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Review

Animal models for the study of influenza pathogenesis and therapy

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ABSTRACT

Influenza A viruses causes a variety of illnesses in humans. The most common infection, seasonal influenza, is usually a mild, self-limited febrile syndrome, but it can be more severe in infants, the elderly, and immunodeficient persons, in whom it can progress to severe viral pneumonitis or be complicated by bacterial superinfection, leading to pneumonia and sepsis. Seasonal influenza also occasionally results in neurologic complications. Rarely, viruses that have spread from wild birds to domestic poultry can infect humans; such “avian influenza” can range in severity from mild conjunctivitis through the rapidly lethal disease seen in persons infected with the H5N1 virus that first emerged in Hong Kong in 1997. To develop effective therapies for this wide range of diseases, it is essential to have laboratory animal models that replicate the major features of illness in humans. This review describes models currently in use for elucidating influenza pathogenesis and evaluating new therapeutic agents.

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1. Introduction

Influenza A viruses infecting humans are responsible for a gamut of illnesses ranging from inapparent infections to pneumonia and severe acute respiratory syndrome. The typical infection one associates with seasonal influenza is a mild, self-limited febrile syndrome. As with other respiratory viruses, influenza viruses can cause more severe infections in infants, the elderly, and immunodeficient persons. In those individuals whose immune system is compromised or not fully developed, influenza-associated disease will often lead to severe viral pneumonitis or be complicated by bacterial superinfection, leading to pneumonia and sepsis. Seasonal influenza also occasionally results in neurologic complications. Even in non-pandemic years, more than 40,000 deaths annually are attributable to influenza infections in the United States (Dushoff et al., 2006). Much more rarely, humans can become infected by viruses that have spread directly from wild birds to domestic poultry. Such “avian influenza” can range in severity from mild conjunctivitis through the rapidly lethal disease seen in persons infected with the recently emerged Southeast Asian H5N1 virus. However, the potential for such highly virulent, lethal viruses to spread globally, as in the 1918 “Spanish Flu” pandemic, has resulted in a tremendous effort to develop vaccines and antiviral drugs to block the spread of virus and to treat the disease in humans, so as to prevent death.

The basic virology, clinical syndromes, epidemiology, and approved antiviral drugs for influenza virus infections are presented

in Table 1. Efforts have intensified in recent years to understand the pathogenesis of the various forms of influenza virus infection and to develop new treatments (Beigel and Bray, 2008). To develop effective antiviral therapies for this wide range of diseases, it is essential to have laboratory animal models that replicate the major features of illness in humans and provide selective, sensitive and reproducible results. Selecting the appropriate laboratory animal infection is very important in the drug development process. Some animals, such as pigs and ferrets, are naturally susceptible to infection by influenza viruses, but some of the other species that have been used for influenza studies are not. For the latter, the virus requires adaptation before it can replicate in the animal and/or cause disease (e.g., human seasonal influenza viruses in mice, rats). Once influenza viruses infect an animal, the virus may cause non-lethal disease or lethal disease. The type of disease that is induced can be manipulated by the investigator and is dependent on a variety of factors including virus strain, amount of virus in the inoculum, route of inoculation, time allowed for the disease to develop and the animal’s immune status.

This paper reviews animal models currently in use for the study of influenza pathogenesis and immune responses to influenza virus infections and to assess vaccine and antiviral drug efficacy. Models that will be reviewed will include: models of benign influenza, typical severe seasonal influenza and pneumonia, influenza-associated sepsis, models using H5N1 viruses, models in which acute respiratory distress syndrome (ARDS) is induced, models of influenza-associated neurologic disease, models for virus

Table 1
Influenza A virus infections: the basics.

Classification and structure	Influenza viruses are spherical or pleomorphic, single-stranded, negative-sense RNA viruses belonging to the family <i>Orthomyxoviridae</i> . Influenza A and B viruses contain eight separate ribonucleoprotein (RNP) segments, while influenza C virus contains seven, each of which encodes 1 or 2 proteins. The internal antigens (M1 and NP proteins) are the type-specific antigens used to determine if a particular virus is A, B or C, while the external hemagglutinin (HA) and neuraminidase (NA) are the subtype- and strain-specific antigens.
Infection cycle	Influenza virus binds to receptors on the surface of the host cell via the HA protein. It is internalized into endosomes, after which pH-dependent fusion and uncoating release the viral RNPs into the cytoplasm, where they are transported into the nucleus for replication. Viral messenger RNAs are exported out of the nucleus for protein synthesis, and some of the resulting proteins are transported back into the nucleus to assist in replication and RNP assembly. New RNPs assemble with other virus proteins at the M1 matrix to form virions, which bud from the plasma membrane.
Epidemiology	Influenza A viruses cause chronic, asymptomatic infection in the gastrointestinal tracts of wild birds, but are also able to infect and cause disease in a variety of mammals. Such avian viruses cause a range of illness in humans, ranging from conjunctivitis through the fulminant illness caused by the recently emerged H5N1 virus. On rare occasions, an influenza A virus is introduced into human populations and spreads rapidly to cause a global pandemic. This can occur either when an avian virus with a novel HA protein adapts to human-to-human transmission, or when an avian virus undergoes genomic reassortment during co-infection of an influenza virus-infected mammal such as a pig. As a pandemic virus circulates, it undergoes progressive antigenic drift in its HA and NA proteins, permitting to re-infect the same populations in regular outbreaks of “seasonal” influenza.
Clinical syndromes	Symptoms of seasonal influenza typically include high fever, chills, headache, sore throat, dry cough, myalgia, anorexia, and malaise. Complications include primary viral pneumonia, secondary bacterial pneumonia, or combined bacterial and viral pneumonia. Severe infections caused by the recently emerged avian influenza A H5N1 virus are characterized by rapid development of diffuse interstitial pneumonia, viremia and shock leading to death.
Vaccines	Inactivated vaccines (Fluzone®, Fluvirin™) obtained from infected chicken embryos are most commonly used. Attenuated vaccines include FluMist®.
Approved therapeutics	Approved therapeutics for seasonal influenza A virus infections include the neuraminidase inhibitors oseltamivir phosphate (Tamiflu®) and zanamivir (Relenza®) and the M2 ion channel blockers amantadine (Symmetrel®) and rimantadine (Flumadine®).

transmission studies, immunocompromised models, and models of host resistance.

2. Seasonal influenza

2.1. Models of benign influenza

Influenza is usually an acute, self-limited respiratory tract infection that begins with the sudden onset of high fever, followed by inflammation of the upper respiratory tree and trachea, with coryza, cough, headache, prostration, malaise and other signs and symptoms that persist for 7–10 days (Taubenberger and Morens, 2008). The virus replicates in both the upper and lower respiratory tract. In experimental infections in healthy volunteers, influenza A viral replication peaks approximately 48 h after inoculation into the nasopharynx, declining thereafter, with usually little or no virus shed after 6 days. However, viral antigen can still be detected in cells from the respiratory tract and secretions of infected individuals by enzyme immunoassay for several days after infectious virus can no longer be recovered (Wright et al., 2007). A summary of current animal models available for studying benign seasonal influenza is presented in Table 2.

2.1.1. Mice

In mice infected with influenza viruses of low pathogenicity, a mild illness occurs with few of the signs and symptoms that are seen in humans. The major parameters of mild to moderate disease are weight loss and the detection of virus, primarily in the lungs (Sidwell and Smee, 2004). In such a model, a polymer-bound 6'-sialyl-N-acetylactosamine was found to ameliorate weight loss in treated animals (Gambaryan et al., 2005).

2.1.2. Ferrets

Less virulent strains of influenza virus cause mild influenza-like symptoms in ferrets that rarely end in death. For example ferrets infected with A/Aichi/2/68 (H3N2) did not lose weight (Svitek et al., 2008). Only mild respiratory disease occurred in Aichi-infected animals with occasional sneezing and little serous nose exudate expressed. Virus infection elicits a rapid and strong upregulation of IFN- α , IFN- γ , and TNF- α , while more virulent strains induced significantly lower levels of IFN- α during the first 2 days after infection. During the first 4 days after infection, only IL-8 was detected in samples from animals inoculated with A/Aichi/2/68, whereas IL-6 expression was associated with the more virulent viruses (Svitek et al., 2008).

The utility of a ferret model in which a benign infection was induced is illustrated by the results from the following vaccine study. Ferrets immunized with virosome-based intranasal

influenza vaccine consisting of hemagglutinins from influenza A/Beijing/262/95like (H1N1), A/Sydney/5/97 (H3N2), and influenza B/Harbin/7/94 and then challenged with the A/Sydney/5/97 (H3N2) strain had less viral shedding in nasal secretions and the vaccine elicited a vaccine-specific antibody response (Lambkin et al., 2004). In addition, the vaccine regimen protected against fever, weight loss, and infiltration of inflammatory cells.

2.1.3. Rats

“True” rat species have been evaluated to determine their suitability as models for influenza disease and to ascertain whether genetic background impacts their susceptibility to infection. In one study, Brown Norway (BN), Fischer-344 (F344) and Sprague–Dawley (SD) rats were challenged with a rat-adapted influenzaA/Port/Chalmers/173 (H3N2) virus (Daniels et al., 2003). The virus was adapted to rats by 11 successive passages thorough infected lung homogenate. The F344 and SD rats were most sensitive to the infection, with 100-fold higher lung virus titers than seen in the BN rats. Alveolar macrophages, lactate dehydrogenase activity, and total lung protein concentrations (an indicator of pulmonary edema) were higher in the BN rats. Neutrophil numbers, interleukin 6 levels, and TNF- α activity were greatest in the bronchoalveolar lavage fluids from F344 and SD rats. Nevertheless, the infection was not lethal and few pathologic abnormalities were noted in the lungs. Although the study provided insights into factors of importance in protecting the host from influenza virus infections, rat models probably have not been characterized well enough to be recommended for use in evaluating anti-influenza therapies.

Recently Alarcon et al. (2007) demonstrated the use of the Brown Norway rat in evaluating influenza vaccines. Using microneedle technology for i.d. administration of three different types of influenza vaccines (Fluzone[®] 2003–2004 formulations and DNA plasmid-based vaccine) the investigators demonstrated in a rat model, that a whole inactivated virus elicited antibody responses to the corresponding wild type parent H3N2 and B strains. Animals treated with multiple doses of DNA plasmid vaccine also responded with antibody response to the parent strains.

2.1.4. Pigs

Because influenza A viruses frequently adapt to efficient transmission among pigs, these animals have occasionally been used as a model for testing vaccines (van der Laan et al., 2008). Signs of illness include fever, loss of appetite, labored breathing and coughing. However, the animals rarely die from the disease unless virus is directly inoculated into the trachea, in which case they exhibit signs of pneumonia. Pigs have been found to have low susceptibility to recently emerged H5N1 strains (Lipatov et al., 2008). To date,

Table 2
Summary of current animal models available for studying benign disease associated with seasonal influenza.

Virus	Disease Model	Animal Model	References
Influenza A strains Aichi/2/68 (H3N2) A/NIB/26/90M (H3N2) A/NIB/23/89M (H1N1) A/NIB/23/89-MA (mouse adapted)	Mild Pathogenesis	Mouse BALB/c/CitMoise (B/c) A/Snj/CitMoise CBA/CaLacSto C57BL/6LacSto	Gambaryan et al. (2005)
Influenza A/Aichi/2/68 (H3N2) Influenza A/Port/Chalmers/173 (rat adapted after 11 passages in rats)	Mild Pathogenesis Mild Pathogenesis	Ferret Rat (Rattus) Brown Norway Fischer-344 Sprague–Dawley	Svitek et al. (2008) Daniels et al. (2003)
Swine influenza A strains X98 H3N2 A/SW/CO/23619/99 H3N2 A/SW/IA/00239/2004 rH1N1	Mild Pathogenesis	Pig	Vincent et al. (2007)
Influenza A H1N1 A/Texas/36/91	Mild Pathogenesis/Pneumonia	Pigtailed macaque	Baas et al. (2006)

the main use of the pig has been in the development of vaccines against swine influenza (reviewed by van der Laan et al., 2008).

Problems that may preclude the use of pigs for influenza studies include caging, the complexities of animal husbandry and waste management. Thus, the recent development of a model in Ellegaard Göttingen minipigs appears to offer an alternative, if these animals can be shown to develop a fulminant pneumonia using a less intrusive route than intratracheal inoculation. However, as in the case of common pigs, minipigs were not susceptible to infection with H5N1 strains such as A/chicken/Yamaguchi/7/04 (Isoda et al., 2006). As with normal pigs one must always be cautious to obtain animals with no previous exposure to influenza viruses, since minipigs can be hosts for swine influenza A viruses (Hansen et al., 1997).

2.1.5. Nonhuman primates

Because nonhuman primates are much more closely related to humans than small animals typically used to study influenza, they have been used as models for human disease. In particular, rhesus macaques have been used to study pathogenesis and evaluate therapeutic and prophylactic strategies (see Baas et al., 2006 for review). In addition, the suitability of pigtailed macaques as models of influenza in the context of transcriptional studies has been evaluated (Baskin et al., 2004). A recent study examined the innate immune response in affected lung tissue with viral genetic material present (Baas et al., 2006). The authors used histopathological analysis of lung tissue, immunohistochemistry, viral and host gene expression by microarray analysis, proteomics, gene expression in circulating blood cells, and quantitative real-time RT-PCR to study individual animal responses until the end of the experiment. The infections were mild, without pneumonia or significant lung pathology. The investigators were able to demonstrate significant differences in gene expression within regions in influenza virus-induced lesions, based on the presence or absence of viral mRNA, and correlated them with transcriptional markers of early disease in peripheral white blood cells (Baas et al., 2006).

The use of viruses causing benign infections in macaques has primarily been used for vaccine studies. For example, Rimmelzwaan et al. (2001) demonstrated that protective immunity induced by immune stimulating complex (ISCOM)-based vaccines consisting of the membrane glycoproteins of A/Philippines/2/82 did not protect macaques from a challenge infection with A/Netherlands/18/94. However, vaccination of monkeys, which had had a prior infection with an influenza A/Philippines/2/82-like virus with a single dose of ISCOM vaccine, induced long-lasting protective antibody

immunity against challenge infection with the homologous virus A/Netherlands/18/94.

2.2. Models of severe seasonal influenza and pneumonia

Occasionally in healthy individuals, but much more often in the very young, the elderly, and the immunocompromised, acute seasonal influenza develops into hemorrhagic bronchitis and viral pneumonia, characterized by dyspnea, hemoptysis, pulmonary edema and cyanosis. Death can occur within 48 h after the onset of symptoms. A summary of animal models available to study such infections is presented in Table 3.

In cases of severe influenza pneumonia in humans, histological changes in the lungs include capillary and small vessel thromboses, necrotizing bronchitis and bronchiolitis, interstitial edema and inflammatory infiltrates, the formation of hyaline membranes in alveoli and alveolar ducts, varying degrees of acute edema between the alveoli with or without hemorrhaging, and diffuse alveolar damage. The later stages of the disease are characterized by organizing diffuse alveolar damage, fibrosis, epithelial regeneration, and squamous metaplasia. If concomitant bacterial or fungal infection is present, there may be pronounced neutrophil infiltration into alveolar air spaces, with a lesser degree of alveolar hemorrhage and edema than in primary influenza pneumonia (Taubenberger and Morens, 2008).

2.2.1. Mice

The mouse remains the primary model for evaluating the antiviral therapy of influenza pneumonia, due to the inexpensiveness of the animals and their caging and the general fidelity of the illness in mice to the human disease (Sidwell and Smees, 2004). In addition, many reagents are available to study the effects of virus replication and treatment on the mouse immune system. The laboratory mouse can be experimentally infected with both seasonal influenza A viruses and influenza B viruses, but this usually requires some adaptation by multiple lung passage. The adapted virus can then infect murine lung cells, probably as a result of amino acid changes in the surface hemagglutinins that enable it to bind to cell-surface alpha 2,3-linked sialic acid molecules (Ibricevic et al., 2006). A recent advance in influenza A modeling has been the finding that the reconstructed 1918 pandemic influenza virus is lethal for mice (Jeffery et al., 2001; Taubenberger, 2006).

A number of parameters may be used to monitor influenza virus infection in mice, including change in body weight, decline in arterial oxygen saturation, increase in serum alpha-1-acid gly-

Table 3

Summary of currently available animal models for studying severe seasonal influenza pneumonia.

Virus	Animal Model	References
Influenza A H3N2 strains A/Shangdong/09/93 A/Victoria/3/79	Mouse (BALB/c)	Reviewed by Sidwell and Smees (2000)
Influenza B strains B/HongKong/5/72 B/Lee/40		
B/Sichuan/379/99	BALB/c	Smees et al. (2006)
Influenza A H1N1 strains A/WSN/33 (H1N1) virus containing the HA gene of A/New Caledonia/20/99	BALB/c	Smees et al. (2008)
A/New Caledonia/20/99	BALB/c	Smees et al. (2006)
Influenza A strains A/Charlottesville/31/95 (H1N1)	Ferret	Smees et al. (2008)
A/USSR/90/77 (H1N1)	Ferret	Svitek et al. (2008)
A/Port Chalmers/1/73 (H3N2)		
Influenza A H3N2 A/Wuhan/359/95	Cotton Rat	Ottolini et al. (2005), Review by Eichelberger (2007)

coprotein, mean time to death, and lung weight, viral titer and pathology scores (Sidwell and Smees, 2000). Disease manifestations often depend on the infectivity and challenge dose of the virus and (for seasonal influenza virus strains) how well the virus has adapted to the host. If a virus replicates in mice without causing apparent illness, the effects of therapy can be monitored using parameters such as lung viral titers, increase in lung weight and increase in 1-acid glycoprotein (1-AG), all of which increase in nonlethally infected mice (Ottolini et al., 2005; Sidwell and Smees, 2000).

Most of the histopathological features of influenza viral pneumonia listed above are also seen in mice, but some signs and symptoms of human influenza are rarely observed. Mice do not show outward signs of fever and do not have increased rectal temperatures, nor is dyspnea, cyanosis or hemoptysis easily, if ever detected in mice infected with strains other than the H5N1 virus. However, as in humans, reduced blood oxygen saturation levels, a measure of lung function, can be measured in mice, and these levels are dramatically lower as pneumonia progresses and the mice approach death (Barnard et al., 2007). Weight loss is also a good marker of disease severity (Sidwell and Smees, 2000). The reader is referred to the following recent papers that show the utility of the seasonal influenza mouse model for evaluating vaccines (reviewed by van der Laan et al., 2008; Hagenaars et al., 2008; Hai et al., 2008) and antiviral drugs (Dimmock et al., 2008; Gilbert and McLeay, 2008; Reading et al., 2008; Smees et al., 2008; Wang et al., 2008).

2.2.2. Ferrets

Because influenza-virus-infected ferrets develop many of the typical signs of infection in humans, including nasal discharge, anorexia, watery eyes, otologic symptoms and fever, they are now being used as an animal model for influenza-like pneumonia (Sidwell and Smees, 2000; Govorkova et al., 2007; Smees et al., 2008; Svitek et al., 2008). Virus in high titers can be recovered from the respiratory tract (Smith and Sweet, 1988; Potter et al., 1976). Although ferrets mimic seasonal influenza A infections in humans, they appear to be less responsive to infections by influenza B viruses (Pinto et al., 1969).

A limitation of ferret studies is the lack of specific reagents for studying the ferret immune system, compared with similar resources for mice. However, this issue will probably be resolved in the near future with the development of reagents such as cross-reactive monoclonal antibodies (mAb). For example, CD8+ cells in mouse bronchoalveolar lavages from pneumonic ferrets were identified with mAb to human CD8 (Rutigliano et al., 2008). Caging can also be a problem, because most animal facilities require that 1–3 ferrets be housed in rabbit-style cage systems. Proper caging for work with H5N1 influenza virus is at the final stages of development, but not yet available for use. Access to specific pathogen-free (influenza virus seronegative) ferrets is also an issue, and will become increasingly so as the demand for ferret studies grows. Finally, although ferrets are relatively passive in temperament relative to their mink cousins, they can become fairly aggressive after being exposed to invasive procedures.

As a result of the studies described above, a number of studies have been done to show the adequacy of ferrets as models for testing vaccine efficacy (reviewed by van der Laan et al., 2008; (Huber et al., 2008; Parks et al., 2007) and the effectiveness of antiviral drug therapy (Smees et al., 2008; Oxford et al., 2007; Malakhov et al., 2006).

2.2.3. Cotton rats

Cotton rats (*Sigmodon hispidus*) have several advantages over mice and ferrets as a model for human influenza, including the availability of reagents to study immunological responses. When compared to mice of any strain, cotton rats appear to have all the innate and adaptive immune responses seen in humans, such as

Mx gene-mediated responses. Viruses isolated from humans do not have to be adapted to cotton rats to cause disease (Eichelberger, 2007). The disadvantages of the cotton rat are primarily animal availability and the aggressiveness of species, regardless of gender.

The influenza A cotton rat model is a nasal and pulmonary infection in adult inbred cotton rats. Animals infected intranasally with doses of a recent H3N2 influenza strain had increased breathing rates accompanied by weight loss and decreased temperature (Ottolini et al., 2005). Virus replication peaked within 24 h in the lung, with peak titers proportional to the infecting dose. Although virus was cleared from the lung by day 3, replication persisted in nasal tissues for 6 days. Pulmonary pathology included early bronchiolar epithelial cell damage, followed by extensive alveolar and interstitial pneumonia detectable in animals for up to about three weeks. Cytokine levels were typical of an inflammatory response to a lung infection; their upregulation appeared to coincide with increased virus replication. Since this model has not been gained wide acceptance due to the limitations described above, there are a paucity of papers using cotton rats to vaccine efficacy (Straight et al., 2008) and antiviral efficacy (Eichelberger et al., 2004; Stertz et al., 2007) against influenza virus infections.

3. Models of influenza-associated sepsis

Although influenza virus by itself can cause pneumonia, secondary bacterial pneumonia beginning either during or shortly after recovery from the primary virus infection is much more common (Speshock et al., 2007). Co-infections with bacteria in seasonal influenza pandemics have been associated with approximately 25% of all influenza-related deaths (Gupta et al., 2008). A recent paper concluded “that influenza A virus infection in conjunction with bacterial infection led to most of the deaths during the 1918–1919 pandemic” (Morens et al., 2008). A number of animal models have been developed to evaluate the susceptibility of influenza-infected individuals to bacterial superinfections, such as those caused by *Neisseria meningitidis* (Alonso et al., 2003) and *Streptococcus pneumoniae* (Hayashi et al., 2006; Peltola et al., 2006; Rosseau et al., 2007; Smith et al., 2007) (Table 4). Thus, efforts have been undertaken to establish animal models to mimic influenza infections complicated by bacterial infections, primarily pneumonia (McCullers and English, 2008).

Sun and Metzger (2008) recently proposed a mechanism whereby secondary bacterial infection can occur. They found that interferon-gamma produced in the lungs of mice during T-cell responses to influenza virus infection inhibited bacterial clearance by alveolar macrophages. The higher the levels of interferon-gamma, the greater the suppression of phagocytosis, leading to enhanced susceptibility to secondary pneumococcal infection.

3.1. Pneumococcal superinfection

The infectious agent most often complicating influenza is *Streptococcus pneumoniae*. In a superinfection model, pneumococcal pneumonia after influenza virus infection was established in six-week old mice using sublethal doses of both microbes (Hayashi et al., 2006). *S. pneumoniae* was inoculated intranasally 7 days after exposure to influenza virus and death in the presence of both agents only began at day 4 post bacterial exposure. The efficacy of several quinolones against bacteria-induced pneumonia in this model was evaluated, with gatifloxacin used successfully to treat the pneumococcal infection.

In another model of secondary pneumococcal pneumonia during influenza A virus infection, the pathology and immunology that led to fatal disease was studied by specifically looking at cytokine profiles (Smith et al., 2007). Influenza-infected mice challenged

Table 4

Summary of current animal models available for studying models of sepsis associated with influenza virus infections.

Virus	Bacterial Infection	Disease Model	Animal Model	References
Influenza A H3N2 A/Scotland/20/74	<i>Neisseria meningitidis</i>	Sepsis model	Mouse (BALB/c)	Alonso et al. (2003)
Influenza A H1N1 A/PR/8/34	<i>Streptococcus pneumoniae</i>	Sepsis model	Mouse (BALB/c)	Hayashi et al. (2006)
Influenza A H1N1 A/PR/8/34	<i>Streptococcus pneumoniae</i>	Sepsis model	Mouse (BALB/c)	Smith et al. (2007)
Influenza A H3N2 A/Sydney/5/97 A/Fujian/411/02 Influenza A H1N1 ATaiwan/1/86 Influenza B B/Singapore/222/79	<i>Streptococcus pneumoniae</i>	Secondary bacterial infection model	Ferret	Peltola et al. (2006)
Influenza A H3N2 Wuhan/359/95	<i>Staphylococcus aureus</i>	Bacterial synergy model	Ferret	Braun et al. (2007)

with each of 3 serotypes of *S. pneumoniae* developed a severe, necrotic pneumonia, characterized by markedly elevated levels of both pro- and anti-inflammatory molecules in the lungs, accompanied by a massive influx of neutrophils. Death was associated with the development of pneumonia and lung inflammation, but not with bacteremia. Thus, this model may be used to delineate factors associated with the pathogenesis of severe mixed lung infections.

3.2. Meningococcal superinfection

In the meningococcal superinfection model, BALB/c mice were inoculated intranasally with a sublethal dose of mouse-adapted influenza A/Scotland/20/74 (H3N2), followed by intranasal inoculation with *Neisseria meningitidis* serogroup C (Alonso et al., 2003). Fatal meningococcal pneumonia and bacteremia were observed in influenza-infected mice superinfected with bacteria on day 7, but not on day 10. Thus, the investigators were able to model human meningococcemia with fatal sepsis, and were also able to examine the role of various bacterial virulence factors leading to sepsis.

3.3. Secondary bacterial infection in ferrets

A bacterial superinfection model was also established in ferrets, in which influenza more closely resembles that found in humans (Peltola et al., 2006). The goals of the study were to better understand the various sequelae of *S. pneumoniae* infection, including otitis media, sinusitis, and pneumonia, and to determine if the frequency and character of secondary pneumococcal infections differed depending on the strain of influenza virus that preceded bacterial challenge, as has been reported in humans. The viruses studied were A/Taiwan/1/86 (H1N1), the H3N2 viruses A/Sydney/5/97 and A/Fujian/411/02, and influenza B virus B/Singapore/222/79. In seven-week-old ferrets inoculated intranasally with virus and 5 days later with *S. pneumoniae*, all viruses increased bacterial colonization of the nasopharynx. However, 9 of 10 ferrets infected with H3N2 subtype influenza A viruses developed either sinusitis or otitis media, while only 1 of 11 infected with an H1N1 virus or an influenza B virus did so. These data support observations in humans that bacterial complication rates are higher during seasons when H3N2 viruses predominate (Bhat et al., 2005). This animal model could be useful for further study of the mechanisms that underlie viral–bacterial disease synergism.

Secondary infection by *Staphylococcus aureus* has also been evaluated in cotton rats (Braun et al., 2007). Although *S. aureus* superinfections are a relatively rare complication of influenza, the case fatality rate is extremely high. In addition, all three influenza pandemics of the 20th century included cases of co-infection with *S.*

aureus in healthier and younger patients (Braun et al., 2007). Therefore, physiologic and pathologic changes in cotton rats infected with both *S. aureus* and influenza A/Wuhan/359/95 (H3N2) was evaluated and compared to the infections in cotton rats with each agent alone. Peribronchiolitis, interstitial pneumonitis as well as alveolitis were observed. Although pathology scores began to decline in the co-infected on day 7 p.i., they remained significantly higher than all other infected cohorts. This was true even in the cohort co-infected with inactivated *S. aureus* and influenza virus. Co-infection resulted in higher mortality than infection with either agent alone, and was associated with more marked hypothermia, lung pathology an enhancement of proinflammatory cytokine expression in the lungs (Braun et al., 2007). In addition, bacteremia was prolonged and bacterial lung titers were higher in co-infected cotton rats.

4. H5N1 avian influenza

In contrast to the human-adapted influenza A viruses that cause benign seasonal influenza in the majority of patients, the recently emerged Southeast Asian H5N1 virus causes acute viral pneumonia aggravated by ARDS, toxic shock and multiple organ failure in previously healthy, immunocompetent individuals (Peiris et al., 2007; Beigel et al., 2005). Its markedly enhanced virulence for humans appears at least in part to reflect a difference in viral tropism for cells of the lower respiratory tract, but until recently, those target cells were unknown for both humans and other mammals (Rimmelzwaan et al., 2006; van Riel et al., 2006). In a recent study (van Riel et al., 2006), an H5N1 virus was found to preferentially bind to type II pneumocytes, alveolar macrophages, and nonciliated bronchiolar cells in the lower respiratory tract of cats, similar to the clinical scenario in humans (Matrosovich et al., 2004). How well various animal models resemble human disease may thus reflect the difference in distribution of infection in the respiratory tract. Because the H5N1 virus infects a wide range of mammalian species without prior adaptation, a number of animal models are available for study (Table 5).

4.1. Mice

Isolates of H5N1 virus recovered from human patients can cause lethal disease in BALB/c mice without prior adaptation (Evseenko et al., 2007; Hatta et al., 2007; Hatta and Kawaoka, 2005; Isoda et al., 2006; Katz et al., 2000b; Gubareva et al., 1998a). Although the infection is primarily pulmonary, there is strong evidence of systemic spread to solid organs, including the brain (Gubareva et al., 1998a; Katz et al., 2000a). Most of these viruses cause necrosis in respiratory epithelium of the nasal cavity (Hatta et al., 2007),

Table 5
Summary of current animal models available for studying human disease caused by highly pathogenic H5N1 avian viruses.

Virus	Disease Model	Animal Model	References
A/duck/Tuva/01/06 (H5N1)	Interstitial pneumonia	Mouse (BALB/c)	Evseenko et al. (2007)
A/Vietnam/1203/2004 VN1204PB2-627Lys (mutant)		(BALB/c)	Hatta et al. (2007)
A/Vietnam/1204/2004 VN1203PB2-627Glu (mutant)			
A/chicken/Vietnam/NCVD5/2003 A/muscovy/duck/Vietnam/NCVD18/2003			
A/Vietnam/1194/04 A/Vietnam/1203/04 (VN1203) A/Vietnam/1204/04 (VN1204)		(BALB/c)	Hatta and Kawaoka (2005)
A/duck/Vietnam/NCVD-5/03 A/duck/Vietnam/NCVD-18/03 A/duck/Vietnam/NCVD-25/03 A/chicken/Vietnam/NCVD-8/03 A/chicken/Vietnam/NCVD-15/03 A/chicken/Vietnam/NCVD-30/03			
A/chicken/Yamaguchi/7/04		(BALB/c)	Isoda et al. (2006)
H5N1 viruses A/Hong Kong/481/97 A/HongKong/483/97 A/Hong Kong/485197 A/Hong Kong/1486197 A/Hong Kong/507/97		(BALB/c)	Katz et al. (2000b)
H5N1 viruses A/ck/HK/220/97 A/ck/HK/728/97 A/HK/156/97 A/ck/Scotland/59 A/tk/England/91		BALB/c	Dybing et al. (2000)
A/HongKong/156/97		(BALB/c)	Gubareva et al. (1998a)
Influenza A H5N1 A/Hong Kong/486/97 A/Hong Kong/483/97	Pathogenesis/Pneumonia	Ferret	Zitzow et al. (2002)
Influenza B clinical isolate related to B/Beijing/184/93 and B/Guangdong/8/93			Gubareva et al. (1998b)
Influenza A H5N1 A/Vietnam/1194/04	Pathogenesis/Pneumonia	Cat	Rimmelzwaan et al. (2006)
Influenza A H5N1 A/HongKong/156/97	Pathogenesis/Pneumonia	Cynomolgus macaque	Kuiken et al. (2003) Rimmelzwaan et al. (2003)

trachea (Hatta et al., 2007), bronchi, and bronchioles (Katz et al., 2000b; Hatta and Kawaoka, 2005) with accompanying inflammation and accumulation of fibrin and neutrophils (Katz et al., 2000b). The pathology in the lungs can be characterized as a peribronchial alveolitis with intra-alveolar serofibrinous exudate, erythrocytes and neutrophils, and increased numbers of alveolar macrophages (Katz et al., 2000b). Often the infection results in a bronchointerstitial pneumonia and alveolar edema or in severe cases it may become a diffuse interstitial pneumonia affecting entire lung lobes (Dybing et al., 2000). Body temperatures usually decrease by four of five degrees and animals lose at least 20% or more body when they succumb to the effects of the infection.

In another study, a recent H5N1 isolate was shown to promote significantly higher levels of pro-inflammatory cytokines in whole lungs and primary human macrophages probably resulting in early and excessive infiltration of macrophages and neutrophils in the lungs of mice (Perrone et al., 2008). Another study has suggested that in addition to the typical proinflammatory cytokines, TNF- α may also contribute to the morbidity during H5N1 influenza virus infection (Szretter et al., 2007).

Because of the severity of H5N1 infections in humans caused by highly pathogenic avian influenza viruses, numerous vaccine and antiviral efficacy studies have recently been done in mice. See the following recent references for vaccine studies (reviewed by van der Laan et al., 2008; Chen et al., 2008; Chen et al., 2009; Hickman et al., 2008; de Vries et al., 2008) and for antiviral efficacy studies

(Barnard et al., 2007; Boltz et al., 2008a; Ilyushina et al., 2008; Zheng et al., 2008).

4.2. Ferrets

Two H5N1 viruses isolated in Hong Kong during the 1997 outbreak were found to readily infect ferrets (Zitzow et al., 2002). Since then, more H5N1 isolates that are highly pathogenic for birds have been found to replicate in ferret lungs without prior host adaptation. The infections generally have been characterized by severe lethargy, fever, weight loss, transient lymphopenia, and virus replication in the upper and lower respiratory tract and in multiple organs including the brain. More importantly, the illness induced by these agents was more severe than that induced by recently isolated human H3N2 viruses. The lungs of H5N1-infected ferrets showed diffuse inflammation of interalveolar septa with infiltrates of mononuclear cells and intra-alveolar edema, regardless of the time post infection; in contrast to the scattered infiltrates seen with other influenza A viruses, these changes were observed throughout the lungs (Maines et al., 2005). Viral antigens were detected in alveolar bronchial cells or bronchioles in the majority of animals by day 3 post virus exposure. Brain tissues showed mononuclear cell infiltrates in the meninges, choroid plexus, and brain parenchyma, and viral antigens were detected in neurons in the same areas.

In an effort to determine why the H5N1 virus is more virulent for ferrets than seasonal influenza A isolates, Cameron et al. (2008) ana-

lyzed the expression of host innate immune response genes during the course of lethal infection. The authors found that many interferon response genes, including IFI44, ISG15 (G1P2), MX2, OAS1, OAS2, STAT1, TAP1, and UBE1L, were significantly upregulated in ferrets infected with the H5N1 virus, in comparison to an H3N2 isolate. CXCL10, a chemoattractant of activated Th1 lymphocytes and natural killer cells, was also upregulated to a much greater extent in an H5N1 infection than with the H3N2 virus. CXCL10 and its receptor CXCR3 are thought to play a role in the temporal development of innate and adaptive immunity in concert with type I and II interferons (Neville et al., 1997). In support of that hypothesis, Cameron et al. (2008) found that treatment of H5N1-infected ferrets with AMG487, a CXCR3 antagonist, markedly reduced the severity of symptoms and delayed death, compared to untreated animals.

Vaccines (reviewed by Subbarao and Luke, 2007; Chen et al., 2008; Lalor et al., 2008; Mahmood et al., 2008) and a number antiviral compounds (Malakhov et al., 2006; Govorkova et al., 2007; Boltz et al., 2008b; Yun et al., 2008) have been tested in ferrets infected with H5N1 viruses very often have verified findings in mice (Malakhov et al., 2006; Govorkova et al., 2007; Boltz et al., 2008b; Yun et al., 2008).

4.3. Cats

Rimmelzwaan et al. (2006) assessed the virulence of a H5N1 virus in cats by infecting them by intratracheal inoculation or through feeding on virus-infected chicks. Within 2 days, most animals developed fever, conjunctivitis, lethargy, and labored breathing. Virus was detected in the throat, nasal, and rectum, regardless of the original site of infection. The virus spread systemically and was detected in the respiratory and digestive tracts, liver, kidney, heart, brain, and lymph nodes. Cellular damage in infected tissues correlated with the detection of viral proteins. Histopathological examination of the lungs revealed multiple or coalescing foci of inflammation and necrosis in the bronchioles. The alveolar and bronchiolar lumina were infiltrated with alveolar macrophages, neutrophils, and erythrocytes, mixed with fibrin, edema fluid, and cellular debris. More importantly, some alveoli were covered by hyaline membranes, which are also seen human lungs upon autopsy. Hyaline membrane formation has not been observed in mice or other animal models. The epithelium of bronchiolar and alveolar walls, which were moderately thickened, also had evidence of both necrosis and hyperplasia. There was also edema and moderate accumulation of mononuclear cells around pulmonary artery branches. Although there has been some work in defining the cat influenza model for evaluating pathogenesis of H5N1 infections, few if any studies have been published which describe the suitability of the cat for doing vaccine (Karaca et al., 2005; Vahlenkamp et al., 2008) and antiviral efficacy studies.

4.4. Dogs

Dogs can also be infected with the H5N1 avian influenza virus. Because these animals are often in close contact with both wild and domestic birds and with humans, this raises the possibility that the virus could adapt to dogs and be transmitted to humans while retaining virulence. To date it has been found that dogs are susceptible to experimental infection but are not capable of transmitting the virus to other mammals (Giese et al., 2008). The disease in dogs was characterized by the development of conjunctivitis and fever within 2 days after virus exposure, which resolved with no other adverse events by day 7. Dogs may be useful more as sentinel for human disease than as a model for human influenza disease (Cleaveland et al., 2006).

4.5. Nonhuman primates

A cynomolgus macaque (*Macaca fascicularis*) model of H5N1 (A/HongKong/156/97) virus infection with pneumonia and ARDS has been developed (Kuiken et al., 2003; Rimmelzwaan et al., 2003). The principal change in the lung was a necrotizing bronchial interstitial pneumonia, similar to that described for primary human influenza pneumonia. In contrast to human H5N1 cases, detection of influenza virus antigen was limited to pulmonary tissue and tonsils. The model was used to evaluate the efficacy of intravenous zanamivir, which has a longer half-life than oseltamivir (Stittelaar et al., 2008). Drug levels in the epithelial lining fluids of the lungs were equivalent to those in plasma. Treated macaques had lower gross pathology scores and less lung pathology than untreated animals. However, there was considerable variability in viral lung titers and gross pathology within groups of macaques, and scattering of foci of lung infection from animal to animal, making it difficult to establish statistically significant differences. The authors pointed out that such variability probably reflects what happens within the human population. Some vaccine studies with macaques have also been done (de Vries et al., 2008; Kreijtz et al., 2008; Ruat et al., 2008 among others).

5. Models of ARDS

ARDS is characterized by a localized hyperinflammatory response to an infectious agent in the lung, resulting in damage to the alveolus–capillary interface (Hammerbeck et al., 2007). H5N1 virus-infected humans initially present with clinical signs of influenza-like illness, then develop progressive pneumonia and may eventually die of ARDS (Grose and Chokephaibulkit, 2004). Thus, some research has been done to develop a model of ARDS in H5N1 virus-infected mice.

In one ARDS model using six to eight-week-old BALB/c mice inoculated intranasally with influenza virus A H5N1 (A/Chicken/Hebei/108/2002), the animals developed typical ARDS, with highly edematous lungs, dramatic increases in lung weights, progressively more hypoxemia and 80% mortality by day 8 (Xu et al., 2006). Histological examination revealed inflammatory cell infiltrates in the alveoli with a significant increase in neutrophils, interstitial edema and hemorrhage. Levels of TNF- α and L-6 in bronchiolar lavage fluids were significantly increased. The model thus displayed some of the more significant signs and symptoms associated with ARDS in humans. A summary of current animal models available for studying ARDS in H5N1 influenza is shown in Table 6.

6. Models of influenza-associated neurologic disease

Because a number of studies have described neurological sequelae from influenza virus infections, an animal model in which such complications can be studied and treatment can be tested would be of importance (Mizuguchi et al., 2007; Steininger et al., 2003). A summary of current animal models available for studying influenza virus-associated neurotropic disease is presented in Table 7.

Table 6

Summary of current animal models available for studying acute respiratory distress syndrome (ARDS) developing in the setting of H5N1 avian influenza.

Virus	Animal Model	References
Influenza A H5N1 A/Chicken/Hebei/108/2002	Mouse (BALB/c)	Xu et al. (2006)
Influenza A H5N1 A/HongKong/156/97	Cynomolgus macaques	Kuiken et al. (2003) Rimmelzwaan et al. (2003)

Table 7
Summary of current animal models available for studying influenza virus-associated neurotropic disease.

Virus	Animal Model	References
Influenza A H1N1 A/WSN/33	Mouse (C57BL/6-BJ6)	Aronsson et al. (2003)
Influenza A H5N1 Recombinant R404BP A/WSN/33 X A/Aichi/2/68	Mouse (C57BL/6)	Mori et al. (2002)
Influenza A H5N1 A/Hong Kong/483/97	Mouse (BALB/cA Jcl)	Park et al. (2002)

6.1. Seasonal influenza

Mori et al. (2002) have used a highly neurovirulent recombinant R404BP strain of influenza A (H1N1) virus to test amantadine therapy in mice. Stereotaxic microinjection of virus into the olfactory bulb of the animal results in outward spread to neurons in broad areas of the brain parenchyma, leading to apoptotic neurodegeneration and fatal edema, beginning on day 8. However, amantadine treatment beginning as late as 7 days after virus injection protected the animals. The disadvantage of this clinically relevant model is that only researchers having stereotaxic microinjection equipment could use it.

A second study in mice used a neurovirulent A/WSN/33 strain of H1N1 influenza A virus administered intranasally, resulting in infection of olfactory neurons and anterograde axonal transport to the olfactory bulbs (Aronsson et al., 2003). Using immunodeficient mutant mice to examine how the immune system controls the spread and persistence of the virus, the authors showed that an absence of T and B cells allowed the infection to spread fully into the brain.

6.2. H5N1 avian influenza

In a simplified neurovirulence model, Park et al. (2002) demonstrated that simple intranasal instillation of influenza A (H5N1) virus isolated from a patient during the 1997 Hong Kong outbreak could result in the infection of the murine central nervous system. After initial replication in the respiratory mucosa, the virus apparently traveled through afferent fibers of the olfactory, vagal, trigeminal, and sympathetic nerves, where it was detected by immunohistochemical and *in situ* hybridization. This study demonstrates the feasibility of using intranasal installation of viruses isolated directly from patients to establish an encephalitis-like infection, an ideal way for quickly evaluating antiviral inhibitors.

Yun et al. (2008) injected peramivir intramuscularly into ferrets infected intranasally with the highly neurovirulent

A/Vietnam/1203/04 (H5N1) influenza virus and were able to show that peramivir delivered in this manner ameliorated virus-induced disease by reducing infectious virus titers in the lungs and brains and promoted survival in ferrets.

7. Animal models for the study of drug-resistant influenza virus infections

The potential for influenza viruses to develop resistance to amantadine and rimantadine has been well established in clinical studies (Monto and Arden, 1992; Hayden and Hay, 1992; Englund et al., 1998) and in mice (Sidwell et al., 2003). By contrast, viruses that have acquired resistance to zanamivir and oseltamivir generally display a significant reduction in infectivity in mice, compared to the parent viruses (Gubareva et al., 2000; Herlocher et al., 2004; Yen et al., 2005). This reduction in virulence has been evaluated by several means. In one study, mice were infected in parallel with equal quantities of the parental and the mutant viruses, and lungs were removed early in the infection (Gubareva et al., 1997). The virus titer in each was then determined and the titers of the challenge viruses needed to induce a 50% infection were compared or the amount of virus produced in the lungs by equivalent concentrations of the viruses was quantified (Tai et al., 1998). Alternatively, using a mouse-adapted influenza virus, the relative virulence of the mutant virus was assessed using parameters such as increased lung weight, lung pathogenesis scores, lung histopathology, and decreased SaO₂ levels of the lungs (Sidwell et al., 1995).

Ferrets have been used extensively to study the infectivity and virulence of influenza virus mutants resistant to the neuraminidase inhibitors (Blick et al., 1998; Gubareva et al., 1998b; Barnett et al., 2000). Blick et al. (1998) found that a zanamivir-resistant influenza virus with an NA and HA mutation was not resistant to zanamivir in ferrets, even though a small decrease in sensitivity to the drug had been shown in mice, indicating some potential differences in the two models when studying drug-resistant viruses. A summary of current animal models available for studying drug-resistant influenza virus infections is presented in Table 8.

8. Models of influenza in the immunocompromised host

Influenza can be particularly serious in individuals with congenital or acquired immunodeficiency because of aging, cancer treatment, organ transplantation, or human immunodeficiency virus infection (Lee and Barton, 2007). It is therefore important to have models in which to evaluate the effects of chemotherapy on influenza virus infections in immunosuppressed animals (Table 9).

Cyclophosphamide treatment has been used to immunosuppress mice to assess the efficacy of a neuraminidase inhibitor

Table 8
Summary of current animal models available for studying drug-resistant influenza virus infections.

Virus	Animal Model	Drug	References
Influenza A H4N2 A/turkey/Minnesota/833/80	Mouse (BALB/CBYJ)	Neuraminidase inhibitors	Gubareva et al. (1997)
Influenza A H1N1 NWS/G70C4-G	Mouse (C57BL6 X C57BL10) Ferret	Zanamivir	Blick et al. (1998)

Table 9
Summary of current animal models available for studying the immunocompromised host infected with influenza virus.

Virus	Disease Model	Animal Model	References
Influenza A H1N1, NWS/33	Cyclophosphamide immunosuppression Immunocompromised mouse	Mouse (BALB/c) SCID Mouse	Sidwell et al. (2003)
Influenza A H2N2, Japan/305/57	Immunocompromised mouse	SCID Mouse	Ison et al. (2006)

(peramivir) in an immunocompromised host (Sidwell et al., 2003). Cyclophosphamide was injected intraperitoneally at a dose of 100 mg/kg every 4 days for varying lengths of time. Peramivir was capable of significantly ameliorating the influenza virus infection in mice immunosuppressed by short-term or prolonged cyclophosphamide therapy.

Severe, combined immunodeficient (SCID) mice also provide a model for evaluating anti-influenza treatments in an immunocompromised host. In a recent study, SCID mice infected with an influenza A virus and treated with either oral oseltamivir or the experimental compound A-322278 showed reduced viral replication, limited weight loss and prolonged survival, so long as the treatments persisted (Sidwell et al., 2003). However, once treatment was discontinued, the animals had detectable, progressive viral replication with subsequent clinical decline. More importantly, drug-resistant virus was detected in the chronically infected, drug-treated mice, but not in the placebo-treated animals that died rapidly from infection.

9. Models of host resistance (immunocompetence after therapy)

Effective immune clearance of influenza virus requires an immune system that is intact and functioning normally. Host resistance models have therefore been used to evaluate the effect of an antiviral treatment on virus clearance, as an indicator of the immunocompetence of an animal during or after treatment and to test for the immunotoxicity of a particular agent (Burlison and Burlison, 2007). Mechanistic studies to detect immunotoxicity should include measuring innate, humoral and cell mediated immune responses, through parameters such as cytokine and interferon production, macrophage function, and natural killer cell function and cytotoxic T lymphocyte activity, as well as influenza-specific antibody during or after drug treatment. For example, the compound 3M-011, a synthetic human TLR7/8 agonist, was evaluated for antiviral efficacy and immunotoxicity in a rat HR model, which parallel human influenza infection, both with respect to time course and development of respiratory tract lesions (Hammerbeck et al., 2007; Burlison and Burlison, 2007). Intranasal (IN) administration of 3M-011 significantly inhibited H3N2 influenza viral replication in the nasal cavity when animals were pretreated or therapeutically treated with the compound. Inhibition correlated closely with induction of type I interferon and to a lesser extent with other cytokines such as TNF- α , IL-12, and IFN- γ from rat peripheral blood mononuclear cells, and seemed to have no negative effect on the immune response to infection.

10. Animal models of influenza A virus transmission

To pose a significant threat to humans, a novel influenza A virus must be able to replicate in the human respiratory tract and be transmitted efficiently from person to person. This is clearly demonstrated by the recently emerged H5N1 avian influenza virus, which causes rapidly overwhelming pneumonia, but has only rarely spread from a patient to close contacts. Animal models have been developed to evaluate the pandemic potential of influenza viruses, especially for the H5N1 agent. In one recent study, Yen et al. (2007) housed ferrets infected with a neuraminidase inhibitor resistant recombinant A/Vietnam/1203/04 (H5N1) influenza virus together with uninfected ferrets and monitored illness, detectable virus shedding lung viral titers and death. They observed only limited transmission of infection to health contacts, which correlated with low amounts of virus in respiratory secretions. The authors concluded that molecular determinants in addition to a virus's receptor binding affinity were responsible for influenza virus transmission among mammalian species (Yen et al., 2007).

The guinea pig has also been evaluated as a transmission model mammalian host for influenza virus. Guinea pigs were found to be highly susceptible to infection with the unadapted virus A/Panama/2007/99 (H3N2) virus, which replicated to high titers in the upper respiratory tract (Lowen et al., 2006). Although the animals did not sneeze or cough, virus was shed in nasal washings. In experiments performed at an ambient temperature of 20 °C and under low humidity, virus was transmitted from infected to non-infected guinea pigs housed either in the same cage, in an adjacent cage, or in a cage placed 91 cm away. Transmission appeared to be by droplet spread, and was more efficient at lower ambient temperature and humidity.

11. Conclusion

As shown in this review, several species of laboratory animals have proved to be very useful for research on influenza. Mice, particularly the BALB/c strain, are currently intensively employed for studying pathogenesis, for demonstrating the efficacy of antiviral drugs, and for preliminary efficacy studies for vaccines. Studies with antiviral agents have shown the infection in mice to be predictive of efficacy in humans. For example, all of the currently approved drugs for treating influenza virus infections were first shown to be efficacious in mice. Although mice are not readily susceptible to newly isolated human seasonal influenza viruses and require virus adaptation, many highly pathogenic avian influenza strains require no adaptation and readily infect mice to cause lethal disease. Mice also do not exhibit some of the clinical symptoms detected in humans such as nasal exudate, fever, sneezing, and coughing. However, many clinical signs that seem to be markers of disease severity can be measured in mice to study influenza disease, such as saturated oxygen levels, virus lung titers, cytokine levels, serum and acute phase proteins. In addition, the use of mouse models enable the investigator to rapidly evaluate the efficacy of antiviral agents and vaccines at relatively lesser costs than other animal models.

Ferrets are now being widely used to supplement or validate studies in mice, especially for H5N1 virus studies in order to comply with new FDA "two animal rule". A tremendous advantage of ferrets is that these animals display many clinical symptoms of human influenza. The model also has a similar complexity to that of humans. However, as with the mouse model, many host factors found associated with humans are missing or have not yet been discovered in the ferret. The costs of ferrets, space needed for housing, temperament of the animal once handled experimentally, and the availability of animals without prior exposure to influenza remain of concern.

The other animal species that could be used as models have not been sufficiently studied for their suitability for routine use in studying influenza disease and influenza pathogenesis. In addition, immune reagents for some of these other species remain yet to be developed. The availability of some of the species is very limited, and the costs for housing and handling of some of the larger species are great.

Despite the evidence of the great influenza pandemic of 1918, which brought about the death of some 50 million people, influenza is often viewed as a "nuisance" disease, for which adequate countermeasures are already available. However, the recent emergence of the highly virulent H5N1 avian influenza virus in Southeast Asia has served as a wake-up call, reminding the medical community that novel influenza A viruses can unexpectedly enter the human population and that even the currently circulating seasonal strains are responsible for hundreds of thousands of deaths worldwide each year. Influenza A viruses, far from being benign respiratory pathogens, are a major public health problem that calls for a serious and sustained research response. An essential part of that process

is the development of laboratory animal models of influenza virus infection for the study of pathogenesis and the evaluation of new drugs and vaccines. As described in this paper, a number of animal models have been developed, but many gaps remain. Many of the current models remain incompletely characterized, and none has been employed to its full potential. New and better models are needed to provide more accurate answers to critical questions in pathogenesis and therapy. It is hoped that this paper will contribute to the better use of laboratory animal models to combat the continuing threat of influenza.

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References

- Alarcon, J.B., Hartley, A.W., Harvey, N.G., Mikszta, J.A., 2007. Preclinical evaluation of microneedle technology for intradermal delivery of influenza vaccines. *Clin. Vaccine Immunol.* 14, 375–381.
- Alonso, J.-M., Guiyoule, A., Zarattonelli, M.L., Ramiere, F., Pires, R., Antignac, A., Deghmane, A.E., Huerre, M., van der Werf, S., Taha, M.-K., 2003. A model of meningococcal bacteremia after respiratory superinfection in influenza A virus-infected mice. *FEMS Microbiol. Lett.* 222, 99–106.
- Aronsson, F., Robertson, B., Ljunggren, H.G., Kristensson, K., 2003. Invasion and persistence of the neuroadapted influenza virus A/WSN/33 in the mouse olfactory system. *Viral Immunol.* 16, 415–513.
- Baas, T., Baskin, C.R., Diamond, D.L., Garcia-Sastre, A., Bielefeldt-Ohmann, H., Tumpey, T.M., Thomas, M.J., Carter, V.S., Teal, T.H., Van Hoven, N., Prohl, S., Jacobs, J.M., Caldwell, Z.R., Gritsenko, M.A., Hukkanen, R.R., Camp II, D.G., Smith, R.D., Katze, M.G., 2006. Integrated molecular signature of disease: analysis of influenza virus-infected macaques through functional genomics and proteomics. *J. Virol.* 80, 10813–10828.
- Barnard, D.L., Wong, M.-H., Bailey, K., Day, C.W., Sidwell, R.W., Hickok, S.S., Hall, T.J., 2007. Effect of oral gavage treatment with ZnAL42 and other metallo-ion formulations on influenza A H5N1 and H1N1 virus infections in mice. *Antivir. Chem. Chemother.* 18, 125–132.
- Barnett, J.M., Cadman, A., Gor, D., Dempsey, M., Walters, M., Candlin, A., Tisdale, M., Morley, P.J., Owens, I.J., Fenton, R.J., Lewis, A.P., Claas, E.C., Rimmelzwaan, G.F., De Groot, R., Osterhaus, A.D., 2000. Zanamivir susceptibility monitoring and characterization of influenza virus clinical isolates obtained during phase II clinical efficacy studies. *Antimicrob. Agents Chemother.* 44, 78–87.
- Baskin, C.R., Garcia-Sastre, A., Tumpey, T.M., Bielefeldt-Ohmann, H., Carter, V.S., Nistal-Villan, E., Katze, M.G., 2004. Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (*Macaca nemestrina*). *J. Virol.* 78, 10420–10432.
- Beigel, J.H., Farrar, J., Han, A.M., Hayden, F.G., Hyer, R., de Jong, M.D., Lochindarat, S., Nguyen, T.K., Nguyen, T.H., Tran, T.H., Nicoll, A., Touch, S., Yuen, K.Y., 2005. Avian influenza A (H5N1) infection in humans. *N. Engl. J. Med.* 353, 1374–1385.
- Beigel, J., Bray, M., 2008. Current and future antiviral therapy of severe seasonal and avian influenza. *Antiviral Res.* 78, 91–102.
- Blick, T.J., Sahasrabudhe, A., McDonald, M., Owens, I.J., Morley, P.J., Fenton, R.J., McKimm-Breschkin, J.L., 1998. The interaction of neuraminidase and hemagglutinin mutations in influenza virus in resistance to 4-guanidino-Neu5Ac2en. *Virology* 246, 95–103.
- Bhat, N., Wright, J.G., Broder, K.R., Murray, E.L., Greenberg, M.E., Glover, M.J., Likos, A.M., Posey, D.L., Klimov, A., Lindstrom, S.E., Balish, A., Medina, M.-j., Wallis, T.R., Guarner, J., Paddock, C.D., Shieh, W.-j., Zaki, S.R., Sejvar, J.J., Shay, D.K., Harper, S.A., Cox, N.J., Fukuda, K., Uyek, T.M., for the Influenza Special Investigations Team, 2005. Influenza-associated deaths among children in the United States, 2003–2004. *N. Engl. J. Med.* 353, 2559–2567.
- Boltz, D.A., Ilyushina, N.A., Arnold, C.S., Babu, Y.S., Webster, R.G., Govorkova, E.A., 2008a. Intramuscularly administered neuraminidase inhibitor peramivir is effective against lethal H5N1 influenza virus in mice. *Antiviral Res.* 80, 150–157.
- Boltz, D.A., Rehg, J.E., McClaren, J., Webster, R.G., Govorkova, E.A., 2008b. Oseltamivir prophylactic regimens prevent H5N1 influenza morbidity and mortality in a ferret model. *J. Infect. Dis.* 197, 1315–1323.
- Braun, L.E., Sutter, D.E., Eichelberger, M.C., Pletneva, L., Kokai-Kun, J.F., Blanco, J.C., Prince, G.A., Ottolini, M.G., 2007. Co-infection of the cotton rat (*Sigmodon hispidus*) with *Staphylococcus aureus* and influenza A virus results in synergistic disease. *Microb. Pathog.* 43, 208–216.
- Burleson, G.R., Burleson, F.G., 2007. Influenza virus host resistance model. *Methods* 41, 31–37.
- Cameron, C.M., Cameron, M.J., Bermejo-Martin, J.F., Ran, L., Xu, L., Turner, P.V., Ran, R., Danesh, A., Fang, Y., Chan, P.-K.M., Mytle, N., Sullivan, T.J., Collins, T.L., Johnson, M.G., Medina, J.C., Rowe, T., Kelvin, D.J., 2008. Gene expression analysis of host innate immune responses during lethal H5N1 infection in ferrets. *J. Virol.* 82, 11308–11317.
- Chen, Q., Kuang, H., Wang, H., Fang, F., Yang, Z., Zhang, Z., Zhang, X., Chen, Z., 2009. Comparing the ability of a series of viral protein-expressing plasmid DNAs to protect against H5N1 influenza virus. *Virus Genes.* 38, 30–38.
- Chen, Z., Santos, C., Aspelund, A., Gillim-Ross, L., Jin, H., Kemble, G., Subbarao, K., 2009. Evaluation of live attenuated influenza A virus h6 vaccines in mice and ferrets. *J. Virol.* 83, 65–72.
- Cleaveland, S., Meslin, F.X., Breiman, R., 2006. Dogs can play useful role as sentinel hosts for disease. *Nature* 440, 605.
- Daniels, M.J., Selgrade, M.K., Doerfler, D., Gilmour, M.I., 2003. Kinetic profile of influenza virus infection in three rat strains. *Comp. Med.* 53, 293–298.
- de Vries, J.J., Bungener, L., Ter Veer, W., van Alphen, L., van der Ley, P., Wilschut, J., Huckriede, A., 2009. Incorporation of LpxL1, a detoxified lipopolysaccharide adjuvant, in influenza H5N1 virosomes increases vaccine immunogenicity. *Vaccine* 27, 947–955.
- Dimmock, N.J., Rainsford, E.W., Scott, P.D., Marriott, A.C., 2008. Influenza virus protecting RNA: an effective prophylactic and therapeutic antiviral. *J. Virol.* 82, 8570–8578.
- Dushoff, J., Plotkin, J.B., Viboud, C., Earn, D.J., Simonsen, L., 2006. Mortality due to influenza in the United States—an annualized regression approach using multiple-cause mortality data. *Am. J. Epidemiol.* 163, 181–187.
- Dybing, J.K., Schultz-Cherry, S., Swayne, D.E., Suarez, D.L., Perdue, M.L., 2000. Distinct pathogenesis of Hong Kong–origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. *J. Virol.* 74, 1443–1450.
- Eichelberger, M.C., Prince, G.A., Ottolini, M.G., 2004. Influenza-induced tachypnea is prevented in immune cotton rats, but cannot be treated with an anti-inflammatory steroid or a neuraminidase inhibitor. *Virology* 322, 300–307.
- Eichelberger, M.C., 2007. The cotton rat as a model to study influenza pathogenesis and immunity. *Viral Immunol.* 20, 243–249.
- Englund, J.A., Champlin, R.E., Wyde, P.R., Kantarjian, H., Atmar, R.L., Tarrand, J., Yousuf, H., Regnery, H., Klimov, A.I., Cox, N.J., Whimby, E., 1998. Common emergence of amantadine- and rimantadine-resistant influenza A viruses in symptomatic immunocompromised adults. *Clin. Infect. Dis.* 26, 1418–1424.
- Evseenko, V.A., Bukin, E.K., Zaykovskaya, A.V., Sharshov, K.A., Ternovoi, V.A., Ignatyev, G.M., Shestopalov, A.M., 2007. Experimental infection of H5N1 HPAI in BALB/c mice. *J. Virol.* 81, 77.
- Gambaryan, A.S., Boravleva, E.Y., Matrosovich, T.Y., Matrosovich, M.N., Klenk, H.D., Moiseeva, E.V., Tuzikov, A.B., Chinarev, A.A., Pazynina, G.V., Bovin, N.V., 2005. Polymer-bound 6'-sialyl-N-acetylglucosamine protects mice infected by influenza virus. *Antiviral Res.* 68, 116–123.
- Giese, M., Harder, T.C., Teifke, J.P., Klopffleisch, R., Breithaupt, A., Mettenleiter, T.C., Vahlenkamp, T.W., 2008. Experimental infection and natural contact exposure of dogs with avian influenza virus (H5N1). *Emerg. Infect. Dis.* 14, 308–310.
- Gilbert, B.E., McCleay, M.T., 2008. MegaRibavirin aerosol for the treatment of influenza A virus infections in mice. *Antiviral Res.* 78, 223–229.
- Govorkova, E.A., Ilyushina, N.A., Boltz, D.A., Douglas, A., Yilmaz, N., Webster, R.G., 2007. Efficacy of oseltamivir therapy in ferrets inoculated with different clades of H5N1 influenza virus. *Antimicrob. Agents Chemother.* 51, 1414–1424.
- Grose, C., Chokephaibulkit, K., 2004. Avian influenza virus infection of children in Vietnam and Thailand. *Ped. Infect. Dis. J.* 23, 793–794.
- Gubareva, L.V., Robinson, M.J., Bethell, R.C., Webster, R.G., 1997. Catalytic and framework mutations in the neuraminidase active site of influenza viruses that are resistant to 4-guanidino-Neu5Ac2en. *J. Virol.* 71, 3385–3390.
- Gubareva, L.V., McCullers, J.A., Bethell, R.C., Webster, R.G., 1998a. Characterization of influenza A/HongKong/156/97 (H5N1) virus in a mouse model and protective effect of zanamivir on H5N1 infection in mice. *J. Infect. Dis.* 178, 1592–1596.
- Gubareva, L.V., Matrosovich, M.N., Brenner, M.K., Bethell, R.C., Webster, R.G., 1998b. Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. *J. Infect. Dis.* 178, 1257–1262.
- Gubareva, L.V., Kaiser, L., Hayden, F.G., 2000. Influenza virus neuraminidase inhibitors. *Lancet* 355, 827–835.
- Gupta, R.K., George, R., Nguyen-Van-Tam, J.S., 2008. Bacterial pneumonia and pandemic influenza planning. *Emerg. Infect. Dis.* 14, 1187–1192.
- Hagenaars, N., Mastrobattista, E., Glansbeek, H., Helden, J., van den Bosch, H., Schijns, V., Betheder, D., Vromans, H., Jiskoot, W., 2008. Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model. *Vaccine* 26, 6555–6563.
- Hai, R., Martinez-Sobrido, L., Fraser, K.A., Ayllon, J., Garcia-Sastre, A., Palese, P., 2008. Influenza B virus NS1-truncated mutants: live-attenuated vaccine approach. *J. Virol.* 82, 10580–10590.
- Hammerbeck, D.M., Burleson, G.R., Schuller, C.J., Vasilakos, J.P., Tomai, M., Egging, E., Cochran, F.R., Woulfe, S., Miller, R.L., 2007. Administration of a dual toll-like receptor 7 and toll-like receptor 8 agonist protects against influenza in rats. *Antiviral Res.* 73, 1–11.
- Hansen, A.K., Farlov, H., Bollen, P., 1997. Microbiological monitoring of laboratory pigs. *Lab. Anim.* 31, 193–200.
- Hatta, M., Kawaoka, Y., 2005. A clue to the molecular mechanism of virulence of highly pathogenic H5N1 avian influenza viruses isolated in 2004. *Uirusu* 55, 55–61.
- Hatta, M., Hatta, Y., Kim, J.H., Watanabe, S., Shinya, K., Nguyen, T., Lien, P.S., Le, Q.M., Kawaoka, Y., 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. *PLoS Pathog.* 3, 1374–1379.
- Hayashi, K., Kadowaki, S.-e., Takei, M., Fukuda, H., 2006. Efficacy of quinolones against secondary pneumococcal pneumonia after influenza virus infection in mice. *Antimicrob. Agents Chemother.* 50, 748–751.

- Hayden, F.G., Hay, A.J., 1992. Emergence and transmission of influenza A viruses resistant to amantadine and rimantadine. *Curr. Top. Microbiol. Immunol.* 176, 119–130.
- Herlocher, M.L., Truscon, R., Elias, S., Yen, H.L., Roberts, N.A., Ohmit, S.E., Monto, A.S., 2004. Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. *J. Infect. Dis.* 190, 1627–1630.
- Hickman, D., Hossain, M.J., Song, H., Araya, Y., Solorzano, A., Perez, D.R., 2008. An avian live attenuated master backbone for potential use in epidemic and pandemic influenza vaccines. *J. Gen. Virol.* 89, 2682–2690.
- Huber, V.C., Kleimeyer, L.H., McCullers, J.A., 2008. Live, attenuated influenza virus (LAIV) vehicles are strong inducers of immunity toward influenza B virus. *Vaccine* 26, 5381–5388.
- Ibricevic, A., Pekosz, A., Walter, M.J., Newby, C., Battaile, J.T., Brown, E.G., Holtzman, M.J., Brody, S.L., 2006. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. *J. Virol.* 80, 7469–7480.
- Ilyushina, N.A., Hay, A., Yilmaz, N., Boon, A.C., Webster, R.G., Govorkova, E.A., 2008. Oseltamivir–ribavirin combination therapy for highly pathogenic H5N1 influenza virus infection in mice. *Antimicrob. Agents Chemother.* 52, 3889–3897.
- Isoda, N., Sakoda, Y., Kishida, N., Bai, G.R., Matsuda, K., Umemura, T., Kida, H., 2006. Pathogenicity of a highly pathogenic avian influenza virus. A/chicken/Yamaguchi/7/04 (H5N1) in different species of birds and mammals. *Arch. Virol.* 151, 1267–1279.
- Ison, M.G., Mishin, V.P., Braciale, T.J., Hayden, F.G., Gubareva, L.V., 2006. Comparative activities of oseltamivir and A-322278 in immunocompetent and immunocompromised murine models of influenza virus infection. *J. Infect. Dis.* 193, 765–772.
- Jeffery, K.T., Ann, H.R., Thomas, A.J., Thomas, G.F., 2001. Integrating historical, clinical and molecular genetic data in order to explain the origin and virulence of the 1918 Spanish influenza virus. *Philos. Trans. R. Soc. B: Biol. Sci.* 356, 1829–1839.
- Karaca, K., Swayne, D.E., Grosenbaugh, D., Bublot, M., Robles, A., Spackman, E., Nordgren, R., 2005. Immunogenicity of fowlpox virus expressing the avian influenza virus H5 gene (TROVAC AIV-H5) in cats. *Clin. Diagn. Lab. Immunol.* 12, 1340–1342.
- Katz, J.M., Lu, X., Tumpey, T.M., Smith, C.B., Shaw, M.W., Subbarao, K., 2000a. Molecular correlates of influenza A H5N1 virus pathogenesis in mice. *J. Virol.* 74, 10807–10810.
- Katz, J.M., Lu, X., Frace, A.M., Morken, T., Zaki, S.R., Tumpey, T.M., 2000b. Pathogenesis of and immunity to avian influenza A H5 viruses. *Biomed. Pharmacother.* 54, 178–187.
- Kreijtz, J.H., Suezter, Y., de Mutsert, G., van den Brand, J.M., van Amerongen, G., Schrieler, B.S., Kuiken, T., Fouchier, R.A., Lower, J., Osterhaus, A.D., Sutter, G., Rimmelzwaan, G.F., 2009. Recombinant modified vaccinia virus Ankara expressing the hemagglutinin gene confers protection against homologous and heterologous H5N1 influenza virus infections in macaques. *J. Infect. Dis.* 199, 405–413.
- Kuiken, T., Rimmelzwaan, G.F., Van Amerongen, G., Osterhaus, A.D., 2003. Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (*Macaca fascicularis*). *Vet. Pathol.* 40, 304–310.
- Lambkin, R., Oxford, J.S., Bossuyt, S., Mann, A., Metcalfe, I.C., Herzog, C., Viret, J.-F., Glück, R., 2004. Strong local and systemic protective immunity induced in the ferret model by an intranasal virosome-formulated influenza subunit vaccine. *Vaccine* 22, 4390–4396.
- Lee, I., Barton, T.D., 2007. Viral respiratory tract infections in transplant patients: Epidemiology, recognition and management. *Drugs* 67, 1411–1427.
- Lipatov, A.S., Kwon, Y.K., Sarmento, L.V., Lager, K.M., Spackman, E., Suarez, D.L., Swayne, D.E., 2008. Domestic pigs have low susceptibility to H5N1 highly pathogenic avian influenza viruses. *PLoS Pathog.* 4, e1000102.
- Lalor, P.A., Webby, R.J., Morrow, J., Rusalov, D., Kaslow, D.C., Rolland, A., Smith, L.R., 2008. Plasmid DNA-based vaccines protect mice and ferrets against lethal challenge with A/Vietnam/1203/04 (H5N1) influenza virus. *J. Infect. Dis.* 197, 1643–1652.
- Lowen, A.C., Mubareka, S., Tumpey, T.M., Garcia-Sastre, A., Palese, P., 2006. The guinea pig as a transmission model for human influenza viruses. *PNAS* 103, 9988–9992.
- Mahmood, K., Bright, R.A., Mytle, N., Carter, D.M., Crevar, C.J., Achenbach, J.E., Heaton, P.M., Tumpey, T.M., Ross, T.M., 2008. H5N1 VLP vaccine induced protection in ferrets against lethal challenge with highly pathogenic H5N1 influenza viruses. *Vaccine* 26, 5393–5399.
- Maines, T.R., Lu, X.H., Erb, S.M., Edwards, L., Guarner, J., Greer, P.W., Nguyen, D.C., Szretter, K.J., Chen, L.M., Thawatsupha, P., Chittaganpitch, M., Waichareon, S., Nguyen, D.T., Nguyen, T., Nguyen, H.H., Kim, J.H., Hoang, L.T., Kang, C., Phuong, L.S., Lim, W., Zaki, S., Donis, R.O., Cox, N.J., Katz, J.M., Tumpey, T.M., 2005. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. *J. Virol.* 79, 11788–11800.
- Malakhov, M.P., Aschenbrenner, L.M., Smees, D.F., Wandersee, M.K., Sidwell, R.W., Gubareva, L.V., Mishin, V.P., Hayden, F.G., Kim, D.H., Ing, A., Campbell, E.R., Yu, M., Fang, F., 2006. Sialidase fusion protein as a novel broad-spectrum inhibitor of influenza virus infection. *Antimicrob. Agents Chemother.* 50, 1470–1479.
- Matrosovich, M.N., Matrosovich, T.Y., Gray, T., Roberts, N.A., Klenk, H.-D., 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. *PNAS* 101, 4620–4624.
- McCullers, J.A., English, B.K., 2008. Improving therapeutic strategies for secondary bacterial pneumonia following influenza. *Future Microbiol.* 3, 397–404.
- Mizuguchi, M., Yamanouchi, H., Ichiyama, T., Shiomi, M., 2007. Acute encephalopathy associated with influenza and other viral infections. *Acta Neurol. Scand.* 115, 45–56.
- Monto, A.S., Arden, N.H., 1992. Implications of viral resistance to amantadine in control of influenza A. *Clin. Infect. Dis.* 15, 362–367 (discussion 368–369).
- Morens, D.M., Taubenberger, J.K., Fauci, A.S., 2008. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J. Infect. Dis.* 198, 962–970.
- Mori, I., Liu, B., Hossain, M.J., Takakuwa, H., Daikoku, T., Nishiyama, Y., Naiki, H., Matsumoto, K., Yokochi, T., Kimura, Y., 2002. Successful protection by amantadine hydrochloride against lethal encephalitis caused by a highly neurovirulent recombinant influenza A virus in mice. *Virology* 303, 287–296.
- Neville, L.F., Mathiak, G., Bagasra, O., 1997. The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. *Cytokine Growth Factor Rev.* 8, 207–219.
- Ottolini, M.G., Blanco, J.C.G., Eichelberger, M.C., Porter, D.D., Pletneva, L., Richardson, J.Y., Prince, G.A., 2005. The cotton rat provides a useful small-animal model for the study of influenza virus pathogenesis. *J. Gen. Virol.* 86, 2823–2830.
- Oxford, J.S., Lambkin, R., Guralnik, M., Rosenbloom, R.A., Petteruti, M.P., Digian, K., LeFante, C., 2007. In vivo prophylactic activity of QR-435 against H3N2 influenza virus infection. *Am. J. Ther.* 14, 462–468.
- Park, C.H., Ishinaka, M., Takada, A., Kida, H., Kimura, T., Ochiai, K., Umemura, T., 2002. The invasion routes of neurovirulent A/Hong Kong/483/97 (H5N1) influenza virus into the central nervous system after respiratory infection in mice. *Arch. Virol.* 147, 1425–1436.
- Parks, C.L., Latham, T., Cahill, A., O'Neill, R.E., Passarotti, C.J., Buonagurio, D.A., Bechert, T.M., D'Arco, G.A., Neumann, G., Destefano, J., Arendt, H.E., Obregon, J., Shutyak, L., Hamm, S., Sidhu, M.S., Zamb, T.J., Udem, S.A., 2007. Phenotypic properties resulting from directed gene segment reassortment between wild-type A/Sydney/5/97 influenza virus and the live attenuated vaccine strain. *Virology* 367, 275–287.
- Peltola, V.T., Boyd, K.L., McAuley, J.L., Rehg, J.E., McCullers, J.A., 2006. Bacterial sinusitis and otitis media following influenza virus infection in ferrets. *Infect. Immun.* 74, 2562–2567.
- Peiris, J.S.M., de Jong, M.D., Guan, Y., 2007. Avian influenza virus (H5N1): a threat to human health. *Clin. Microbiol. Rev.* 20, 243–267.
- Perrone, L.A., Plowden, J.K., Garcia-Sastre, A., Katz, J.M., Tumpey, T.M., 2008. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. *PLoS Pathog.* 4, e1000115.
- Pinto, C.A., Haff, R.F., Stewart, R.C., 1969. Pathogenesis of and recovery from respiratory syncytial and influenza infections in ferrets. *Arch. Gesamte Virusforsch.* 26, 225–237.
- Potter, C.W., Phair, J.P., Vodinelich, L., Fenton, R., Jennings, R., 1976. Antiviral, immunosuppressive and antitumour effects of ribavirin. *Nature* 259, 496–497.
- Reading, P.C., Bozza, S., Gilbertson, B., Tate, M., Moretti, S., Job, E.R., Crouch, E.C., Brooks, A.G., Brown, L.E., Bottazzi, B., Romani, L., Mantovani, A., 2008. Antiviral activity of the long chain Pentraxin PTX3 against influenza viruses. *J. Immunol.* 180, 3391–3398.
- Rimmelzwaan, G.F., Baars, M., van Amerongen, G., van Beek, R., Osterhaus, A.D.M.E., 2001. A single dose of an ISCOM influenza vaccine induces long-lasting protective immunity against homologous challenge infection but fails to protect *Cynomolgus macaques* against distant drift variants of influenza A (H3N2) viruses. *Vaccine* 20, 158–163.
- Rimmelzwaan, G.F., Kuiken, T., van Amerongen, G., Bestebroer, T.M., Fouchier, R.A., Osterhaus, A.D., 2003. A primate model to study the pathogenesis of influenza A (H5N1) virus infection. *Avian Dis.* 47, 931–933.
- Rimmelzwaan, G.F., van Riel, D., Baars, M., Bestebroer, T.M., van Amerongen, G., Fouchier, R.A.M., Osterhaus, A.D.M.E., Kuiken, T., 2006. Influenza A Virus (H5N1) Infection in Cats Causes Systemic Disease with Potential Novel Routes of Virus Spread within and between Hosts. *Am. J. Pathol.* 168, 176–183.
- Rosseau, S., Hocke, A., Mollenkopf, H., Schmeck, B., Suttorp, N., Kaufmann, S.H.E., Zerrahn, J., 2007. Comparative transcriptional profiling of the lung reveals shared and distinct features of *Streptococcus pneumoniae* and influenza A virus infection. *Immunology* 120, 380–391.
- Ruat, C., Caillet, C., Bidaut, A., Simon, J., Osterhaus, A.D.M.E., 2008. Vaccination of macaques with adjuvanted formalin-inactivated influenza A virus (H5N1) vaccines: protection against H5N1 challenge without disease enhancement. *J. Virol.* 82, 2565–2569.
- Rutigliano, J.A., Doherty, P.C., Franks, J., Morris, M.Y., Reynolds, C., Thomas, P.G., 2008. Screening monoclonal antibodies for cross-reactivity in the ferret model of influenza infection. *J. Immunol. Methods* 336, 71–77.
- Subbarao, K., Luke, C., 2007. H5N1 viruses and vaccines. *PLoS Pathog.* 3, e40.
- Sidwell, R.W., Bailey, K.W., Wong, M.H., Huffman, J.H., 1995. In vitro and in vivo sensitivity of a non-mouse-adapted influenza A (Beijing) virus infection to amantadine and ribavirin. *Chemotherapy* 41, 455–461.
- Sidwell, R.W., Smees, D.F., 2000. In vitro and in vivo assay systems for study of influenza virus inhibitors. *Antiviral Res.* 48, 1–16.
- Sidwell, R.W., Bailey, K.W., Morrey, J.D., Wong, M.H., Baldwin, T.J., Smees, D.F., 2003. Inhibition of influenza virus infections in immunosuppressed mice with orally administered peramivir (BCX-1812). *Antiviral Res.* 60, 17–25.
- Sidwell, R.W., Smees, D.F., 2004. Experimental disease models of influenza virus infections: recent developments. *Drug Discov. Today Dis. Models* 1, 57–63.
- Smees, D.F., Wong, M.H., Bailey, K.W., Sidwell, R.W., 2006. Activities of oseltamivir and ribavirin used alone and in combination against infections in mice with recent isolates of influenza A (H1N1) and B viruses. *Antivir. Chem. Chemother.* 17, 185–192.
- Smees, D.F., Bailey, K.W., Wong, M.-H., O'Keefe, B.R., Gustafson, K.R., Mishin, V.P., Gubareva, L.V., 2008. Treatment of influenza A (H1N1) virus infections in mice and ferrets with cyanovirin-N. *Antiviral Res.* 80, 266–271.

- Smith, H., Sweet, C., 1988. Lessons for human influenza from pathogenicity studies with ferrets. *Rev. Infect. Dis.* 10, 56–75.
- Smith, M.W., Schmidt, J.E., Rehg, J.E., Orihuela, C.J., McCullers, J.A., 2007. Induction of pro- and anti-inflammatory molecules in a mouse model of pneumococcal pneumonia after influenza. *Comp. Med.* 57, 82–89.
- Speshock, J.L., Doyon-Reale, N., Rabah, R., Neely, M.N., Roberts, P.C., 2007. Filamentous influenza A virus infection predisposes mice to fatal septicemia following superinfection with *Streptococcus pneumoniae* serotype 3. *Infect. Immun.* 75, 3102–3111.
- Steininger, C., Popow-Kraupp, T., Laferl, H., Seiser, A., Godl, I., Djamshidian, S., Puchhammer-Stockl, E., 2003. Acute encephalopathy associated with influenza A virus infection. *Clin. Infect. Dis.* 36, 567–574.
- Stertz, S., Dittmann, J., Blanco, J.C., Pletneva, L.M., Haller, O., Kochs, G., 2007. The antiviral potential of interferon-induced cotton rat Mx proteins against orthomyxovirus (influenza), rhabdovirus, and bunyavirus. *J. Interferon Cytokine Res.* 27, 847–855.
- Stittelaar, K.J., Tisdale, M., van Amerongen, G., van Lavieren, R.F., Pistor, F., Simon, J., Osterhaus, A.D., 2008. Evaluation of intravenous zanamivir against experimental influenza A (H5N1) virus infection in cynomolgus macaques. *Antiviral Res.* 80, 225–228.
- Straight, T.M., Ottolini, M.G., Prince, G.A., Eichelberger, M.C., 2008. Antibody contributes to heterosubtypic protection against influenza A-induced tachypnea in cotton rats. *Virology* 376, 53–59.
- Sun, K., Metzger, D.W., 2008. Inhibition of pulmonary antibacterial defense by interferon- γ during recovery from influenza infection. *Nat. Med.* 14, 558–564.
- Svitek, N., Rudd, P.A., Obojes, K., Pilet, S., von Messling, V., 2008. Severe seasonal influenza in ferrets correlates with reduced interferon and increased IL-6 induction. *Virology* 376, 53–59.
- Szretter, K.J., Gangappa, S., Lu, X., Smith, C., Shieh, W.-J., Zaki, S.R., Sambhara, S., Tumpey, T.M., Katz, J.M., 2007. Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. *J. Virol.* 81, 2736–2744.
- Tai, C.Y., Escarpe, P.A., Sidwell, R.W., Williams, M.A., Lew, W., Wu, H., Kim, C.U., Mendel, D.B., 1998. Characterization of human influenza virus variants selected in vitro in the presence of the neuraminidase inhibitor GS 4071. *Antimicrob. Agents Chemother.* 42, 3234–3241.
- Taubenberger, J.K., 2006. The origin and virulence of the 1918 “Spanish” influenza virus. *Proc. Am. Philos. Soc.* 150, 86–112.
- Taubenberger, J.K., Morens, D.M., 2008. The pathology of influenza virus infections. *Ann. Rev. Pathol.: Mech. Dis.* 3, 499–522.
- Vahlenkamp, T.W., Harder, T.C., Giese, M., Lin, F., Teifke, J.P., Klopfleisch, R., Hoffmann, R., Tarpey, I., Beer, M., Mettenleiter, T.C., 2008. Protection of cats against lethal influenza H5N1 challenge infection. *J. Gen. Virol.* 89, 968–974.
- van der Laan, J.W., Herberts, C., Lambkin-Williams, R., Boyers, A., Mann, A.J., Oxford, J., 2008. Animal models in influenza vaccine testing. *Exp. Rev. Vaccines* 7, 783–793.
- van Riel, D., Munster, V.J., de Wit, E., Rimmelzwaan, G.F., Fouchier, R.A.M., Osterhaus, A.D.M.E., Kuiken, T., 2006. H5N1 Virus attachment to lower respiratory tract. *Science* 312, 399.
- Vincent, A.L., Ma, W., Lager, K.M., Janke, B.H., Webby, R.J., Garcia-Sastre, A., Richt, J.A., 2007. Efficacy of intranasal administration of a truncated NS1 modified live influenza virus vaccine in swine. *Vaccine* 25, 7999–8009.
- Wang, R., Song, A., Levin, J., Dennis, D., Zhang, N.J., Yoshida, H., Koriazova, L., Madura, L., Shapiro, L., Matsumoto, A., Yoshida, H., Mikayama, T., Kubo, R.T., Sarawar, S., Cheroutre, H., Kato, S., 2008. Therapeutic potential of a fully human monoclonal antibody against influenza A virus M2 protein. *Antiviral Res.* 80, 168–177.
- Wright, P.F., Neumann, G., Kawaoka, Y., 2007. Orthomyxoviruses. In: Knipe, D.M., Howley, P.M. (Eds.), *Fields Virology*, fifth ed. Lippincott Williams & Wilkins, Philadelphia, pp. 1691–1740.
- Xu, T., Qiao, J., Zhao, L., Wang, G., He, G., Li, K., Tian, Y., Gao, M., Wang, J., Wang, H., Dong, C., 2006. Acute respiratory distress syndrome induced by avian influenza A (H5N1) virus in mice. *Am. J. Respir. Crit. Care Med.* 74, 1011–1017.
- Yen, H.L., Herlocher, L.M., Hoffmann, E., Matrosovich, M.N., Monto, A.S., Webster, R.G., Govorkova, E.A., 2005. Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. *Antimicrob. Agents Chemother.* 49, 4075–4084.
- Yen, H.L., Ilyushina, N.A., Salomon, R., Hoffmann, E., Webster, R.G., Govorkova, E.A., 2007. Neuraminidase inhibitor resistant recombinant A/Vietnam/1203/04 (H5N1) influenza viruses retain their replication efficiency and pathogenicity in vitro and in vivo. *J. Virol.* 81, 12418–12426.
- Yun, N.E., Linde, N.S., Zacks, M.A., Barr, I.G., Hurt, A.C., Smith, J.N., Dziuba, N., Holbrook, M.R., Zhang, L., Kilpatrick, J.M., Arnold, C.S., Paessler, S., 2008. Injectable peramivir mitigates disease and promotes survival in ferrets and mice infected with the highly virulent influenza virus A/Vietnam/1203/04 (H5N1). *Virology* 374, 198–209.
- Zheng, B.J., Chan, K.W., Lin, Y.P., Zhao, G.Y., Chan, C., Zhang, H.J., Chen, H.L., Wong, S.S., Lau, S.K., Woo, P.C., Chan, K.H., Jin, D.Y., Yuen, K.Y., 2008. Delayed antiviral plus immunomodulator treatment still reduces mortality in mice infected by high inoculum of influenza A/H5N1 virus. *Proc. Natl. Acad. Sci. U.S.A.* 105, 8091–8096.
- Zitzow, L.A., Rowe, T., Morken, T., Shieh, W.-J., Zaki, S., Katz, J.M., 2002. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. *J. Virol.* 76, 4420–4429.