IND05-NA and one of IND05-HA, IND05-HA$_{N182K}$, IND05-HA$_{Q222L,G224S}$ or IND05-HA$_{N182K,Q222L,G224S}$ were produced. The virus with IND05-HA$_{N182K,Q222L,G224S}$ and IND05-NA could not be rescued using a standard protocol. Upon addition of exogenous *Vibrio cholerae* neuraminidase to the supernatant of transfected 293T cells and inoculated MDCK cells, virus was rescued with a low hemagglutination titer. However, in each of three attempts, the rescued virus

contained either a K153E substitution or a K153 deletion in HA. Because of this mutation, replication efficiency of this virus was not determined. Of note, when these virus stocks were tested in a HA assay with modified TRBC, it was shown that the substitution/deletion at position 153 of HA resulted in decreased receptor binding, but not in a shift in receptor specificity from α2,6-linked SA to α2,3-linked SA.

The replication efficiency of viruses with the IND05-HA_{N182K} or IND05-$HA_{Q222L,G224S}$ and IND05-NA was determined in MDCK cells and compared to that of a virus with IND05-HA and IND05-NA. As shown in figure 3B, the IND05-HA_{N182K} or IND05-$HA_{Q222L,G224S}$ viruses replicated to a more than 100-fold lower titer with IND05-NA than with PR8-NA. However, this was also the case with IND05-HA and is thus probably not related to the receptor specificity of HA.

Upon replication of avian influenza viruses in poultry, a deletion can occur in the stalk region of neuraminidase, as has been the case with HPAI H5N1 virus.[136] Hypothesizing that this adaptation to replication in poultry may hamper efficient replication in mammalian cells, especially in combination with a HA with α2,6-linked SA specificity, the stalk fragment of IND05-NA was replaced by that of A/HongKong/213/03 NA, resulting in IND05-NA_{stalk}. The effect of this insertion in NA on the replication efficiency of viruses with IND05-HA, IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$ or IND05-$HA_{N182K,Q222L,G224S}$ was determined in MDCK cells. The insertion of the stalk in IND05-NA did not have an effect on the replication of virus with IND05-HA or on IND05-HA_{N182K}

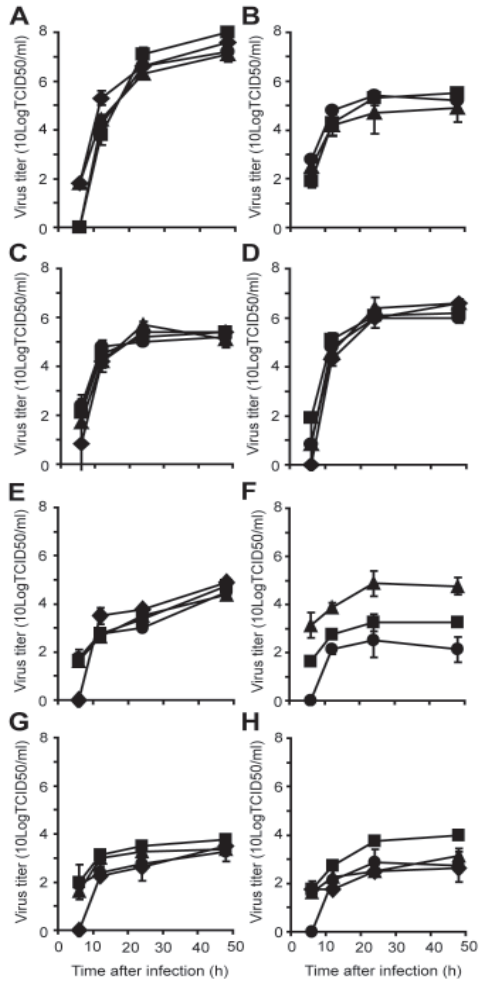

Figure 3: *Replication kinetics of viruses with IND05-HA with altered receptor specificity and different NA genes in MDCK (A-D) and A549 (E-H) cells. Cells were inoculated at an moi of 0.01 TCID50/cell of virus with the IND05-HA (●), IND05-HA_{N182K} (■), IND05-$HA_{Q222L,G224S}$ (▲) or IND05-$HA_{N182K,Q222L,G224S}$ (♦) in combination with (A,E) NA of influenza virus PR8; (B,F) NA of influenza virus IND05; (C,G) NA of influenza virus IND05 with stalk insertion (NA_{stalk}) or (D,H) NA of influenza virus NL07 (H1N1). Remaining gene segments originated from influenza virus PR8. Supernatants from 6, 12, 24 and 48 hours after inoculation were titrated by end-point dilution in MDCK cells. Geometric mean titers were calculated from two independent experiments; error bars indicate standard deviation.*

(Figure 3C). However, the IND05-$HA_{Q222L,G224S}NA_{stalk}$ mutant replicated to a ten-fold higher virus titer than the IND05-$HA_{Q222L,G224S}$NA and the IND05-$HA_{N182K,Q222L,G224S}NA_{stalk}$ mutant could be rescued efficiently using a standard protocol, indicating that the lack of virus production and generation of mutations in HA was a result of incompatibility of the $HA_{N182K,Q222L,G224S}$ variant with IND05 NA.

Next, the mutant HAs were combined with N1 of influenza virus A/NL/26/07 (NL07-NA), a recent seasonal H1N1 isolate. The virus with IND05-HA and NL07-NA replicated to slightly higher titers than IND05-HANA (Figure 3D). All viruses with mutant HA also replicated to higher titers with NL07-NA than with IND05-NA, with the smallest effect seen in combination with IND05-HA_{N182K} (~6-fold increase) and the largest effect, an approximately 50-fold increase in virus titer, with IND05-$HA_{Q222L,G224S}$ (Figure 3D). When these experiments were repeated in A549 cells, the effect of changing NA on replication efficiency of the viruses was not as pronounced. Only the virus with IND05-HA_{N182K} and NL07-NA replicated to a 10-fold higher virus titer than the same virus with IND05-NA (Figure 3F-H).

Discussion

Although the current H1N1 pandemic may have distracted the attention from the continuing circulation of HPAI H5N1 virus in large parts of the world, its pandemic threat has not decreased. Here, we show that amino acid substitutions in HA of the H5N1 IND05 virus may increase its pandemic potential. Not only do these substitutions change the receptor specificity of these viruses to α2,6-linked SA, these viruses also have an attachment pattern in the human respiratory tract that resembles that of human influenza A viruses.

Despite reports of isolated clusters of human-to-human transmission,[32] the HPAI H5N1 virus so far has not been capable of efficient human-to-human transmission, an absolute requirement for an influenza A virus to become pandemic. It is generally accepted that a switch in receptor specificity from α2,3-linked SA to α2,6-linked SA and a resultant shift to replication in the upper respiratory tract, is necessary for any respiratory transmission of influenza A viruses to occur.[60] The HPAI H5N1 virus has continuously circulated in poultry in Indonesia since 2004 and has caused 163 human cases there since 2005, identifying Indonesia as a hotspot for the human-animal interface of the HPAI H5N1 virus, together with Egypt where the HPAI H5N1 virus has also caused a relatively large number of human cases. When comparing the effect of the amino acid substitutions in IND05-HA on the α2,3-/α2,6-linked SA binding specificities as determined in the present study to those observed in the original studies, it is clear that the substitutions in the IND05 background result in a much more pronounced effect, or absence thereof (Table 2). The original studies described residual α2,3-linked SA binding in combination with the newly acquired α2,6-linked SA binding, whereas only 3 out of 27 IND05-HA mutants showed this dual

receptor specificity. Of the remaining mutants, 10 bound α2,3-linked SA exclusively and 11 resulted in exclusive α2,6-linked SA binding. For two substitutions, A134V and E186D we could not detect binding to either α2,3-linked SA or α2,6-linked SA. This could be due to the fact that these HAs have a very low receptor affinity or to the fact that VCNA is able to remove all α2,3- and α2,6-linked SA from the TRBC, but not all conformations of SA can be restored by the sialyltransferases used to resialylate these TRBC. Our results suggest that IND05-like viruses are more predisposed to acquiring solely α2,6-linked SA receptor preference. With several mutant viruses, large differences in receptor specificity were observed between the original and the present study. The IND05-$HA_{S129V,A134V}$, IND05-HA_{Q192R} and IND05-HA_{G139R} viruses showed less α2,6-linked SA binding in the IND05-HA background, whereas the IND05-HA_{N182K}, IND05-HA_{Q222L}, IND05-HA_{G224S} and IND05-$HA_{Q222L,G224S}$ viruses bound more efficiently to α2,6-linked SA than the same amino acid substitutions in their original background. This seems to be due to the genetic context in which these substitutions were introduced (IND05-HA) rather than the assay used to determine receptor specificity, since both less and more abundant α2,6-linked SA binding were observed here with amino acid substitutions originating from the same study. For instance, the N182K substitution described by Yamada et al. resulted in limited α2,6-linked SA binding in the original study, but abundant α2,6-linked SA binding in the IND05-HA background, whereas the Q192R substitution described by the same authors resulted in α2,6-linked SA binding in the original study but did not lead to a change in receptor preference in the IND05-HA background.[128]

Although the modified TRBC assay provides a simple and easy to interpret test to quickly determine the receptor specificity of a certain virus isolate or laboratory-created mutant, it does not distinguish between different glycan topologies that may be of importance for attachment to and replication in the upper respiratory tract.[122;137] Therefore, we used virus histochemistry to determine the attachment pattern to upper and lower respiratory tract tissues of the mutant H5 viruses with a preference for α2,6-linked SA. In contrast to glycan arrays, virus histochemistry is a qualitative assay in which it is very difficult to quantify the observed attachment. On top of that, it only shows the type of cells in the respiratory tract to which a virus attaches, but not through which exact receptor. However, an advantage of virus histochemistry over glycan arrays is that it shows biologically relevant attachment whereas the localization of glycans used in the arrays in the human respiratory tract is currently unknown. Three mutant viruses, IND05-HA_{N182K}, IND05-$HA_{Q222L,G224S}$ and IND05-$HA_{N182K,Q222L,G224S}$, showed an attachment pattern to the human upper and lower respiratory tract that resembled that of human influenza viruses rather than avian viruses (Figure 2). Nevertheless, small differences were observed between these mutants and human influenza A viruses. For instance, the three mutant viruses showed less abundant attachment to the trachea than the H3N2 virus, albeit more abundant than the IND05 virus. Why the attachment to the trachea was less abundant despite the fact that attachment to the nasal turbinates was in the same range as that of H3N2 is unclear. One possible explanation is that the mutant viruses

have a preference for a slightly different glycan topology than the H3N2 virus and the distribution of the different glycan topologies expressed in the nasal turbinates and trachea is not identical. Because of these small differences in attachment pattern, it is impossible to predict which - if any - of the three receptor binding mutants is most likely to transmit efficiently via contact, aerosols or respiratory droplets. Testing these viruses in an animal model for transmission is required to demonstrate whether the changes in receptor specificity observed in the modified TRBC and virus histochemistry assay are sufficient for efficient human-to-human transmission.

Increased virus titers were detected in MDCK cells when the viruses with a preference for the human receptor were combined with NA originating from a human influenza A virus (Figure 3). Although this effect was seen with only one out of three mutants in A549 cells, this may be due to the fact that virus replication in A549 cells was very inefficient. The results in MDCK cells suggest that, due to the need for a balance between receptor binding of HA and sialic acid removal by NA,[134;135] amino acid substitutions or reassortment of NA will likely enhance the ability of the HPAI H5N1 virus to replicate efficiently in humans. This fits with observations that the substrate specificity of NA of the pandemic H2N2 1957 virus acquired increased affinity for α2,6-linked SA over time through amino acid substitutions.[138 139]

Likewise, several adaptations in the internal genes of avian influenza viruses to replication in humans have been described (reviewed[140]). The majority of these substitutions are in the influenza virus polymerase complex, indicating its importance in adaptation to a new host species. One of the well-described substitutions is the E627K substitution in PB2, that has been described as a host range determinant and major pathogenicity factor.[141-145] Moreover, the aerosol transmission efficiency of a human H3N2 influenza A virus was directly related to the presence of a 627K or 701N residue in PB2[146] and contact transmission of A/duck/Guangxi/35/01 (H5N1) was dependent on a combination of the ability of HA to bind α2,6-linked SA and the presence of a 701N residue in PB2.[147]

A likely effect of the change in receptor specificity from α2,3- to α2,6-linked SA is changing the site of primary virus replication of H5N1 viruses from the lower to the upper respiratory tract. This could result in a less pathogenic virus, with severe pneumonia being a less frequent complication as observed in seasonal influenza A virus cases. Indeed, a HPAI H5N1 virus with increased affinity for α2,6-linked SA showed a reduced pathogenicity and limited systemic spread in a mouse model.[148] These results may however have been affected by the virtual absence of α2,6-linked SA from the mouse lung[105] and reduced binding of human influenza viruses to mouse alveolar epithelium compared to avian influenza viruses.[107] Thus, it would be of interest to determine the effect of the substitutions in HA described here on the pathogenicity of the HPAI H5N1 virus in a ferret model, since it has been shown that ferrets have one of the most similar attachment patterns to humans with respect to avian as well as human influenza A viruses.[107]

The *in vitro* data described here have to be confirmed by *in vivo* experiments using viruses with HA with basic cleavage site and the internal genes of A/Indonesia/5/05. Of note, such viruses with the N182K; Q222L, G224S or N182K, Q222L, G224S substitutions in HA showed sole α2,6-linked SA receptor binding preference in the modified TRBC assay (data not shown). However, the results presented here suggest that one or a few amino acid substitutions in HA, possibly in combination with changes in NA, could result in a virus capable of efficient replication in humans and potentially sustained human-to-human transmission, thereby stressing the need for continuous surveillance of circulating HPAI H5N1 viruses.

Acknowledgements
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Chapter 6

Highly pathogenic avian influenza virus H5N1 infects alveolar macrophages without virus production or excessive TNF-alpha induction

Based on:
Debby van Riel, Lonneke M. E. Leijten, Menno van der Eerden, Henk Hoogsteden, Leonie A. Boven, Bart N. Lambrecht, Ab D.M.E. Osterhaus and Thijs Kuiken

In preparation

Abstract

Highly pathogenic avian influenza virus (HPAIV) of the subtype H5N1 causes severe, often fatal pneumonia in humans. The pathogenesis of HPAIV H5N1 infection is not completely understood, although the alveolar macrophage (AM) is thought to play an important role. HPAIV H5N1 infection of macrophages cultured from monocytes leads to high percentages of infection accompanied by virus production and an excessive pro-inflammatory immune response. However macrophages cultured from monocytes are different from AM, both in phenotype and in response to seasonal influenza virus infection. Consequently, it remains unclear whether the results of studies with macrophages cultured from monocytes are valid for AM. Therefore we infected AM and, for comparison macrophages cultured from monocytes, with seasonal H3N2 virus, HPAIV H5N1 or pandemic H1N1 virus, and determined the percentage of cells infected, virus production and induction of TNF-alpha a pro-inflammatory cytokine. HPAIV H5N1 infection of AM compared to that of macrophages cultured from monocytes resulted in a lower percentage of infected cells (up to 25% vs up to 84%), lower virus production and lower TNF-alpha induction. Infection of AM with H3N2 or H1N1 virus resulted even in lower percentages of infected cells (up to 7%) than that with HPAIV H5N1, while virus production and TNF-alpha production were comparable. In conclusion, this study reveals that macrophages cultured from monocytes are not a good model to study the interaction between AM and influenza virus. Furthermore, the interaction between HPAIV H5N1 and AM could contribute to the pathogenicity of this virus in humans, due to the relative high percentage of infected cells rather than virus production or an excessive TNF-alpha induction.

Introduction

Seasonal, pandemic and zoonotic influenza A virus infections cause substantial morbidity and mortality in humans. Seasonal influenza virus infections in humans are usually mild, causing pneumonia in a minority of infected individuals. Pandemic influenza virus infections vary in their disease outcome. The 1918 Spanish flu caused pneumonia more often than the currently ongoing pandemic H1N1 (pH1N1) virus. Zoonotic influenza virus infections in humans vary from self-limiting conjunctivitis to severe, often fatal pneumonia. The currently ongoing outbreak of highly pathogenic avian influenza virus (HPAIV) of the subtype H5N1 in poultry is sporadically transmitted to humans, in which it causes severe pneumonia with a case-fatality rate of about 60%.[27;28;102]

The differences in pathogenesis of different influenza virus infections in humans, and thereby their disease outcome are only partly understood. Early events after infection of the human alveoli—the site of pneumonia—contribute to protection against or the development of pneumonia. Previously we have shown that different influenza viruses attach to different cell types in the human alveoli. Seasonal influenza viruses and pH1N1 virus attach predominantly to type I pneumocytes,[107] whereas HPAIV H5N1 attaches predominantly to type II pneumocytes and alveolar macrophages (AM).[66] This fits with the detection of influenza A virus antigens in type II pneumocytes in lung tissues from HPAIV H5N1 fatal infections.[28-30] Furthermore, in experimental HPAIV H5N1 infections of *ex vivo* lung cultures, influenza virus antigen was detected in type II pneumocytes and alveolar macrophages.[63;69] Therefore, the response of alveolar epithelial cells and AM early after HPAIV H5N1 infection is likely important in the development of disease. In the present study we focus on the AM, an important cell of the innate immune system in the human alveolus.

Lung macrophages can be distinguished in alveolar, pleural, interstitial and intravascular macrophages, but the AM is the most important cell of the innate immune system of the human alveoli.[10] The phenotype of the AM is induced by the micro-environment of the human alveoli, which includes interactions with epithelial cells and the presence of SP-A and SP-D.[11;12] Precursors of AM are monocytes that enter the lung,[10] after which they mature into the AM phenotype.[149]

The AM has a protective role in the lung to prevent loss of function. Therefore, immune responses need to be balanced to protect the lungs from invading pathogens without excessive inflammation.[149] AM phagocytose virus particles and apoptotic cells by which they protect the lung from influenza induced damage.[150;151] Depletion of AM leads therefore to enhanced virus replication and more severe disease during influenza virus infection.[88;152]

The role of the AM in the pathogenesis of HPAIV H5N1-induced pneumonia has been studied by *in vitro* infections of macrophages cultured from monocytes. HPAIV H5N1 infects up to 100% of these macrophages, resulting in a productive infection. Furthermore, infection with HPAIV H5N1 results in an excessive immune response, marked by the high induction of the pro-inflammatory cytokine TNF-alpha, which is not observed after infection with seasonal H1N1 or H3N2 virus.[80;153;154] However, macrophages cultured from monocytes respond differently to influenza virus infections than AM as was shown by Ettensohn and Roberts.[155] In that study, macrophages cultured from monocytes produced significantly higher levels of interferon than AM. Another study revealed that only up to 20% of AM were infected with seasonal influenza viruses, and did not result in a productive infection.[156] Therefore, it is unclear whether macrophages cultured from monocytes are suitable substitutes for studying the interaction between influenza virus and AM.

To address this question, we compared the effect of seasonal H3N2 virus, pH1N1 virus or HPAIV H5N1 infection both on AM from broncho-alveolar lavages (BAL) of healthy volunteers, and on macrophages cultured from monocytes. In addition, we determined the percentage of AM infected in experimentally infected *ex vivo* cultured lung biopsies. Also we examined whether monocytes cultured in the presence of GM-CSF—which is abundantly present in the alveolar lining fluid—instead of human serum would develop a phenotype more similar to that of the AM.[157]

Materials and methods

Viruses

Viruses used for these experiments were seasonal H3N2 virus (A/Netherlands/213/03), a pandemic H1N1 virus (A/NL/602/09) and a HPAIV H5N1 (A /Vietnam/1194/04). All virus stocks were prepared on Madin-Darby Canine Kidney cells (MDCK).

Percentage of AM infected in ex vivo lung cultures

Surgically removed lung tissues, without any histological evidence for respiratory disease, were used for *ex vivo* infections. Biopsies were made with a 3-mm-biopsy punch and cultured overnight in F12K (Gibco, Breda, The Netherlands) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 5% FCS. After washing, to remove FCS, lung biopsies were infected with 10^7 tissue culture infectious dose $(TCID)_{50}$/ml of seasonal H3N2 virus, pH1N1 virus or HPAIV H5N1 at room temperature on a rocker for 1 hour. Lung biopsies were washed and cultured in F12K supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine at 37°C in 95% O_2 and 5% CO_2. After 24 hours, biopsies were collected in 10% neutral-buffered formalin and embedded in paraffin.

All lung biopsies were stained for pancytokeratin to determine the condition of the alveolar epithelium. Lung biopsies of which the epithelium in more than 50% of the alveoli was desquamated were excluded. Lung biopsies infected with seasonal H3N2 virus (n=5), pH1N1 virus (n=3), HPAIV H5N1 (n=6), or uninfected lung biopsies were stained for influenza A virus nucleoprotein as described earlier.[54] To determine the percentage of AM infected, sections were double-stained for influenza A virus nucleoprotein and HAM65 (macrophage marker). Slides were incubated with an antibody against influenza A virus nucleoprotein (IgG2a, Clone Hb65, American Type Culture Collection, Wesel, Germany), followed by incubation with an antibody against HAM65 (IgM, DAKO, Glostrup, Denmark). Primary antibodies were detected with a mixture of peroxidase-labeled goat-anti-mouse IgG2a (Southern Biotech, Birmingham, AL, USA) and alkaline phosphatase-labeled goat-anti-mouse IgM (Southern Biotech). Peroxidase activity was revealed with 3,3'–diaminobenzidine-tetrachlorhydrate (DAB) (Sigma, St. Louis, MO, USA), resulting in a brown precipitate. Alkaline phosphatase was revealed with SIGMA*FAST*™Fast Red TR/ Naphthol AS-MX (Sigma), resulting in a pink precipitate. All macrophages present in the alveolar lumina were counted as influenza A positive or -negative.

Isolation and culture of alveolar macrophages

Six non-smoking volunteers (age > 18 years) free of any respiratory symptoms, with a normal lung function (FEV_1 > 85% predicted, Tiffeneau-index > 0.7), and a normal thorax X-ray underwent a BAL. The BAL was performed according to standard clinical procedures. A total of 3 times 50 ml warm, sterile 0.9% NaCl was instilled and aspirated sequentially. The middle pulmonary lobe was selected for sampling in all cases. All procedures were approved by the Dutch Medical Ethical Committee (MEC-2008-018). From the BAL, debris and mucus was filtered and cells were plated in the required concentrations in serum-free macrophage medium (SFM, Gibco) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. After 2 hours, non-adherent cells were removed. To confirm that cells isolated were macrophages, cells were stained for CD68 (KP-1, Dako) using the same protocol as for the influenza nucleoprotein staining described below.

Culture of monocyte-derived macrophages from heparine blood

From donors that underwent the BAL, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using a Lymphoprep gradient (density 1.007 ± 0.001 g/ml (Axis-shield PoC AS)). Subsequently, monocytes were purified using a Percoll gradient (density 1.063 ± 0.002 g/ml (GE healthcare, London, UK)). Monocytes were cultured in suspension at a concentration of 1*106 cells/ml in teflon flasks (Nalgene, Roskilde, Denmark) in RPMI-1640 (Lonza, Walkersville, MD, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine and 5% human AB serum. After 7 days, monocyte-derived macrophages (MM) were harvested from the teflon flask and seeded in flat-bottom culture plates. After 2 hours, non-adherent cells were removed. Cells were stained for CD68 as described below.

Culture of monocyte-derived macrophages from blood bank donors

Blood was diluted in PBS and centrifuged for 10 minutes at 220g. The top layer, mainly consisting of thrombocytes, was discarded. After this extra procedure, PBMC and monocytes were subsequently isolated and purified as described above. For comparison, half of the monocytes were cultured in teflon flasks as described above, and the other half was cultured in RPMI supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, 5% FCS and GM-CSF (25 ng/ml, Invitrogen, Paisley, UK). GM-CSF cultured MM (GM-MM) were harvested after 7 days. Cells were stained for CD68 as described below.

Percentage of cells infected

AM (n=6), MM (n=10, 6 paired samples with AM and 4 from blood bank donors) and GM-MM (n=4) were seeded in a concentration of 50 000 cells per wells. Cells were infected with a multiplicity of infection (MOI) of 2 with seasonal H3N2 virus, pH1N1 virus or HPAIV H5N1 for 1 hour. After 8 hours post infection (hpi), cells were fixed in the plate with 80% acetone and stored at -20°C. Plates were thawed, and washed with PBS before incubation with the primary antibody, a mouse-anti-influenza A nucleoprotein (Hb65) followed by peroxidase-labeled goat-anti-mouse IgG2a. Peroxidase was revealed using AEC resulting in a red precipitate. Influenza virus positive and -negative cells were counted in five 40x high power fields.

Virus production

AM (n=6), MM (n=6) and GM-MM (n=4) were seeded in a concentration of 100 000 cells per well and infected with a MOI of 0.1 with seasonal H3N2 virus, pH1N1 virus or HPAIV H5N1 for 1 hour. Supernatants were sampled at 4, 8 and 24 hpi. Virus titrations on these supernatants were performed by end-point titration in MDCK cells as described previously.[71]

Cytokine production

AM (n=6) and MM (n=6) were seeded in a concentration of 100 000 cells per well and infected with an MOI of 2 with seasonal H3N2 virus, pH1N1 virus of HPAIV H5N1 for 1 hour. At 8 and 24 hpi, cells were harvested in lysis buffer, and mRNA was isolated using an mRNA capture kit (Roche Diagnostics Netherlands, Almere, Netherlands) according to manufacturer's instructions. Subsequently, cDNA was made using Superscript III reverse transcriptase (Invitrogen). Taqman analysis was used to determine target gene RNA expression as described previously.[158] Gene expression levels were corrected for GAPDH mRNA and ubiquitin C (UBC) mRNA levels and PCR efficiency, and expressed in fold induction (FI) over uninfected cells sampled at the same time point. Sequences of the PCR primers (Eurogentec, Maastricht, The Netherlands) are: forward primer 5'-TCCACTGGCGTCTTCAC, reverse primer 5'-GGCAGAGATGATGACCCTTTT for GAPDH; forward primer 5'-GGCAAAGATCCAAGATAAGGAA, reverse primer 5'-GGACCAAGTGCAGAGTGGAC for UBC; forward primer 5'-

GAGCCCAAGGGCTACCAT, reverse primer 5'-GGGTTATGCTGGTTGTACAGG for TGF-beta; forward primer 5'-CAGCCTCTTCTCCTTCCTGAT, reverse primer 5'-GCCAGAGGGCTGATTAGAGA for TNF-alpha. Probes from the universal probe library from Roche were used; probe 45 for GAPDH; probe 11 for UBC; probe 29 for TNF-alpha and TGF-beta.

Statistics

Statistical analyses were performed using SPSS 15 software.

Results

Percentage of cells infected in ex vivo lung biopsies

All three viruses infected AM in experimentally infected *ex vivo* lung cultures, although percentages varied. In the double-staining, influenza virus antigen was present in the nucleus, indicative for virus replication, whereas HAM65 was present in the cytoplasm (Figure 1a). HPAIV H5N1 infected significantly more AM than seasonal H3N2 or pH1N1 viruses in *ex vivo* cultured lung biopsies (Figure 1b).

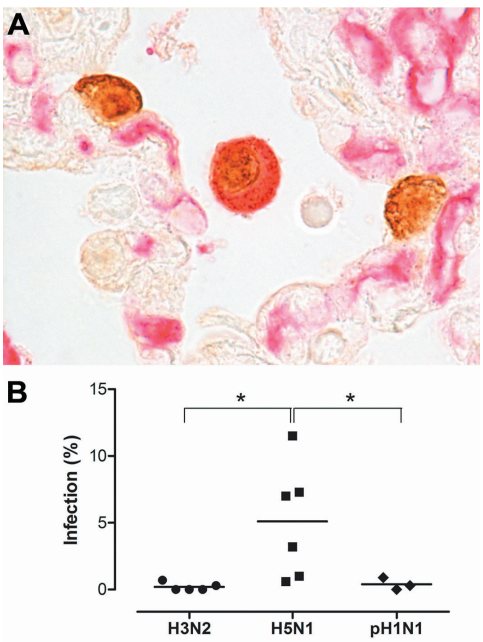


Figure 1: *Percentage of alveolar macrophages (AM) infected in ex vivo lung cultures. Double staining of an AM for influenza A virus nucleoprotein (brown nuclear staining) and a macrophage marker (red/pink, cytoplasmic staining. The two cells expressing influenza virus nucleoprotein on the alveolar wall are likely type II pneumocytes. (a). Percentages of AM infected after 8 hpi with seasonal H3N2 virus, HPAIV H5N1 or pH1N1 virus (b). Mean values are represented by horizontal lines. * indicates a statistical ($p < 0.05$) difference.*

Phenotype of macrophages

AM had a round shape (Figure 2a). The shape of macrophages cultured from monocytes depended on the culture medium in which they differentiated. Most MM (cultured in the presence of human serum) were spindle-shaped with a few round cells (figure 2b); in contrast, most GM-MM (cultured in the presence of GM-CSF) were round with a few spindle-shaped cells (Figure 2c). All cells were confirmed to be of the macrophage/monocyte lineage by CD68 staining.

Percentage of AM, MM and GM-MM infected

Influenza A antigen was visible as nuclear and cytoplasmic red staining (Figure 2). Significant less AM than MM from the same donor were infected with either seasonal

H3N2 virus or HPAIV H5N1 (Figure 3a). The percentage of AM infected by influenza virus declined significantly in the following order: HPAIV H5N1 > seasonal H3N2 virus > pH1N1 virus (Figure 3a). The same pattern was observed for MM and GM-MM (Figure 3b). For each of the three viruses tested, the percentage of cells infected did not differ significantly between AM and GM-MM (Figure 3b).

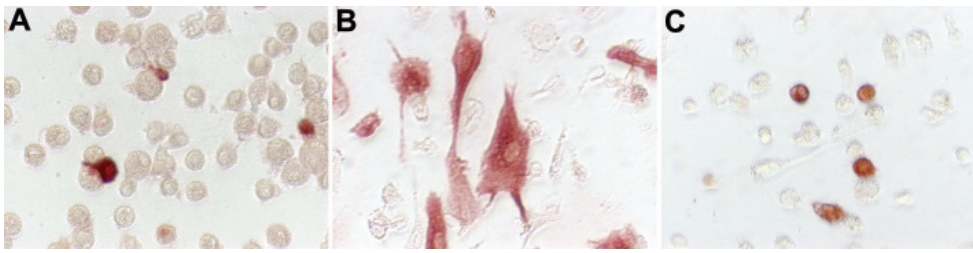


Figure 2: *Influenza A virus nucleoprotein (red) staining in alveolar macrophages (a), macrophages cultured from monocytes in the presence of human serum (b) and macrophages cultured from monocytes in the presence of GM-CSF (c).*

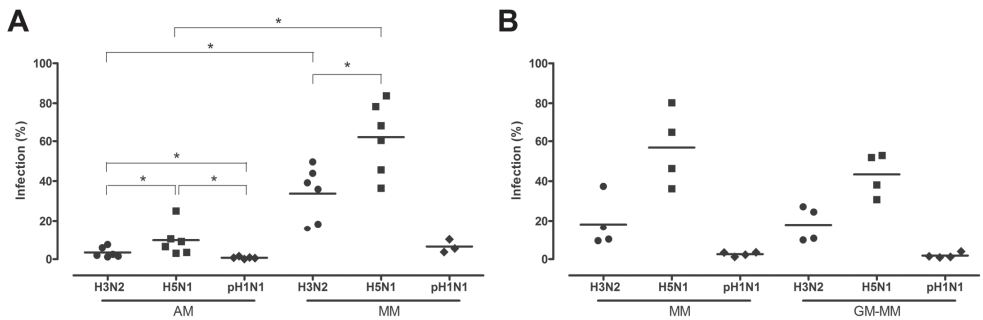


Figure 3: *Percentage of infected alveolar macrophages (AM) and macrophages cultured in the presence of human serum (MM), from the same donor (a). Percentages of infected MM or macrophages cultured in the presence of GM-CSF (GM-MM), cultured from blood bank donors (b). Mean values are represented by horizontal lines. * indicates a statistical (p < 0.05) difference.*

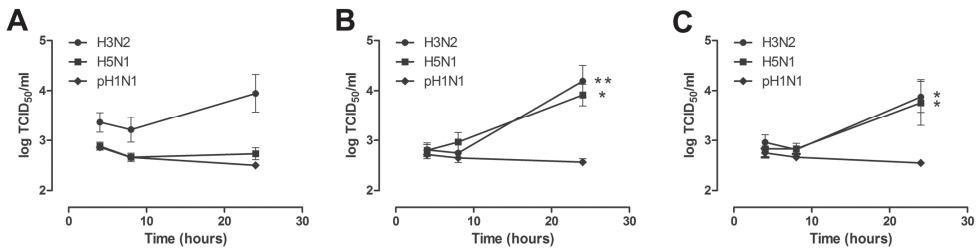


Figure 4: *Virus production of alveolar macrophages (AM) (a), macrophages cultured in the presence of human serum (MM) (b), and macrophages cultured in the presence of GM-CSF (GM-MM) (c), after infection with seasonal H3N2 virus, HPAIV H5N1 or pandemic H1N1 virus. Geometric mean titers were calculated from independent experiments; error bars indicate standard deviation. * indicates a statistical (p < 0.05) difference. ** indicates a statistical (p < 0.01) difference.*

Virus production in AM, MM and GM-MM

There was no significant influenza virus production in AM regardless of the virus used (figure 4a). In contrast, there was significant production of seasonal H3N2 virus and HPAIV H5N1 in both MM and GM-MM (Figure 4b and 4c). There was no significant pH1N1 virus production in any of the cell types used.

Cytokine production in AM and MM

HPAIV H5N1 infection of AM did not induce significant more TNF-alpha mRNA levels than seasonal H3N2 virus or pH1N1 virus (Figure 5a and 5b). In contrast, and as found previously,[80] this was the case for HPAIV H5N1 virus infection of MM. There was a trend ($p = 0.055$) for HPAIV H5N1 infection to induce more TNF-alpha in MM than AM, both at 8 and 24 hpi (Figure 5a and 5b). The results for TGF-beta were similar for those of TNF-alpha, although overall the levels of induction were lower: HPAIV H5N1 infection did not induce significantly more TGF-beta than seasonal H3N2 virus or pH1N1 virus, but that of MM did (Figure 5a and 5b). Interestingly, pH1N1 virus infection of MM induced significantly more TGF-beta than that of AM, both at 8 hpi and 24 hpi.

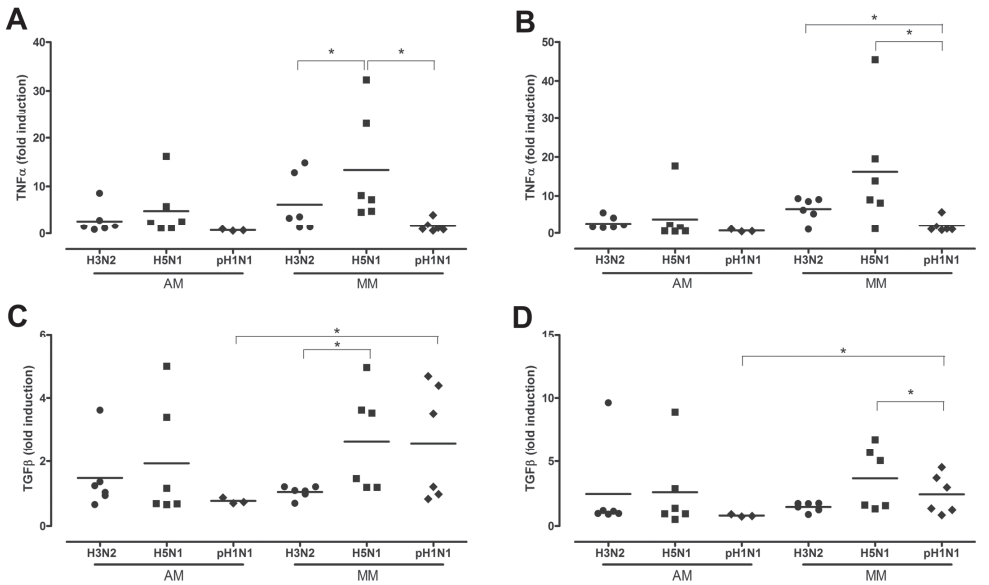


Figure 5: *Cytokine mRNA levels. TNF-alpha mRNA levels expressed in fold-induction over non-infected cells after seasonal H3N2 virus, HPAIV H5N1 or pandemic H1N1 virus infection in alveolar macrophages (AM) and macrophages cultured in the presence of human serum (MM) at 8 hpi (a) and 24 hpi (b). The same for TGF-beta mRNA levels at 8 hpi (c) and 24 hpi (d). Mean values are represented by horizontal lines. * indicates a statistical ($p < 0.05$) difference.*

Discussion

This is the first time that the role of AM during HPAIV H5N1 virus infection has been studied. This study shows that AM are more susceptible to HPAIV H5N1 infection than to seasonal H3N2 or pH1N1 virus infection but, that this infection results neither in virus production nor in an excessive immune response.

Macrophages cultured from monocytes, do not respond the same way as AM to influenza virus infection: there are significant differences between MM and AM in the percentages of cells infected, cytokine response and virus production. Therefore, MM are not suitable to study the interaction between AM and influenza virus.

HPAIV H5N1 infected more AM than seasonal H3N2 or pH1N1 viruses, in both the *ex vivo* lung cultures and in AM collected from BAL. This fits with the results of our virus attachment studies, in which HPAIV H5N1 attached more abundantly to AM than H3N2 virus[107] and pH1N1 (unpublished data). Infection of an AM likely hampers its protective function and, since approximately one AM is found in each alveolus,[10] this could lead to substantial loss of protection in the alveoli.

HPAIV H5N1 infection in AM did not result in significant higher induction of TNF-alpha mRNA compared to seasonal H3N2 or pH1N1 virus. This is in contrast with the significantly higher TNF-alpha mRNA levels in MM after HPAIV H5N1 virus infection compared to seasonal H3N2 and pH1N1 virus infection observed in our and previous studies.[80;153;154] TNF-alpha, and the cascade it induces, is known to attract neutrophils and other leukocytes in the lung and is thought to play an important role in the pathogenesis of acute respiratory distress syndrome and multiple organ dysfunction syndrome.[159] The lack of excessive TNF-alpha induction in AM after HPAIV H5N1 infection indicates that, in contrast to previous conclusions based on studies with MM, the AM does not contribute to the excessive immune response after HPAIV H5N1 infections.

The lack of virus production in AM after influenza virus infection corresponds with a previous study in which no virus release in AM was detected after infection with a seasonal H1N1 or H3N2 virus.[156] The lack of virus production indicates that AM do not contribute to a productive infection in the human alveolus.

During influenza virus pneumonia, there is an influx of a variety of leukocytes, including monocytes.[27] The monocytes that enter the alveoli during influenza virus pneumonia most likely respond differently than resident AM. We cannot exclude that these monocytes will respond in a similar way as MM, with high percentages of cells infected, a productive infection and the induction of TNF-alpha. Unfortunately, the interaction between monocytes and influenza virus has never been studied, so their role remains to be elucidated.

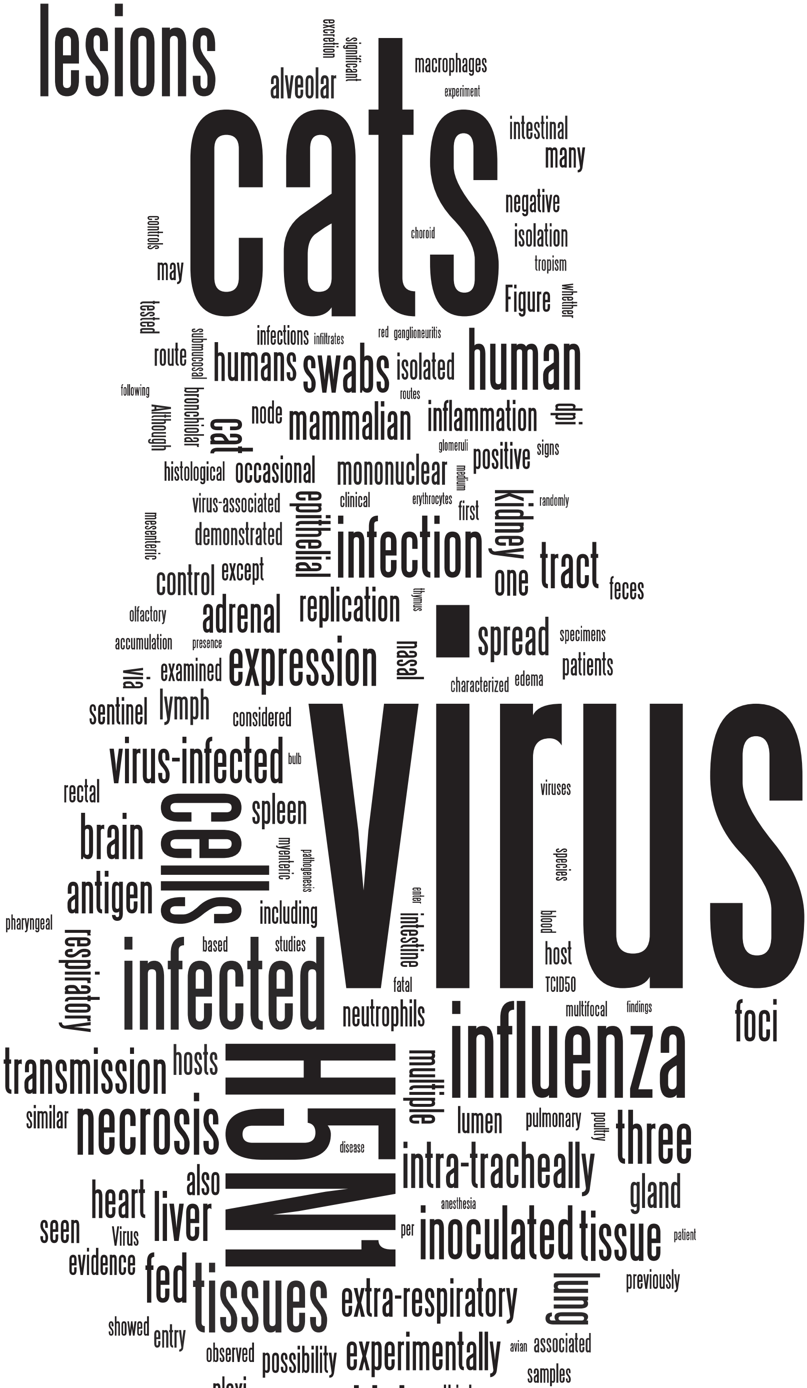
Interestingly, macrophages that were cultured in the presence of GM-CSF, resembled AM in shape, but differed in their response to influenza virus infection. In fact, the percentages of cells infected of GM-MM resembled that of the MM more closely. Although GM-CSF is abundantly present in the human alveolus and is thought to be responsible for the AM phenotype,[157;160;161] addition of GM-CSF was not sufficient to transform the phenotype of monocytes to that of AM, with respect to their response to influenza virus infections. It is likely that other factors present in the alveolus are required for this transformation.

Overall, pH1N1 virus did not infect high percentages of either AM or MM. Even compared to seasonal H3N2 virus, pH1N1 virus infected lower percentages of cells. Whether this is a unique feature of pH1N1 virus or a common feature of pandemic influenza viruses remains to be determined. Overall, this observation fits with the relatively mild disease caused by pH1N1 virus infection.[133]

In conclusion, we have shown that the MM are unsuitable to study the interaction between AM and influenza virus. In contrast to MM, AM do not induce excessive TNF-alpha after HPAIV H5N1 infection. However, AM are more abundantly infected by HPAIV H5N1 than by seasonal H3N2 virus or pH1N1 virus. This relatively high percentage of AM infection by HPAIV H5N1 may contribute to the unusual high pathogenicity of HPAIV H5N1 for the human lung.

Acknowledgements

We thank M. den Bakker and A. Kleinjan for the providing the human lung tissues; M. van Meurs, F. van de Panne, D. van de Vijver and M. van de Bildt for technical advises and assistance; and W. Lim for providing the H5N1 virus isolate.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Chapter 7

Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts

Based on:
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Am J Pathol. 2006; 168:176-83

Abstract

The ongoing outbreak of avian influenza A virus (subtype H5N1) infection in Asia is of great concern because of the high human case fatality rate and the threat of a new influenza pandemic. Case reports in humans and felids suggest that this virus may have a different tissue tropism from other influenza viruses, which are normally restricted to the respiratory tract in mammals. To study its pathogenesis in a mammalian host, domestic cats were inoculated with H5N1 virus intra-tracheally (n = 3), by feeding on virus-infected chicks (n = 3), or by horizontal transmission (n = 2), and examined by virological and pathological assays. In all cats, virus replicated not only in the respiratory tract but also in multiple extra-respiratory tissues. Virus antigen expression in these tissues was associated with severe necrosis and inflammation seven days after inoculation. In cats fed on virus-infected chicks only, virus-associated ganglioneuritis also occurred in the submucosal and myenteric plexi of the small intestine, suggesting direct infection from the intestinal lumen. All cats excreted virus not only via the respiratory tract, but also via the digestive tract. This study in cats demonstrates that H5N1 virus infection causes systemic disease and has potential novel routes of spread within and between mammalian hosts.

Introduction

Avian influenza A viruses of the H5N1 subtype responsible for the ongoing outbreak of fowl plague in South East Asia have caused fatal infections in humans and other mammal species.[162-164] Out of 89 laboratory-confirmed patients, 52 have died of H5N1 virus infection as of 4 May 2005 (http://wwwwhoint/csr/disease/avian_influenza/country/en/). In addition, H5N1 virus also has caused fatal infection in domestic cats, leopards, and tigers, species previously considered to be resistant to disease from influenza A virus infection.[35;90] The transmission of avian influenza A virus to mammalian species is of great concern because this may allow the virus to adapt to mammalian hosts and acquire pandemic potential. So far, however, there is only evidence for limited human spread.[32] The pathogenesis of H5N1 virus infection in humans and other mammalian hosts is poorly understood, including how the virus spreads within the host and from one host to another.

When bird-to-human transmission of H5N1 virus was first recorded in 1997, with 6 deaths out of 18 hospitalized patients,[43;165;166] the question of why this virus was so pathogenic was raised. One hypothesis was that H5N1 virus had expanded its tissue tropism in humans beyond its normal location in the respiratory tract, resulting in systemic infection,[167] as is the usual situation in poultry infected with highly pathogenic avian influenza virus.[168] Although support for this hypothesis was provided neither by studies of patients who died in 1997[67] nor by experimental infections in cynomolgus macaques,[54;92] the recent isolation of H5N1 virus from a patient with severe neurological symptoms[31] once again raises the question of extra-respiratory tissue tropism of H5N1 virus in humans and other mammalian hosts.

Related to the question of tissue tropism is the question whether H5N1 virus can spread from one mammalian host to another and, if so, how. Although most human H5N1 virus infections have been due to contact with infected poultry or poultry products, probable person-to-person transmission has been recorded for two patients.[32] The possibility of mammal-to-mammal transmission of this virus was confirmed by studies in cats and tigers.[36;90] By which routes H5N1 virus could be spread from human to human is not known. The accepted transmission route of human influenza virus infection—by inhalation of virus-infected aerosol—is based on the infection being restricted to the respiratory tract. However, fecal-oral transmission is considered the most important route of transmission of influenza A virus in birds.[168] The isolation of H5N1 virus from a rectal swab of a human patient presenting with diarrhea suggests that transmission by infected feces in humans also could occur.[31]

In order to investigate the pathogenesis of H5N1 virus infection in a mammalian host, we performed detailed virological and pathological studies of domestic cats infected experimentally with H5N1 virus by different routes of inoculation. We have previously used this model to show that cats can be infected with H5N1 virus both by horizontal transmission and by feeding on virus-infected birds, and that infected cats develop severe virus-associated pneumonia.[90] In the present study we determined whether

extra-respiratory spread of H5N1 virus occurred, and, if so, with which lesions this was associated. The results are discussed in the light of viral spread within the host and between hosts.

Materials and methods

Virus preparation

A virus stock was prepared of influenza virus A/Vietnam/1194/2004 (H5N1), which was isolated from a fatal human case. The virus (passage xE1) was kindly provided by Dr. W. Lim, Queen Mary Hospital, Hong Kong and propagated once in Madin-Darby canine kidney (MDCK) cells (xE1MDCK1). It was titrated according to standard methods[71] and reached an infectious virus titer of 106.2 median tissue culture infectious dose (TCID50) per ml.

Experimental protocol

Four-to-six-month-old specified-pathogen-free European short hair cats were purchased from a commercial breeder (Harlan USA). During the entire experiment the animals were housed in negatively pressurized isolator units. Three cats (#1 to 3) were infected intra-tracheally by use of a catheter with 2.5x104 TCID50 of H5N1 virus under anesthesia with ketamine. Two days after cats #1 to 3 were inoculated intra-tracheally, two sentinel cats (#4 and 5) were placed in the same enclosure to investigate whether cats could be infected via horizontal transmission. A third group of three cats (#6 to 8) were fed with infected birds. To this end, one-day-old chicks were infected intratracheally with 2.5x104 TCID50 of H5N1 virus At 1 dpi, the chicks were euthanized and were fed to cats 6 to 8 (one chick per cat). Liver and lung homogenates obtained from infected chicks contained >109 TCID50 per gram tissue and cloaca swabs reached titers of 104-107.5 TCID50/ml. Two cats (#9 and 10) fed with chicks sham-infected with phosphate buffered saline served as negative controls. Before and at 1,3,5, and 7 dpi, pharyngeal, nasal and rectal swabs were collected under anesthesia and submersed in 3 ml virus transport medium (minimal essential medium with antibiotics). All cats were euthanized at 7 dpi by exsanguination under ketamine anesthesia, except for one cat that died on day 6 pi. Experimental procedures were approved by an independent Animal Care and Use Committee.

Pathological examination and immunohistochemistry

Necropsies and tissue sampling were carried out according to a standard protocol. After fixation in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were stained with hematoxylin and eosin for histological evaluation or with an immunohistological method using a monoclonal antibody against the nucleoprotein of influenza A virus as a primary antibody for detection of influenza viral antigen.[54] Lung tissue of an experimentally infected cynomolgus macaque (Macaca fascicularis) experimentally infected with influenza virus A/Hong

Kong/156/1997 (H5N1) was included as a positive control. Isotype- and omission controls were included as negative controls. The following tissues were examined by these two methods: conjunctiva, nasal concha, trachea, lung (nine specimens), tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, tonsil, tracheo-bronchial lymph node, mesenteric lymph node, spleen, thymus, heart, liver, pancreas, kidney, adrenal gland, urinary bladder, olfactory bulb, cerebrum (at level of hippocampus), cerebellum, and brain stem.

Virus titrations

Tissue samples were weighed and homogenized in 3 ml of transport medium with a homogenizer (Kinematica Polytron, Lucerne, Switzerland). Tenfold serial dilutions of the tissue suspensions and swabs were inoculated in MDCK cells in quadruplicate as described previously.[71] All experiments were carried out under biosafety level 3+ conditions.

Bacteriology

Lung swabs were tested by routine bacteriologic methods (with blood agar, chocolate agar, and MacConkey agar) under aerobic and anaerobic conditions for bacterial pathogens by the Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, The Netherlands.

Results

Clinical signs

All intra-tracheally inoculated cats and cats fed on virus-infected chicks showed clinical signs, including raised body temperature, decreased activity, protrusion of the third eyelid, conjunctivitis, and labored breathing, from 2 dpi onwards. Clinical signs in sentinel cats were similar, except that they developed symptoms later (from 5 dpi onwards) and protrusion of the third eyelid was not observed. Negative control cats showed no clinical signs.

Virology and bacteriology

Virus was isolated from pharyngeal, nasal, and rectal swabs of all intra-tracheally inoculated cats, cats fed on virus-infected chicks, and sentinel cats (Figure 1). The only exception was the failure to isolate virus from nasal swabs of one sentinel cat (#4). In general, swabs first tested positive at 3 dpi and remained positive until the end of the experiment at 7 dpi. The titers in the pharyngeal swabs of the intra-tracheally inoculated cats continued to increase up to $10^{4.5}$ $TCID_{50}$/ml during the course of the experiment. Peak titers in nasal swabs ranged from $10^{2.5}$-$10^{5.0}$ $TCID_{50}$/ml. The virus titers in the rectal swabs varied widely. No virus was isolated from any of the swabs collected from the negative control cats.

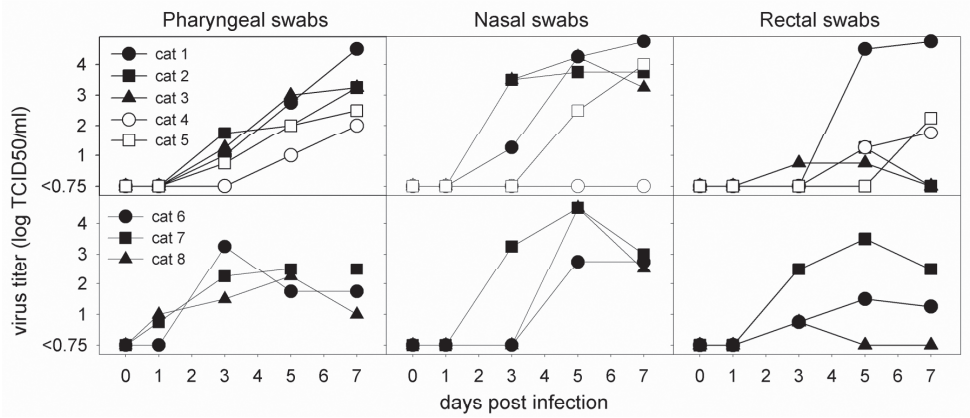

Figure 1: *Virus titers in pharyngeal, nasal and rectal swabs obtained from cats infected with influenza A virus (H5N1) by the intra-tracheal route (upper panels, solid symbols), by horizontal transmission (upper panels, open symbols), or by feeding on virus-infected chicks (lower panels, solid symbols) at various time points post infection. No virus was isolated from any swabs of negative control cats (data not shown).*

Seventeen different organs were tested for the presence of virus by virus-isolation procedures (Figure 2). Both in intra-tracheally inoculated cats and cats fed on virus-infected chicks, virus replication was demonstrated in extra-respiratory tissues, most often brain, liver, kidney, and heart (Figure 2). There was no clear difference in virus distribution between these two groups, except that the tracheo-bronchial lymph node was positive in all three intra-tracheally inoculated cats and negative in all three cats fed on virus-infected chicks. The stomach, eyelid, mesenteric lymph node, and spleen specimens were negative in all cats, except for cat #1, in which all tissues were positive. The respiratory tract specimens of the two sentinel cats (#4 and 5) also tested positive, indicating that they became productively infected after contact with the intra-tracheally inoculated cats. No virus was isolated from any of the tissues collected from the negative control cats. No bacteria were isolated from lung specimens by bacteriologic culture.

Gross pathology

All intra-tracheally inoculated cats, cats fed on virus-infected chicks, and sentinel cats had multifocal or coalescing pulmonary lesions, which were red-purple, slightly raised, and firmer than normal. The estimated percentage of lung tissue involved per cat varied: 95 (cat #1), 80 (#2), 66 (#3), 5 (#4), 33 (#5), 33 (#6), 95 (#7), and 33 (#8). Negative control cats (#9 and 10) had no pulmonary lesions.

The only gross lesions in extra-respiratory organs were seen in cats fed on virus-infected chicks. All three cats had enlargement of and multifocal petechial hemorrhages in the tonsils, mandibular lymph nodes, retropharyngeal lymph nodes, or a combination of these tissues. Two of three cats (#6 and 7) had multiple petechial hemorrhages in the liver. In one of these (#6), this was associated with generalized icterus, characterized by yellow staining of multiple tissues.

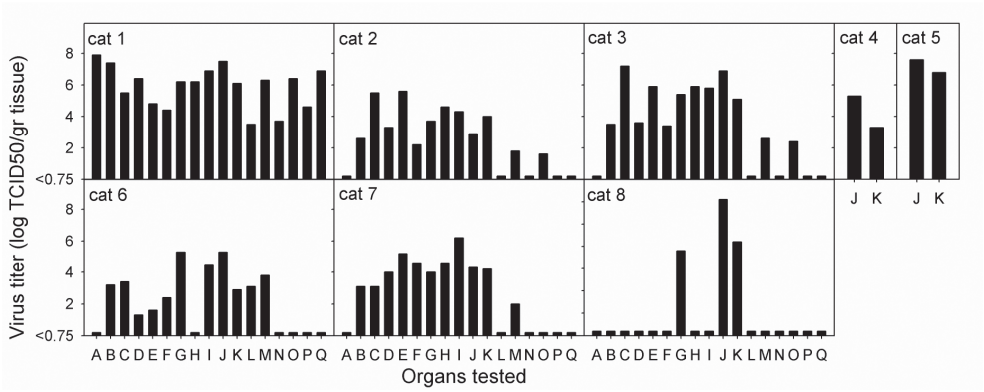

Figure 2: *Infectious virus titers in organ tissue homogenates obtained from cats infected with influenza A virus (H5N1) by the intra-tracheal route (cats #1-3), by horizontal transmission (cats #4 and #5) or by feeding on virus-infected chicks (cats #6-8) seven days post infection. Organs that were tested included A. Stomach, B. Liver, C. Kidney, D. Heart, E. Cerebrum, F. Cerebellum, G. Brain stem, H. Olfactory bulb, I. Nasal concha, J. Lung, K. Trachea, L. Jejunum, M. Tonsil, N. Eyelid, O. Tracheo-broncheal lymph node, P. Mesenteric lymph node and Q. Spleen.*

Histopathology

Intra-tracheally inoculated cats had histological lesions in the lung, brain, heart, kidney, liver, and adrenal gland (Figure 3). In the lung, all three cats had multiple or coalescing foci of inflammation and necrosis, centered on the bronchioles. In these foci, the alveolar and bronchiolar lumina were filled with alveolar macrophages, neutrophils, and erythrocytes, mixed with fibrin, edema fluid, and cellular debris. Some alveoli were covered by hyaline membranes. The epithelium of bronchiolar and alveolar walls had evidence of both necrosis and hyperplasia. The bronchiolar and alveolar walls were moderately thickened. Some foci in the lung parenchyma showed coagulation necrosis, while others had evidence of beginning alveolar fibroplasia and bronchiolitis obliterans. The bronchi were much less severely affected. There was edema and moderate accumulation of mononuclear cells around pulmonary artery branches.

In the brain, all three cats had multiple randomly distributed foci of necrosis and inflammation, characterized by aggregates of neutrophils and glial cells, interstitial edema, neuronal necrosis, and perivascular mononuclear infiltrates. The leptomeninges had multifocal to diffuse mononuclear infiltrates. One cat (#1) had segmental loss of ependymal cells lining the lateral ventricle, with increased cellularity and edema in the underlying parenchyma. Another cat (#3) had mononuclear infiltrates in the choroid plexi of lateral and fourth ventricles. The lesions were generally more marked in the cerebrum than in the brain stem, and mild or absent in the cerebellum.

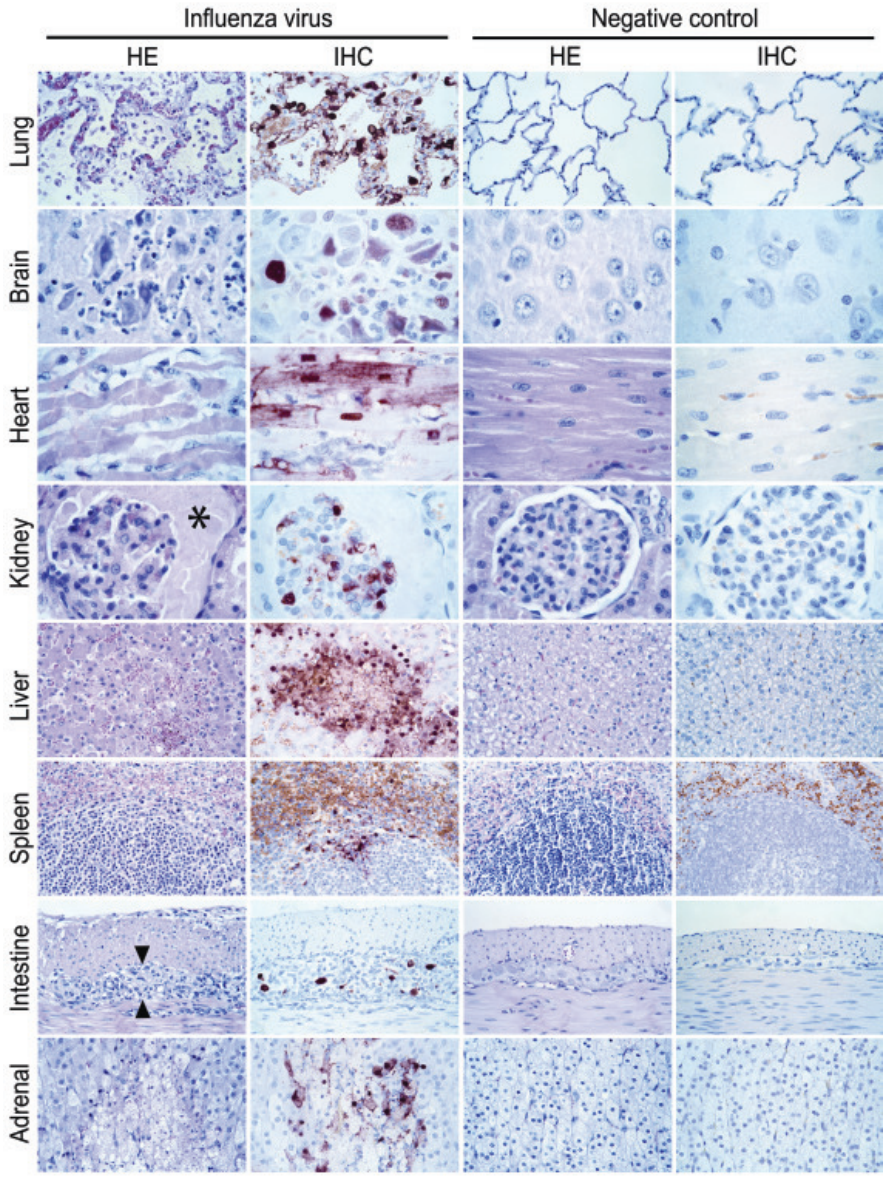

Figure 3*: Cats infected with influenza A virus (H5N1) (cats #1-8) have lesions associated with virus replication in multiple tissues. Necrotizing and inflammatory changes are seen in multiple tissues, except the spleen, of cats infected with H5N1 virus (first column). See the text for a more detailed description of these lesions. The asterisk indicates protein exudate in the Bowman's capsule of a renal glomerulus. The two arrowheads point to the inflamed myenteric plexus in the intestinal wall. Serial sections of these tissues (second column) show that these lesions are closely associated with the expression of influenza virus antigen. Tissues of negative control cats (cats #9 and 10) show neither lesions (third column) nor influenza virus antigen expression (fourth column). Tissues were stained either with hematoxylin and eosin or by immunohistochemistry using a monoclonal antibody against the nucleoprotein of influenza.*

In the heart, two of three cats (#2 and #3) had multiple foci of necrosis and inflammation in the myocardium, mainly near to the lumen of left or right ventricle. These foci were characterized by myocyte necrosis and accumulation of mononuclear cells. In the kidney, one of three cats (#1) had multiple foci of tubular epithelial necrosis and presence of protein exudate in the Bowman's capsule of many glomeruli. In the liver, one of three cats (#1) had multiple randomly located foci of necrosis and inflammation, characterized by hepatocytic necrosis, accumulation of erythrocytes, and infiltration by macrophages and neutrophils. In the adrenal gland, all three cats had multiple foci of necrosis and inflammation in the cortex, with necrosis of adrenal cortical cells, accumulation of erythrocytes, and infiltration by neutrophils. The other tissues examined had no significant lesions.

Both sentinel cats had similar histological lesions to the intra-tracheally inoculated cats, with the following differences. In the lung, there were relatively more neutrophils and fewer macrophages in alveoli and bronchioles. There was no evidence of regeneration of alveolar and bronchiolar epithelium, alveolar fibroplasia, bronchiolitis obliterans, or perivascular cuffing. In the liver, the necrotic foci were infiltrated with relatively more neutrophils and fewer macrophages. Only one of two cats (#4) had lesions in the adrenal gland similar to those in the intra-tracheally inoculated cats, while neither had significant lesions in the heart, kidney, spleen, intestine, or other tissues examined. The brain of contact cats was not examined.

All three cats fed on virus-infected chicks had similar histological lesions to the intra-tracheally inoculated cats, with the following differences. In the brain, the character and distribution of lesions was similar, except that lesions were not observed in ependyma and choroid plexus. Only two of three cats (#6 and #7) had lesions in the heart. In the small intestine were lesions found only in cats fed on virus-infected chicks (and of these, only in cats #6 and #7). These consisted of multifocal to diffuse necrotic and inflammatory changes in the submucosal (Meissner's) and myenteric (Auerbach's) plexi. Affected plexi had loss and necrosis of ganglion cells, and infiltration by neutrophils and mononuclear cells. In the adrenal gland, two of three cats (#7 and #8) had multiple lesions in the medulla similar in character to those in the cortex. None of the cats had significant lesions in kidney, spleen, or other tissues examined. Negative control cats had no significant lesions in the lung, brain, heart, kidney, liver, spleen, intestine, adrenal gland, or other tissues examined.

Immunohistochemistry

Influenza virus antigen expression was visible as diffuse to granular red staining, which was usually darker in the nucleus than in the cytoplasm (Figure 3). Influenza virus antigen expression was seen most frequently in liver, heart, brain, lung, and adrenal gland (Table 1). In the lung (Figure 3), virus antigen expression was seen in many type 1 pneumocytes, type 2 pneumocytes, bronchiolar and bronchial epithelial cells, occasional alveolar macrophages, and rare endothelial cells and smooth muscle cells of pulmonary vein branches. In the brain, virus antigen expression

Table 1: *Influenza virus antigen expression in tissues of cats infected with influenza A virus (H5N1) by different inoculation routes.*

Tissue*	Number of animals positive per method of inoculation		
	Intra-tracheal (n = 3)	Sentinel (n = 2)	Fed on virus -infected chicks (n = 3)
Liver	3	2	1
Kidney	1	0	1
Heart	3	0	2
Cerebrum	3	n.d.†	3
Cerebellum	2	n.d.	1
Brain stem	3	n.d.	3
Olfactory bulb	3	n.d.	2
Lung	3	2	3
Duodenum	0	n.d.	2
Ileum	0	n.d.	1
Mesenteric lymph node	1	0	0
Spleen	2	2	0
Thymus	1	n.d.	0
Adrenal gland	3	1	3

*No virus antigen expression was seen in the following tissues of the above animals: tongue, nasal concha, nasal septum, trachea, eyelid, third eyelid, thyroid, salivary gland, tonsil, tracheo-bronchial lymph node, retropharyngeal lymph node, mandibular lymph node, bone marrow, esophagus, stomach, pancreas, jejunum, cecum, colon, and urinary bladder.
†Not done.

was seen in many neurons and glial cells, and occasional choroid epithelial cells, ependymal cells, and leptomeningeal epithelial cells. Virus antigen expression was seen in many cardiac myocytes and occasional endocardial endothelial cells in the heart; in many glomerular cells (visceral epithelial and/or mesangial cells; cat

#1 only) and occasional distal tubular epithelial cells (cat #6 only) in the kidney; in many hepatocytes in the liver; in occasional large mononuclear cells (mainly at the border between red pulp and follicles) in the spleen; in occasional ganglion cells and Schwann cells in submucosal and myenteric plexi in duodenum and ileum; and in many cortical cells and occasional pheochromocytes in the adrenal gland. Additionally, virus antigen expression was seen in a few medullary epithelial cells in the thymus and in a few large mononuclear cells in the germinal center of secondary follicles of the mesenteric lymph node in cat #1 only.

In all tissues except spleen, thymus, and mesenteric lymph node, influenza virus expression was closely associated with the presence of histological lesions. In general, influenza virus antigen expression was strongest at the transition of normal and necrotic tissue. In larger lesions, this resulted in a ring of antigen expression at the edge of the lesion, and lack of antigen expression in the center (liver in Figure 3).

Influenza virus expression was present in positive control tissues, and absent in both isotype and omission controls. Erythrocytes showed non-specific light brown staining, probably due to pseudoperoxidase,[169] most prominent in the red pulp of the spleen.

Discussion

Our study in experimentally inoculated cats shows that there is extensive extra-respiratory spread of H5N1 virus, including to tissues of the nervous, cardiovascular, urinary, digestive, lymphoid, and endocrine systems. In these tissues, we demonstrated both active virus replication and severe pathological changes, consisting of necrosis and inflammation. Our study also shows that infected cats excrete H5N1 virus via the rectum, suggesting that cat-to-cat transmission of H5N1 virus could occur through infected feces.

Although it has been shown previously that cats can be experimentally infected with influenza A viruses,[34;90;170;171] to our knowledge this is the first time that systemic replication has been demonstrated in this species. The extensive extra-respiratory virus replication and its association with severe necrosis and inflammation in these cats suggest that the high pathogenicity of this H5N1 virus for mammalian species, including humans, may be related to its wide tissue tropism. Our findings in cats correspond with previous findings from experimentally infected ferrets[172;173] and mice.[174-176] Evidence for extra-respiratory replication of H5N1 virus in humans is the isolation of H5N1 virus from the cerebrospinal fluid of a human patient diagnosed clinically with acute encephalitis.[31] The extensive virus-associated liver necrosis and inflammation we observed in the cats is compatible with the increased serum levels of alanine aminotransferase and aspartate aminotransferase, indicative of liver

damage,[177] seen in 6 of 7 human patients.[32;178] The virus-associated ganglioneuritis of the intestinal nervous plexi we observed in the cats is noteworthy considering the diarrhea diagnosed in 9 out of 12 human patients.[31;178] Although ganglioneuritis from influenza virus infection has never been reported to our knowledge, ganglioneuritis from other causes, including virus infections, may result in diarrhea.[179] Another example of extra-respiratory infection by avian influenza viruses in mammalian hosts involves the infection of human conjunctivae by viruses of the H7N7 subtype.[44;180-182]

There was evidence for at least two routes of virus entry, from blood and from the intestinal lumen, into extra-respiratory tissues of the cats. Evidence for virus entry from blood comes first from the pattern of virus infection in renal glomeruli and randomly located foci in liver and adrenal gland. This pattern closely resembles that seen in blood-borne spread of bacteria, e.g., Salmonella septicemia.[183] Second, the predominance of virus-associated myocardial lesions adjacent to the lumen of the heart ventricles and, third, the expression of virus antigen by endothelial cells of pulmonary veins and endocardium also point towards blood-borne spread of H5N1 virus. This route of virus spread corresponds to the detection of H5N1 virus in the serum of a human patient.[31] In contrast to the above tissues, the submucosal and myenteric plexi in the intestine may have been infected directly from the intestinal lumen. We only observed virus expression by immunohistochemistry and associated lesions in the intestine of cats fed on virus-infected chicks. Although virus entry by this route has not been demonstrated before for influenza virus, herpes simplex virus has been shown to enter the submucosal and myenteric plexi from the intestinal lumen via nerve fibers projecting through the mucosa and interacting directly with surface epithelial cells.[184] Entry of influenza virus into host tissues directly from the intestinal lumen remains to be conclusively demonstrated, because we cannot exclude the possibility that the cats fed on virus-inoculated chicks received some inoculum through the respiratory tract while feeding. Virus entry via the intestinal lumen is of interest for humans because of reports of fatal H5N1 virus infection after consuming raw poultry products (Avian influenza, human—East Asia (64): Viet Nam, http://www.promedmail.org, 4 April 2005) or after using the mouth to suck exudate from the upper respiratory tract of fighting cocks (Avian influenza, human—Thailand (06), http://www.promedmail.org ,9 September 2004). Park and others[185] demonstrated a third route of influenza virus entry in extra-respiratory tissues of experimentally infected mice. They showed that influenza virus could enter the brain from the nasal cavity by way of the olfactory nerves—which penetrate the cribriform plate—and olfactory bulb. We have no evidence for this route of entry in the cats, because the virus-associated lesions in the olfactory bulb and adjacent cerebral tissue did not appear more chronic or more frequent than those elsewhere in the brain. However, more detailed studies at earlier time points after infection would be needed to exclude this possibility.

Cat-to-cat spread of H5N1 virus may have occurred due to virus excretion from a number of organ systems. The first and most obvious possibility is excretion from the respiratory tract, based on virus isolation from nasal and pharyngeal swabs as well as from trachea and lung samples. This also has been found for H5N1 virus infection in human patients[31;32;178] and in experimentally infected ferrets.[172;173] The second possibility, previously considered important only for influenza virus infection in birds,[168] is excretion from the digestive tract. This is based on virus isolation from rectal swabs as well as from jejunum samples. Surprisingly, we did not find immunohistochemical evidence for virus replication in the epithelium of the digestive tract at any of the seven levels (esophagus, stomach, duodenum, jejunum, ileum, cecum, colon) tested, even though this is an important site of influenza virus replication in birds.[168] Transmission of H5N1 virus by infected feces should be considered in humans. The virus has been isolated from a rectal swab of a human patient.[31] Also, a high proportion of H5N1 influenza patients presented with severe and watery diarrhea, which increases the risk of transmission via feces. H5N1 virus also has been isolated from the feces of experimentally infected ferrets.[172] The third and previously not considered possibility of virus excretion is from the urinary tract. This is based on virus isolation from kidney samples of 5 out of 6 cats, and virus antigen detection in the glomeruli or renal tubular epithelial cells of 2 out of 8 cats. Virological examination of urine samples of H5N1 virus-infected hosts is necessary to determine whether urine could form a source of H5N1 virus. It cannot be excluded that there were other sources of excreted virus, like blood and saliva.

Our results on the pathogenesis and replication of H5N1 virus in experimentally infected cats has several implications for the clinical and epidemiological management of the ongoing H5N1 virus outbreak. First, the severity of the lesions associated with H5N1 virus replication outside the respiratory tract could partly explain the increased pathogenicity of this virus for mammalian hosts, including humans. If so, supportive measures in patients with severe disease should be directed not only to the respiratory tract but also to other organ systems. Second, the potential for H5N1 virus to enter mammalian hosts via the intestine provides a possible explanation for how this virus could present clinically in absence of respiratory tract disease, as suggested by the recent findings in two patients with encephalitis and diarrhea.[31] Therefore, H5N1 virus infection needs to be included in the differential diagnosis of a broader range of clinical presentations than is currently done. Finally, the possibility for the spread of H5N1 virus via feces in mammals needs to be taken into account in the epidemiological control of H5N1 virus infections. The above measures may limit the risk of H5N1 virus developing into a pandemic influenza virus.

Acknowledgments

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HPAIV
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Chapter 8

Highly pathogenic avian influenza virus H7N7 isolated from a fatal human case causes respiratory disease in cats, but does not spread systemically

Based on:
Debby van Riel, Guus F. Rimmelzwaan, Geert van Amerongen, Albert D.M.E. Osterhaus and Thijs Kuiken

American Journal of Pathology. 2010; In press.

Abstract

Highly pathogenic avian influenza viruses (HPAIV) of the H5 and H7 subtypes primarily infect poultry but are occasionally transmitted to humans and other mammalian species, often causing severe disease. Previously we have shown that HPAIV H5N1 causes severe systemic disease in cats. Here we investigated whether an HPAIV H7N7 isolated from a fatal human case also is able to cause disease in cats. Additionally, we compared the cell tropism of both viruses by immunohistochemistry and virus histochemistry. Three domestic cats were inoculated intratracheally with HPAIV H7N7. Virus excretion was restricted to the pharynx. At necropsy, 7 days post inoculation, lesions were restricted to the respiratory tract in all cats. Lesions consisted of diffuse alveolar damage and co-localized with virus antigen expression in type II pneumocytes and non-ciliated bronchiolar cells. The attachment patterns of HPAIV H7N7 and H5N1 were similar: both viruses attached to non-ciliated bronchiolar epithelial cells, type II pneumocytes and alveolar macrophages. These data show for the first time that a non-H5 HPAIV is able to infect and cause respiratory disease in cats. The failure of HPAIV H7N7 to spread beyond the respiratory tract was not explained by differences in cell tropism compared to HPAIV H5N1. This suggests that HPAIV H5N1 possesses other characteristics that allow it to cause systemic disease in humans and cats.

Introduction

Although the natural reservoir for influenza A viruses are free-ranging waterbirds, influenza virus lineages can be found in many other species, including humans, swine, horses and dogs. Three pandemics in humans have been recorded in the previous century, as the result of introduction of a new subtype of influenza A virus that was efficiently transmissible among humans. Of these three pandemics, the H1N1 virus outbreak in 1918 was the worst, claiming more than 40 million lives worldwide.[45] The first influenza pandemic of this century, which started in 2009, was also caused by an H1N1 virus (www.who.int/csr/don/2009_05_29/en/index.html).

Highly pathogenic avian influenza viruses (HPAIV) primarily infect poultry, but occasionally also humans. Fatal disease in humans has been reported for HPAIV infections of the H5N1 and H7N7 subtypes. During an outbreak of HPAIV of the H7N7 subtype in the Netherlands in 2003, virus was transmitted to 89 people, of whom one died.[44] During the ongoing outbreak of HPAIV of the H5N1 subtype, virus has been transmitted to 492 people, resulting in 291 deaths (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_03_30/en/index.html). Since 2003, HPAIV H5N1 has also been transmitted to tigers,[35;36] leopards,[35] domestic cats,[186-189] a stone marten,[190] Owston's palm civets,[191] domestic dogs,[192] an American mink[4] and swine.[38] These repeated transmissions to mammals, including humans, might facilitate viral adaptations to the mammalian host, which could potentially lead to a pandemic.

Of particular interest are the frequent transmissions of HPAIV H5N1 to felids, which were previously considered to be resistant to influenza A virus disease.[170;187] Early in the outbreak of HPAIV H5N1, two tigers and two leopards at a zoo in Thailand became fatally infected with H5N1 virus.[35] Experimental studies subsequently proved that domestic cats developed severe disease after H5N1 virus infection, with demonstration of felid-to-felid transmission.[90] Interestingly, although influenza viruses are normally restricted to the respiratory tract in mammals, cats infected with HPAIV H5N1 had extensive extra-respiratory spread of the virus.[55] This is relevant to the disease in humans, where extra-respiratory disease from HPAIV H5N1 also has been demonstrated.[27-31]

The pathogenesis of HPAIV infections in humans is not completely understood, partly because data on human HPAIV infections are often from a late stage of disease, which is not representative for events that happen early after infection. Attachment studies have shown that the attachment pattern of avian influenza viruses in cats resembles that of humans closely.[66;107] Therefore, studies on influenza A virus infections in cats can provide insight into the pathogenesis of influenza A virus infections in humans.

All data on HPAIV infections in cats are from the HPAIV H5N1 virus outbreak. It is unknown whether disease from HPAIV infections in cats, including its systemic

spread, is unique for HPAIV H5N1. To gain more insight into the pathogenesis of HPAIV infection in cats, domestic cats were infected with a non-H5 HPAIV, a H7N7 subtype. This virus was isolated from a fatal human case during the HPAIV outbreak in 2003 in the Netherlands and is known to be highly pathogenic in mouse and ferret models.[142;25] Furthermore, pathological investigations on chickens infected during this outbreak showed a similar pathogenesis to that from other HPAIV infections, including HPAIV H5N1.[21 193] Our main objective was to determine if cats could become infected and develop disease from this HPAIV of the H7N7 subtype. Furthermore, we wanted to determine if the infection was restricted to the respiratory tract, or spread systemically. To investigate observed differences between HPAIV H5N1 and HPAIV H7N7 infections, we subsequently compared the attachment pattern of this HPAIV H7N7 isolate with that of HPAIV H5N1 in cats.

Material and Methods

Virus preparation

A virus stock was prepared from the influenza A virus A/NL/219/03 (H7N7), which was isolated from a fatal human case during the 2003 outbreak in the Netherlands.[44] The virus was propagated in Madin-Darby canine kidney (MDCK) cells. The stock was titrated according to standard methods.[71]

Experimental protocol

Four- to six-month-old specific pathogen-free European shorthair cats were purchased from a commercial breeder (Harlan, Indianapolis, IN). Throughout the experiment the animals were housed in negatively pressurized isolator units. Three cats (cat 1-3) were anesthetized with ketamine and infected intratracheally using a catheter containing 2.5 x 10^4 $TCID_{50}$ of H7N7 virus. In parallel, two cats were inoculated intratracheally with PBS. Before and at 1, 3, 5 and 7 days post infection (dpi), pharyngeal, nasal and rectal swabs were collected from cats under anesthesia (ketamine) and submersed in 3 ml of virus transport medium (minimal essential medium with antibiotics). Body temperature was measured daily using subcutaneous probes. All cats were euthanized 7 dpi by exsanguination under ketamine anesthesia. Experimental procedures were approved by an independent Animal Care and Use Committee.

Pathological examination and immunohistochemistry

Necropsies and tissue sampling were performed according to a standard protocol. The following tissues were collected: conjunctiva, trachea, lung (nine specimens), tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, tonsil, tracheo-bronchial lymph node, mesenteric lymph node, spleen, thymus, heart, liver, pancreas, kidney, adrenal gland, urinary bladder, olfactory bulb, cerebrum (at the level of the hippocampus), cerebellum, and brain stem. After fixation in 10% neutral-buffered formalin and embedding in paraffin, tissue sections were stained with

hematoxylin and eosin for histological evaluation. For the detection of influenza A virus antigen, all tissues were stained with a primary antibody against the influenza A virus nucleoprotein (HB-65, ATCC Wesel, Germany) as described previously,[54] with the following modifications: Tissue sections were pre-incubated with 0.1% protease for 10 minutes at 37° C (Sigma, St. Louis, MO). Binding of the primary antibody was detected using a peroxidase labeled goat-anti-mouse IgG2a (Southern Biotech, Birmingham, AL). Peroxidase was revealed using 3-Amino-9-ethyl-carbazole (AEC, Sigma) resulting in a bright red precipitate. In each staining procedure an isotype control was included as a negative control, and influenza A virus-positive tissue as positive control.

Virus titrations

The following tissues were sampled for virus titration: stomach, liver, kidney, heart, cerebrum, cerebellum, brain stem, olfactory bulb, nasal concha, trachea, jejunum, tonsil, eyelid, tracheo-bronchial lymph node, mesenteric lymph node, spleen and lung. Tissues were weighed and homogenized in 3 ml of transport medium by use of a homogenizer (Kinematica Polytron, Lucerne, Switzerland). Ten-fold serial dilutions of the tissue suspensions and swabs were inoculated in MDCK cells in quadruplicate as described previously.[71] All experiments were performed under biosafety level 3 conditions.

Virus histochemistry

The attachment of HPAIV H7N7 (A/NL/219/03) and HPAIV H5N1 (A/Vietnam/1194/2004) was determined on lung, liver and cerebrum tissues from 3 healthy non-infected domestic cats. Virus histochemistry was performed as described previously.[66;107;121]

Results

Clinical signs

None of the three cats showed any severe clinical signs. Two of the cats appeared less active from day 1 onwards and one cat appeared lethargic at day 7. All three cats showed a body temperature rise of 1 to 1.5 degree Celsius at 2 dpi that lasted until 3-6 dpi.

Virology

In all infected cats, virus was isolated from the pharyngeal swabs. No virus was isolated from any of the nasal or rectal swabs. The maximum titer in the pharyngeal swabs ($10^{0.75}$ - $10^{2.5}$ $TCID_{50}$/ml) reached its peak at 5 dpi in all three cats (Figure 1). At necropsy on 7 dpi, virus was isolated from the lung ($10^{3.1}$ – $10^{7.7}$ $TCID_{50}$/g) and the trachea ($10^{4.0}$ – $10^{4.4}$ $TCID_{50}$/g) in all three cats (table 1). In two of the cats (cat 2-3), virus was isolated from the tonsil ($10^{2.7}$ – $10^{4.5}$ $TCID_{50}$/g). In cat 1 virus was

isolated from the heart ($10^{2.3}$ $TCID_{50}$/g), and in cat 3 virus was isolated from the tracheo-bronchial lymph node ($10^{1.1}$ $TCID_{50}$/g) (Table 1). No virus could be isolated from any of the swabs from the sham-infected cats.

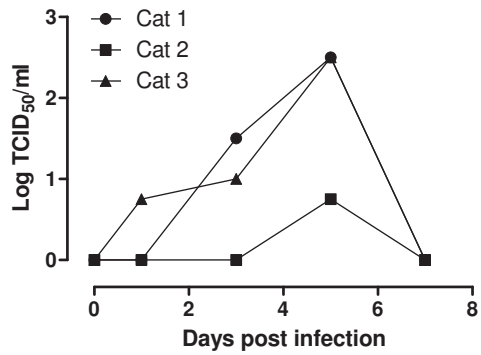


Figure 1: *Virus titers in pharyngeal swabs from individual cats infected with HPAIV H7N7 virus.*

Gross Pathology

All cats had multifocal or coalescing pulmonary lesions, which were red, slightly raised and firmer than normal. The estimated lung volume affected ranged from 25 to 80%. All cats had enlarged tracheo-bronchial lymph nodes and cat 2 had enlarged tonsils. The stomachs of cat 1 and 2 were half-filled, while the stomach of cat 3 was empty. In cat 2, the pelvis of the left kidney was distended and filled with urine and the upper one-third of the left ureter was distended.

Histopathology

In the lungs of all three cats, there were multiple foci of sub-acute to chronic necrosis and inflammation in the lung parenchyma centered on the bronchioles. The alveolar and bronchiolar lumina were filled with variable numbers of large mononuclear cells, neutrophils and lymphocytes, mixed with edema fluid, fibrin, erythrocytes and cell debris. The alveolar and bronchiolar walls had lost their epithelium focally. In the alveoli, there was partial to complete re-epithelialization with low to high cuboidal epithelial cells (type II pneumocyte hyperplasia). The alveolar and bronchiolar walls were mildly thickened with edema fluid, macrophages, neutrophils and lymphocytes. The connective tissue around the pulmonary arteries and veins was widened and contained few macrophages, neutrophils and lymphocytes (Figure 2A and 2B).
In cat 3, there was evidence of moderate lymphocyte hyperplasia in lymph nodes. In the conjunctiva of cat 1, there was diffuse loss of histologic architecture, intra- and intercellular edema, and infiltration with moderate numbers of neutrophils. Lesions considered unrelated to the H7N7 virus infection were a hydronephrosis in the kidney and a focal chronic hematoma in the spleen in cat 2 and a small lingual ulcer on the tongue in cat 3. Other tissues examined had no significant lesions.

Immunohistochemistry

Influenza virus antigen was visible as diffuse to granular red staining which was most prominent in the nucleus of the cell. In all three cats, influenza virus antigen was only present in the lungs. Lesions in the lung were more widespread than the distribution of virus antigen. Virus antigen was present predominantly in type II pneumocytes in the alveoli (Figure 2C) in the alveoli and occasionally in non-ciliated epithelial cells in the bronchioles (Figure 2D). The bronchiolar epithelium in domestic cats consists more than 95% of non-ciliated cells and less than 5% of ciliated cells.[194;195]

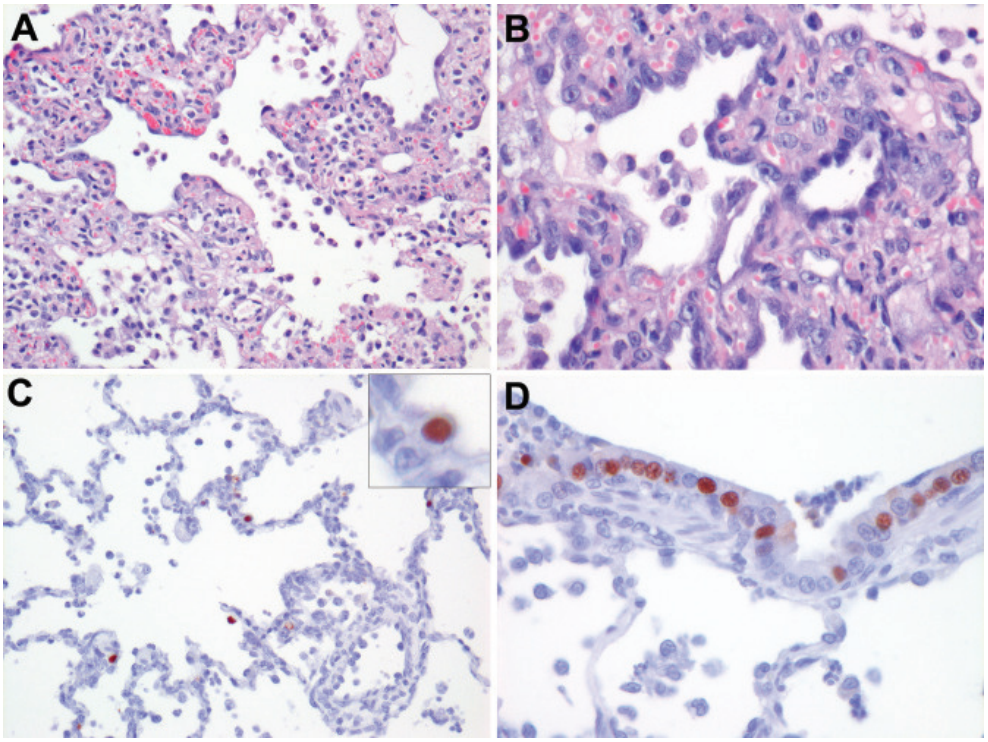


Figure 2: *Histologic changes (A and B) and virus antigen distribution (C and D) in lung tissue of cat no. 1 at 7 days after experimental infection with HPAIV H7N7. A: Alveoli with a thickened wall, covered by hyperplastic type II pneumocytes. Alveolar lumina are filled with mononuclear cells, neutrophils and lymphocytes, mixed with edema fluid, fibrin, erythrocytes and cell debris (original magnification 200x). B: Alveolus covered by hyperplastic type II pneumocytes (original magnification 400x). C: Influenza virus antigen expression in a few type II pneumocytes in alveoli at the edge of a lesion (original magnification 200x) Insert: Influenza virus antigen expression in a type II pneumocyte. D: Non-ciliated bronchiolar cells expressing influenza A virus antigen (original magnification 400x).*

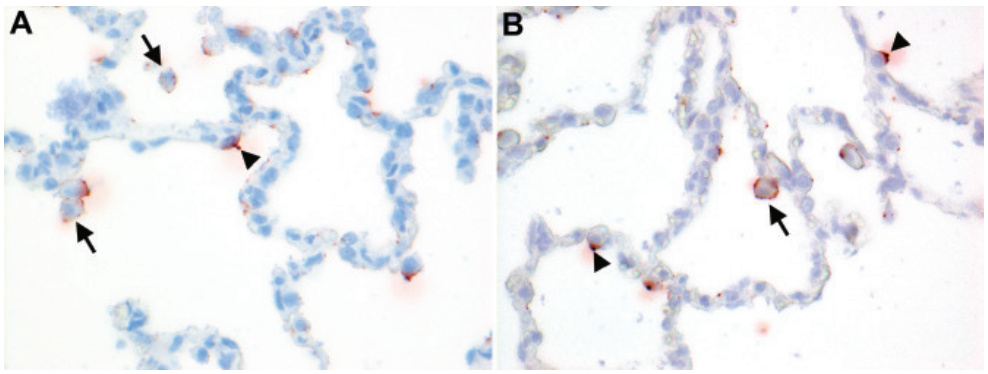


Figure 3: *Virus attachment, visible as red precipitate, of HPAIV H7N7 (A) and H5N1 (B) in alveoli of uninfected cats. Both viruses attach to type II pneumocytes (arrowheads) and alveolar macrophages (arrows).*

Virus histochemistry

To determine if the lack of systemic dissemination of HPAIV H7N7 –which we had observed in HPAIV H5N1 infected cats– could be due to differences in cell tropism we compared the pattern of virus attachment of the two viruses in respiratory and extra-respiratory tissues of non-infected healthy cats. In the lung, both viruses attached predominantly to non-ciliated cuboidal cells in the bronchioles and to type II pneumocytes and alveolar macrophages in the alveoli (Figure 3). Neither of the viruses attached to cells in liver or cerebrum.

Table 1: *Virus titers (log^{10} $TCID_{50}$/gram tissue) from tissues collected at 7 days post infection. Brain, eye, jejunum, kidney, liver, mesenteric lymph node, nasal concha, spleen and stomach all tested negative.*

Tissue	Cat 1	Cat 2	Cat 3
Heart	2.3	<1	<1
Lung	5.1	7.7	3.1
Trachea	4	4	4.4
Tracheo-bronchial lymph node	<1	<1	1.1
Tonsil	<1	2.7	4.5

Discussion

This study shows for the first time that cats are susceptible to disease after infection with a HPAIV of another subtype than H5N1. Cats developed respiratory disease after intratracheal infection with a HPAIV H7N7, isolated from a fatal human case during the 2003 outbreak in the Netherlands. Interestingly, H7N7 virus replication and associated lesions were limited to in the respiratory tract. This is in contrasts with HPAIV H5N1 infection in cats, which resulted in systemic virus replication associated with severe necrosis and inflammation.[55;90;186-188]

The fact that both HPAIV H5N1 and HPAIV H7N7 are able to infect cats implies that cats might be susceptible to infection by multiple subtypes of HPAIV. (Recent demonstration of pandemic H1N1 virus infection in cats indicates that cats might be susceptible to infection by human influenza viruses as well.[196;197]) The role of cats should therefore be considered during outbreaks of any HPAIV subtype since they may transmit virus from one poultry farm to another poultry farm, or to other species (including humans).[198] Furthermore, the ability of HPAIV to infect cats could facilitate adaptations to the mammalian host, and thereby increase the pandemic potential of the virus.

The difference in histological pulmonary lesions between HPAIV H5N1 and H7N7 virus infected cats seems to be associated with the stage of infection. HPAIV H7N7 Infection was resolving at 7 dpi, as indicated by the lack of virus excretion and the few cells that contained virus antigen in the pulmonary lesions. In contrast, HPAIV H5N1 infection seemed to be ongoing at 7 dpi, with still increasing virus titers from the pharynx and many influenza virus antigen containing cells in the pulmonary lesions.[55;90]

The differences in virus distribution between HPAIV H7N7 and H5N1 infected cats could not be explained by differences in genome, pattern of virus attachment, or pattern of virus replication. Regarding genome, there were no obvious differences between the two viruses. For example, both viruses have a lysine at position 627 of PB2, which is associated with increased pathogenicity of influenza virus infection in mammals.[142] Regarding pattern of virus attachment, both viruses attached to non-ciliated epithelial cells in the bronchioles, and to type II pneumocytes and alveolar macrophages in the alveoli. Regarding pattern of virus replication, both viruses replicated in the same cell types, non-ciliated bronchiolar epithelial cells and type II pneumocytes, as detected by immunohistochemistry at 7 dpi. The only difference was infection of alveolar macrophages: these were not observed in HPAIV H7N7 infected cats, and were occasionally observed in HPAIV H5N1 infected cats. It cannot be excluded that this difference contributes to the difference in pathogenicity between these two viruses.

Other factors, such as replication efficiency and mechanisms to escape innate immune response, might contribute to the observed difference in virus distribution between HPAIV H5N1 and H7N7. Virus isolation from the heart of one cat indicated there might be some spillover of HPAIV H7N7 from the lung into the bloodstream. Since there was no virus antigen detected by immunohistochemistry in the heart or any other extra-respiratory tissues, there is no evidence for active virus replication at these sites.

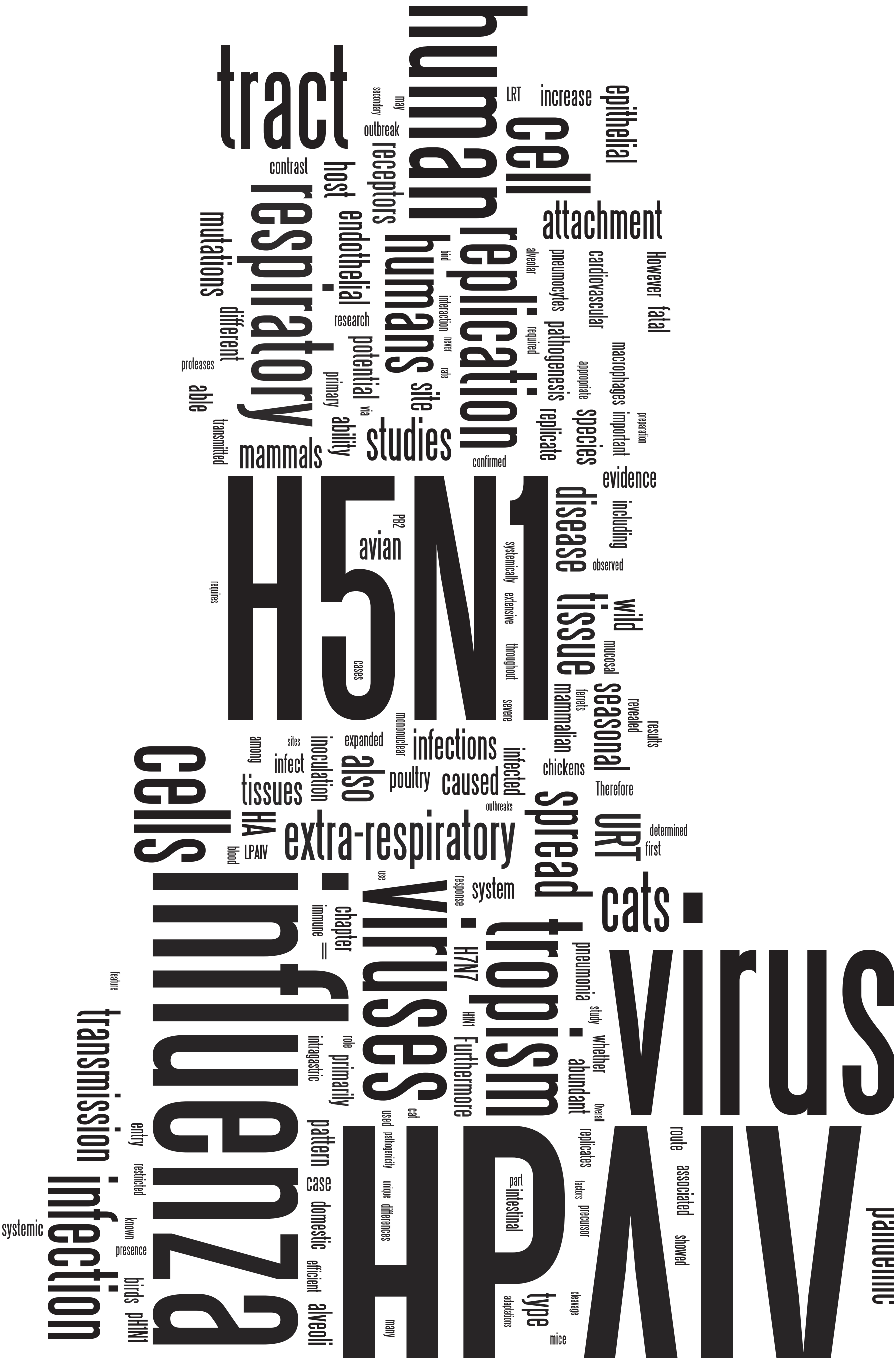
The differences in tissue tropism between HPAIV H7N7 and HPAIV H5N1 in other experimental animal species corresponds with our findings in cats. In both mice and ferrets, HPAIV H7N7 was detected in extra-respiratory tract tissues by virus isolation. However, virus replication in these tissues could not be confirmed by immunohistochemistry in mice,[142] while immunohistochemistry was not performed in ferrets.[193] In comparison, HPAIV H5N1 did show virus replication in extra-respiratory tissues by immunohistochemistry in both mice and ferrets.[53;175]

The lesions found in the fatal human case, from which the HPAIV H7N7 was isolated, are similar to the lesions observed in these experimentally infected cats. In the human fatal case, who died 15 days after infection, the main histological lesion observed was diffuse alveolar damage (DAD), without any significant lesions in any other organs by gross or histologic examination.[27;44] Although virus was isolated from the lungs, virus antigen could not be detected in the lungs or any other organs. In the cats, which were euthanized at 7 dpi, the main histological lesion was also DAD, without evidence for systemic dissemination of the HPAIV H7N7. Virus antigen – albeit scarce - was associated with the histological lesions. The difference in the presence of virus antigen may be because the infection in the fatal human case was about 1 week more chronic than that in cats.[44]

The extra-respiratory spread of HPAIV H5N1 in cats, which was not observed in HPAIV H7N7 infected cats, fits with the extra-respiratory of HPAIV H5N1 in some of the confirmed human cases. In HPAIV H5N1 virus infected humans, virus has been detected in blood by virus isolation,[31;199] and in brain, intestine, liver and placenta by immunohistochemistry and *in situ* hybridization.[28;30] Since influenza A viruses are normally restricted to the respiratory tract in humans and other mammals, HPAIV H5N1 seems to have a unique pathogenesis, even when compared to other HPAIV.

Acknowledgements

We thank Theo Bestebroer, Lonneke Leijten, Frank van de Panne and Robert Dias d'Ullois for excellent technical assistance, and Emmie de Wit and Ron Fouchier for helpful comments.



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Chapter 9

General Discussion

Summarizing discussion

Cell tropism of influenza viruses throughout the respiratory tract

The first part of this thesis focussed on the role of cell tropism in the pathogenesis of influenza. Although this disease is common in the human population worldwide, its pathogenesis and, more specifically, the cell tropism of influenza A virus in humans only had been studied to a limited extent.[50] The emergence of highly pathogenic avian influenza virus (HPAIV) H5N1 infection in humans increased the awareness of this gap in our knowledge. Therefore, we focussed on the cell tropism of seasonal influenza A viruses, pandemic H1N1 (pH1N1) virus and avian influenza A viruses, including HPAIV H5N1, throughout the human respiratory tract.

The cell tropism of an influenza virus is its ability to productively infect a certain cell type. This is largely, but not solely, dependent on the first step, attachment of the virus to its host cell. We set up a technique, which we called virus histochemistry, to visualize the attachment of influenza viruses to host cells in tissue sections. By use of this technique, the studies described in chapter 2 to 4 showed that seasonal influenza viruses and pH1N1 virus have a completely different pattern of attachment in the human respiratory tract than avian influenza viruses, including HPAIV H5N1. Seasonal and pH1N1 viruses attached to epithelial cells throughout the human respiratory tract. Attachment was most abundant to ciliated epithelial cells in the airways of the upper respiratory tract (URT) and lower respiratory tract (LRT). In the alveoli, seasonal influenza viruses attached predominantly to type I pneumocytes. In contrast, HPAIV H5N1 attached mainly to epithelial cells in the deepest part of the LRT. Attachment was absent or sparse to ciliated epithelial cells in the airways of the URT and LRT, but abundant to non-ciliated cuboidal cells in the bronchioles, and to type II pneumocytes and alveolar macrophages (AM) in the alveoli. Pathological studies on HPAIV H5N1 fatal cases and *ex vivo* infected human lung cultures confirmed that HPAIV H5N1 not only attaches abundantly to type II pneumocytes and AM in human alveoli but also replicates in these cell types.[63]

These observations led to the hypothesis that tropism of HPAIV H5N1 for type II pneumocytes and AM in the human alveoli—the primary site of pneumonia—helps to explain why HPAIV H5N1-associated pneumonia has a higher case fatality rate than seasonal influenza virus-associated pneumonia.[69] Infection of type II pneumocytes could hamper the re-epithelialization of the damaged alveolar wall, resorption of fluid or production of surfactants. Furthermore, type II pneumocytes are metabolically very active, which could lead to the production of high virus titers in the alveoli. Infection of AM could be important because of their role in limiting viral replication and in the immune response to viral infection.

One part of this hypothesis—the tropism of HPAIV H5N1 for AM—was studied in chapter 6. Earlier studies had used macrophages cultured from monocytes to examine the interaction with HPAIV H5N1. These studies had shown high percentages of productive infection and an excessive pro-inflammatory immune response. However, macrophages cultured from monocytes are phenotypically different from AM and may not be suitable surrogates. Therefore, we used AM obtained from human volunteers by broncho-alveolar lavage to study the interaction between influenza virus and AM. The first main result was that, in contrast to infection of macrophages cultured from monocytes, infection of AM with HPAIV H5N1 did not result in virus production and did not induce higher levels of TNF-alpha mRNA than after infection with seasonal H3N2 virus or pandemic H1N1 virus. Therefore, our study revealed that macrophages cultured from monocytes are not a good model to examine the interaction between AM and influenza virus. The second main result was that infection of AM with HPAIV H5N1 resulted in a higher percentage of cells infected than infection with seasonal H3N2 virus or pandemic H1N1 virus. This higher rate of infection could inhibit the function of AM in balancing immune responses in the alveoli and in phagocytosing progeny virus particles and apoptotic cells in patients with HPAIV H5N1 infection.

The observations of the pattern of influenza virus attachment in the human URT (chapter 4) also led to the hypothesis that efficient transmission of influenza virus among humans is associated with tropism for epithelium of the human URT. Seasonal influenza viruses and pH1N1 virus, which are transmitted efficiently among humans, bound abundantly throughout the human URT. In contrast, avian influenza viruses, including HPAIV H5N1, which are not transmitted efficiently among humans, did not. We used this association to screen for mutations in the HA of HPAIV H5N1 that would increase the tropism for the human URT, and thereby potentially increase its pandemic potential (chapter 5). Indeed, we found mutations that resulted in abundant attachment of HPAIV H5N1 to the human URT. Whether this increased attachment in the URT also results in abundant replication in the URT and in efficient transmission requires further research.

The attachment pattern of influenza viruses to the LRT varied among experimental animal species used in models for influenza virus pneumonia in humans (chapters 2 and 3). Overall, the pattern of virus attachment in ferret and pig respiratory tracts resembled that in the human respiratory tract most closely for seasonal influenza viruses. The pattern of HPAIV H5N1 virus attachment in respiratory tracts of the ferret and cat resembled that in the human respiratory tract most closely. For studies on the pathogenesis of influenza virus pneumonia it is important to consider these differences, as attachment to different tissues or cell types is known to influence the pathogenesis of influenza virus infection.

Before we started these studies, the attachment pattern of different influenza viruses in the human respiratory tract was largely unknown. The general consensus was that the human respiratory tract lacked receptors for avian influenza viruses.[62] However, this did not fit with the ability of HPAIV H5N1 to infect humans, which was first detected in 1997.[43] The studies presented in this thesis revealed that the human respiratory tract does have receptors for HPAIV H5N1, but that they are restricted mainly to the distal parts. Furthermore, these studies revealed that seasonal and avian influenza viruses not only have a different tissue tropism throughout the human respiratory tract, but also a different cell tropism within the human alveolus. Overall, these studies improve our insight in the pathogenesis of influenza virus infections in humans and the transmission of influenza viruses among humans.

Further studies should reveal which events are responsible for the development of, or protection against influenza virus pneumonia. Among other things, they should explain whether the differences in the pathogenesis of seasonal influenza virus, pH1N1 virus or HPAIV H5N1 are more quantitative of qualitative. The interaction between influenza virus and specific respiratory cell types (e.g., AM, type I pneumocyte, type II pneumocyte, Clara cell) could be studied by use of *in vitro* cell cultures or *ex vivo* tissue cultures. Furthermore, it also is important to study the interaction of influenza virus in a complete and functional respiratory tract by use of *in vivo* animal models, since the respiratory tract, and particularly the alveoli, form a complex system with a unique micro-environment. Comparing human seasonal influenza viruses, pH1N1 virus and HPAIV H5N1 in such studies would give us more insight into the differences in the pathogenesis of these influenza virus infections.

HPAIV infections in cats

The second part of this thesis focussed on HPAIV infections in the domestic cat, a species that was previously considered not to be susceptible to disease from influenza virus infection. Experimental infection studies, in which we combined virological and pathological methods, showed in which tissues and, more specifically, in which cell types HPAIV replicated. Furthermore, we revealed a potential novel route of entry for HPAIV H5N1 (chapters 7 and 8).

In 2003, tigers (*Panthera tigris)* and leopards (*Panthera pardus*) in a zoo died from HPAIV H5N1 infection after feeding on infected chicken carcasses,[35] and the following year there were anecdotal reports of domestic cats dying from HPAIV H5N1 virus infection after contact with chickens. Before this, fatal influenza virus infection in felids never had been never reported. By experimental infections in domestic cats, we confirmed that HPAIV H5N1 indeed caused severe respiratory disease, as well as extensive extra-respiratory disease (chapter 7).

Furthermore, by feeding infected chicks to cats, we revealed that the intestinal tract is a potential novel route of entry for influenza virus (chapter 7). This was later confirmed by intragastric inoculation of HPAIV H5N1 into cats (Reperant, in

preparation), ferrets and mice.[200] Interestingly, intragastric inoculation of HPAIV H5N1 in cats led to extensive virus replication in endothelial cells of almost all important organs and was associated with hemorrhages. It contrasts to the pattern of virus replication in intratracheally inoculated cats, in which HPAIV H5N1 also spread systemically, presumably via blood, but did not or only rarely replicate in endothelial cells. Although endothelial cell tropism of HPAIV H5N1 is a common feature of HPAIV infection in chickens, this is the first time that it has been seen so extensively in a mammalian species. Whether this phenomenon is related to the virus isolate, route of entry, inoculation dose, or some other feature remains to be determined.

In another study (chapter 8), we showed that not only HPAIV H5N1 but also HPAIV H7N7 caused severe disease in domestic cats. Remarkably, however, HPAIV H7N7 replication and associated disease were restricted to the respiratory tract and did not spread systemically, as was observed in HPAIV H5N1-infected cats. This difference in the extent of virus spread could not be explained by differences in the pattern of virus attachment between these viruses. Further studies are needed to understand which factors are required for the extra-respiratory tract disease caused by HPAIV H5N1; possibly, differences in replication efficiency or host immune responses contribute to this phenomenon.

The susceptibility of cats for two different HPAIV raises the question whether cats are susceptible for all HPAIV and what their role might be during outbreaks of HPAIV. During such outbreaks, cats could spread virus from one location to another, or transmit virus to humans. Furthermore, HPAIV H5N1 infection in cats could be an opportunity for the virus to adapt to the mammalian host, and thereby increase its pandemic potential.

Besides the importance of HPAIV infections in cats for their own sake, they might also mimic human infections with respect to extra-respiratory spread and the potential of virus entry via the intestinal tract. As in cats, HPAIV H5N1 infection in humans is regularly associated with extra-respiratory disease,[27;28;30] while there was no evidence for extra-respiratory spread of virus in the fatal human case of HPAIV H7N7 infection,[44] Virus entry via the digestive tract could possibly explain a human case of HPAIV H5N1 encephalitis without any evidence of respiratory tract disease.[31]

Further research in cats or other experimental animals should reveal which mechanisms are responsible for the extra-respiratory spread of HPAIV H5N1, and whether these mechanisms are universal for all mammalian species. Furthermore, it would be interesting to know why intragastric inoculation of HPAIV H5N1 in cats results in an endothelial cell tropism, and intratracheal inoculation does not.

HPAIV H5N1 outbreak

The outbreak of HPAIV H5N1 has many features that have not or only very rarely been observed. First, it is the largest HPAIV outbreak in poultry that has ever been recorded. Since 2003, HPAIV H5N1 has spread to poultry in over 22 countries in Asia, the Middle East, Africa and Europe, and has caused the death or culling of more than 400 million chickens and other poultry.

Second, HPAIV H5N1 virus is able to infect and spread in wild bird species. During previous HPAIV outbreaks, virus occasionally spilled back from poultry to wild birds, but there was no evidence that wild birds played any role in virus spread. In 2005, HPAIV H5N1 caused an outbreak in Lake Qinghai causing extensive mortality in several wild bird species,[23;24] after which HPAIV H5N1 spread westwards to India, Europe and Africa. There is enough evidence from field and laboratory studies that HPAIV H5N1 is able to infect and spread in wild waterbird species. However, it is not clear whether HPAIV H5N1 can be maintained in wild waterbird populations, as is the case with LPAIV.[25]

Third, HPAIV H5N1 is repeatedly transmitted directly from birds to humans, causing severe and often fatal disease. Before 1997, transmissions of avian influenza viruses from birds to humans were very uncommon and resulted in mild disease.[27] Since 2003, there have been over 500 human infections with HPAIV H5N1, with a case fatality rate of 60% (http://www.who.int/csr/disease/avian_influenza/country/cases_table_2010_06_08/en/index.html).

Fourth, HPAIV H5N1 has infected and caused disease in a wide range in both wild bird species and mammals, many of which were previously not considered to be susceptible to influenza. So far, natural infections in mammals have been observed primarily in carnivores including tigers, leopards, domestic cats, domestic dogs, Owston's palm civets (*Chrotogale owstoni*), a stone marten (*Mustela foina*), and an American mink (*Mustela vison*), but also in domestic pigs.[4] Disease caused by HPAIV H5N1 infection in these species varied from mild disease in swine to severe and often fatal disease in tigers, leopards, cats, a dog and Owston's palm civets.[4]

Finally, HPAIV H5N1 has a rather unique tissue- and cell tropism compared to other influenza viruses. Unlike most influenza viruses, HPAIV H5N1 is able to replicate extensively in extra-respiratory tract tissues not only in poultry, in which systemic virus replication occurs with all HPAIV infections, but also in several wild bird and mammalian species. Furthermore, this expanded tissue- and cell tropism of HPAIV H5N1 might have led to novel routes of entry, namely the intestinal tract.[31] The expanded tissue tropism of HPAIV H5N1 is discussed in more detail below.
Not all individual features discussed above are unique for HPAIV H5N1. HPAIV H5N3 infection in common terns (*Sterna hirundo*) from South Africa in 1961 is an example of a previous HPAIV outbreak in wild birds. Also, avian influenza viruses

have been transmitted before to humans and other mammals, and influenza A viruses also have caused extra-respiratory disease in humans before. A unique feature is the circulation of HPAIV H5N1 in poultry during several years in a large number of countries. Possibly, this widespread distribution and abundant presence of HPAIV H5N1 in poultry increases the chance of its transmission to wild birds, humans and other mammals. However, this could not explain the expanded tissue- and cell tropism of HPAIV H5N1. Even so, when all the above-mentioned features are taken together, they make the outbreak of HPAIV H5N1 very different from all other HPAIV outbreaks reported.

Expanded tissue- and cell tropism of HPAIV H5N1

The tissue- and cell tropism of HPAIV H5N1 is different from that of seasonal influenza viruses, pH1N1 virus, swine influenza viruses, equine influenza viruses, low pathogenic avian influenza viruses (LPAIV) and, to a lesser extent, other HPAIV. In general, mammalian influenza viruses and LPAIV have a tropism for epithelial cells of mucosal surfaces. The site where they infect mucosal epithelial cells varies. In mammals, including humans, pigs, dogs and horses, influenza viruses replicate primarily in the epithelial cells of the mucosal surfaces of the respiratory tract.[63;69;103;201-203] In contrast, in birds, LPAIV replicate primarily in the epithelial cells of the mucosal surfaces of the intestinal tract (Daoust in preparation).[204]

HPAIV H5N1 is unusual in that it has expanded its tropism to many non-epithelial cells types in poultry, wild birds and mammals. In chickens, it primarily replicates in endothelial cells and, later in infection, in mononuclear cells and parenchymal cells of multiple organs.[5] In wild duck species, HPAIV H5N1 replicates in parenchymal cells of the brain, heart, pancreas, liver, adrenal gland, and lung. However, there is no evidence for replication in endothelial cells as observed in chickens.[26] In humans, HPAIV H5N1 primarily replicates in epithelial cells of the lung, but is also able to replicate in neurons in the brain, epithelial cells and mononuclear cells in the intestines, Kupffer cells in the liver and Hofbauer cells of the placenta.[27;28;30] Similarly, in other mammals such as the cat, ferret and mouse, HPAIV H5N1 primarily replicates in epithelial cells of the lung, but also is able to replicate in parenchymal cells of many important organs, after intratracheal, intranasal or natural infection.[55;173] Interestingly, intragastric inoculation of HPAIV H5N1, which could occur after consumption of infected meat, results in systemic disease in ferrets, mice and cats.[200] In cats, intragastric inoculation results in a changed cell tropism of HPAIV H5N1 into a preference for endothelial cells (Reperant, in preparation), which was never observed before in mammals.

The tissue- and cell tropism of a HPAIV H7N7, which caused fatal disease in a human case, is more restricted than that of HPAIV H5N1. In chickens, we have shown that this HPAIV H7N7 has a tissue tropism comparable to HPAIV H5N1,

with replication in both respiratory and extra-respiratory tissues, primarily involving endothelial cells and mononuclear cells.[21] In mammals, however, evidence for extra-respiratory spread is rare. There is no evidence for extensive extra-respiratory tract replication in the cat (chapter 8). In ferrets and mice, HPAIV H7N7 was isolated from extra-respiratory tract tissues, but replication could not be confirmed by immunohistochemistry in mice and was not tested in ferrets.[142;193] In the human fatal case, there was no evidence for extra-respiratory tract replication by virus isolation or immunohistochemistry.[44] Overall, the tissue- and cell tropism of HPAIV H7N7 seems to be restricted mainly to the epithelial cells of the respiratory tract in mammals.

Many factors are involved in determining the tissue- and cell tropism of influenza viruses. The expanded tissue tropism of HPAIV H5N1 is most likely caused by multiple factors, which are discussed below.

Determinants for tissue- and cell tropism

Tissue-and cell tropism of an influenza virus depends partly on primary site of replication, ability to spread systemically, and ability to replicate at secondary sites. An important factor that determines the primary site of replication is the route of entry. In humans, seasonal influenza viruses are transmitted via large droplets, aerosols or fomites. This leads to exposure of the mucosal surface of the human respiratory tract, which fits with the replication site of seasonal influenza A viruses in humans. In contrast, the route of transmission for LPAIV in free ranging waterbirds is primarily fecal-oral. This leads to exposure of mucosal surfaces of the intestinal tract and, to a lesser extent, the respiratory tract,[204] which again fits with the replication sites of LPAIV in waterbirds. The importance of route of transmission for primary site of replication is illustrated by experimental infections with alternative routes of transmission. For example, inoculation of influenza virus into the vagina leads to virus replication in the vaginal epithelium.[205] Furthermore, intragastric inoculation of HPAIV H5N1 in ferrets results in widespread virus replication, but not in the respiratory tract, which is the primary site where HPAIV H5N1 replicates after intranasal inoculation.[200]

After virus replication at the primary site, the ability to spread to secondary replication sites will contribute to the dissemination of the virus within the host. The cardiovascular system is the most likely route for systemic spread, either in cells or as free virus particles in the blood. Although influenza viruses are able to infect mononuclear cells,[30;80] there is no substantial evidence in the mammalian host for cell-associated systemic spread, as seen for HIV or measles virus. The detection of influenza virus antigen in parenchymal cells but not in mononuclear cells of extra-respiratory tract tissues suggests that cell-associated systemic spread is not likely.[53;55] The mechanism by which virus particles could enter the cardiovascular system in large numbers from its primary site of replication would be either via lymph, or directly into blood in the cardiovascular system. Via lymph, virus would have to pass the filtering lymph nodes before it enters the cardiovascular system. Whether

direct release of the virus into blood in the cardiovascular system is possible, by for example basolateral release of virus—which is not common for influenza virus, including HPAIV H5N1[206]—is not clear. In the severely damaged alveoli, an open connection with the alveolar capillaries could possibly also lead to spill-over of virus into the cardiovascular system. Furthermore, endothelial cell infection could lead to virus release into blood in the cardiovascular system. Endotheliotropism is known for HPAIV infection in chickens, but is uncommon in mammals. Exceptions are the abundant replication of HPAIV H5N1 in endothelial cells in intragastrically infected cats (Reperant, in preparation), and HPAIV H5N1 replication in human endothelial cells *in vitro.*[206]

Once systemic spread has occurred, different factors affect the ability of influenza virus to replicate at secondary sites, including presence of appropriate receptors for virus attachment, presence of appropriate proteases for cleavage of hemagglutinin (HA), and character of the local innate immune response. First, the ability of the influenza virus to attach to a host cell requires the presence appropriate receptors on the host cell surface. Since extra-respiratory spread has not been an important feature of human influenza virus infections until the emergence of HPAIV H5N1 infection, the distribution of these receptors in extra-respiratory tract tissues has never been studied and is not known. Second, virus infectivity depends on the cleavage of precursor HA. All mammalian influenza viruses and LPAIV have a HA that is cleaved extracellularly, which requires the presence of appropriate proteases at the site of replication. These proteases are present in the mammalian respiratory tract and the avian intestinal tract. In contrast, HPAIV do not require extracellular proteases. This is because they have an insertion in the basic cleavage site, by which the precursor HA can be cleaved by furin-like enzymes, which are present in nearly all cell types.[207] By this mechanism, the precursor HA of HPAIV, including HPAIV H5N1, can be cleaved intracellularly. Therefore, cleavage of the precursor HA is not restricted to tissues of the respiratory and intestinal tract, where the appropriate extracellular proteases are present, but can take place in many tissues. Third, the character of the innate immune response at the secondary site may play a role. In wild-type mice, type I interferon expression was abundant in the extra-respiratory tissues but low in the respiratory tract, resulting in restriction of WSN/33 influenza virus infection to the respiratory tract. However, in mice in which the type I interferon response had been knocked out, influenza virus was able to spread from the respiratory tract to extra-respiratory tissues, resulting in systemic virus replication.[208]

Taken together, the tissue- and cell tropism of influenza viruses is a multifactorial trait, of which a few are discussed above. Further studies are required to obtain a better insight into the tissue- and cell tropism of influenza viruses in general, and the expanded tissue- and cell tropism of HPAIV H5N1. First, the distribution of influenza virus receptors in extra-respiratory tract tissues should be determined. This could be done by virus histochemistry, a technique we have used to determine the tissue tropism of influenza viruses in the respiratory tract. Second, it should be

determined how influenza viruses reach the cardiovascular system, from which they can spread systemically. Third, it would be interesting to focus on the endothelial cell tropism of influenza viruses. In particular, the observation that HPAIV H5N1 is able to infect endothelial cells in intragastrically infected cats and in chickens, but not in intratracheally infected cats required further research.

Pandemic potential of HPAIV

HPAIV H5N1 has circulated in poultry since 2003 and has been transmitted to over 500 people without the emergence of a HPAIV H5N1 pandemic. This raises the question which adaptations HPAIV H5N1 has to make to increase its pandemic potential, and whether such adaptations are feasible. Overall, the proteins HA, PB2 and NS1 are recognised as major determinants in the adaptation of influenza viruses to humans.[15] Therefore, mutations in the genes coding for these proteins may increase the pandemic potential of HPAIV H5N1.

Mutations in the HA gene of HPAIV H5N1 that switch its receptor affinity from alpha-2,3-linked sialic acids (avian-type receptors) to alpha-2,6-linked sialic acids (human-type receptors) may increase its ability to replicate in the human URT, because human-type receptors are more abundant there. Virus replication in the URT is thought to be a prerequisite for efficient transmission among humans. Viruses with an increased affinity for human-type receptors have been isolated from humans with HPAIV H5N1 infection.[128] We have confirmed that one of the mutations in the HA (N182K) of one of these virus isolates indeed increases the attachment of HPAIV H5N1 to the human URT (chapter 5). These results indicate that the HA of HPAIV H5N1 is able to adapt to some extent to replication in the human URT during natural infections in humans.

Mutations in the PB2 gene are involved in the host range, replication, pathogenicity and transmission of influenza viruses. A lysine at position 627 and an asparagine at position 701 of PB2 are found in most human and pandemic influenza viruses, are known to favour HPAIV replication in mammals,[140] and increase transmission of both human and avian influenza viruses in the guinea pig transmission model.[146;147] Interestingly, both mutations have been found in HPAIV H5N1 isolated from human cases, indicating that the PB2 of HPAIV H5N1 also is able to adapt to replication and transmission in humans during natural human infections.[77]
The above studies on HA and PB2 show that HPAIV H5N1 has made some adaptations to the human host without resulting in a pandemic. Therefore, the question remains which mutations are still required for this to happen. Until now, research has not yet revealed which mutations in HPAIV H5N1 would facilitate efficient human-to-human transmission. From the opposite standpoint, infection experiments in ferrets have shown that only two mutations in the pandemic 1918 Spanish influenza virus are sufficient to abolish transmission. However, whether

these two mutations were the only ones required for adaptation of the ancestor avian H1N1 virus to the human host seems unlikely.

Another question is whether the pathogenicity of HPAIV H5N1 would decrease with increasing adaptation to humans? Phrased differently, would the tropism of HPAIV H5N1 for the human LRT decrease with an increased tropism for the URT? The most likely answer is: yes. Since a tropism for the human URT requires the recognition of human-like receptors, which are most abundant in the URT, this would decrease the recognition of avian-like receptors, which are primarily located in the LRT. However, influenza viruses with a preference for human-like receptors still seem to differ in their ability to cause pneumonia. For example, the estimated case fatality rate of clinical cases was 1% for 1918 Spanish influenza virus and 0.048% for the 2009 pH1N1 virus, [209] even though both have a preference for the human-like receptor. How strongly this difference depends on their receptor preference remains to be determined.

Further research to reveal the pandemic potential of HPAIV H5N1 should include continued screening for new mutations in HPAIV H5N1 viruses isolated from humans and other mammals. Subsequently, the impact of these new mutations on pathogenicity, tissue tropism, and transmission should be determined. Another approach would be to experimentally adapt a HPAIV H5N1 virus to URT tropism and efficient transmission in a representative mammalian animal model by sequential passages, and determine which mutations are responsible for these adaptations.

General conclusion

In conclusion, the research described in this thesis set out to understand the unusual severity of disease caused by HPAIV H5N1, a virus that emerged in the human population in Hong Kong in 1997 and continues to cause sporadic fatal cases in humans to this day. The first approach was to examine the pattern of attachment of HPAIV H5N1 virus and other influenza viruses along the full length of the respiratory tract, from nose to alveoli, by use of virus histochemistry techniques. These studies showed that the unusual severity of HPAIV H5N1 pneumonia was associated with a predilection for cells in the most distal part of the human respiratory tract: non-ciliated cuboidal cells, type II pneumocytes, and alveolar macrophages, and that the ability of HPAIV H5N1 to infect alveolar macrophages might reduce their protective functions in the alveoli. They also showed that efficient human-to-human transmission of seasonal human and pandemic H1N1 influenza viruses was associated with a predilection for ciliated epithelial cells in the URT and trachea. From this, it follows that mutations in the HA of HPAIV H5N1 that increase its predilection for the human URT also may increase its pandemic potential.

The second approach was to combine virological and pathological methods to study the pathogenesis of influenza virus infection in a novel host, the domestic cat. These studies showed that, in contrast to a seasonal human influenza virus, both H7N7 and H5N1 subtypes of HPAIV caused severe pulmonary disease in cats, with histopathological changes resembling those in corresponding fatal human cases. Remarkably, H5N1 but not H7N7 also spread systemically in cats, with extensive virus replication and associated inflammation in multiple extra-respiratory tissues. This extra-respiratory spread and replication of HPAIV H5N1 compared to HPAIV H7N7 points to a special property of HPAIV H5N1 that is not related to its pattern of virus attachment, cleavability of the precursor HA, or other known pathogenicity markers. Together, these approaches, combining virological, pathological, and immunological techniques, show that the cell- and tissue tropism of influenza viruses are critical determinants of their host range, pathogenesis, transmission, and pandemic potential.


virus
vogelgriepvirus
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influenza
luchtwegen
seizoensgriep
Mexicaanse
veroorzaken
griepvirussen
longontsteking
mensen
bindingspatroon
ernstige
griep
infecties
virussen
ziekte
ziektebeeld
onderzoek
veroorzaakt
vogelgriep
H5N1
cellen
vogelgriepvirussen
verschillen
binding
bovenste
katten
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pandemie
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Introductie

Influenza A virussen, of griepvirussen, worden onderverdeeld op basis van twee oppervlakte eiwiten, haemagglutinine (HA) en neuraminidase (NA). Er zijn 16 verschillende typen HA en 9 verschillende typen NA. De combinatie van het type HA en NA bepalen de naam van het virus (bv. H5N1 of H3N2).

Influenza A virussen circuleren in mensen, paarden, varkens, honden en vogels. Ondanks het feit dat al deze diersoorten een 'eigen' influenza virus(sen) hebben, kan zo'n virus soms overspringen naar een nieuwe soort, en daarin ziekte veroorzaken.

Influenza A virussen die in mensen circuleren veroorzaken seizoensgriep. Dit zijn jaarlijkse epidemieën die vaak seizoens gebonden zijn en veroorzaken meestal een mild ziektebeeld. Slechts een minderheid van de infecties resulteert in een ernstige longontsteking.

Influenza A virussen kunnen ook van andere diersoorten overspringen naar mensen en ziekte veroorzaken. Dit noemen we zoönotische griep. Het belangrijkste voorbeeld hiervan is het hoog pathogene aviaire H5N1 virus, of het vogelgriepvirus. Sinds 2003 is dit virus verantwoordelijk voor uitbraken in pluimvee in Azië, van waaruit het zich verder heeft verspreid naar Europa en Afrika. Het vogelgriepvirus veroorzaakt ernstige ziekte in pluimvee, maar kan ook worden overgedragen op mensen. Ondanks dat deze overdracht naar mensen niet efficiënt is zijn er al meer dan 500 gevallen van bekend in mensen, waarvan meer dan 60% met dodelijke afloop. Tot op heden wordt dit virus niet efficiënt van mens op mens overgedragen waardoor een pandemie vooralsnog uitblijft.

Een influenza pandemie wordt veroorzaakt door de introductie van een nieuw influenza virus in mensen, dat efficiënt wordt overgedragen en zich zo wereldwijd kan verspreiden. De vorige eeuw kende drie influenza pandemieën; de Spaanse griep in 1918, de Aziatische griep in 1957 en de Hongkong griep in 1968. De eerste pandemie van deze eeuw is de Mexicaanse griep die begon in 2009. Ziekte veroorzaakt door deze pandemische griepvirussen verschilt enorm. Zo veroorzaakte het Spaanse griepvirus vaker een longontsteking dan de andere pandemische griepvirussen. Naar schatting heeft de Spaanse griep ongeveer 40 miljoen doden geëist, terwijl het dodental van de Aziatische griep en Hongkong griep wordt geschat tussen 1 en 4 miljoen. De Mexicaanse griep pandemie veroorzaakt tot nu toe relatief lage sterfte in mensen.

Meerdere influenza virussen zijn dus in staat om ziekte te veroorzaken in mensen, hoewel de ernst van deze ziekte erg kan verschillen per virus. In tegenstelling tot het relatief mild ziekteverloop van de seizoensgriep, leidt infectie met het vogelgriepvirus meestal tot een ernstige longontsteking, die vaak fataal is. Het onderzoek beschreven in dit proefschrift richtte zich op de vraag wat de oorzaken

zijn van deze verschillen in ziektebeeld. Om deze vraag te kunnen beantwoorden hebben we ons gericht op het celtropisme van influenza virussen. Het tropisme van een influenza virus is de voorkeur voor het infecteren van specifieke celsoorten of weefseltypen. De luchtwegen zijn erg divers, en bestaan uit een aantal anatomische locaties die allemaal verschillende functies hebben. Verschillen in tropisme tussen influenza virussen kunnen dus leiden tot verschillen in de locatie van virus replicatie, met als gevolg daarvan verschillen in het ziektebeeld.

Bindingspatroon van influenza virussen

Om het celtropisme van influenza virussen te bepalen hebben we gekeken naar de eerste stap van de virusreplicatiecyclus, namelijk de binding van het influenza virus aan de gastheercel. Hiervoor hebben we een techniek opgezet waarbij we direct kunnen visualiseren waar een influenza virus bindt in weefselcoupes. Hiermee hebben we bepaald aan welke celtypen seizoensgriep- en vogelgriepvirussen binden in de luchtwegen van mensen en andere zoogdieren.

In de eerste plaats hebben we onderzocht waar seizoensgriep-, Mexicaanse griep-, en vogelgriepvirussen binden in de humane luchtwegen (hoofdstuk 2, 3, en 4). Hieruit bleek dat seizoensgriep- en Mexicaanse griepvirussen een heel ander bindingspatroon laten zien dan het vogelgriepvirus. Zo binden de seizoensgriep- en de Mexicaanse griepvirussen vooral aan cellen in de bovenste luchtwegen, terwijl het vogelgriepvirus vooral aan cellen, in de longblaasjes diep in de longen bind, namelijk aan type II pneumocyten en alveolaire macrofagen. Binding van seizoensgriep- en Mexicaanse griepvirussen aan de bovenste luchtwegen, komt overeen met het ziektebeeld van deze virusinfecties en het feit dat deze virussen gemakkelijk overdraagbaar zijn van mens op mens. Binding van het vogelgriepvirus aan cellen diep in de longen verklaart mogelijk waarom het een ernstige longontsteking kan veroorzaken. Daarnaast kan het gebrek aan binding in de bovenste luchtwegen verklaren waarom het vogelgriepvirus niet gemakkelijk van mens op mens wordt overgedragen.

Tevens hebben we bepaald wat het bindingspatroon van seizoensgriep- en vogelgriepvirussen in de luchtwegen van de muis, fret, makaak, varken en kat is (hoofdstuk 3). Deze dierensoorten worden regelmatig gebruikt voor onderzoek naar griepvirussen. Net als in de luchtwegen van de mens geeft het seizoensgriepvirus een ander bindingspatroon dan het vogelgriepvirus in de luchtwegen van deze diersoorten. Tussen de verschillende diersoorten waren er grote verschillen in het bindingspatroon van zowel het seizoensgriep virus en het vogelgriep virus. In de fret en het varken vertoonde het seizoensgriepvirus een bindingspatroon dat vergelijkbaar was met het patroon in de mens. In de kat en fret had het vogelgriepvirus een bindingspatroon dat vergelijkbaar was met het patroon in de mens. Deze bevindingen moeten in acht worden genomen bij het uitvoeren van

influenza onderzoek in dieren, aangezien verschil in tropisme een verschil in het ziekteverloop kan veroorzaken.

In hoofdstuk 5 hebben we gekeken of bepaalde mutaties in het oppervlakte-eiwit HA van het vogelgriepvirus, zorgen voor meer binding in de bovenste luchtwegen de mens. Deze mutaties zouden ervoor kunnen zorgen dat het virus gemakkelijker van mens op mens kan worden overgedragen, hetgeen de kans op een pandemie aanzienlijk zou vergroten. In deze studie vonden we dat er inderdaad mutaties zijn die zorgen voor meer binding van het vogelgriep virus in de bovenste luchtwegen van de mens. Of deze mutaties ook daadwerkelijk leiden tot een efficiëntere overdracht tussen mensen moet verder onderzoek uitwijzen.

In hoofdstuk 2 en 3 hebben we aangetoond dat het vogelgriepvirus aan alveolaire macrofagen bindt, terwijl het seizoensgriepvirus dit niet doet. Deze alveolaire macrofagen zijn belangrijke immuuncellen die de longblaasjes diep in de longen beschermen. Wij hebben in hoofdstuk 6 aangetoond dat het vogelgriep virus deze cellen vaker infecteert dan seizoens griep virus. Mogelijk verliezen alveolaire macrofagen hierdoor hun beschermende rol, hetgeen de kans op een ernstige longontsteking vergroot.

Influenza virus infecties in katten

In hoofdstuk 7 en 8 hebben we hoog pathogene influenzavirus infecties in katten bestudeerd, en het H5N1 vogelgriepvirus vergeleken met het H7N7 vogelgriepvirus. Het H5N1 vogelgriepvirus veroorzaakte een ernstige longontsteking en repliceerde daarnaast ook in weefsels buiten de luchtwegen, zoals in het hart, lever, nier en het centrale zenuwstelsel. Toen we katten onderzochten die geïnfecteerd waren met het H7N7 vogelgriep virus bleek dat dit virus ook in staat was om een longontsteking te veroorzaken. Dit virus repliceerde echter niet in weefsels buiten de luchtwegen. Deze bevindingen komen overeen met H5N1 en H7N7 vogelgriepvirus infecties de mens. Hieruit blijkt dat het H5N1 vogelgriepvirus intrinsiek meer pathogeen is in katten dan het H7N7 vogelgriep virus.

Dankzij de studies beschreven in dit proefschrift hebben we meer inzicht gekregen in de pathogenese van seizoensgriep-, Mexicaanse griep- en vogelgriepvirus infecties de mens. Infectie studies in katten geven ons meer inzicht in de pathogenese van het H5N1 vogelgriepvirus in de mens. Het is belangrijk om goed te begrijpen wat er gebeurt tijdens influenza virus infecties in de mens, omdat deze kennis nodig is voor de ontwikkeling van nieuwe interventie strategieën.


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Boet
Lobke
Tineke
Oma
Thijs
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Lonneke
Frans
Peter
Pebbles
Ab
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Emmie
Chantal
Femke
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Curriculum Vitae

De auteur van dit proefschrift werd geboren in op 25 juli 1976 te Goirle. In 1994 behaalde ze haar HAVO diploma aan het Cobbenhagen College te Tilburg. In datzelfde jaar begon ze aan de studie Hoger Laboratorium Onderwijs, met als specialisatie Histologie, aan Hogeschool Brabant te Etten-Leur. In het laaste jaar van haar studie heeft ze haar 2 onderzoeksstages doorlopen op de Afdeling Celbiologie aan de Vrije Universiteit van Amsterdam en het Patterson Institute for Cancer Research in Manchester, Engeland.Na het behalen van haar diploma in januari 1999, begon ze te werken op een gezamenlijk project tussen de afdelingen Immunologie en Virologie van het Erasmus MC, waarna ze in 2001 volledig in dienst trad van de afdeling Immunologie. In 2004 verhuisde ze naar de afdeling Virologie waar ze in september 2005 startte met een M.Sc. Virology aan de John Moores University in Liverpool, Engeland die in juni 2008 werd afgerond ‘with distinction’. Daarnaast startte ze in 2005 met haar promotieonderzoek aan de afdeling Virologie onder begeleiding van Prof.dr. Thijs Kuiken en Prof.dr. Ab Osterhaus, resulterend in dit proefschrift.

Publicaties

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PhD portfolio

Name PhD student:	Debby van Riel
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Research school:	Post-graduate Molecular Medicine
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Education

2006 – 2010 Ph.D., Erasmus MC, Rotterdam, The Netherlands. Title of thesis: A critical role of cell tropism for the pathogenesis of influenza.

2006 – 2008 M.Sc., Liverpool John Moores University, England. Study: Virology.

1994 – 1999 B.Sc., Hogeschool Brabant, Etten-Leur, The Netherlands. Study: Medical Laboratory Sciences. Specialisation: Histology.

In-depth courses

2010 Course on Laboratory Animal Science, 3-week course, Erasmus MC, Rotterdam, The Netherlands.

2009 Course in Biomedical Wrinting and Communications, 15-week course, Erasmus MC, Rotterdam, The Netherlands.

Basic introduction course on SPSS, 2-day course, Erasmus MC, Rotterdam, The Netherlands.

2008 In vivo Imaging, from molecule to organism, 1-week course, Erasmus MC, The Netherlands.

2006 Health and Evolution, 3-day course, Erasmus MC, Rotterdam, The Netherlands.

Course in Virology, 1-week course, Erasmus MC, Rotterdam, The Netherlands.

Poster presentations

2010 Molecular Medicine Day, Rotterdam, The Netherlands. *Site of attachment in human respiratory tract associated with severity of disease and transmission.*

2009 Molecular Medicine Day, Rotterdam, The Netherlands. *Highly pathogenic avian influenza virus H7N7 isolated from a fatal human case causes respiratory disease in cats, but does not spread systemically.*

2008 ESWI, 3rd European Influenza Conference, Villamoura, Portugal. *Highly pathogenic avian influenza virus H7N7 isolated from a fatal human case causes respiratory disease in cats, but does not spread systemically.*

2007 Molecular Medicine Day, Rotterdam, The Netherlands. *Human and avian influenza A viruses target different sites and cell types in the human respiratory tract.*

2006 Respiratory viruses of animals causing disease in humans, Singapore*. Human and avian influenza A viruses target different sites and cell types in the human respiratory tract.*

2005 3rd Orthomyxovirus Conference, Cambridge, England. *Distribution of highly pathogenic avian influenza A (H5N1) virus in cats: A systemic infection.*

Oral presentations

2009 Dutch Annual Pathology Days, Zeist, The Netherlands. *Highly pathogenic avian influenza H7N7 virus causes respiratory disease in cats.*

5th Orthomyxovirus Conference, Freiburg, Germany. *Influenza virus attachment in the human upper respiratory tract.*

2008 Dutch Annual Virology Days, Amsterdam, The Netherlands. *Human and avian influenza A viruses target different sites in the respiratory tract.*

Dutch Annual Pathology Days, Zeist, The Netherlands. *Human and avian influenza A viruses target different sites and cell types in the respiratory tract.*

2007 4rd Orthomyxovirus Conference, Woodshole, MA, USA. *Human and avian influenza A viruses target different sites in the respiratory tract.*

2006 Molecular Medicine Day. Rotterdam, The Netherlands. *Distribution of H5N1 in cats: systemic infection and a possible novel route for virus entry.*

Comparative Pathology meeting, Rotterdam, The Netherlands. *Human and avian influenza A viruses target different sites in the respiratory tract.*

Grants & Awards

2009 Travel grant for young scientists, 5th Orthomyxovirus Conference, Freiburg, Germany.

Award for best oral presentation, 5th Orthomyxovirus Conference, Freiburg, Germany.

2008 ESWI Young Scientist travel grant, 3rd European Influenza Conference, Villamoura, Portugal.

2006 Keystone Symposia Scholarship travel grant, Respiratory Viruses of Animals Causing Disease in Humans, Singapore.


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Farrar
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acid
Bestebroer
acute
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