Monographs in Virology

Vol. 27

Series Editor

H.W. Doerr Frankfurt a.M.

Avian Influenza

Volume Editors

H.-D. Klenk Marburg
M.N. Matrosovich Marburg
J. Stech Greifswald

35 figures, 16 in color, and 12 tables, 2008

KARGER

Basel · Freiburg · Paris · London · New York · Bangalore ·
Bangkok · Shanghai · Singapore · Tokyo · Sydney

Hans-Dieter Klenk
Institute of Virology
Philipps University
Hans-Meerwein-Strasse 2
DE-35043 Marburg (Germany)

Mikhail N. Matrosovich
Institute of Virology
Philipps University
Hans-Meerwein-Strasse 2
DE-35043 Marburg (Germany)

Jürgen Stech
Friedrich-Loeffler-Institute
Federal Research Institute for Animal Health
Institute of Molecular Biology
Südufer 10
DE-17493 Greifswald-Insel Riems (Germany)

Library of Congress Cataloging-in-Publication Data

Avian influenza / volume editors, H.-D. Klenk, M.N. Matrosovich, J. Stech.
p. ; cm. – (Monographs in virology ; v. 27)
Includes bibliographical references and indexes.
ISBN 978-3-8055-8501-9 (hard cover : alk. paper)
1. Avian influenza. I. Klenk, H.-D. (Hans-Dieter), 1938– II. Matrosovich, M. N. (Mikhail N.) III. Stech, J. (Jürgen) IV. Series.
[DNLM: 1. Influenza in Birds–epidemiology. 2. Influenza A virus–pathogenicity. 3. Influenza, Human–prevention & control. W1 MO569P v.27 2008 / SF 995.6.I6 A95652 2008]
SF995.6.I6A94 2008
636.5089'6203–dc22

2008022013

Bibliographic Indices. This publication is listed in bibliographic services, including Current Contents® PubMed/MEDLINE

www.karger.com
Printed in Switzerland on acid-free and non-aging paper (ISO 9706) by Reinhardt Druck, Basel
ISSN 0077–0965
ISBN 978–3–8055–8501–9
e-ISBN 978–3–8055–8502–6

Contents

Foreword

Avian influenza has been known since the last decades of the 19th century, when E. Perroncito reported on outbreaks of a severe and highly contagious new poultry disease in Northern Italy. Two other Italian scientists, E. Centanni and E. Savonuzzi, discovered in 1901 that the disease which they called fowl plague was caused by a filterable agent (Centanni, E., Savonuzzi, E.: La peste aviaria. Comm. Acad. Med. Ferrara, 1901). The exact nature of fowl plague virus (FPV) remained obscure, however, until W. Schäfer in Germany identified it in 1955 as an influenza A virus (Schäfer, W.: Vergleichende sero-immunologische Untersuchungen über die Viren der Influenza und der klassischen Geflügelpest. Z Naturforsch 1955;10b:81-91). Within the last 130 years, more than 30 outbreaks of fowl plague have been reported worldwide. They were caused by viruses belonging to either subtype H5 or H7, and the birds affected with lethal illness were primarily chickens, turkeys and other species of the order *Galliformes*. Most of them have been confined to specific geographical areas and were eliminated in less than a year through comprehensive eradication programs. The H5N1 outbreak ongoing since 1996 has been unprecedented by its length, by its spread over large parts of the world and by occasional virus transmission to humans with a high case-fatality rate. When it became clear that FPV was an influenza A virus, other avian influenza viruses were identified. Usually they were isolated during mild disease outbreaks from a wide variety of wild and domestic birds, and they included all known serotypes (H1-H16, N1-9). These low pathogenic avian influenza (LPAI) viruses have therefore to be distinguished from highly virulent FPV or FPV-like viruses which are now called highly pathogenic avian influenza (HPAI) viruses. There is consent that wild aquatic birds are the natural hosts of all LPAI viruses providing a large genetic pool from which the HPAI viruses as well as the human and the other mammalian influenza A viruses are derived.

Because of its high impact on both animal and human health, avian influenza has become a matter of increasing public concern and growing scientific interest within the

last decade. This volume gives an overview of the most important results of these research efforts and provides information about the ecology and epidemiology of avian influenza with particular emphasis on recent H5N1 outbreaks in China, Siberia, and Europe. Molecular biology, culminating in the generation of influenza viruses by recombinant DNA technology, was instrumental for unravelling the role of the viral hemagglutinin and the polymerase as well as cellular signalling pathways and innate immunity in pathogenicity and interspecies transmission. Several articles deal with new vaccination strategies, use of antivirals and other control measures to combat outbreaks of avian influenza. Finally, the threat of a pandemic originating from avian influenza viruses is illustrated by the example of the Spanish influenza of 1918.

H.-D. Klenk
M.N. Matrosovich
J. Stech

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 1–10

Epidemiology of Avian Influenza

Albert D.M.E. Osterhaus · Vincent J. Munster · Ron A.M. Fouchier

Department of Virology, Erasmus MC, Rotterdam, The Netherlands

Abstract

While in the past decade the epidemiology of both low and highly pathogenic avian influenza (LPAI/HPAI) has changed considerably, much was learned from newly established global surveillance systems to monitor LPAI and HPAI viruses in migratory birds. LPAI H9N2 viruses that cause health problems in poultry in Eurasia and Africa could not be brought under control. Several major outbreaks of HPAI occurred in Europe and Canada, with serious implications for animal and often human health. The unprecedented spread of the HPAI H5N1 viruses throughout Eurasia and Africa became a major public health threat, as hundreds of people were hospitalised and more than 210 died. The risk that the current HPAI H5N1 or another emerging avian influenza virus may soon create the first influenza pandemic of the 21st century is widely appreciated. The current spread of H5N1 viruses also has major implications for animal health, animal welfare, food supplies, economies and biodiversity. Intervention strategies should focus foremost on the surveillance of LPAI and HPAI viruses in animals and humans, to provide early warning systems and virus repositories that will allow the timely establishment of seed viruses for candidate animal and human vaccines.

Introduction

Influenza viruses are negative-strand RNA viruses with a segmented genome belonging to the *Orthomyxoviridae* family. On the basis of antigenic differences they are divided into influenza virus types A, B and C. Only viruses of the influenza A type are known to infect birds [1]. Influenza A viruses are classified on the basis of the antigenic properties of their haemagglutinin (HA) and their neuraminidase (NA) surface glycoproteins [2]: to date, 16 HA (H1-H16) and 9 NA (N1-N9) subtypes have been identified, which are found in the majority of possible combinations (e.g. H1N1, H3N2…) in wild and domestic birds [3, 4]. All influenza A virus subtypes identified to date have been isolated from birds [5]. They have been identified in more than 100 bird species but also in various mammalian species, such as humans, pigs, horses, cats, mink and marine mammals [1, 6]. Most of these influenza A viruses have the so-called

low pathogenic avian influenza (LPAI) phenotype for poultry, since the disease they cause is mild in infected poultry. The only exception to this rule is H9N2 infection, which has caused major problems in poultry over extended geographical areas [5].

Upon introduction of the H5 and H7 subtypes into domestic poultry, these viruses may change their LPAI phenotype into a highly pathogenic avian influenza (HPAI) phenotype with up to 100% mortality for domestic poultry. This is the result of the insertion of multiple basic amino acid residues in the HA cleavage site. This predisposes these viruses to replicate systemically in their host and cause outbreaks of HPAI in domestic poultry [5–8]. Although outbreaks of HPAI or 'fowl plague' as it was formerly termed, have been recognized in domestic poultry for many decades, the recent and unprecedented spread of viruses of the HPAI H5N1 virus subtype, which had originally been identified as the cause of 18 severe human influenza cases in Hong Kong in 1997 [9–12], from Asia towards Russia, the Middle East, Europe and Africa in the past five years has attracted a lot of attention and public concern. This spread was not only associated with outbreaks of HPAI in domestic poultry but also with outbreaks and cases of avian influenza in wild birds and cases of avian influenza in mammals including humans. It has dramatically changed our views on the actual and potential threat of avian influenza to animal and human health: not only the direct impact on animal and public health, but also the threat to food supplies, economies and biodiversity are major issues of concern.

To date more than 340 hospitalized human HPAI H5N1 cases have been reported, of whom more than 210 were fatal [13]. Recently there have been major outbreaks of HPAI by other H5 and H7 subtypes in Italy, The Netherlands and Canada in which millions of poultry had to be culled [5]. Especially the massive HPAI H7N7 virus outbreak in The Netherlands in 2003, in which more than 30 million poultry died or had to be culled and 89 clinical human cases of the infection were reported (one fatal in spite of all the precautions taken) [14, 15], has also contributed to our fundamentally changed perception of the impact of avian influenza on animal and human health.

Avian Influenza in Birds

Low Pathogenic Avian Influenza Viruses

Although many different wild bird species may harbour influenza A viruses, birds belonging to the orders *Anseriformes* (duck, geese and swan species) and *Charadriiformes* (shorebird species, including gulls), which are distributed globally except for the most arid regions of the globe, are the principle reservoirs of avian influenza viruses [16–18]. All influenza A virus subtypes and the majority of all the possible HA/NA combinations have been identified in the wild bird reservoirs [5]. These viruses, which are of the LPAI phenotype, have been identified in more than 100 different wild bird species of more than 25 different families [19]. They preferentially replicate in the cells of the respiratory and intestinal tracts, and are excreted in

high concentrations in their faeces. It has been shown that influenza viruses remain infectious in lake water up to 4 days at 22°C and more than 30 days at 0°C and the relatively high virus prevalence in birds living in aquatic environments may be due at least in part to the efficient transmission through the faecal-oral route via surface waters. Transmission of avian influenza A viruses between birds is thought to occur primarily via the faecal-oral route, although other routes are possible [16]. Many species of the *Anseriformes* and the *Charadriiformes* are regular long-distance migrators, thereby potentially distributing LPAI viruses over long distances between countries or even continents. It is important to realize that the transmission of the LPAI viruses and their geographical spread is largely dependent on the ecology of the migrating hosts. Although migration patterns are largely confined to the 'major flyways', there are numerous exceptions where populations migrate differently from the common patterns. Within the major continents and along the major flyways, migration connects many bird populations in time and space along migration pathways and during stopovers where they may aggregate to rest and forage. Many species aggregate at favourable stopover or wintering sites, resulting in high local densities of birds of several species. Such areas may be important for transmission of the LPAI viruses between wild bird populations of different species. Also spread of LPAI viruses to domestic poultry may thus occur as a result of direct or indirect contacts with wild birds [16–19].

Although most of the LPAI viruses when transmitted from wild birds to domestic poultry cause no serious outbreaks and do not become endemic over large geographical areas, H9N2 LPAI viruses are an exception to this rule [5]. Since the second half of the 1990s, numerous H9N2 outbreaks occurred in poultry in several European countries, South Africa, the Middle East and Asia. In spite of the implementation of extensive vaccination programs, infections with viruses of this LPAI subtype have become endemic in domestic poultry in Eurasia and Africa [5].

Highly Pathogenic Avian Influenza Viruses

Influenza A viruses containing HA of subtypes H5 and H7 may become highly pathogenic upon introduction from wild birds into poultry. Before the unprecedented spread of HPAI H5N1 viruses, which has been found in free-living birds numerous times, there has only been one report on the isolation of a HPAI virus from a free living bird – a tern – that could not be associated with outbreaks of HPAI in poultry [20]. The change from the LPAI phenotype to HPAI phenotype is thought to occur primarily as the result of the insertion of multiple basic amino acid residues in the HA cleavage site, which is used to process the HA precursor protein (HA_0) to the functional components HA_1 and HA_2 [7, 21, 22]. A basic cleavage site allows cleavage of HA by cellular proteases that do not cleave LPAI HAs, and facilitates replication of the virus in a wider range of organs in its hosts. Thus, HPAI viruses can in contrast to LPAI viruses replicate throughout the body of the bird, since their HA can be cleaved by intracellular ubiquitous proteases. How and why mutation from LPAI to HPAI

viruses takes place in poultry is not fully understood. In some instances these mutations occurred 'overnight' whereas in other cases the LPAI virus gradually acquired the relevant mutations while it circulated in poultry [5, see also Garten and Klenk, this volume]. Viruses of subtype H5 and H7 lacking the multibasic cleavage site and viruses of all other HA subtypes are generally considered LPAI viruses, although a definitive classification as LPAI or HPAI requires analysis of the intravenous pathogenicity index (IVPI) in chickens [23]. HPAI viruses have been isolated mainly from gallinaceous birds (chickens, turkeys and quails) and cause an acute generalized disease, for which mortality in poultry may be as high as 100% [21, 24, 25]. The last decade has seen a marked increase in HPAI outbreaks in poultry all over the world.

The deaths of 6 people in Hong Kong in 1997 from an HPAI H5N1 virus infection was the first indication that a purely avian influenza virus could cause respiratory disease and death in humans [10–12]. After the 1997 H5N1 HPAI virus infections in Hong Kong, such a virus reappeared in 2002 in an outbreak in waterfowl and various other bird species at two waterfowl parks in Hong Kong [26, 27]. In 2003 the H5N1 HPAI were transmitted again to humans leading to at least 1 fatal case [28, 29]. The viruses resurfaced again in 2004 to spread over a large part of SE Asia, including Cambodia, China, Hong Kong, Indonesia, Japan, Laos, Malaysia, South Korea, Thailand and Vietnam [30]. The ongoing circulation of HPAI H5N1 viruses in SE Asia since 1997 has had a devastating impact on the poultry industry in that area. Until recently, HPAI H5N1 viruses have been isolated only sporadically from wild birds [27]. In 2005, however the virus was isolated during an outbreak among migratory birds in Lake Qinghai (Koko Nor), China, and subsequently appeared in Mongolia, Kazakhstan, Russia, Turkey, Romania, and Croatia [29–31]. While in some of these outbreaks transmission of the virus via movement of poultry, poultry products and humans cannot be excluded, dispersal via wild migratory birds seems to be a likely route for several of the outbreaks. There are indications that the susceptibility and pathogenesis of the HPAI H5N1 virus infection in different wild bird species may vary considerably, depending on bird species and previous exposure to viruses of the same or other influenza a virus subtypes. Upon experimental HPAI H5N1 virus infection, some duck species proved to develop minor if any disease signs while still excreting the virus predominantly from the respiratory tract, whereas other species developed a largely fatal infection that would not allow them to spread the virus over any distance efficiently [32]. Furthermore, it may be speculated that pre-exposure to LPAI viruses of the same H5 or N1 subtypes may result in circulating protective virus neutralising antibodies. Such pre-existing immunity might partially protect birds from developing severe infection but still allow spreading of the virus.

The spread of HPAI H5N1 viruses from SE Asia to migratory and domestic birds in Mongolia, Kazakhstan, Russia, Turkey, Romania and Croatia increased the risk for transmission of these viruses to an even larger geographical area. Probably as a result of spill back and forth of HPAI H5N1 viruses between poultry and migratory wild birds the virus has now spread to other parts of Asia and Europe, the Middle East and

Africa [13, 30]. HPAI H5N1 viruses have for instance been identified in wild birds and domestic poultry at least a dozen times in seven European countries in 2007. This raises the question whether these infections have indeed become endemic in non-migratory wild bird populations in Europe or are being re-introduced repeatedly by migratory birds or human activities. Whether these HPAI H5N1 viruses will eventually also cross the Atlantic or the Pacific Oceans to reach the Americas is still a matter of speculation.

A massive HPAI H7N7 virus outbreak among poultry occurred in The Netherlands in 2003. LPAI virus ancestors of the virus had been identified in mallards in a wild bird influenza surveillance network that had been started and implemented in the Northwestern Europe in the years preceding the outbreak. Due to the exceptionally high poultry density in the outbreak area, the outbreak was difficult to control and it eventually also spread to neighbouring areas within and outside the country. Of the total number of about 100 million poultry in The Netherlands, more than 30 million died or had to be culled. Unexpectedly, and in spite of all the precautionary measures taken, a large number of poultry workers developed symptomatic infections, there seemed to be limited human-to-human spread, and one veterinarian died due to the H7N7 infection [14, 15].

Since the HPAI H5N1 viruses that currently spread over Eurasia and Africa are already highly pathogenic, they do not need to mutate to cause serious disease outbreaks among poultry, and therefore represent an acute threat to meat production and food supply, public health, the economy and biodiversity. This environmental impact of HPAI H5N1 viruses is of special concern for vulnerable wild bird populations. For instance, the highly endangered population of bar-headed geese (*Anser indicus)* suffered an estimated 8–10% decrease through a mass die off due to HPAI H5N1 virus infection in China in 2005 [16].

Influenza Virus Surveillance in Birds

It is now well recognized that real-time global influenza virus surveillance in wild birds should play a key role in our understanding of the way in which LPAI viruses of the different subtypes, but most importantly of the H5 and H7 subtypes, are spreading worldwide and may thus pose a threat to our domestic poultry [16–19, 33]. For instance, panels of reference reagents required for testing of animals and humans can be updated when needed. Importantly, the global surveillance of avian influenza may shed new light on questions related to the temporal and spatial variation in circulating influenza viruses and their epidemiology, ecology and evolution. Recent extensive surveillance studies carried out among migratory birds of more than 250 species representing most of the major bird families in different geographical areas around the globe have shed light on the prevalence of influenza A viruses in wild migratory birds. Overall, influenza A viruses were detected in approximately 2% of the samples collected. However, at selected locations and during peak periods, the prevalence in specific bird species was much higher, up to 60% [19]. Such studies also indicated

that avian influenza A viruses have a taxonomically wider reservoir spectrum than previously known, and that for instance auks (*Alcidae*) and other seabirds could possibly function as a mixing vessel for influenza viruses of American and Eurasian avian lineages [34]. Such surveillance studies in wild birds have also identified several influenza A viruses of the H5 and H7 subtypes, containing various NAs. For each of the recorded H5 and H7 HPAI outbreaks in Europe in the past decade a thus established influenza A virus collection contained closely related virus isolates recovered from wild birds, as determined by sequencing and phylogenetic analyses of the HA gene and antigenic characterization of the HA glycoprotein. The minor genetic and antigenic diversity between the viruses recovered from wild birds and those causing HPAI outbreaks indicate that influenza A virus surveillance studies in wild birds are indeed useful to generate prototypic vaccine candidates and to design and evaluate diagnostic tests, prior to the occurrence of outbreaks in animals and humans [35, 36]. These data have also provided further evidence that HPAI outbreaks in poultry originate from LPAI viruses of wild birds, upon mutation to the HPAI form in poultry.

Influenza A virus surveillance of wild birds has now become even more important than before, as an 'early warning system' for the introduction of HPAI H5N1 viruses in countries or regions where they have not been found before.

Avian Influenza in Non-Avian Species

Avian Influenza in Mammals

It has long been known that besides birds, also various mammalian species, such as pigs, horses, mink and marine mammals [1] may be infected directly by avian influenza viruses under natural circumstances. Introduction of avian influenza viruses into some species like harbour seals has been associated with acute disease outbreaks with high mortality, after which the virus disappeared from the population [1, 6]. In other species like horses and pigs, influenza viruses of avian origin may after their introduction eventually persist in the population causing recurrent epidemic outbreaks of influenza similar to those observed in humans. However, the exact transmission history is often not known and interspecies transmission between different mammalian host species may occur. The latter was recently shown by the introduction of an H3N8 virus endemic in horses, into dogs [37]. This caused serious disease outbreaks in dog populations in the USA. Introduction of avian influenza viruses into the human population was until recently only supposed to be possible after passage of the virus through another susceptible mammalian species like the pig.

Disease and mortality due to HPAI H5N1 virus infection in areas, where these viruses were identified in poultry, wild birds and humans (see below), were also reported to occur in an ever-increasing number of other wild and domestic mammalian species [30]. Among the naturally infected mammalian species are pigs,

domestic and feral cats, large captive felids, civets, domestic dogs and mustellid species. Infection of most of these animals occurred upon contact with infected poultry, poultry carcasses or poultry meat, or by predation on infected wild birds. It became clear that in areas where the virus is present in wild birds, domestic or feral cats may serve as excellent sentinel animals that may detect and show the presence of virus very early on, by developing serious disease [38–40]. On the other hand, they may also be the cause of virus spread to poultry, other mammals and humans. Especially the infection of increasing numbers of mammals raised the fear that the adaptation to mammalian species could contribute to its further adaptation to replication in, and transmission among humans. This may eventually lead to a pandemic outbreak of influenza in humans that will cost the lives of many millions of people. Under experimental laboratory conditions, HPAI H5N1 virus has infected several other mammalian species like ferrets, cats, macaques and mice. It is interesting to note that infection with viruses of this HPAI subtype in different species may have quite different outcomes depending not only on animal species, but also on virus clade, as well as route and dose of infection. For instance, experimental infection of cats may lead to generalised often fatal disease, as is for example also seen in feral cats feeding on chicken carcasses [38–40]. This is in contrast to what is found under similar conditions in experimentally infected macaques, which seem to suffer from serious infection that is largely confined to the respiratory tract [41, 42]. Also in humans, HPAI H5N1 infection seems to be largely confined to the respiratory tract, although serious cases have been described in which the virus was found in the central nervous system and the enteric tract [43].

Avian Influenza in Humans

When in 1997 from a tracheal aspirate of a 3-year-old boy who had died in Hong Kong of acute influenza pneumonia and acute respiratory distress syndrome (ARDS) a HPAI H5N1 virus was isolated [9–12], it was realised that a purely avian influenza virus could cause respiratory disease and death in humans. The same virus was identified in an additional 17 humans in Hong Kong, causing 5 more deaths [9, 12]. Two viruses isolated from humans during the 1997 outbreak in Hong Kong showed a different pathogenicity for mice. The virus isolated from a fatal case was lethal for mice, whereas a virus isolated from a patient with relatively mild disease caused a non-lethal infection in mice. The difference in pathogenicity was mainly determined by an E627K substitution in PB2. An I227S substitution in HA also increased the virulence of this virus in mice [44]. In 2003, HPAI H5N1 viruses were again identified in humans in association with at least 1 fatal case [28]. In that same year an outbreak of HPAI H7N7 occurred in domestic poultry in The Netherlands, probably as a direct consequence of the transmission of a LPAI H7N7 virus from wild mallards, that subsequently mutated into a HPAI virus [14, 15, 18, 36]. During this outbreak in which more than 30 million chickens died or had to be culled 89 poultry workers and their family members ($n = 3$) developed a symptomatic H7N7 infection, one of which was

fatal. This in spite of all the precautionary measures that were taken. The fatal case was a veterinarian who died of severe pneumonia. The virus isolated from the lungs showed 14 amino acid substitutions as compared to viruses from milder cases [45]. In virus-attachment experiments, marked differences were found between these viruses in binding to the lower respiratory tract of humans [46]. Also HPAI H5N1 viruses had been shown to preferentially attach in the lower respiratory tract. In a mouse model, the HA gene of the H7N7 virus from the fatal case was a determinant of tissue distribution [45]. The lysine at position 627 of basic polymerase 2 (PB2) of this virus was the major determinant of pathogenicity and tissue distribution. Although other markers of pathogenicity have been identified [for review, see 47], remarkable similarities were revealed between recent HPAI H5N1 and H7N7 viruses. Consequently, influenza virus HA and PB2 genes should be the prime targets for molecular surveillance during outbreaks of zoonotic HPAI viruses. It should however be realized that besides pathogenicity for humans, efficient transmissibility from human to human is the second major condition for an avian virus to become a human pandemic virus. Molecular correlates of transmissibility of H5N1 viruses between humans can only be addressed in animal models, and their predictive value for the human situation remains to be shown.

From 2003 onward, more than 340 serious human infections with the HPAI H5N1 virus have been reported, more than 210 of which were fatal [13]. Almost all became infected through direct or indirect contacts with domestic poultry or their products in the areas where the virus had been found in poultry and wild birds. No efficient human-to-human transmission of H5N1 and H7N7 viruses has so far been found.

Conclusions

Avian influenza has long been a disease that attracted little attention of the veterinary, let alone the medical profession. HPAI and LPAI disease outbreaks in poultry were limited and could be controlled well. In the past decade the scene has changed dramatically. On the one hand, LPAI viruses of the H9N2 subtype, that did cause health problems in poultry in ever-expanding areas, could not be brought under control. On the other hand, the unprecedented spread of the HPAI H5N1 viruses throughout Eurasia, into the Middle East and Africa proved to become a major public health threat, as several hundreds of people became hospitalised of whom more than 210 have died to date. Furthermore, the risk that the current HPAI H5N1 viruses or any of the other emerging LPAI or HPAI viruses may be at the basis of the first pandemic influenza outbreak of the 21st century is considered more and more seriously. In this light it is important to realise that HPAI viruses were not at the basis of the previous pandemics that we know. Finally the outbreaks caused by the emerging avian influenza viruses proved to have major implications for animal health and welfare, food supplies, economies and biodiversity.

The reasons why avian influenza viruses have changed their epidemiological features so suddenly and dramatically are poorly understood. The chances for interspecies transmission of both LPAI and HPAI viruses between wild birds and domestic poultry, and vice versa, have probably increased in the past decade due to changes in poultry husbandry practices and in trade routes for poultry products. Intervention strategies should focus foremost on the surveillance of LPAI and HPAI viruses in animals and humans, to provide early warning systems for the emergence of major animal and human pathogens and to allow the establishment of virus repositories that will allow the timely establishment of seed viruses for candidate animal and human vaccines.

References

1 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–179.

2 Cox NJ, Fuller F, Kaverin N, et al: Orthomyxoviridae in virus taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. San Diego, Academic Press, 2000.

3 Fouchier RA, Munster V, Wallensten A, et al: Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005;79:2814–2822.

4 WHO: A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull World Health Organ 1980;58:585–591.

5 Alexander DJ: An overview of the epidemiology of avian influenza. Vaccine 2007;25:5637–5644.

6 Hinshaw VS, Air GM, Gibbs AJ, Graves L, Prescott B, Karunakaran D: Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. J Virol 1982;42:865–872.

7 Banks J, Speidel ES, Moore E, et al: Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 2001;146: 963–973.

8 Rott R: The pathogenic determinant of influenza virus. Vet Microbiol 1992;33:303–310.

9 Claas EC, Osterhaus AD: New clues to the emergence of flu pandemics. Nat Med 1998;4:1122–1123.

10 Claas EC, Osterhaus AD, van Beek R, et al: Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472–477.

11 De Jong JC, Claas EC, Osterhaus AD, Webster RG, Lim WL: A pandemic warning? Nature 1997; 389:554.

12 Suarez DL, Perdue ML, Cox N, et al: Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 1998;72:6678–6688.

13 Cumulative number of confirmed human cases of influenza A (H5N1) since 1 November 2005 (accessed at http://www.who.int/csr/disease/avian_ influenza/country/cases_table_2005_11_01/index.html).

14 Fouchier RA, Schneeberger PM, Rozendaal FW, et al: Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci USA 2004;101:1356–1361.

15 Koopmans M, Wilbrink B, Conyn M, et al: Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in The Netherlands. Lancet 2004; 363:587–593.

16 Munster VJ, Veen J, Olsen B, Vogel R, Osterhaus AD, Fouchier RA: Towards improved influenza A virus surveillance in migrating birds. Vaccine 2006;24:6729–6733.

17 Wallensten A, Munster VJ, Latorre-Margalef N, et al: Surveillance of influenza A virus in migratory waterfowl in northern Europe. Emerg Infect Dis 2007;13:404–411.

18 Munster VJ, Baas C, Lexmond P, et al: Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 2007;3:e61.

19 Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA: Global patterns of influenza a virus in wild birds. Science 2006;312: 384–388.

20 Becker WB: The isolation and classification of tern virus: influenza A-Tern South Africa-1961. J Hyg (Lond) 1966;64:309–320.

21 Alexander DJ: A review of avian influenza in different bird species. Vet Microbiol 2000;74:3–13.

22 Suarez DL, Senne DA, Banks J, et al: Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerg Infect Dis 2004;10:693–699.

23 Avian Influenza: Manual of diagnostic tests and vaccines for terrestrial animals, 2004 (accessed at http://www.oie.int/eng/normes/mmanual/A_summry.html).
24 Capua I, Alexander DJ: Avian influenza: recent developments. Avian Pathol 2004;33:393–404.
25 Perkins LE, Swayne DE: Pathobiology of A/chicken/Hong Kong/220/97 (H5N1) avian influenza virus in seven gallinaceous species. Vet Pathol 2001;38:149–164.
26 Ellis TM, Bousfield RB, Bissett LA, et al: Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002. Avian Pathol 2004;33:492–505.
27 Sturm-Ramirez KM, Ellis T, Bousfield B, et al: Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J Virol 2004;78:4892–4901.
28 Peiris JS, Yu WC, Leung CW, et al: Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 2004;363:617–619.
29 Liu J, Xiao H, Lei F, et al: Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 2005;309:1206.
30 Update on avian influenza in animals, 2005 (accessed at http://www.oie.int/downld/AVIAN%20INFLUENZA/A_AI-Asia.htm).
31 Avian influenza-Eurasia (44) Europe, 2005 (accessed at http://www.promedmail.org).
32 Keawcharoen J: Experimental infection of six wild duck species with HPAI virus (H5N1). Emerg Infect Dis 2008 (in press).
33 Fouchier RA, Osterhaus AD, Brown IH: Animal influenza virus surveillance. Vaccine 2003;21:1754–1757.
34 Wallensten A, Munster VJ, Elmberg J, Osterhaus AD, Fouchier RA, Olsen B: Multiple gene segment reassortment between Eurasian and American lineages of influenza A virus (H6N2) in guillemot (*Uria aalge)*. Arch Virol 2005;150:1685–1692.
35 De Wit E, Munster VJ, Spronken MI, et al: Protection of mice against lethal infection with highly pathogenic H7N7 influenza A virus by using a recombinant low-pathogenicity vaccine strain. J Virol 2005;79:12401–12407.
36 Munster VJ, Wallensten A, Baas C, et al: Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerg Infect Dis 2005;11:1545–1551.
37 Crawford PC, Dubovi EJ, Castleman WL, et al: Transmission of equine influenza virus to dogs. Science 2005;310:482–485.
38 Amonsin A, Songserm T, Chutinimitkul S, et al: Genetic analysis of influenza A virus (H5N1) derived from domestic cat and dog in Thailand. Arch Virol 2007;152:1925–1933.
39 Kuiken T, Fouchier R, Rimmelzwaan G, Osterhaus A, Roeder P: Feline friend or potential foe? Nature 2006;440:741–742.
40 Songserm T, Jam-on R, Sae-Heng N, et al: Domestic ducks and H5N1 influenza epidemic, Thailand. Emerg Infect Dis 2006;12:575–581.
41 Kuiken T, Rimmelzwaan G, van Riel D, et al: Avian H5N1 influenza in cats. Science 2004;306:241.
42 Rimmelzwaan GF, van Riel D, Baars M, et al: Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol 2006;168:176–183.
43 De Jong MD, Tran TT, Truong HK, et al: Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med 2005;353:2667–2672.
44 Hatta M, Gao P, Halfmann P, Kawaoka Y: Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 2001;293:1840–1842.
45 Munster VJ, de Wit E, van Riel D, et al: The molecular basis of the pathogenicity of the Dutch highly pathogenic human influenza A H7N7 viruses. J Infect Dis 2007;196:258–265.
46 Van Riel D, Munster VJ, de Wit E, et al: Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol 2007;171:1215–1223.
47 De Wit E, Fouchier RAM: Emerging influenza. J Clin Virol 2008;41:6.

Prof. A.D.M.E. Osterhaus, DVM, PhD
Head, Department of Virology, Erasmus MC, 's Gravendijkwal 230
Faculty Building, PO Box 2040
NL–3000 CA Rotterdam (The Netherlands)
Tel. +31 10 408 8066, Fax +31 10 408 9485, E-Mail a.osterhaus@erasmusmc.nl

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 11–26

H5N1 in Asia

Hui-Ling Yen[a] · Yi Guan[b] · Malik Peiris[b] · Robert G. Webster[a]

[a]St. Jude Children's Research Hospital, Department of Infectious Diseases, Division of Virology, Memphis, Tenn., USA, and [b]University of Hong Kong, Queen Mary Hospital Compound, Hong Kong, SAR, China

Abstract

The highly pathogenic H5N1 influenza virus that emerged in Southeastern Asia a decade ago has evolved into multiple genetic clades, spread from Asia to Europe and Africa, greatly compromised the poultry industry and endangered human health, and now poses a serious pandemic threat. The unique ecology of the long-established influenza epicenter of Southeastern Asia continues to play a role in driving the genetic diversity and possibly the persistence of H5N1 virus. Long-term surveillance in this region has provided insight into the emergence of H5N1 in Asia, establishment of the virus in this region, and potential routes of spread to other regions. Although highly pathogenic H5N1 virus can be lethal to wild waterfowl, this natural reservoir of all subtypes of influenza A virus is more resistant to the infection and demonstrates the ability to select antigenic variants with attenuated virulence in these species. Other unique characteristics of the H5N1 virus include an expanded host range to many *Carnivora* species and increased pathogenicity in mammals. Because multiple factors contribute to the spread and perseverance of H5N1 virus, integration of multifaceted strategies and global collaboration are necessary for effective control.

Introduction

It has been more than a decade since the highly pathogenic (HP) H5N1 influenza emerged in domestic geese in Guangdong, China [1]. During this time, the virus has evolved rapidly and spread essentially throughout the entire Eurasian continent. The virus has caused devastating outbreaks of lethal disease in domestic poultry and has infected a limited number of humans, causing the death of more than half of those infected. A particular concern is that we may be witnessing in real time the evolution of an influenza virus with pandemic potential both for people and poultry. In addition to humans, lethal H5N1 infection has been reported in an expanded range of species, including felids (tigers, leopards, and domestic cats), dogs, stone martens, and viverrids in nature as well as mice and ferrets in laboratory settings [1–4]. Although seroconversion was detected in a small percentage (range 0.25–10%) of

pigs in Southern Asia [5, 6], lethal infection of H5N1 in pigs has not been documented.

It is important to consider the pandemic potential of HP H5N1 avian influenza in conjunction with the other subtypes of influenza A virus that have transmitted transiently to humans (e.g., influenza virus of H9N2, H7N7, and H7N3 subtypes) [1]. Although H5N1 is currently at the top of the 'hit list' for pandemic threat, these other viruses continue to circulate, and H9N2 viruses that recognize both avian-like sialyl receptors (sialic acid linked to galactose by α-2,3 linkage) and human-like sialyl receptors (sialic acid linked to galactose by α-2,6 linkage) are now ubiquitous in domestic chickens across much of Eurasia [1].

Although HP H5N1 prompts continued concern because of its pandemic potential, it is noteworthy that this virus and its variants already have devastated the poultry industry in many countries and, either directly or indirectly, has decreased both economic and social well-being. The continued circulation and evolution of a novel HP influenza virus for more than a decade is a unique phenomenon. Previously reported HP outbreaks of H5 and H7 influenza in domestic poultry have either been stamped out or burnt out and disappeared [7, 8]. The current HP H5N1 has been stamped out in Japan, South Korea, Thailand, and many other countries in Asia, Africa and Europe – only to return during the cooler months. What, then, drives the diversity, persistence, and resurgence of HP H5N1?

Here we consider the origin and evolution of HP H5N1 viruses in Asia and whether Southeast Asia continues to be the epicenter for the emergence of influenza viruses. We also discuss the features that promote the antigenic and biologic diversity of HP H5N1 viruses and address their ultimate reservoirs.

Reservoirs of Influenza Viruses in Nature

The established reservoirs of all 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes of influenza A viruses are the aquatic birds of the world [9, 10]. In this reservoir, the viruses replicate primarily in the intestine and live in apparent harmony with their hosts, causing no disease signs. Although diverse animal species are susceptible to influenza virus infection, current information suggests that host-specific lineages have been established in birds, pigs, horses, and humans. The possibility exists that the established host-specific influenza viruses may be introduced and further established into new species, an example would be the establishment of H3N8 equine influenza virus in greyhounds in the USA since 2000 [11]. Phylogenetic analyses suggest that mammalian influenza viruses derive from the avian influenza reservoir. The perpetuation of a large gene pool in wild migratory birds and the dynamic genetic reassortment within the gene pool allow the influenza virus to persist as a successful microbe.

Of the 16 HA subtypes, only 2 (H5 and H7) are known to have the capacity to become highly pathogenic. The HP phenotype is related, but not restricted, to the

presence of multiple basic amino acids at the HA cleavage site [12, 13]. The biologic mechanism for the formation of this motif includes influenza's error-prone RNA polymerase and subsequent selection of viruses with highly cleavable HAs (as demonstrated through continue passage of a low-pathogenicity (LP) H5 isolate in trypsin-free chicken embryo fibroblast cultures), secondary RNA structure, and recombination events between the HA and other viral gene segments or between HA and host cell 28S ribosomal RNA [13]. To date, there is no convincing evidence for the perpetuation of HP H5 or H7 influenza viruses in wild migratory waterfowl reservoirs, as HP H5 viruses have only been detected from dead waterfowl in the vicinity of wild migrating bird aggregations. Prospective surveillance of migratory waterfowl in Canada and the USA for more than 30 years [14] has failed to detect HP H5 or H7 influenza viruses in migrating birds despite multiple outbreaks of H5N2 in domestic fowl in the USA and Central America and H7N3 outbreaks in Canada, Chile, and Peru [7]. Similar extensive studies in Europe [15] have failed to detect HP H5 or H7 influenza viruses in wild aquatic birds despite HP H7 outbreaks in The Netherlands, Germany, and Italy [7, 8]. Studies in Asia, including Japan and Siberia, also support this hypothesis [16, 17]. The available evidence indicates that HP H5 and H7 influenza viruses evolve from LP H5 and H7 precursors in wild aquatic bird reservoirs [7]. Wild waterfowl or wild birds may directly or indirectly introduce LP H5 or H7 influenza virus into domestic poultry among which HP H5 or H7 emerged. Factors influencing the emergence of HP H5 and H7 in domestic poultry include host adaptation, husbandry practices, and the intensified poultry industry. Previous studies suggest that HP H5 or H7 influenza virus did not replicate well in mallards; however, the currently widely spread HP H5N1 virus has the capacity to cause lethal infection in both domestic and wild waterfowl.

The Ecology of Influenza Virus in Asia

Southern China is the hypothetical pandemic epicenter of influenza. Among the four pandemics that occurred during the last century, the 1957 Asian and 1968 Hong Kong viruses originated from this region [13]. In tropical and subtropical areas, human influenza can be detected year-round. The warm winter in Southeast Asia attracts migratory birds from northern climes to spend the winter in this region. The high density of human population and prevalence of backyard poultry (ducks, geese, and chickens) and pigs provide the opportunity for close interaction between these influenza reservoir animals and create a unique environment for influenza virus ecology. In addition, the live-poultry market ('wet market') system provides optimal conditions for influenza virus evolution, with transmission between avian species and possible infection of humans [1, 18]. Transmission between different host species and serologic evidence of human infection with H4, H5, H6, H7, H10, and H11 subtypes of avian influenza virus were documented in this region prior the 1997 Hong Kong H5N1 outbreak [1].

Since the emergence of HP H5N1 in Southern China in 1996 (see 'Emergence of H5N1 Influenza Viruses in Asia'), a great proportion of countries in Asia are currently affected by HP H5N1 virus, many of them suffered from severe loss in poultry industry and continued threat for human health (fig. 1). Although various poultry industry practices may have aided the spread of H5N1 virus, the high prevalence of backyard poultry and close interaction between different influenza reservoirs hosts in many Asian countries provided opportunity for the virus to persevere and to continue evolving. The continued circulation and evolution of HP H5N1 in this region suggest that Southeastern Asia continues to serve as the epicenter for HP H5N1 virus.

Emergence of H5N1 Influenza Viruses in Asia

Surveillance data show that prior to 1996, LP H5 avian influenza viruses had been isolated from domestic ducks and geese in Southeastern China but not from chickens [18]. Neutralizing antibodies to H5 virus were detected in pig sera from Southeastern China collected in 1977–1982 (2 of 127 samples) and 1998 (10 of 101 samples) [1], suggesting that pigs in this region had been exposed to influenza virus of the H5 subtype.

Genetic evidence showed that the precursor virus (A/Goose/Guangdong/1/96) for the currently circulating HP H5N1 virus was first detected in domestic geese in Guangdong China in 1996 [1]. To date, the precursor(s) of this virus is unknown, although the eight gene segments are closely related to those from LP H5 viruses isolated from migratory birds or wild ducks in Hokkaido, Japan [16, 17].

The index human case of H5N1 influenza occurred in May 1997 and the causative virus was identified in August 1997 [1] as the first HP avian influenza virus known to cause lethal infection in humans. During the remainder of 1997, 17 additional human cases were detected; a total 6 patients succumbed to H5N1 infection. Surveillance and epidemiological studies established that poultry markets were the source of human H5N1 infection, as H5N1 virus was isolated from approximately 20% of fecal samples from chickens and from approximately 2% of fecal samples from ducks and geese in the market [18]. Subsequent genetic analysis of the index human virus revealed that the six internal genes were closely related to those in A/Quail/HK/G1/97 (H9N2) and that the NA gene was genetically similar to that of A/Teal/HK/W312/97 (H6N1), raising the possibility that reassortment between these viruses was involved in the genesis of the HP H5N1 virus [1]. The failure to detect an increased death rate among chickens in the 6 Hong Kong live-bird markets studied was surprising, given that HP H5N1 isolates were isolated at each of the markets. Although market-stall owners likely removed dead birds before opening, an ongoing outbreak of HP influenza is difficult to hide due to rapid development of the classic signs of hemorrhage of the head and legs and neurological signs in some cases. One of the possible explanations for the unexpectedly low death rate among these chickens in the market is that cell-mediated immunity provided some cross-protection between the H5N1 and H9N2

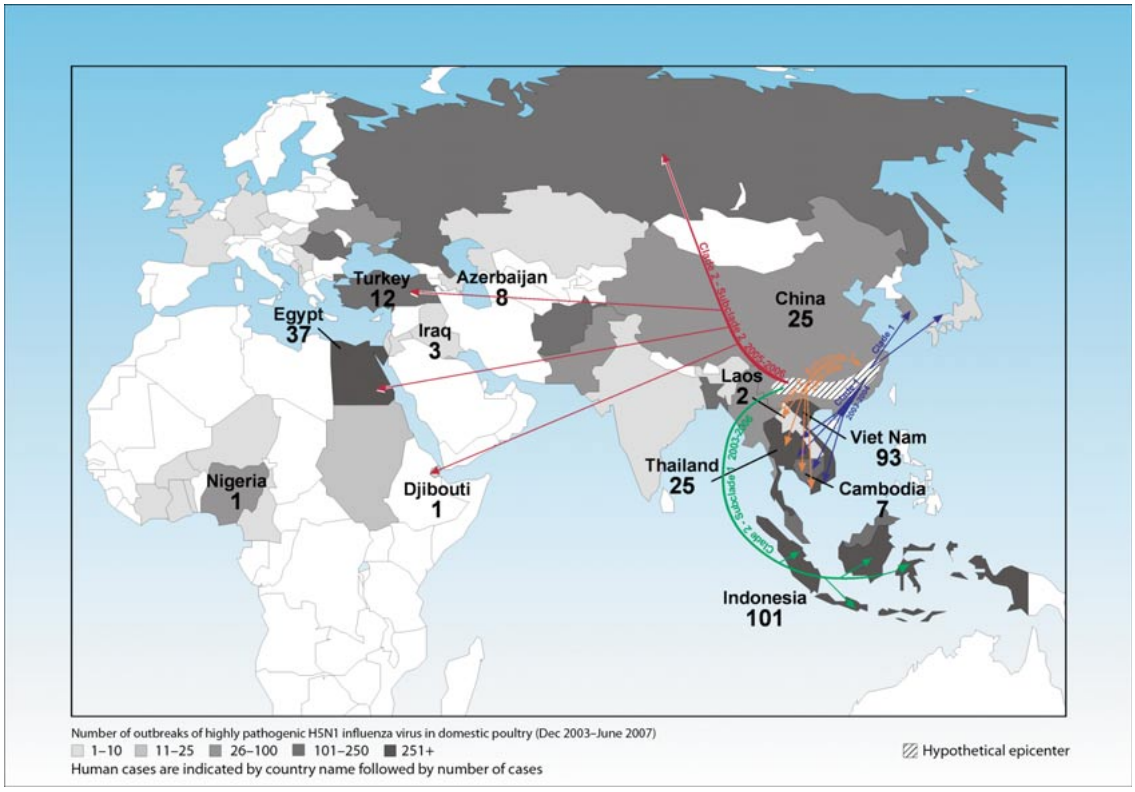


Fig. 1. The spread of H5N1 virus from Southeastern Asia to other continents. HP H5N1 influenza first emerged from Southern China in 1996. To date, the virus has spread to many countries in Asia, Europe, and Africa, with continued establishment of the virus mostly in Southeastern Asia and West Africa. Highlighted countries have reported HP H5N1 outbreaks from late 2003 through June 2007. Countries with reported human H5N1 infections are indicated by the number of confirmed human cases. More than 50% of infected humans have died. Multiple clades and sub-clades differing antigenically and molecularly have emerged since 2003. Source of data: World Organization of Animal Health (OIE; http://www.oie.int/downld/avian%20influenza/A_AI-Asia.htm) and World Health Organization (WHO; http://www.who.int/csr/disease/avian_influenza/country/en/).

viruses which shared six closely related internal genes and co-circulated in the markets during the outbreak in 1997.

The culling of all poultry in Hong Kong effectively eradicated that particular genotype of HP H5N1 influenza virus. There were no more human cases in Hong Kong, but H5N1 viruses continued to circulate among apparently healthy domestic ducks in the costal provinces of China between 1999 and 2002 [1]. Detections of HP H5N1 virus were also documented in geese in live-poultry markets in Vietnam in 2001 and from duck meat exported from China to Korea and Japan in 2001 and 2003, respectively [1]. During 2001 and 2002, multiple H5N1 genotypes were detected in poultry

in Southern China [1]. These viruses had the HA typical of the A/Goose/Guangdong/1/96-like lineage but with a plethora of different internal genes. In addition, the NA genes of these variant H5N1 viruses were typical of that of A/Goose/Guangdong/1/96 but frequently had deletions of amino acids in the stalk region [1]. In 2002, HP H5N1 outbreaks of lethal disease in waterfowl occurred in Penfold Park and Kowloon Park in Hong Kong; many different aquatic species as well as tree sparrows and pigeons were killed [1].

The next key event in the development of H5N1 viruses was its re-emergence in humans in 2003. The daughter of a Hong Kong family died on visiting Fujian Province of China in February 2003. On their return to Hong Kong, her father and brother were diagnosed to have H5N1 infection [1]; the father subsequently died, but the brother recovered.

In late 2003 to early 2004, outbreaks of HP H5N1 viruses in domestic poultry were reported in South Korea, Japan, Vietnam, Lao PDR, Cambodia, and Indonesia (fig. 1). During this period, avian-to-human transmission had occurred and resulted in lethal H5N1 human infection in Vietnam and Thailand (fig. 1). Serologic evidence suggests that limited human infections occurred in Japan and South Korea during the 2003–2004 H5N1 outbreaks. Genetic analysis showed that the viruses that spread to Japan and South Korea belonged to the V genotype, which had a PA gene that was distinct from that of the Z genotype that became dominant in Vietnam, Thailand, Cambodia, Indonesia, and Southern China [1].

Qinghai Lake Outbreak of H5N1 Infection and Spread to Europe and Africa

Qinghai Lake in Western China is a leading breeding site of migratory waterfowl. In April 2005, a lethal outbreak of H5N1 influenza occurred at Qinghai Lake that affected bar-headed geese *(Anser indicas)*, great black-headed gulls *(Larus ichthyaetus)*, brown-headed gulls *(Larus brunnicephalus)*, ruddy shelducks *(Tadorna ferruginea)*, and great cormorants *(Phalacrocorax carbo)* and killed more than 6,000 migratory waterfowl [1, 19]. Other wild birds that have been affected by HP H5N1 include whooper swans *(Cygnus cygnus)*, black-necked cranes *(Grus nigricollis)*, and pochards (diving ducks that belong to the subfamily Aythyinae) [1]. This event was the first major outbreak of H5N1 influenza virus in wild migratory birds. At least four genotypes of H5N1 virus were detected in the waterfowl at Qinghai Lake, but one genotype (genotype c) became dominant and rapidly spread to wild and domestic birds in Siberia (July 2005), Mongolia and Kazakhstan (August 2005), and Europe including Croatia, Romania, and Turkey (October 2005) [19]. The precursors of the dominant Qinghai H5N1 virus were detected in mallard ducks at Poyang Lake, China, in March 2005 [20] and may have came from domestic poultry. A notable feature of the dominant Qinghai H5N1 virus is that it had a mutation of the PB2 gene (residue E627K) that is conserved in human influenza viruses and associated

with increased viral pathogenicity in mice [19, 21]. Genetic and epidemiological evidence (see 'Emergence of Multiple Clades and Subclades of H5N1 Influenza Viruses') suggest that Qinghai-like H5N1 virus was remarkably successful in spreading to Europe and Africa, infecting domestic and wild waterfowl, as well as humans, cats, and stone martens. The likely carrier of the virus was migrating ducks, because experimental studies showed that the Qinghai H5N1 virus killed 100% of geese but most mallard ducks survived. In Europe, dead domestic and wild geese and swans served as sentinel animals for the presence of H5N1 virus, but it is unlikely that they spread the virus [22]. Although duck species differ in their susceptibility to the Qinghai H5N1 virus, some species show limited evidence of infection [1, Keawcharoen et al., unpubl. data].

A second notable property of the Qinghai lineage of H5N1 viruses is that in both domestic and wild ducks and other waterfowl, greater numbers of virus are shed from the respiratory tract than in fecal droppings [28]. This property of respiratory shedding applies to different clades and subclades of H5N1 viruses and must be considered when studying the ecology of this H5N1 virus in migratory birds. Collecting both oral and cloacal samples from birds therefore is critical for surveillance purposes.

The resurgence of HP H5N1 influenza during the cooler months was noted during the surveillance of live-poultry markets in Southern China [20, 23]. This seasonality of H5N1 was repeated in winter 2006–2007, when the Qinghai-like H5N1 virus reemerged in many Eurasian and African countries, particularly Japan, South Korea, and Thailand, which had previously stamped out the virus. An association between decreased environmental temperatures and the onset of H5N1 outbreaks in wild birds were also observed, suggesting a possible correlation between rapid weather changes and immunosuppression due to physiologic stress [24]. Although the HP Qinghai-like H5N1 virus can transiently infect migratory waterfowl, available surveillance evidence does not indicate perpetuation of this virus in this natural influenza reservoir. The ultimate reservoir of HP H5N1 virus during the warmer months needs to be elucidated.

Emergence of Multiple Clades and Subclades of H5N1 Influenza Viruses

In 2005, two major clades with non-overlapping geographic distributions were identified on the basis of HA sequence analysis [25]. Viruses isolated from Thailand, Cambodia, and Vietnam during 2004–2005 outbreaks were clustered into clade 1, whereas viruses isolated from China and Indonesia during 2003–2004 outbreaks were clustered into clade 2. Since 2005, clade 1 viruses continued to be detected in Vietnam and Thailand although they largely were superseded by clade 2 viruses, which continue to evolve into three major subclades that differ in geographic distribution. One subclade (2.2) contains the H5N1 virus that caused the large-scale lethal

outbreak in wild birds at Qinghai Lake in Central China during summer 2005 and HP H5N1 viruses that subsequently spread to the Middle East, Europe, and Africa, suggesting a potential role of migratory birds in virus spreading [25, 26]. Indonesian H5N1 viruses isolated since 2004 continue to cluster into a distinct subclade (2.1), suggesting a single introduction of the virus into Indonesia and its endemicity in this region since 2004 [1, 25–27]. The endemicity of the H5N1 viruses in Southern China initially resulted in diverse regional sublineages, but dominant Fujian-like H5N1 viruses emerged during 2005–2006 and replaced most of the previously established sublineages. These Fujian-like viruses formed a separate subclade (2.3) and further spread to Hong Kong, Malaysia, Laos, and Thailand, causing outbreaks in wild birds or domestic poultry in 2006 [23, 25]. The nomenclature for phylogenetic relationships among the HA gene of H5N1 viruses is currently (February 2008) being revised. H5N1 viruses circulated during the past decade can be separated into ten phylogenetic clades [26].

Overall, phylogenetic analysis provided information regarding the evolution and the spread of the HP H5N1 virus: multiple introduction or re-introduction of the H5N1 virus likely occurred in several Asian countries that have previously stamped out the virus before the re-emergence of HP H5N1 in 2006 or 2007; single introductions of the H5N1 virus into Indonesia, where the virus evolves into its own lineage (subclades), and the spread of Qinghai-like HP H5N1 virus to Europe and Africa [1, 25]. The use of vaccines in poultry, migratory bird flyways, and poultry trade (legal or illegal) between regions are also potential factors influencing the formation of different genetic clades.

Unique Features of Re-Emergent H5N1 Viruses: Changing Patterns

As the H5N1 viruses continued to spread and evolve during the past decade, we have learned of and observed several unique features about the virus. After multiple genotypes emerged in China during 1997–2002, a change in the pattern was first noticed in winter 2002, when H5N1 viruses were isolated from dead wild birds in Hong Kong. These 2002 H5N1 isolates were highly lethal to mallard ducks and could cause neurological symptoms [28]. Although HP H5 viruses are highly lethal in chickens, they had rarely been reported to be pathogenic in wild birds. The only recorded incident prior to the Hong Kong H5N1 event was reported in 1961, when an H5N3 virus (A/Tern/South Africa/61) caused deaths in terns. We have further learned that although some of the H5N1 virus isolated since 2002 were initially highly lethal to mallard ducks, antigenic variants with decreased pathogenicity can be selected rapidly in this natural influenza reservoir [29].

The re-emergence of human H5N1 infections in 2004 was accompanied by several unique characteristics of the virus, including an increased host range and increased pathogenicity in mammalian species. Although cats can experimentally be infected

with influenza virus, the first report of natural influenza virus infection in felids was caused by the HP H5N1 virus in a zoo in Thailand: tigers and leopards that were fed H5N1-infected poultry carcasses showed severe pneumonia and succumbed to infection [2]. Further laboratory study confirmed the susceptibility of domestic cats to HP H5N1 infection as well as experimental transmission among cats [3]. In addition to cats, the fatal infection of a dog fed with H5N1-infected duck carcasses in Thailand was reported [4]. Stone martens, a wild mammalian species that, like ferrets, belongs to the *Mustelidae* family, also were infected during an H5N1 outbreak in wild birds in Germany, and H5N1 infection in Owston's palm civet (*Chrotagale owstoni*) was reported in Vietnam [1]. These cases highlight the potential threat of H5N1 in wild mammalian species.

In addition to increased host range, increased viral pathogenicity in mammalian species was associated with H5N1 viruses isolated from human infection [30]. Host range determinants and factors contributing to the high pathogenicity of human H5N1 isolates in mammalian species include viral surface glycoproteins, presence of K at residue 627 in the PB2 protein, the ability to evade the host immune response through viral NS1 protein [21], and viral polymerase activity [31, 32].

Although transmission of the H5N1 viruses among avian species is highly efficient, interspecies transmission from avian species to mammalians remained infrequent. Various mammals (pigs, mice, cats, and ferrets) have been used to study the transmission of HP H5N1 viruses, but their transmission between mammals remains a rare event, except that transmission between cats seems more efficient [3, 33].

Overall, the widely spread HP H5N1 virus has several unique characteristics that should be taken into account for the control of the virus. First is the ability of HP H5N1 viruses to replicate in both the respiratory and gastrointestinal tracts and cause lethal infection in waterfowl reservoirs. Second is that in domestic ducks and waterfowl reservoirs, selection of antigenic variants with decreased pathogenicity to these species can occur. Domestic ducks or waterfowl that harbor the selected variants without apparent symptoms may transmit the virus to chickens or other wild birds (geese or swans), which are highly susceptible to infection, thus causing outbreaks (fig. 2). Third, HP H5N1 virus with increased host range to carnivora species may provide the virus opportunities to further adapt in mammals, including humans (fig. 2).

Drivers of Diversity

The multiple clades and subclades of H5N1 that continue to evolve have been traced phylogenetically to the region of Southern China [34]. Emergence of these phylogenetically distinct lineages is a consequence of the continued circulation of viruses and the selection of those best fitted for continued circulation. The drivers of diversity for

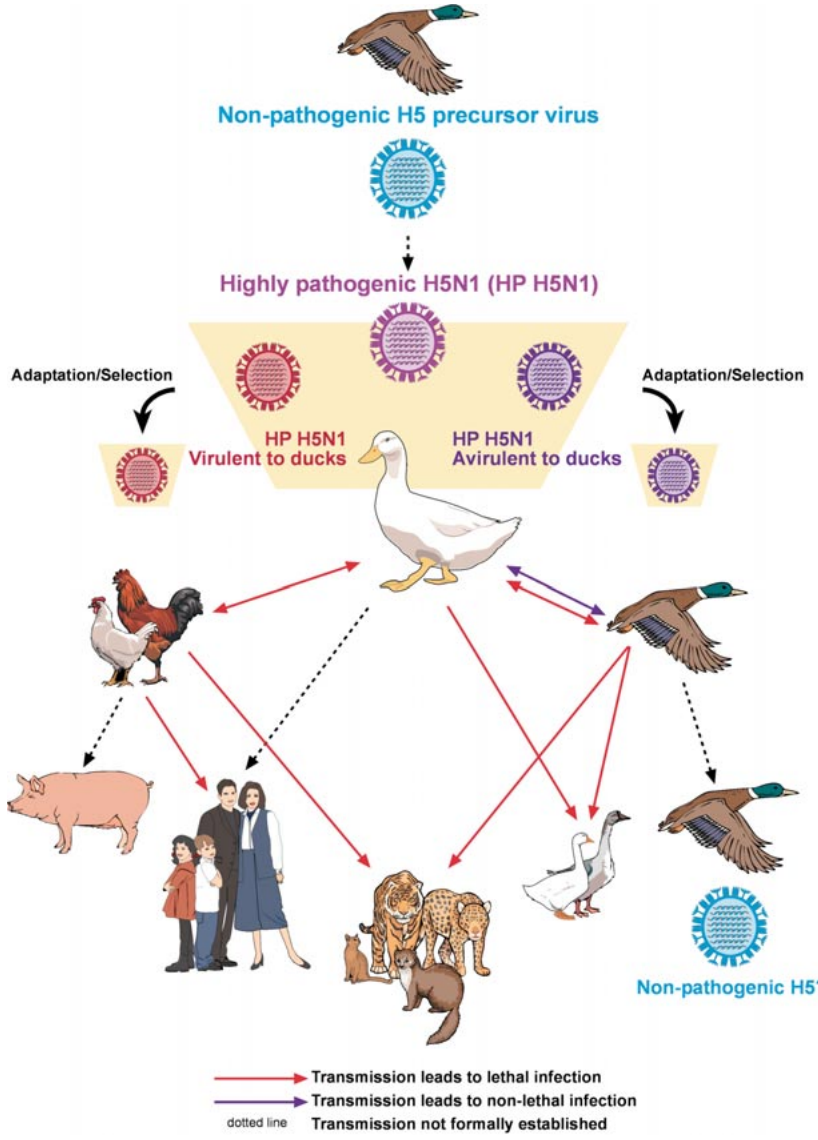


Fig. 2. Drivers of diversity for H5N1 virus. HP H5N1 influenza viruses evolved from non-pathogenic H5 precursors preserved in wild aquatic-bird reservoirs; their eight gene segments derived from the Eurasia influenza gene pool. The proximity of multiple influenza reservoirs and the endemicity of the H5N1 avian influenza virus in Southeastern Asia since 1996 have provided numerous opportunities for the viruses to interact with various avian and mammalian species. Because the selection pressure on H5N1 viruses varies with the host, interspecies transmission events may have driven both antigenic and host range diversity of the virus. Although H5N1 viruses isolated from various animals species are universally highly pathogenic to chickens, these isolates demonstrate variable pathogenicity in other mammals and mallard ducks. Human H5N1 isolates show increased pathogenicity to mammalian species (mice and ferrets), whereas antigenic variants with decreased pathogenicity in mallard ducks can be selected from this natural influenza reservoir.

influenza viruses are conditions that select for antigenic variants and change in host range. The mechanism involved includes accumulation of point mutations and reassortment. From 2000 to 2003, reassortment was rampant in H5N1 viruses in Asia, with the resulting generation of multiple genotypes and eventual selection of the dominant Z genotype. Further reassortment led to the genesis of the Qinghai H5N1 strain (discussed previously).

Selection of antigenic variants classically is associated with immune selection, with both antibodies and T-cell immunity. Experimental studies have shown that ducks that survived infection with H5N1 viruses developed protective immunity and select antigenically distinct viruses that retain high pathogenicity [29]. Thus during the genesis of the Qinghai H5N1 viruses, antigenic and host range variants likely were co-evolving.

The other driver of diversity that is suspected – but not formally established – for the Asian H5N1 viruses is the use of poultry vaccines. Undoubtedly, vaccination can be one of the powerful control measures for eliminating HP influenza outbreaks, as vaccination has been shown to increase resistance to field challenge, reduce shedding levels in vaccinated birds, and reduce transmission among chickens [35]. However, a successful vaccination campaign depends on multiple factors, including the use of high-quality vaccines and continued monitoring of vaccinated flocks and virus shedding. The drawback for the improper vaccine use in poultry may result in antigenic diversity. Selection of antigenic variants likely occurs in poultry (especially ducks) that are protected against disease signs but not against virus shedding. China and Vietnam have launched the use of poultry vaccine since 2005. While poultry outbreaks continued reported from China, poultry outbreak as well as human H5N1 infection have ceased in Vietnam during the winter 2005–2006 (only one report in poultry to OIE in January 2006) but re-emerged since August 2006. Genetic analysis revealed that the Fujian-like H5N1 virus became dominant in China since October 2005 and is genetically related to the re-emerged H5N1 virus isolated in Vietnam. It is not clear if the use of poultry vaccine has driven the emergence of Fujian-like H5N1 virus; however, phylogenetic analysis and limited serology data suggested this possibility [23]. Although poultry vaccine was also applied in Indonesia, this appears to have had little effect in controlling the disease in poultry and the disease is now considered endemic in this region.

In contrast, the Hong Kong model for controlling H5N1 influenza in poultry is the use of vaccine together with improved biosecurity (serologic testing and the use of sentinel chickens). Therefore, vaccination of chickens and monitoring for virus shedding can prevent the spread of H5N1 to both poultry and humans. There have been no re-introductions of H5N1 into the poultry markets or farms in Hong Kong, and no antigenic variants have emerged to evade protection afforded by the vaccine even though H5N1 remains active in the wider region, as illustrated by the detection of H5N1 in smuggled chickens and in small terrestrial birds that essentially fell from the sky during the winters of 2006 and 2007.

Taken together, the close interactions between multiple influenza reservoirs as well as the endemicity of the H5N1 avian influenza virus in Southeastern Asia has provided various opportunities for the viruses to interact with different avian and mammalian species (fig. 2). Because the selection pressure for H5N1 viruses varies with the host species, interspecies transmission events may have driven the virus to evolve in different directions (fig. 2). Vaccination of poultry could be beneficial if high-quality vaccines are applied and sentinel chickens are used to monitor the emergence of H5N1 virus. However, the use of substandard vaccines and the application of vaccines that fail to completely block virus shedding (especially in ducks) may contribute to the diversity of H5N1 viruses. Although the H5N1 vaccines used in domestic poultry are largely efficacious in chickens, less work has been done in waterfowl.

Transmission and Spread of H5N1 Virus: Implications for Control

Integrated data on phylogenetic analysis, migratory bird movement, and trade in poultry and wild birds have suggested the pathways for the spread of H5N1 virus to various countries and continents [34]. The spread of H5N1 among Asian countries was related to trade in poultry, spread to most European countries was most likely through migratory birds, and spread to Africa was due to trade in poultry and to migratory birds [34]. However, controversy remains regarding the transmission and spread of HP H5N1 virus both in Asia and to the rest of Eurasia. This controversy concerns the role of humans (including the poultry industry) and whether migratory or domestic waterfowl were (and still are) the main sources of these H5N1 viruses.

Because of various poultry industry practices, humans likely are the ultimate cause of the continuing HP H5N1 epidemic. As noted earlier, the transmissions of the H5N1 viruses after 2000 in both waterfowl and gallinaceous poultry occurred through the respiratory tract in addition to fecal spread. The water supply remains a key component in spread in several countries in Asia, because high concentrations of virus enter water through the respiratory secretions and fecal droppings of H5N1-infected domestic ducks and wild waterfowl. Chicken farms with low-level or no biosecurity measures or 'open housing' and using untreated water, together with exposure to backyard poultry and live-poultry markets ('wet markets'), provided optimal conditions for viral spread and interspecies transmission (fig. 2). Other human activities that have contributed to the spread of H5N1 including trade or smuggling of both domestic and wild birds as well as the movement of fighting cocks [34, 36]. The practice of raising grazing ducks after the rice harvest and the movement of these ducks in trucks was positively associated with the initial spread of H5N1 in Thailand. The banning of duck egg hatching in Vietnam may have played a role in reducing spread in that country. Live-poultry market surveillance across the southern provinces of China from 2004 through 2006 established persistent low-level detection of HP H5N1 in domestic geese and ducks during each month of the year,

with increased frequency during the cool months and an associated increase in the number of H5N1-infected chickens [20, 23]. After domestic ducks were recognized as silent carriers of H5N1 influenza virus, control measures taken by the Thailand government to cull flocks in which virus was detected markedly reduced H5N1 infection.

Phylogenetic analysis supports the role of wild migrating waterfowl in the introduction of the Qinghai Lake H5N1 virus to central Asia and Europe, but there is still no convincing evidence that HP H5N1 viruses are maintained in this natural influenza reservoir. Whether continued attenuation of HP H5N1 may occur in these species through frequent genetic reassortment within the influenza gene pool, eventually leading to non-pathogenic H5 viruses, requires further study. In addition, surveillance at the breeding sites of migratory waterfowl in Northern Europe and Siberia is needed to establish whether HP H5N1 is being perpetuated in migratory waterfowls.

The human H5N1 infections to date predominantly have been due to close contact with infected poultry. Blocking human contact with H5N1-infected poultry is likely the most effective control measure for the prevention of human H5N1 infection. Several control measures – including banning of ducks and geese from live-poultry markets, the enforcement of monthly 'rest days' for these markets, and public education – have dramatically reduced the live-poultry exposure of Hong Kong residents. Together with the use of a poultry vaccine and sentinel chickens, these measures have successfully blocked human H5N1 infection in Hong Kong since 1997. In contrast, backyard poultry and live-bird markets remain leading sources of exposure of humans to live poultry in Vietnam and China. Overall, effective control of H5N1 requires the integration of multifaceted strategies and global collaboration to achieve eradication.

Conclusions

When this chapter was written (mid-2007), multiple sublineages of the HP H5N1 viruses were continuing to cause lethal outbreaks in domestic poultry in Asia and Africa, with sporadic human infections with greater than 50% lethality. In addition to controlling HP H5N1 avian influenza in poultry and preserving a ready source of protein in developing countries, the greatest concern is whether any of the HP H5N1 virus variants will achieve consistent human-to-human transmission. While they continue to circulate and evolve, the possibility exists that HP H5N1 viruses could achieve pandemic potential.

The issues of continuing concerns are:

- Continued circulation in widely separated regions during the summer months, with peak spread during the cooler months
- Re-introduction of HP H5N1 into regions from which the virus had been eradicated (Japan, South Korea, and Thailand)
- Acquisition of changes in HA receptor binding affinity and specificity and in PB2 that promote replication in mammals

- Increased mammalian host range (humans, pigs, felids, dog, and stone martens), which increases the possibility of adaptation of the avian-originated H5N1 virus to mammals
- HP H5N1 variants that are not uniformly lethal to waterfowl (including domestic ducks) and thus can serve as hidden sources ('Trojan horses') for the maintenance and spread of the virus
- The mechanism of selection of attenuated HP H5N1 variants in waterfowl (natural reservoir of all subtypes of influenza A virus) remains to be elucidated. A possible mechanism includes prior exposure to multiple subtypes of influenza A virus that increase the resistance to HP H5N1
- The possibility, timing, and mechanism of HP H5N1 spread to the Americas and Australia
- The availability of effective vaccines and antiviral compounds during an influenza pandemic
- The highly infectious nature of HP H5N1 in domestic chickens is not a regional crisis but a global problem. Collaborations between countries to adopt effective controls measures are essential for the eradication of HP H5N1.

Even if the HP H5N1 virus does not achieve its pandemic potential in humans, preparations for a potential H5N1 pandemic will not be wasted. These efforts will arm the world for dealing with a future influenza virus pandemic – which is inevitable.

Acknowledgements

The authors thank Scott Krauss and Aleksandr Lipatov for comments, Amy Frazier for editorial assistance, and Klo Spelshouse for illustrations. This work was funded in whole or in part with funds from the National Institute Allergy and Infectious Diseases, National Institutes of Health, under Contract No. HHSN266200700005C, by the American Lebanese Syrian Associated Charities and by a Central Allocation Grant from the Research Grants Council of Hong Kong, HKU 1/05C.

References

1 Peiris JS, de Jong MD, Guan Y: Avian influenza virus (H5N1): a threat to human health. Clin Microbiol Rev 2007;20:243–267.

2 Keawcharoen J, Oraveerakul K, Kuiken T, Fouchier RA, Amonsin A, Payungporn S, Noppornpanth S, Wattanodorn S, Theambooniers A, Tantilertcharoen R, Pattanarangsan R, Arya N, Ratanakorn P, Osterhaus DM, Poovorawan Y: Avian influenza H5N1 in tigers and leopards. Emerg Infect Dis 2004;10:2189–2191.

3 Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA, Osterhaus AD, Kuiken T: Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. Am J Pathol 2006;168:176–183.

4 Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Pariyothorn N, Payungporn S, Theamboonlers A, Chutinimitkul S, Thanawongnuwech R, Poovorawan Y: Fatal avian influenza A H5N1 in a dog. Emerg Infect Dis 2006;12:1744–1747.

5 Choi YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, Buranathal C, Chaisingh A, Auewarakul P, Hanh NT, Ma SK, Hui PY, Guan Y, Peiris JS, Webster, RG: Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 2005;79: 10821–10825.
6 Ninomiya A, Takada A, Okazaki K, Shortridge KF, Kida H: Seroepidemiological evidence of avian H4, H5, and H9 influenza A virus transmission to pigs in southeastern China. Vet Microbiol 2002;88: 107–114.
7 Alexander DJ: An overview of the epidemiology of avian influenza. Vaccine 2006;25:5637–5644.
8 Alexander DJ: Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. Avian Dis 2007;51:161–166.
9 Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD: Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005;79:2814–2822.
10 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–179.
11 Crawford, PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM, Donis RO: Transmission of equine influenza virus to dogs. Science 2005;310:482–485.
12 Rott R, Klenk HD, Nagai Y, Tashiro M: Influenza viruses, cell enzymes, and pathogenicity. Am J Respir Crit Care Med 1995;152:S16-S19.
13 Horimoto T, Kawaoka Y: Pandemic threat posed by avian influenza A viruses. Clin Microbiol Rev 2001; 14:129–149.
14 Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, Webster, RG: Influenza A viruses of migrating wild aquatic birds in North America. Vector Borne Zoonotic Dis 2004;4:177–189.
15 Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, Osterhaus AD, Fouchier RA: Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 2007;3:0630–0638.
16 Okazaki K, Takada A, Ito T, Imai M, Takakuwa H, Hatta M, Ozaki H, Tanizaki T, Nagano T, Ninomiya A, Demenev VA, Tyaptirganov MM, Karatayeva TD, Yamnikova, SS, Lvov DK, Kida H: Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. Arch Virol 2000;145:885–893.
17 Duan L, Campitelli L, Fan XH, Leung YH, Vijaykrishna D, Zhang JX, Donatelli I, Delogu M, Li KS, Foni E, Chiapponi C, Wu WL, Kai H, Webster RG, Shortridge KF, Peiris JS, Smith GJ, Chen H, Guan Y: Characterization of low pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. J Virol 2007;81:7529–7539.
18 Shortridge KF, Zhou NN, Guan Y, Gao P, Ito T, Kawaoka Y, Kodihalli S, Krauss S, Markwell D, Murti KG, Norwood M, Senne D, Sims L, Takada A, Webster RG: Characterization of avian H5N1 influenza viruses from poultry in Hong Kong. Virology 1998;252:331–342.
19 Chen H, Li Y, Li Z, Shi J, Shinya K, Deng G, Qi Q, Tian G, Fan S, Zhao H, Sun Y, Kawaoka Y: Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in Western China. J Virol 2006;80: 5976–5983.
20 Chen H, Smith GJ, Li KS, Wang J, Fan XH, Rayner JM, Vijaykrishna D, Zhang JX, Zhang LJ, Guo CT, Cheung CL, Xu KM, Duan L, Huang K, Qin K, Leung YH, Wu WL, Lu HR, Chen Y, Xia NS, Naipospos TS, Yuen KY, Hassan SS, Bahri S, Nguyen TD, Webster RG, Peiris JS, and Guan Y: Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control. Proc Natl Acad Sci USA 2006;103:2845–2850.
21 Neumann G, Kawaoka Y: Host range restriction and pathogenicity in the context of influenza pandemic. Emerg Infect Dis 2006;12:881–886.
22 Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier, RA: Global patterns of influenza a virus in wild birds. Science 2006;312: 384–388.
23 Smith GJ, Fan XH, Wang J, Li KS, Qin K, Zhang JX, Vijaykrishna D, Cheung CL, Huang K, Rayner JM, Peiris JS, Chen H, Webster RG, Guan Y: Emergence and predominance of an H5N1 influenza variant in China. Proc Natl Acad Sci USA 2006;103: 16936–16941.
24 Liu CM, Lin SH, Chen YC, Lin KC, Wu TS, King CC: Temperature drops and the onset of severe avian influenza A H5N1 virus outbreaks. PLoS ONE 2007;2:1–5.
25 World Health Organization: Evolution of H5N1 avian influenza viruses in Asia. Emerg Infect Dis 2005;11:1515–1521.
26 World Health Organization: Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as human vaccines. http://www.who.int/csr/disease/avian_influenza/guidelines/h5n1virus/en/index.html

27 Smith GJ, Naipospos TS, Nguyen TD, de Jong, MD, Vijaykrishna D, Usman TB, Hassan SS, Nguyen TV, Dao TV, Bui NA, Leung YH, Cheung CL, Rayner JM, Zhang JX, Zhang LJ, Poon LL, Li KS, Nguyen VC, Hien TT, Farrar J, Webster RG, Chen H, Peiris JS, Guan Y: Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. Virology 2006;350:258–268.
28 Sturm-Ramirez KM, Ellis T, Bousfield B, Bissett L, Dyrting K, Rehg JE, Poon L, Guan Y, Peiris M, Webster RG: Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J Virol 2004;78:4892–4901.
29 Hulse-Post DJ, Sturm-Ramirez KM, Humberd J, Seiler P, Govorkova EA, Krauss S, Scholtissek C, Puthavathana P, Buranathai C, Nguyen TD, Long HT, Naipospos TS, Chen H, Ellis TM, Guan Y, Peiris JS, Webster RG: Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc Natl Acad Sci USA 2005;102:10682–10687.
30 Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen DT, Hanh TH, Puthavathana P, Hoang TL, Buranathai C, Lim W, Webster RG, Hoffmann E: Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol 2005;79:2191–2198.
31 Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, Hulse-Post DJ, Humberd J, Trichet M, Rehg JE, Webby RJ, Webster RG, Hoffmann E: The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med 2006;203:689–697.
32 Naffakh N, Massin P, Escriou N, Crescenzo-Chaigne B, van der Werf S: Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses. J Gen Virol 2000;81:1283–1291.
33 Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, Ortin J, Falcon A, Nguyen TH, Mai le Q, Sedyaningsih ER, Harun S, Tumpey TM, Donis RO, Cox NJ, Subbarao K, and Katz JM: Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci USA 2006;103:12121–12126.
34 Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP, Daszak P: Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci U S A 2006;103:19368–19373.
35 Capua I, Marangon S: Control of avian influenza in poultry. Emerg Infect Dis 2006;12:1319–1324.
36 Van Borm S, Thomas I, Hanquet G, Lambrecht B, Boschmans M, Dupont G, Decaestecker M, Snacken R, van den BergT: Highly pathogenic H5N1 influenza virus in smuggled Thai eagles, Belgium. Emerg Infect Dis 2005;11:702–705.

Robert G. Webster, PhD
St. Jude Children's Research Hospital, Department of Infectious Diseases, Division of Virology
332 N. Lauderdale, MS 330, Memphis, TN 38105 (USA)
Tel. +1 901 495 3400, Fax +1 901 523 2622, E-Mail robert.webster@stjude.org

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 27–40

Epidemiology and Control of H5N1 Avian Influenza in China

Hualan Chen · Zhigao Bu · Jingfei Wang

Animal Influenza Laboratory of the Ministry of Agriculture and National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, PR China

Abstract

H5N1, the highly pathogenic avian influenza virus, was first detected in a goose in the Guangdong Province of China in 1996. Multiple genotypes of H5N1 viruses have been identified from apparently healthy waterfowl since 1999. In the years 2004, 2005, and 2006, there were 50, 31, and 10 outbreaks in domestic poultry, respectively. These outbreaks occurred in 23 provinces and caused severe economic damage for the poultry industry in China. A culling plus vaccination strategy has been implemented for the control of epidemics beginning in 2004. Since that time, over 34,000,000 poultry have been depopulated, and over 20 billion doses of the different vaccines have been used to control outbreaks. Although it is logistically impossible to vaccinate every single bird in China due to the large poultry population and the complicated rearing styles, there is no doubt that the increased vaccination coverage has resulted in decreased disease epidemic and environmental virus loading. The experience in China suggests that vaccination has played important roles in the protection of poultry from H5N1 virus infection, the reduction of virus load in the environment, and the prevention of H5N1 virus transmission from poultry to humans.

In the past few years, there have been many significant outbreaks of H5N1 avian influenza involving multiple farm flocks in more than 20 provinces in China. The H5N1 viruses affected millions of domestic poultry, including chickens, ducks, and geese, as well as thousands of migratory wild birds [1]. The Chinese government decided to use a culling plus vaccination combined strategy to control avian influenza virus infection in 2004, and several vaccines, including inactivated and live virus-vectored vaccines, have been developed and successfully applied in the field. Here, we will present the epidemiology, vaccines and vaccination, and the control policy and experience of H5N1 avian influenza in China.

The Current Situation of the Poultry Production in China

China is one of the largest countries for poultry production in the world, with the total production of domestic poultry totaling 15.2 billion in 2005, accounting for 20% of the total amount of global poultry production. Among the 15.2 billion poultry, over 60% are bred in small-scale farms or in backyards. China is home to an even larger population of waterfowl that encompasses approximately 70% of the world total. The majority of the waterfowl are ducks that are distributed in the provinces of Southern China, and these ducks are raised in the open field rich in lakes and rivers. During the breeding season, ducks may migrate from one province to another over hundreds of miles. This special breeding style enables domestic waterfowls to contact both wild waterfowls and other domestic animals, such as chickens and pigs, allowing the waterfowl to play an important role as an intermediate host in the transfer of influenza from wild birds to domestic animals. Waterfowl migration also serves to spread influenza from one place to another, which poses huge difficulties for the control of avian influenza in China.

Surveillance of Avian Influenza Virus

Active surveillance of avian influenza virus has been performed on a regular basis since 1994, when an H9N2 influenza virus was first detected in Guangdong Province [2]. H9N2 is the most prevalent influenza A subtype [3], but several other subtypes including H1, H3, H4, H6, H7 [4, 5], H9 and H14, have been identified from chickens, ducks and geese.

In 1996, an infectious disease with high mortality (40%) was observed in an outdoor rearing goose farm in Guangdong Province. Influenza A viruses were isolated from the samples in embryonated chicken eggs and were identified as being an H5N1 subtype by hemagglutination inhibition (HI) and neuraminidase inhibition tests with a panel of antisera provided by Office International des Épizooties (OIE) Reference Laboratory (Veterinary Laboratory Agency, Surrey, UK) [4, 6, 7]. Although the viruses exhibited different virulence in chickens [8], one of the viruses was highly pathogenic for chickens based on the OIE standard, with an intravenous injection index (IVPI) value >1.2. It was designated A/Goose/Guangdong/1/96 (GS/GD/1/96). This virus contained a series of basic amino acids (-RRKKR-) inserted at the cleavage site of the hemagglutinin (HA) protein characteristic of influenza viruses that are highly pathogenic in chickens [see contribution by Garten and Klenk, this volume]. This was the first documented isolation of a highly pathogenic avian H5N1 influenza virus in Mainland China. Since 1999, H5N1 viruses have been repeatedly isolated from apparently healthy waterfowl, mainly ducks, in Southern China [9]. In 2001 and 2003, two H5N1 viruses were isolated from pigs in Fujian Province during routine surveillance [10].

Outbreaks of H5N1 Avian Influenza in China

Outbreaks in 2004

Starting at the end of 2003, H5N1 avian influenza virus has caused outbreaks in domestic poultry in several countries in Southeast Asia (OIE WBS), including Vietnam, Thailand, South Korea, Japan, Cambodia, and Indonesia. On January 27, 2004, a highly pathogenic H5N1 virus caused an outbreak in domestic ducks in Guangxi Province, and then 49 outbreaks occurred within the next month. Multiple species of birds were affected during these outbreaks, including chickens, ducks, geese, quails, and turkeys, and infection of wild zoo birds was also detected in some areas. Although outbreaks were detected in 16 provinces, 41 cases (83% of the total) occurred in the southern provinces of China, including Yunnan, Guangxi, Guangdong, Hubei, Hunan, Jiangxi, Zhejiang, Anhui, and Shanghai. The origin of the epidemic was not established. However, the epidemiological data gathered allowed for the identification of risk factors in the affected farms and markets. The primary risk factors were the presence of the mixed species and the rearing of the birds in the open field. Five months after the large-scale outbreaks in the 16 provinces were controlled, a new outbreak was detected in a backyard chicken farm in Anhui Province. About 143,000 birds were infected, and over 9 million birds in the threatened area were depopulated to control the epidemic of H5N1 avian influenza in 2004 (table 1, fig. 1).

Outbreaks in 2005

On June 7, 2005, 11 months after the 2004 outbreak in Anhui Province, a goose sample from Xinjiang Province was identified as containing highly pathogenic H5N1 avian influenza virus. The outbreak occurred in a small-scale (<2,000 birds), open-field rearing goose farm. To control this outbreak, 79,000 poultry in the threatened area were depopulated. On June 20, 2 weeks after this outbreak, an outbreak of highly pathogenic H5N1 virus infection was again confirmed in another backyard farm that owned a small amount of ducks and geese (<200 birds) in Xinjiang Province. About 149,000 poultry in the surrounding area were killed to control the spread of the disease. These two farms were located in two different cities that are 500 miles away from each other, and yet the two viruses were similar though multiple mutations were detected in all of the genes. This suggested that the H5N1 avian influenza viruses that caused the outbreaks in these two farms originated form the same source, but that the virus that caused the second outbreak was not directly derived from the first one [unpubl. data]. On August 10, a highly pathogenic H5N1 virus was detected in a backyard farm (<200 birds) in Tibet.

Starting on October 19, a total of 28 outbreaks were detected in domestic poultry in ten provinces, including Xinjiang, Inner Mongolia, Liaoning, Shanxi, Ningxia, Hunan, Hubei, Yunnan, Jiangxi, and Sichuan. All of the outbreaks in Southern China occurred in small-scale backyard farms (<2,000 birds) with mixed populations of chickens and ducks or geese. The outbreak that occurred in the Liaoning Province of

Table 1. H5N1 avian influenza outbreaks in China, 2004–2006

Province	2004			2005			2006		
	out-breaks	birds infected	birds slaughtered	out-breaks	birds infected	birds slaughtered	out-breaks	birds infected	birds slaughtered
Hunan	5	5,961	659,701	2	1,001	136,800	1	1,805	217,000
Hubei	10	18,347	747,570	3	8,844	37,800			
Guangxi	2	880	754,005						
Anhui	5	26,461	569,707	2	1,350	294,000	1	13	200
Guangdong	9	6,877	613,560						
Shanghai	1	1,500	365,000						
Yunnan	7	65,093	330,4997	1	2,500	53,000			
Zhejiang	1	550	67,789						
Jiangxi	3	10,301	1,370,752	1	3,100	332,500			
Tibet	1	425	36,188	1	133	78,800	1[a]		
Henan	1	1,500	20,810						
Xinjiang	1	4,058	15,605	11	7,954	1,365,600	2	3,245	357,413
Gansu	1	400	91,543						
Shanxi	2	2,296	138,549						
Tianjin	1	236	288,244						
Jilin	1	49	936						
Qinghai				1[a]		16,000	1[a]		
Liaoning				4	125,010	19,958,500			
Inner Mongolia				3	3,022	118,100	1	985	8,990
Shanxi				1	8,103	67,800	2	17,600	1,657,745
Ningxia				1	294	99,400	2	51,300	653,930
Sichuan				1	1,800	12,900			
Guizhou							1	16,000	42,000
Total	50	144,934	9,044,956	32	163,111	22,571,200	12	90,948	2,937,278

[a]The outbreaks were detected in wild birds.

Northern China was confirmed on November 9, which was at least 2 weeks after the disease was initially noticed in the field. The delayed disease report in this case resulted in the wide spread of the virus. Infections of 39 farms in two cities were confirmed with 125,010 dead chickens. To again control the spread of the disease, 19,958,500 chickens were depopulated.

Outbreaks in 2006

In 2006, there were 10 outbreaks detected in seven provinces (table 1, fig. 1). The first outbreak occurred in Yunnan Province, which resulted in the death of 16,000 quail. In the threatened area, 42,000 poultry were killed to prevent the spread of the disease.

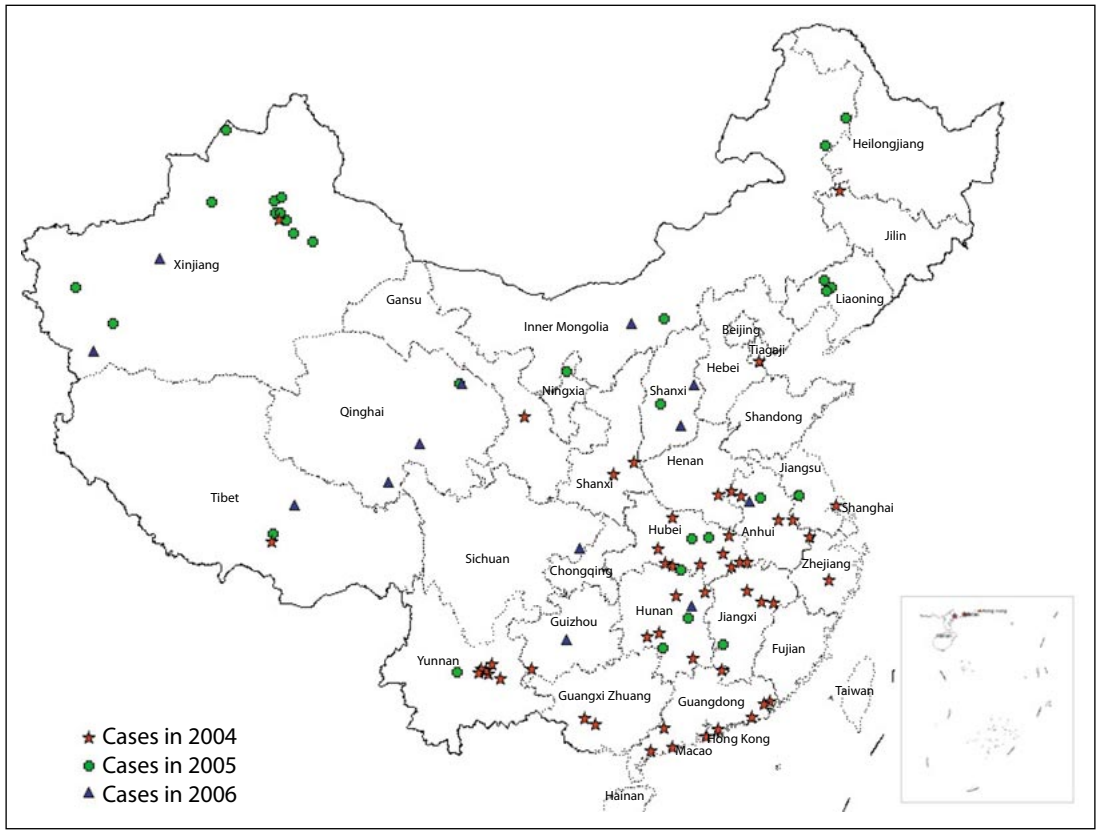


Fig. 1. H5N1 avian influenza outbreaks in China, 2004–2006.

Five outbreaks occurred in small-scale, backyard chicken or duck farms in Hunan, Anhui, Xinjiang and Inner Mongolia. The birds in these farms were not vaccinated, though 100% vaccination of the domestic poultry was encouraged and required by the Chinese government starting at the end of 2005. Four outbreaks in Shanxi and Ningxia Provinces, however, occurred in vaccinated chicken layers. In February 2006, respiratory disease and decreased egg production were noticed in some layer farms in Shanxi Province, and about 10–20% mortality was also recorded in some flocks. Most importantly, the chickens in these farms had been vaccinated with inactivated H5 vaccines and had average HI antibody titers of >8lg2. Samples were sent to the National Avian Influenza Reference Laboratory for disease diagnosis. An H5N1 influenza virus was isolated, but this strain reacted poorly with the antisera raised against GS/GD/1/96 virus-based antigen. Sequence analysis indicated that this H5N1 virus, A/Chicken/Shanxi/2/06 (CK/SX/06), was also genetically quite different from the H5N1 viruses isolated from other locations. This suggested that a new genotype of H5N1 avian influenza virus had been introduced into the chickens in Northern China,

though its origin was still unclear. To control the outbreaks caused by CK/SX/06 virus, over 2,311,000 poultry were depopulated in Shanxi and Ningxia Provinces (table 1). In August 2006, 6 months after the detection of this variant, a new inactivated vaccine was developed by using a recombinant H5N1/PR8 reassortant virus bearing the modified HA and NA genes from the CK/SX/06 virus. The vaccine was applied in selected provinces in Northern China to control CK/SX/06-like viruses.

Outbreaks of H5N1 Avian Influenza in Wild Birds in 2005–2006

Wild aquatic birds are the natural reservoir for all influenza A viruses harboring all 16 HA and all 9 neuraminidase (NA) subtypes. Although influenza viruses are occasionally transmitted from wild aquatic birds to other avian (e.g., chickens and turkeys) and to mammalian (e.g., humans, pigs, horses, minks, whales, and seals) species, where they may produce outbreaks of severe disease, they persist in evolutionary equilibrium (stasis) in their natural reservoir and do not generally cause disease in wild waterfowl [11]. Highly pathogenic H5N1 viruses apparently have not entered wild-bird populations to any appreciable extent until late April to June 2005, when a large outbreak of H5N1 infection occurred in Qinghai Lake in Western China [1, 12], a major breeding site for migratory birds whose flyways extend to Southeast Asia, India, Siberia, Australia, and New Zealand [13].

The islets and wetlands of Qinghai Lake are part of a protected natural reserve for wild birds. More than 100,000 wild birds, representing 189 species, spend the spring and summer at this reserve every year. Since the end of April 2005, bar-headed geese *(Anser indicus)* arriving at Qinghai Lake from Southern Asia have shown signs of disease, including tremor and torticollis. On May 4, 2005, two bar-headed geese were found dead in the wetlands of Qinghai Lake, and 105 geese were reported dead on the following day (fig. 2). On May 13, a total of 437 dead birds were collected. The species identified extended to great black-headed gulls *(Larus ichthyaetus)* and brown-headed gulls *(Larus brunnicephalus)*, whose habitats on the lake overlap closely with those of bar-headed geese. Disease signs and death were observed among ruddy shelducks *(Tadorna ferruginea)* beginning on May 13, with 90 and 12 dead shelducks collected on May 24 and 25, respectively. A limited number of dead great cormorants *(Phalacrocorax carbo)*, gathered on two islets located 2 miles away from concentrations of bar-headed geese and gulls, was first observed on May 16, and a large number of these birds were found dead on May 24–26 and June 1 (fig. 2). Altogether, 6,184 dead gulls, geese, great cormorants, and ruddy shelducks were found from May 4 to June 29, with bar-headed geese accounting for more than half of this total. A limited number of whooper swans *(Cygnus cygnus)*, black-headed cranes *(Grus nigricollis)*, and pochards *(Aythya ferina)* also died during this outbreak.

The epidemiologic information indicated stepwise introduction of the virus into different avian species in the lake. Our sequence analyses revealed that four genotypes of H5N1 influenza viruses contributed to the outbreak and that at least three genotypes of H5N1 viruses were circulating among bar-headed geese, while the

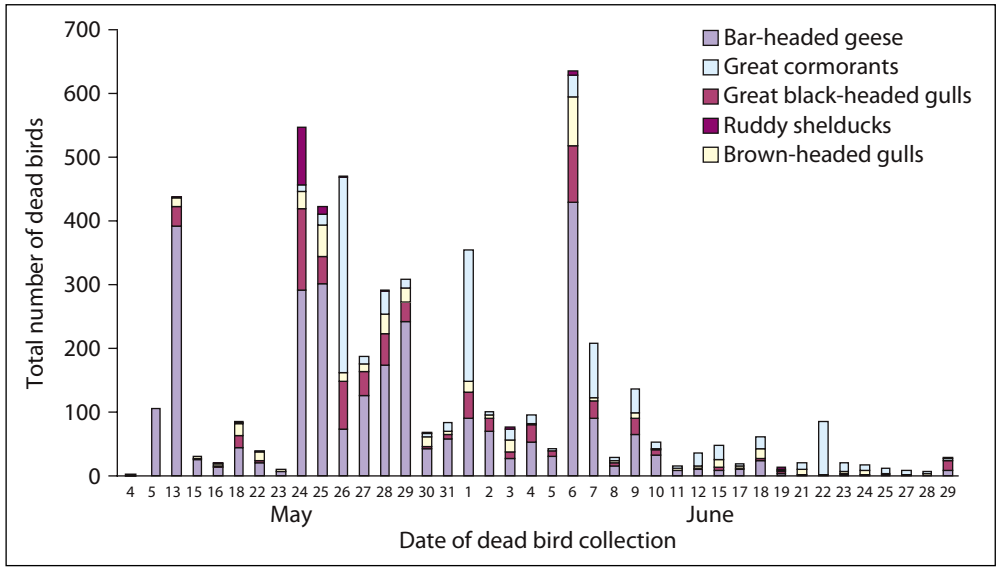


Fig. 2. The H5N1 outbreak in migratory waterfowl at Qinghai Lake. A total of 6,184 dead birds were collected from May 4 to June 29, 2005: 3,282 bar-headed geese, 929 great black-headed gulls, 570 brown-headed gulls, 1,302 great cormorants, and 145 ruddy shelducks.

viruses isolated from great black-headed gulls, brown-headed gulls, great cormorants, and whooper swans were similar to each other and belonged to only one of the genotypes found in bar-headed geese [1]. These data suggested that bar-headed geese infected elsewhere were the species that brought the virus to Qinghai Lake, presumably via the East Asian-Australian flyway or the Central Asian-Indian flyway.

Subsequent to the outbreak in Qinghai Lake from April to June 2005, H5N1 viruses have continued to cause outbreaks in Asia, Europe, and Africa [WHO report, http://www.who.int]. We sequenced the entire genomes of several H5N1 viruses isolated from wild birds in Mongolia in August of 2005, and viral genomes isolated from chickens during major outbreaks in the Liaoning Province and Inner Mongolia in October and November 2005, respectively. Phylogenetic analyses of these viruses and a virus isolated from a wild bird in Russia in August of 2005 showed that these viruses belonged to genotype C [1]. Moreover, all of these viruses possessed a Lys at amino acid position 627 in the PB2 protein. The same genotype caused outbreaks in wild birds in Qinghai and Tibet in 2006 and resulted in the deaths of 3,461 wild birds.

Phylogenetic Analyses of H5N1 Viruses Isolated in China

The H5N1 viruses identified from different dates and locations over the last 10 years are phylogenetically quite different. The HA genes of most H5N1 viruses that were

isolated before 2005 belong to a GS/GD/96-like virus, and all of the isolates had a series of basic amino acids at the cleavage site of HA (-RRKKR-) that is characteristic of influenza viruses that are highly pathogenic in chickens. The viruses could be divided into different forks in the phylogenic tree. The NA genes of the H5N1 viruses isolated in China could be divided into two sub-lineages. The NA genes in one sub-lineage derived from GS/GD/1/96 encode a 20 amino acid deletion in the NA stalk (residues 49–68), whereas the NA gene of GS/GD/96 itself does not. This NA stalk deletion is distinct from, but overlaps with the 19 amino acid deletion found in the HK/97 viruses and the viruses that were detected from eggs of Vietnam waterfowls in 2005 [14, 15].

The phylogenetic trees of the PB2, PB1, PA and NP genes of the H5N1 viruses are very similar. They could be further divided into several sub-lineages. The genes between different lineages showed less than 90% homology, and the genes among different sub-lineages showed about 90–95% homology. The M genes of the H5N1 viruses were relatively conserved, though they also formed multiple forks in the phylogenetic tree. The NS genes of the viruses were separated into two alleles. Some viruses and GS/GD/1/96 were included in the *A* allele, whereas the remainder, including the human HK/97 viruses, were in the *B* allele, which was further divided into two branches. The deduced NS1 amino acid sequence of the viruses in one branch in the *B* allele had a 15 nucleotide deletion resulting in a 5 amino acid (position 80–84) deletion in the NS1 protein. All of the H5N1 genotypes have been described in previous reports [9, 16–18].

Evolution of H5N1 viruses has not only led to multiple genotypes, but also to a large diversity in biological properties. Detailed analysis of these viruses will provide insights into the genetic basis of their host range, antigenicity, and virulence [8, 19].

Control of H5N1 HPAIV in China

A mixed culling plus vaccination strategy is used for the control of outbreaks of highly pathogenic avian influenza in China. After confirmation of a highly pathogenic H5N1 avian influenza infection, all of the poultry within a 3-km radius has to be depopulated. Disinfection and movement control are implemented for 21 days after the poultry depopulation. Any existing live bird market within a 10-km radius will be shut down for at least 21 days. All of the domestic poultry within a buffer zone of a 3- to 8-km ring will be vaccinated immediately. Samples will be taken from the buffer zone for the detection of influenza virus, and negative results are necessary for the retraction of the movement control. The government pays for the vaccines, vaccination implementation and the compensation for the slaughtered poultry.

In 2004, only the birds in the buffer zone were required to be vaccinated. The epidemiological investigation indicated that all of the outbreaks in 2005 occurred in farms that did not vaccinate or vaccinated with unqualified vaccines. Therefore, by

the end of the 2005, the government provided financial support for 100% vaccination coverage in domestic poultry.

Diagnostic Tests

The definitive test for detection and confirmation of the presence of an H5 HPAIV infection in these outbreaks was inoculation of the allantonic cavity of 9- to 11-day-old chicken embryos (OIE manual). It was conducted in the BSL3+ laboratory of the National Avian Influenza Laboratory, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. With many of these outbreaks, confirmation of an H5 infection was possible within 24 h. Surviving embryos were kept for 72 h and repassed if no hemagglutinating activity was detected. HA and NA sequences were usually available within 24 h after the virus was isolated.

H5 Vaccines Developed and Used in China

Vaccine development for the H5 avian influenza has been supported by the government since the detection of HPAIV GS/GD/96 in 1996. During the last 10 years, we have successfully developed several inactivated vaccines using natural low pathogenic H5N2 isolates or low pathogenic high growth reassortants generated by reverse genetics as seed viruses [20, 21]. Two live virus-vectored vaccines using fowlpox virus and Newcastle disease virus as backbone were also developed in China [22, 23]. Most recently, we have generated a HA codon optimized DNA vaccine that is very immunogenic and provides solid HI and NT antibody responses and completely protects chickens against HPAIV H5N1 challenge. Intramuscular injection of two doses of 10 μg of the plasmid induced a very good immune response, and the duration of protective immunity lasted for more than 50 weeks [24].

Inactivated Vaccines

An inactivated oil-emulsified vaccine has been developed using the low pathogenic A/turkey/England/N-28/73 H5N2 virus (kindly provided by Dr. Dennis Alexander) as seed virus. The vaccine was approved to be used in August 2003 in Guangdong Province in chickens that were exported to Hong Kong and Macao. This vaccine was fully evaluated by the Chinese Veterinary Drug Evaluation Committee and certified by the end of 2003. After the H5N1 outbreak in 2004, this vaccine was licensed to nine companies that have Good Manufacture Practice (GMP) facilities and the experience to produce egg cultured vaccines. In total, 2.5 billion doses of H5N2-inactivated vaccine were used in the district where H5N1 outbreaks occurred in 2004.

The H5N2 vaccine played an important role for the rapid control of the H5N1 outbreaks in China in 2004. However, it was still not ideal. First, the vaccine seed virus

exhibited antigenic diversity with the prevalent H5N1 strains in China at the time. Second, the seed virus could not grow to high titers in egg, which severely impaired vaccine production. To solve these problems, we generated a reassortant virus using plasmid-based reverse genetics [25–27], which contained the HA and NA genes from the GS/GD/1/96 virus and the internal genes from the high growth A/Puerto Rico/8/34 (PR8) virus. The multiple basic amino acids (-RRRKKR-) in the cleavage site of the HA protein that are associated with virulence in H5 avian influenza viruses were changed into -RETR-, a characteristic of low pathogenic avian influenza viruses [28, 29, contribution by Garten and Klenk, this volume]. The reassortant virus, Re-1, is completely attenuated in chicken embryos and chickens [20]. It does not kill eggs within 72 h after inoculation and grows to a titer higher than 11log2. Most importantly, the Re-1 virus contains the HA and NA genes of GS/GD/1/96 virus, which antigenically matches well with the H5N1 viruses that circulated in China [9]. This H5N1-inactivated vaccine induced higher HI antibody responses and longer lasting protective immunity in chickens compared with the H5N2 vaccines, and had been shown to be effective in ducks and geese [20]. This vaccine was approved to be used in the field by the end of 2004, and up to the present date, over 10 billion doses of the Re-1 vaccine have been used in China, Vietnam, Mongolia and Egypt.

In early 2006, an H5N1 avian influenza virus was isolated from a chicken flock that had been vaccinated with the H5-inactivated vaccines. The disease in those flocks was recorded as a decrease in egg production and a mortality range of 10–20%. The viruses, represented by CK/SX/06, exhibited a huge antigenic drift from the viruses that were isolated in China previously. Though 187,000 poultry were depopulated to control the spread of this new virus after its first detection in February, the virus was re-isolated in June from Shanxi Province and Ningxia Province. We found that the inactivated H5 vaccines used in China only provided 80% protection to the variant strain in a laboratory challenge study in specific pathogen-free (SPF) chickens, which is quite different from the protective efficacy we reported previously [20]. We therefore developed a new reassortant virus, designated as Re-4, that contained the cleavage site modified HA and NA genes from CK/SX/06 and 6 internal genes from the PR8 virus. This new vaccine was approved for use in Shanxi, Ningxia and several of their neighboring provinces in Northern China in August. A total of 0.84 billion doses were used in 2006 (fig. 3).

Live Virus-Vectored Vaccines

In addition to the inactivated vaccines, we also developed two kinds of recombinant vaccines using fowlpox virus and Newcastle disease virus (NDV) as vectors [21, 23]. After the detection of the GS/GD/96 virus, we started the development of a recombinant fowlpox virus expressing the HA and NA genes of H5N1 virus as a live virus-vectored vaccine. The vaccine efficacy of this recombinant virus was proven in both laboratory and field tests [21, 22]. About 0.7 billion doses of the recombinant fowlpox vaccine have been used in poultry in China since 2005. Whole-virus-inactivated vac-

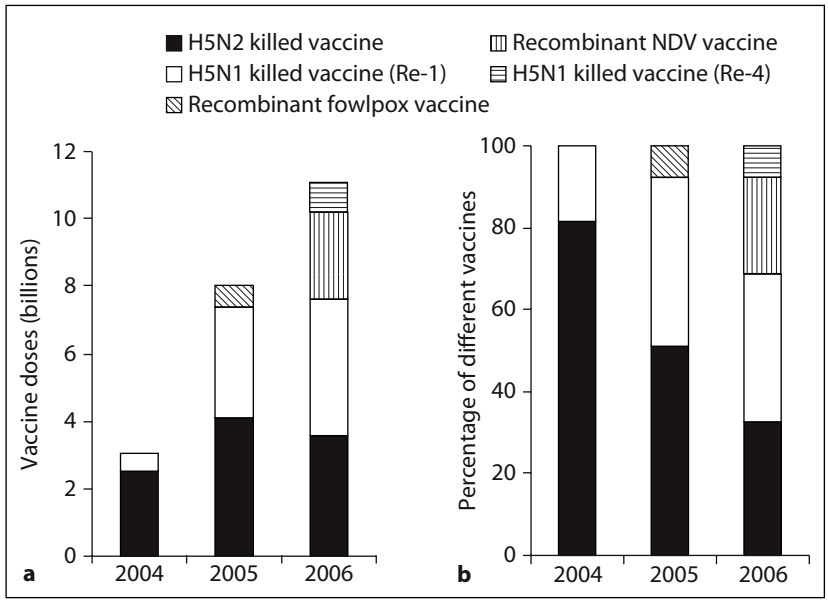


Fig. 3. Vaccines used in China from 2004 to 2006. **a** Doses of the vaccines used in different years. **b** Percentage of different vaccines used in China from 2004 to 2006.

cines and fowlpox virus-based recombinant vaccines have been used as control strategies for highly pathogenic avian influenza in the laboratory and in poultry farms located in different geographic regions in the world [30–33]. However, their high cost of production and the laborious administration of these vaccines are limitations for their wide application in the field.

In 2005, we established a reverse genetics system based on NDV (LaSota) and generated several NDV recombinants expressing HA of several H5N1 viruses from different phylogenetic lineages present in China [pers. unpubl. data]. These viruses included GS/GD/96, A/Anhui/1/05, and A/Bar-Headed Goose/Qinghai/3/05. Recently, we also generated a recombinant NDV expressing the HA gene of the CK/SX/06 virus. We have demonstrated that the recombinant NDV expressing the various influenza HA genes induced strong HI antibody responses to NDV and to H5 avian influenza viruses in chickens. The recombinant NDV vaccinated chickens were protected from disease signs and death from challenge with highly pathogenic NDV. Most importantly, the vaccinated chickens were completely protected from homologous and heterologous H5N1 virus challenges and displayed no virus shedding, signs of disease, or death [23].

The NDV live virus vector vaccine against influenza has several advantages, including the ease of production, the high yield of production, the ease of widespread administration to animals in the field, and the potential for the NDV-based recombinant virus to serve as a bivalent vaccine against two viruses that can decimate bird populations.

The use of NDV as the vaccine backbone should prevent confusion between vaccinated birds and infected birds for surveillance purposes, which is a problematic issue with the use of whole-virus influenza vaccines. Highly pathogenic Newcastle disease has been endemic in China, and >20 billion doses of live vaccines are used in chickens every year in China. In the beginning of 2006, a recombinant NDV virus that expressed the HA gene of GS/GD/96 was approved for use in chickens as a bivalent, live attenuated vaccine for the control of H5N1 avian influenza and highly pathogenic Newcastle disease. By the end of 2006, a total of 2.6 billion doses of this vaccine had been applied in chickens, which dramatically increased the vaccination coverage.

The application of the vaccines played an important role in the control of H5N1 avian influenza in China. Outbreaks and virus circulation were not detected from any of the efficiently vaccinated farms in the last 3 years in China, except for the outbreaks caused by CK/SX/06-like virus in Northern China in 2006. However, it is worthy to note that complete control and eradication of H5N1 HPAIV can only be ultimately achieved by a combination of vaccination, improved biosecurity, extensive surveillance and an effective monitoring program.

Conclusion

Here, we briefly summarized the epidemiology and control of the H5N1 avian influenza in China in the last 10 years. The H5N1 outbreaks in China resulted in the deaths of over 34,952,000 poultry either by infection or depopulation during 2004–2006, and led to severe economic damage for the poultry industry. China employs the culling plus vaccination strategy to control H5N1 avian influenza, and financial support from the government ensures the implementation of this strategy. Billions of doses of the vaccines have been used in the field, and the vaccines are antigenically well matched with the circulating strains. Though the government has required 100% vaccine coverage in domestic poultry since the end of 2005, it is impossible to give every single bird one or two vaccine doses in actual practice as over 70% of the birds are reared in small-scale or backyard farms, often in the open field with ducks and geese. It is apparent that the increased vaccination coverage results in decreased disease outbreaks. There is no doubt that vaccination has played an important role to protect poultry from H5N1 virus infection, reduce the virus load in the environment, and to prevent the transmission of the H5N1 virus from poultry to humans.

Acknowledgements

We thank Dr. Gloria Kelly for editing the manuscript. The authors are supported by the Key Animal Infectious Disease Control Program of the Ministry of Agriculture, the Chinese National S&T Plan Grant 2004BA519A-57, and the Chinese National Key Basic Research Program (973) 2005CB523005 and 2005CB523200.

References

1 Chen H, Li Y, Li Z, Shi J, Shinya K, Deng G, Qi Q, Tian G, Fan S, Zhao H, Sun Y, Kawaoka Y: Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in Western China. J Virol 2006;80:5976–5983.

2 Chen B, Zhang Z, Chen W: Avian influenza research. I. Virus isolation and serologic identification of influenza A virus in chickens. Chin J Vet Med 1994; 20:3–5.

3 Li C, Yu K, Tian G, Yu D, Liu L, Jing B, Ping J, Chen H: Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology 2005;340: 70–83.

4 Tang X, Tian G, Zhao C, Zhou J, Yu K: Isolation and identification of avian influenza viruses circulating in China. Chin J Prev Vet Med 1998;20:1–5.

5 Li Y, Li C, Liu L, Wang H, Wang C, Tian G, Webster RG, Yu K, Chen H: Characterization of an avian influenza virus of subtype H7N2 isolated from chickens in Northern China. Virus Genes 2006;33: 117–122.

6 Chen H, Yu K, Bu Z: Molecular analysis of hemagglutinin gene of goose origin highly pathogenic avian influenza virus. Chin Agric Sci 1999;32:87–92.

7 Xu X, Subbarao K, Cox N, Guo Y: Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 1999;261: 15–19.

8 Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H: The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol 2006;80:11115–11123.

9 Chen H, Deng G, Li Z, Tian G, Li Y, Jiao P, Zhang L, Liu L, Webster RG, Yu K: The evolution of H5N1 influenza viruses in ducks in Southern China. Proc Natl Acad Sci U S A 2004;101:10452–10457.

10 Li H, Yu K, Yang H, Xin X, Chen J, Zhao P, Bi Y, Chen H: Isolation and characterization of H5N1 and H9N2 influenza viruses from pigs in China. Chin J Prev Vet Med 2004;26:1–6.

11 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–179.

12 Liu J, Xiao H, Lei F, Zhu Q, Qin K, Zhang X, Zhang X, Zhao D, Wang G, Feng Y, Ma J, Liu W, Wang J, Gao G: Highly pathogenic H5N1 influenza virus infection in migratory birds. Science 2005;309:1206.

13 Clements JF: Birds of the World: A Checklist. Vista/CA, IBIS, 2000.

14 Chin PS, Hoffmann E, Webby R, Webster RG, Guan Y, Peiris M, Shortridge KF: Molecular evolution of H6 influenza viruses from poultry in Southeastern China: prevalence of H6N1 influenza viruses possessing seven A/Hong Kong/156/97 (H5N1)-like genes in poultry. J Virol 2002;76:507–516.

15 Li Y, Lin Z, Shi J, Qi Q, Deng G, Li Z, Wang X, Tian G, Chen H: Detection of Hong Kong 97-like H5N1 influenza viruses from eggs of Vietnamese waterfowl. Arch Virol 2006;151:1615–1624.

16 Li KS, Guan Y, Wang J, Smith GJD, Xu KM, Duan L, Rahardjo AP, Puthavathana P, Buranathai C, Nguyen TD, Estoepangestie AT, Chaisingh A, Auewarakul P, Long HT, Hanh NT, Webby RJ, Poon LL, Chen H, Shortridge KF, Yuen KY, Webster RG, Peiris JS: Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in Eastern Asia. Nature 2004;430:209–213.

17 Kou Z, Lei F, Yu J, Fan Z, Yin Z, Jia C, Xiaog K, Sun Y, Zhang X, Wu X, Gao X, Li T: New genotype of avian influenza H5N1 viruses isolated from tree sparrows in China. J Virol 2005;79:15460–15466.

18 Wan X, Ren T, Luo K, Liao M, Zhang G, Chen J, Cao W, Li Y, Jin N, Xu D, Xin C: Genetic characterization of H5N1 avian influenza viruses isolated in Southern China during the 2003–2004 avian influenza outbreaks. Arch Virol 2005;150:1257–1266.

19 Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K: Molecular basis associated with replication of duck H5N1 influenza viruses in a mammalian mouse model. J Virol 2005;79:12058–12064.

20 Tian G, Zhang S, Li Y, Bu Z, Liu P, Zhou J, Li C, Shi J, Yu K, Chen H: Protective efficacy in chickens, geese and ducks of an H5N1 inactivated vaccine developed by reverse genetics. Virology 2005;341: 153–162.

21 Qiao C, Yu K, Jiang Y, Jia Y, Tian G, Liu M, Deng G, Wang X, Meng Q, Tang X: Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. Avian Pathol 2003;32:25–32.

22 Qiao C, Tian G, Jiang Y, Li Y, Shi J, Yu K, Chen H: Vaccines developed for H5 highly pathogenic avian influenza in China. Ann NY Acad Sci 2006;1081: 182–192.

23 Ge J, Deng G, Wen Z, Tian G, Wang Y, Shi J, Wang X, Li Y, Hu S, Jiang Y, Yang C, Yu K, Bu Z, Chen H: Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. J Virol 2007;81: 150–158.

24 Jiang Y, Yu K, Zhang H, Zhang P, Li C, Tian G, Li Y, Wang X, Ge J, Bu Z, Chen H: Enhanced protective efficacy of H5 subtype avian influenza DNA vaccine with codon optimized HA gene in a pCAGGS plasmid vector. Antivir Res 2007;75:234–241.
25 Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, Xu X, Lu X, Katz J, Cox N, Matsuoka Y: Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology 2003;305:192–200.
26 Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A: Rescue of influenza A virus from recombinant DNA. J Virol 1999;73:9679–9682.
27 Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y: Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci USA 1999;96:9345–9350.
28 Perdue ML, Garcia M, Senne DA, Fraire M: Virulence associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. Virus Res 1997;49:173–186.
29 Senne DA, Panigrahy B, Kawaoka Y, Pearson JE, Suss J, Lipkind M, Kida H, Webster RG: Survey of the hemagglutinin (HA) cleavage site sequence of H5 and H7 avian influenza viruses: amino acid sequence at the HA cleavage site as a marker of pathogenicity potential. Avian Dis 1996;40:425–437.
30 Capua I, Terregino C, Cattoli G, Mutinelli F, Rodriguez J: Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol 2003;32:47–55.
31 Ellis T, Leung C, Chow M, Bissett L, Wong W, Guan Y, Peiris M: Vaccination of chickens against H5N1 avian influenza in the face of an outbreak interrupts virus transmission. Avian Pathol 2004;33:405–412.
32 Van der Goot J, Koch G, de Jong M, van Boven M: Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens. Proc Natl Acad Sci USA 2005;102:18141–18146.
33 Swayne D, Garcia M, Beck J, Kinney N, Suarez D: Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine 2000;18:1088–1095.

Hualan Chen
Harbin Veterinary Research Institute
427 Maduan Street, Harbin 150001 (PR China)
Tel. +86 451 8276 1925, Fax +86 451 8273 3132, E-Mail hlchen1@yahoo.com

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 41–58

Avian Influenza in Northern Eurasia

Dmitri K. Lvov · Nikolai V. Kaverin

The D.I. Ivanovsky Institute of Virology, RAMS, Moscow, Russia

Abstract

Numerous avian influenza viruses are abundant in the bird populations of Northern Eurasia. All hemagglutinin subtypes except H15 and H16 have been identified in Russia and neighboring countries. Overall, 841 strains were isolated in Russia in 1980–2006 from wild birds. The isolation and characterization of H5 strains is of special interest in connection with the recent spread of highly pathogenic avian H5N1 viruses occasionally causing severe disease in humans. Several H5N2 and H5N3 strains had been isolated in 1976 and 1981 in the Caspian Sea Basin. More recently, in 1991–2001, strains belonging to the same subtypes were isolated in Siberia, indicating continuous circulation of H5 viruses. The highly virulent H5N1 influenza virus first appeared in Russia in summer 2005. Comparative sequence analysis of the genes of probable precursor viruses circulating in wild birds in Russia in the 1990s, of human isolates of highly pathogenic H5N1 virus from Asia and of recent Russian H5N1 viruses isolated from poultry and wild birds supports the concept that these viruses may be the source of a future pandemic virus.

Introduction

The territory of Northern Eurasia, that is Eastern Europe (including the European part of Russia, Ukraine and Byelorussia) together with Siberia, Kazakhstan and the northern part of Central Asia, is rich in wildlife. Numerous species of birds inhabit vast plains and mountains, ranging from arctic deserts through subarctic tundra barrens, north, middle and south taiga forest belts, mixed forests to grass-covered steppes and southern sand deserts. This is the largest nesting area of birds in the world, and it is connected with Asia, Africa, America and Pacific Islands by migration routes. At present we know that numerous avian influenza viruses are abundant in the bird populations of Russia and neighboring countries. However, till the end of the 1960s, data on the presence of influenza viruses in Northern Eurasia were lacking. At that time not the wild birds, but rather poultry was the source of avian influenza virus isolation in the USSR. Large-scale virological and serological studies of wild birds started after the foundation of the National Committee on the Studies of Viruses

Ecologically Linked to Birds in 1969. However, the initial period of Russian avian virology in the 1960s, although restricted to poultry, was by no means futile. One of the first avian viruses isolated in the USSR, A/Duck/Ukraine/1/63, was destined to play an important role in the development of the theory of influenza virus evolution.

Early Studies on Avian Influenza Viruses Isolated from Poultry

We shall begin this chapter with a review of early studies involving mostly the isolation of viruses from poultry and a preliminary characterization of the isolates. Then we shall describe large-scale virus isolation studies which provided information on the circulation of avian influenza viruses in numerous species of wild birds in Northern Eurasia. In the final part of the chapter we shall present recent data on the circulation of the precursors of the highly pathogenic avian H5 viruses in Russia, and, finally, the appearance of H5N1 virus in the Russian territory.

In 1960–1964 a group of researchers in Ukraine isolated several influenza virus strains from ducklings affected with sinusitis. The first three strains were isolated in 1960 in Crimea and in Kharkov Region [1]. The strains were initially designated as Ya-60, B-60 and S-60 [2]. Several other strains (Z-61, C-61, N-62, D-62, D-62, Z-62, S-64 and BV1) were isolated from ducks and chickens in 1961–1964 [3]. The initial characterization of the isolates was performed at the Ukrainian Research Institute of Experimental Veterinary Science in Kharkov. The strains were studied with respect to their pathogenicity for poultry, the ability to accumulate in embryonated chicken and duck eggs, resistance to heating, and stability at storage. However, the most peculiar features of these isolates were revealed in comparative studies performed at the D.I. Ivanovsky Institute of Virology, Moscow. As early as in 1964 the duck strains Ya-60, B-60, Z-61 and C-61 were analyzed with respect to their antigenic specificity in HI test and found to be antigenically distinct from the human H1 and H2 viruses [4]. After the appearance of the H3 pandemic virus in 1968, some of the Ukrainian duck strains were shown to be antigenically related to the new subtype. In 1969, Zakstelskaja et al. [5, 6] demonstrated that duck strains B-60 and BV1 cross-reacted in HI test with the A/Hong Kong/1/68 pandemic strain and other human strains isolated in 1968, whereas strain Ya-60 exhibited a negligible cross-reaction with the human viruses. Moreover, B-60 and BV1 viruses reacted in HI test with human sera, including those collected in 1881–1886 and in 1905–1908 [5]. The authors suggested that an avian virus similar to strains B-60 and BV1 was the precursor of the human pandemic strain, and that this subtype circulated in humans several times in the past.

The strains isolated in Yalta (Crimea) and in Borki (Kharkov Region), initially designated by the first letters of the towns as Ya-60, B-60 and BV1 [2, 3], were later redesignated as A/Duck/Ukraine/1/60, A/Duck/Ukraine/2/60, and A/Duck/Ukraine/1/63, respectively [7]. The latter strain has become widely known, not to say famous. The data indicating a close antigenic relatedness of its hemagglutinin to the hemagglutinin

of the human 1968 pandemic virus were soon confirmed [7, 8]. Later the close resemblance and probable genetic relatedness of the hemagglutinins was proven by peptide analysis [9], oligonucleotide mapping [10], RNA-hybridization studies [11], and sequencing [12]. The relatedness of the hemagglutinin of the A/Duck/Ukraine/1/63 strain to the hemagglutinins of human H3 viruses proved to be one of the first clues to the origin of pandemic viruses as postulated by Webster and Laver [13]. The strain A/Duck/Ukraine/1/60, formerly known as Ya-60, was shown to belong to the H11N2 subtype, whereas A/Duck/Ukraine/2/60 was identified as H3N6 and A/Duck/Ukraine/1/63 as H3N8 [14]. Unfortunately, the Ukrainian duck strains isolated in 1960–1964, with the exception of A/Duck/Ukraine/1/63, were later not studied in detail.

In 1967–1973, avian influenza virus strains belonging to several subtypes were isolated in the USSR from sick chickens and ducks. Highly pathogenic H5N2 and H7N2 strains were isolated from chickens in Moscow Region [8, 15]. Several virus strains producing enteritis in chickens were isolated in 1972 and in 1974 in chicken farms and identified as H6N2 strains [8, 16, 17], which is an unusual antigenic formula for a pathogenic virus affecting poultry. Six H3N2 isolates were obtained in a chicken farm in Kamchatka Region from chickens affected with rhinitis, conjunctivitis, and laryngotracheitis [8, 18]. In 1977, isolates identified as H3N1 viruses were isolated from sick chickens and ducks in Russia [19] and in Uzbekistan [20]. An H8N4 virus was isolated in 1984 in the western part of Ukraine from the lungs of ducklings affected with pneumonia. It was the only isolation of an H8 influenza virus in USSR [D.K. Lvov, unpubl. data].

Overall, the isolation and characterization of the avian influenza viruses from poultry in the USSR enhanced our knowledge on the pathogenic potential of influenza A viruses belonging to different subtypes. The most important basic achievement, however, was the isolation of the strain A/Duck/Ukraine/1/63 and the establishment of a relatedness of the avian and human H3 influenza viruses as revealed by the comparative antigenic analysis.

Influenza Viruses Isolated from Wild Birds

Starting from 1970, a large-scale series of virus isolation from wild birds combined with serologic studies was initiated as a part of the Coordinated Program of the National Committee on the Studies of Viruses Ecologically Linked to Birds together with the Virus Ecology Center of the D.I. Ivanovsky Institute of Virology. In 1974–1991, the isolation of viruses from wild birds was performed by researchers of the Virus Ecology Center or by affiliated groups of researchers all over the territory of the USSR. The viruses isolated by the affiliated groups were characterized at the D.I. Ivanovsky Institute of Virology. After 1991, the work on virus isolation and characterization was continued at the Virus Ecology Department of the D.I. Ivanovsky Institute

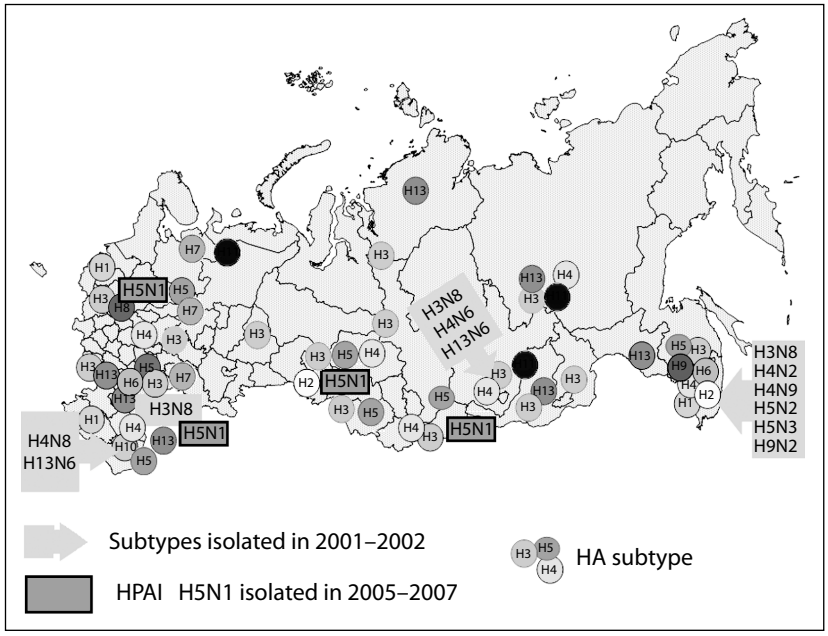


Fig. 1. Avian Influenza A virus subtypes isolated in Russia (1962–2006).

of Virology. The present review describes all data on influenza virus isolation from birds in the USSR and the Russian Federation. It includes studies performed at the D.I. Ivanovsky Institute of Virology, Moscow, in collaboration with the affiliated groups and recent work performed since 2005 by the group in the State Research Center for Virology and Biotechnology Vector, Koltsovo, Novosibirsk Region. By the end of the 1970s the pattern of circulation of avian viruses in the territory of USSR was established [17, 20, 21]. At present all the hemagglutinin subtypes except H15 and H16 have been identified in Russia (fig. 1) and neighboring countries. The data on virus isolation discussed in the review are illustrated in figure 1.

A large-scale serologic survey was started in 1970 in the Russian Far East. The sera collected in spring and autumn of 1970 near Khanka Lake and Peter the Great Bay (Primorsky Region) from 262 birds including 5 species of ducks (mallard, teal, Baikal teal, falcated duck and pintail), and 5 other species (grey heron, snipe, coot, black guillemot and black-tailed gull) were HI-tested against H1, H4, H5, H6, H10 and H11 avian influenza viruses [22]. No antibodies were revealed in the sera of grey heron and coot, and no antibodies against H11 were revealed in any species. Antibodies against all the other subtypes tested were found occasionally in the sera of gull, snipe, black guillemot and 5 species of ducks. In some species, such as teal, falcated duck and black guillemot, antibodies against several subtypes were detected. In 1972, sera were collected in Commander Islands from gulls, cormorants, murres and tufted puffins.

Antibodies against H2, H3, H5 and H7 viruses were detected [23]. In 1970–1972, sera of gulls, cormorants, and murres were collected in Kamchatka, Sakhalin, and Magadan regions, and antibodies to H1, H2, H3, H5, H6 and H7 viruses were detected [17]. In sera collected in 1969–1972 from Arctic terns, loons, ducks, wild geese, skuas and a blue whistling thrush in the White Sea Basin in the estuary of Pechora River (Archangel Region) antibodies against H1, H3, H4, H5 and H7 subtypes were observed [24]. Antibodies against H1, H3 and H5 subtypes were revealed in the sera of birds belonging to 5 orders collected in 1975 in Eastern Kazakhstan [25]. The serologic studies suggested a wide range of avian influenza viruses circulating in wild birds in Northern Eurasia. This concept was confirmed and extended by the isolation of virus strains from wild birds. The viruses were isolated mostly from cloacal and tracheal swabs or, sometimes, from suspensions of internal organs.

Many avian species proved to be the hosts of H1 viruses. A virus belonging to H1N3 subtype was isolated in 1977 from a tern in the southern part of the Caspian Sea Basin [26]. In 1978 an H1N4 strain was isolated from a common teal in Buryatia (Eastern Siberia) [21]. Several H1N1 viruses were isolated in Kazakhstan from waterfowl, including common teal, garganey teal, shoveler and coot in 1979 [27], as well as from a sparrow and a crow in 1980 [21]. In 1979 an H1N1 virus was isolated from a hawfinch in Mongolia [28]. In the same year an H1N2 strain was isolated from a great black-headed gull on an island in the northern part of the Caspian Sea [21].

Avian viruses belonging to H2 subtype seem to be not abundant in Russia. In fact, for a long time the only virological evidence of the presence of this subtype in Russia was the isolation of an H2N3 virus from a pintail in Primorsky Region (Russian Far East) in 1976 [29]. However, serologic data suggested that H2 viruses circulated in wild birds not only in Primorsky Region, but also in other regions of the Russian Far East, including Commander Islands, Kamchatka, Sakhalin and Magadan Regions [17, 23].

Avian influenza viruses belonging to H3 subtype are widespread not only in Russian Far East, but also in several other regions of Northern Eurasia. An H3N2 virus was isolated from a common murre on Sakhalin Island in 1974 [30], and another H3N2 strain was isolated from a pintail in Primorsky Region in 1976 [31]. Two H3N2 strains were isolated in 1974 in Ukraine from hosts unusual for avian viruses, a wagtail and a turtledove [32]. H3N2 strains were also isolated from crows in Middle Volga Basin in 1972 and from a sheldrake in Khazakstan in 1979 [33]. An H3N2 virus was isolated in 1983 in Ukraine from a sparrow [34]. In 1972–1973, H3N3 and H3N8 viruses were isolated from ducks and herons in Khabarovsk Region (Russian Far East). One of them closely resembled a strain isolated one year later in Central Asia. This resemblance demonstrated that the H3N3 viruses circulated in regions fairly distant from each other [35]. The H3N8 viruses were isolated in 1972–1973 in Khabarovsk Region from wild ducks, tufted puffins and horn-billed puffins [33], and in Archangel Region in Pechora estuary (White Sea Basin) from an Arctic tern and a loon [36]. In 1978, H3N8 strains were isolated in Buryatia from a wild duck and a pintail [33], and in Khabarovsk Region from a murre [35] and from gulls [37].

Avian viruses of H4 subtype were isolated in 1970–1980 mostly in a narrow belt stretching from the lower Volga through Kazakhstan to the south of Eastern Siberia. Several H4N6 strains were isolated in 1976 from slender-billed gulls in the Volga estuary [38] and from great black-headed gulls on the islands in the north part of the Caspian Sea [20]. In 1977 an H4N8 virus was isolated from a black tern in Central Kazakhstan [39]. In Buryatia (Eastern Siberia) H4N6 strains were isolated from a teal and a common goldeneye in 1978 [21].

Isolations of H5 influenza viruses from wild birds were scarce. In 1976, several H5N3 strains were isolated from terns (common terns and little terns) and a slender-billed gull in the Volga River delta [38]. Strains belonging to the H6 subtype do not seem to be abundant, but their geographic distribution is wide. An H6N2 strain was isolated in 1972 from an Arctic tern [36] in Archangel Region (White Sea Basin). An H6N4 strain was isolated from a pintail in Primorsky Region (Russian Far East) in 1978 [21], and an H6N8 strain was isolated from a common tern in the Caspian Sea Basin in 1977 [26]. An H7N3 strain was isolated in 1972 in Archangel Region from a sandpiper [36], and an H9N2 virus was isolated in Primorsky Region in 1982 from a mallard [40].

Over 40 strains of H10N5 virus were isolated from a wide array of bird species near Alakol Lake in Southern Kazakhstan in 1979. The strains were isolated from several species of ducks, from shorebirds, passerine birds, two species of harriers, a grebe, a heron, a coot, a plover, a chukar, a crow, and a magpie [21]. This is a rare case of an isolation of closely related viruses from an extremely wide array of avian species. Viruses identified as H11N8 strains were isolated in 1972 from Arctic tern and a red-throated diver in the northern part of European Russia, in the estuary of the Pechora River [17]. Several H11N6 strains were isolated from teals, widgeons, and a golden plover in Eastern Siberia in 1979 [21]. In 1987, H12N2 strains were isolated from mallards, a pintail, and a widgeon in Kyrgyzstan, south of Issyk-Kul Lake [40].

The virus isolation and serologic studies carried out in the territory of the USSR in 1970–1980 suggested a wide circulation of avian influenza viruses in wild birds and allowed construction of a map of avian influenza viruses present in different regions of Northern Eurasia. The general pattern of distribution of influenza virus subtypes in wild birds was fairly evident by the end of the decade. Virus isolation was continued in the following years, and it brought several major results. The isolations were performed mostly in the central and southern parts of European Russia, in Western and Eastern Siberia, and in the Russian Far East [40]. Overall, 841 strains were isolated in Russia in 1980–2006 from wild birds (table 1). About 250 samples were taken every year from 50 to 100 birds in each geographic region. The mean percentage of successful isolations was 3.5–5.7%. Over 50% of the isolates were H13 viruses (H13N2, H13N3, H13N6 and H13N8) obtained mostly from gulls and shorebirds in the north part of the Caspian Sea. The viruses of H3 subtype were isolated in several regions (over 25% of the whole number of isolates). Subtypes H1 and H4 were also relatively abundant (8 and 4% respectively).

Table 1. Isolation of influenza A strains in Northern Eurasia (1980–2006)

HA subtype	NA subtype									Total	
	1	2	3	4	5	6	7	8	9	n	%
1	58		6			4				68	8.1
2		2	1							3	0.4
3	2	13		1		32		159		207	24.6
4	2	1	3	2		18		4	1	31	3.7
5	24	3	9							36	4.3
6		2		1				1		4	0.5
7	8		1				1			10	1.2
8				1						1	0.1
9		6		1						7	0.8
10				12				6		18	2.1
11		1				6		1	4	12	1.4
12		2								2	0.2
13		81	58			289		10		438	52.1
14					3	1				4	0.5
Total, n	94	111	78	18	3	350	1	181	5	841	
%	11.2	13.2	9.3	2.2	0.4	41.6	0.1	21.5	0.6		

Many strains isolated in 1979–1985 on Zhemchuzhny Island in the northeastern part of the Caspian Sea from great black-headed gulls, herring gulls and Caspian terns were not identified at the time of isolation with respect to the subtype of their hemagglutinin. Two isolates were identified as H5N2 strains, whereas the rest (93 isolates) failed to react in HI test with any of the reference immune sera available at the time. In fact the strains belonged to subtype H13. This subtype was first described in 1982 [41], and in 1989 the mysterious Caspian isolates were identified as H13N2 (34 strains), H13N3 (4 strains) and H13N6 (45 strains) [42]. Comparison of strain A/Great black-headed gull/Astrakhan/277/84 with the hemagglutinins of two American strains isolated from a gull and a pilot whale allowed the molecular and antigenic characterization of the subtype H13 [43].

The virus isolation studies in the Northern Caspian Basin were continued in 1986–1999. Samples were collected from great black-headed gulls, herring gulls and Caspian terns in the area of the northern coast of the Caspian Sea from the estuary of the Terek River in the North Caucasus to the Emba River in Western Kazakhstan. Most of the isolated strains (176 out of 182) belonged to subtype H13, including 24 H13N2, 3 H13N3, 141 H13N6 and 8 H13N8 isolates. In addition, 1 H4N3, 1 H4N6, 2 H6N2 and 2 H9N2 strains were isolated [44]. In 1990, a new, previously unrecognized, subtype of influenza virus hemagglutinin designated H14 [45] was described on the basis of the characterization of two strains isolated in 1982 from mallard ducks

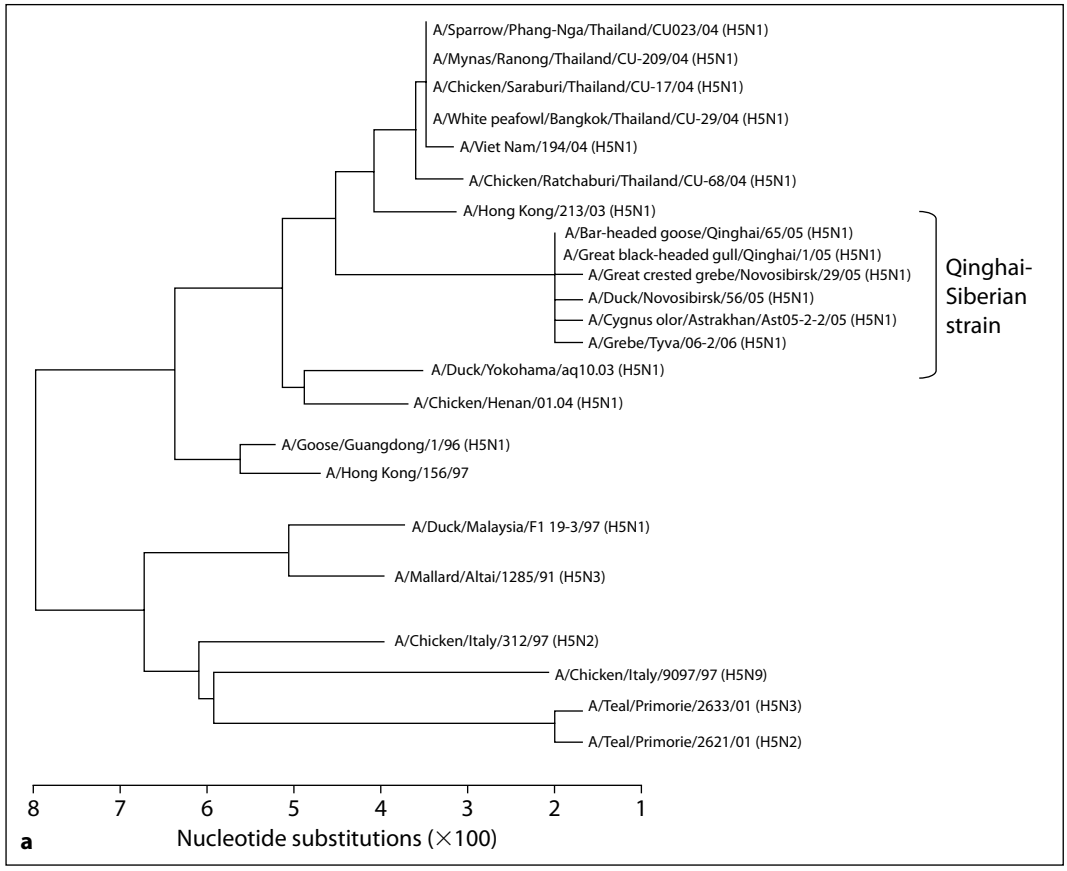
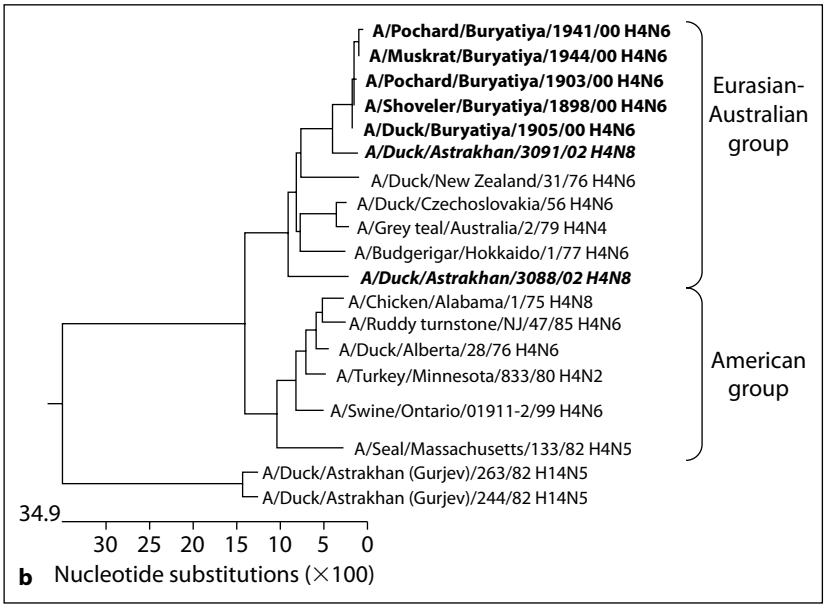


Fig. 2. Phylogenetic trees for (**a**) HA gene of H5 subtype [56, 57], (**b**) HA gene of H4 subtype [40], (**c**) NP gene (1027–1415 fragment) [40], and (**d**) NS gene (600–852 fragment) [40].

in the estuary of the Ural River (Western Kazakhstan). The strains belonging to H14N5 and H14N6 subtypes were isolated from mallards and gulls [44]. Partial sequence analysis revealed that the NS gene of the H14 strains isolated from gulls was closely related to the NS gene of H9 and H13 strains isolated previously from gulls and terns in the Caspian Sea basin and of an H9N4 strain isolated in the Russian Far East. The NS gene of an H14N5 strain isolated from a mallard was much more distantly related to the NS gene of the viruses isolated from gulls [44]. The results suggest that reassortment events play a significant role in the evolution of H14 viruses and that the NS gene is an important determinant of the host range.

Large-scale isolation of avian influenza viruses from fecal samples was performed in 1995–1998 in Eastern Siberia and the Russian Far East by a group including both Russian and Japanese researchers [46]. The samples were collected from ducks, including mallards, teals, and pintails, geese, swans, mew gulls and shorebirds. Although the



samples were taken at 11 different sites, viruses were isolated only in two places in the basin of the Lena River (in Yakutsk and in Kobyaysky area), and only from ducks. 38 strains were isolated, among them 5 H3N8, 20 H4N6, 1 H4N9, 1 H11N1, 2 H11N6, 8 H11N9, and 1 H13N6 virus. Partial sequencing of NP genes of 19 isolates was performed. The NP genes of 5 H11N9 Kobyaysky duck strains had sister-group relationships with the NP genes of H5N1 viruses isolated in Hong Kong in 1997 from humans and chickens, whereas the NP genes of an H3N8 virus isolated in Yakutsk in 1997 was close to the NP gene of viruses isolated from ducks in Hokkaido in 1996. The H4N6 and H11N6 viruses formed a subgroup together with another virus isolated in Hokkaido and an H9N2 virus isolated from a duck in Hong Kong in 1997. The authors considered their findings as evidence for precursor genes of pandemic viruses perpetuating in ducks nesting in Siberia. 23 viruses, among them 2 H3N8, 2 H7N1, 2 H7N8, 2 H13N1, and 15 H13N6 strains, were isolated in a valley of the Sayan Mountains in Southeastern Siberia in summer 2000 [47]. The H3N8 and H7N8 strains were isolated from ruddy sheldrakes and redshanks, the H7N1 strains from pochards, and the H13N1 strains from a shoveler and a grebe. The H13N6 strains were isolated from all of these species, as well as from teals, tufted ducks, and terns.

In 2000–2002, 43 virus strains were isolated in the same region from 1,750 samples taken from 48 bird species. The strains belonged to H3N8, H4N2, H4N6, H4N8, H4N9, H5N2, H5N3, H9N2 and H13N6 subtypes [40]. Interestingly, a strain isolated in Buryatia in 2000 from a muskrat [47] was identified as an H4N6 virus closely resembling the H4N6 strains isolated in the same year and the same region

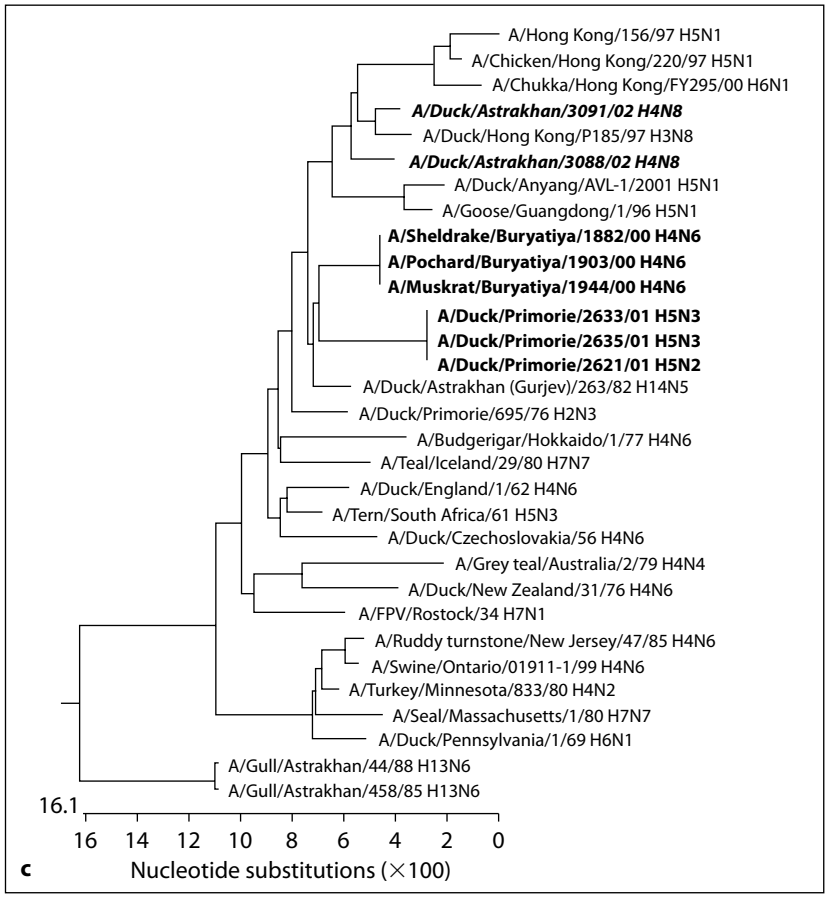
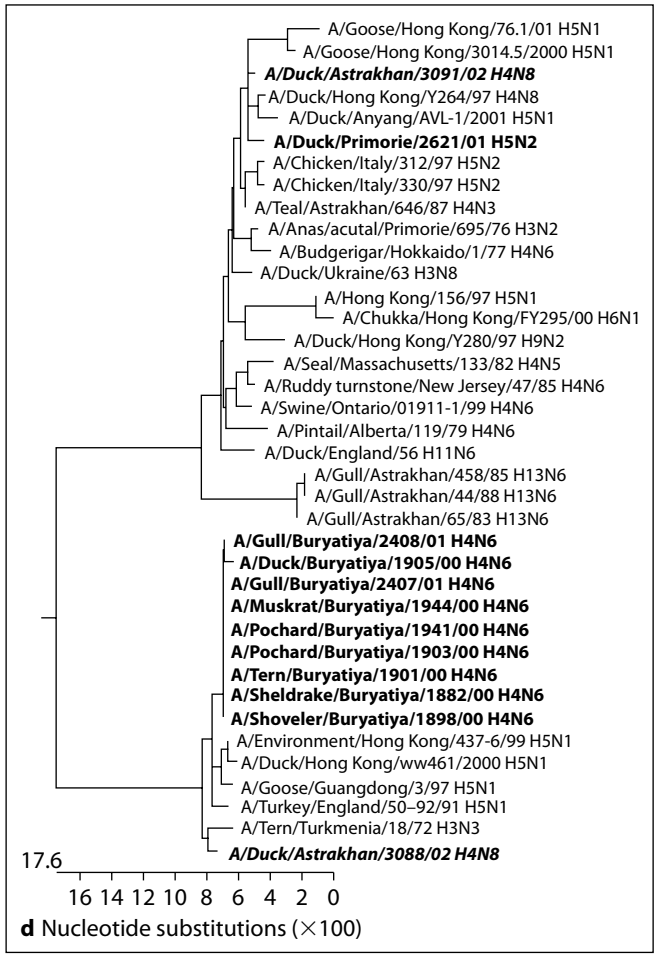


Fig. 2. (continued)

from a sheldrake and a pochard [40]. The isolates and several earlier strains were characterized by partial sequencing (fig. 2). The hemagglutinins of the H4 strains isolated in Buryatia (including the muskrat strain) formed a separate group of the Eurasian-Australian branch in the phylogenetic tree of H4 hemagglutinin (fig. 2b). They had a C-terminal proline residue in the HA1 subunit in contrast to the serine residue of most Eurasian strains. On the other hand, their NP and NS genes were closely related to the NP and NS genes of the H5N1 strains isolated in Southeast Asia (fig. 2c, d). The hemagglutinin genes of the H5N2 isolates had connecting peptides identical to the ones of the low pathogenic strains isolated from ducks in Hong Kong and Malaysia, LRNVPQRETR/GL [48]. On the other hand, the hemagglutinins of the H3 and H4 strains isolated from teals in 2002 and from mallards in 2003 near Chany Lake in Novosibirsk Region (Western Siberia) were related to the hemagglu-



tinins of the European H3 and H4 strains [49, 50]. Unlike the H3 and H4 hemagglutinins, the hemagglutinins of H2 strains isolated in the same area in 2003 from mallards resembled the hemagglutinins of H2 strains isolated in Japan from mallards in 2001 [50]. In 2003, influenza virus strains belonging to the rare subtype H8N6 were isolated in Mongolia from a great cormorant, a white wagtail, and a magpie [51].

Recent Data on the Circulation of Highly Pathogenic H5 Strains and the Appearance of H5N1 Viruses

The isolation and characterization of H5 strains is of special interest in connection with the recent spread of highly pathogenic avian H5N1 viruses occasionally causing

severe disease in humans. Several H5N2 and H5N3 strains had been isolated in 1976 and 1981 in the Caspian Sea Basin [38, 42]. More recently, in 1991–2001, strains belonging to the same subtypes were isolated in Siberia, and their features proved to be relevant for the concept of the H5 viruses circulation. The hemagglutinins of the strains isolated from teals in 2001 in the Primorsky Region (Russian Far East) were shown to be closely related to the H5 strains isolated in 1997 in Italy from poultry [46, 48], whereas the hemagglutinin of the H5N3 strain isolated as early as 1991 in the Altai Region in Southwest Siberia from a wild duck was closely related to the hemagglutinin of the strain isolated in Southeast Asia, A/Duck/Malaysia/F1 19–3/97 (H5N1) (fig. 2a).

Sequence analysis of other genes of the H5 viruses isolated in Russia provided further information on their circulation and on the exchange of their genes. The NP genes of the H5N2 and H5N3 strains isolated in the Primorsky Region in 2001 formed a separate cluster in the phylogenetic tree together with the NP genes of the H4N6 strains isolated in Buryatia in 2000 from a sheldrake and a pochard, an H2N3 strain isolated in the Primorsky Region in 1976 from a pintail, and an H14N5 strain isolated from a wild duck in the Caspian Sea Basin in 1982. However, they were very distantly related to the NP genes of the H3N8, H6N1 and H5N1 strains isolated from poultry and humans in Southeast Asia in 1996–2001, and to the NP genes of H4N8 viruses isolated from wild ducks in 2002 in the Caspian Sea Basin in European Russia. On the other hand, unlike the NP genes, the NS genes of the strains from the Primorsky Region, were closely related to the NS genes of the H5N1 and H4N8 viruses isolated in Southeast Asia in 1997–2001 and of an H4N8 virus isolated in the Caspian Sea Basin in 2002 [40].

The abundance of influenza A subtypes in the avian populations of Northern Eurasia provides excellent conditions for gene exchange. The extent of the exchange is demonstrated by the relatedness of different genes of the Russian isolates to the genes of European strains, on one hand and of South Asian isolates, on the other hand [40, 44, 49, 50]. The exchange is to a certain extent restricted by host specificity, but this restriction is not rigid, and the virus genes are frequently crossing the interspecies barriers. The avian migration routes crossing the Russian territory are an important factor for the gene flow. The extensive intra- and interspecies contacts in the natural habitat of wild birds in Russia stimulate rapid virus evolution and the appearance of new variants through reassortment events and, presumably, through post-reassortment adjustment of genes restoring functional intergenic match [52, 53]. Another factor may be the occurrence of avian influenza viruses in lake water, first registered in Eastern Siberia in 1979 [21]. This phenomenon might provide means for the temporal as well as territorial transfer of genes, as suggested by the recent detection of influenza viral RNA in the ice of high-latitude lakes of the Lena River Basin in Yakutia [54].

Thus, the sequencing data suggest extensive exchange of genes among the avian influenza viruses circulating in Europe, Siberia and Southeast Asia along the avian

migration routes that connect Europe through the Russian territory with Southeast Asia, the cradle of potentially pandemic reassortant viruses. After the highly pathogenic H5N1 viruses started their dissemination from Southeast Asia westward in 2004, their transfer to Russia by migrating birds was therefore to be expected.

The highly virulent H5N1 influenza virus first appeared in Russia in summer 2005 as indicated by an outbreak in the Novosibirsk Region among ducks and chickens in July. It spread quickly with over 90% mortality. Virus isolations in the area were performed independently by two groups of researchers. A group of strains was isolated in Zdvinsky County, Novosibirsk Region, by a team of researchers from The D.I. Ivanovsky Institute of Virology, Moscow. The materials for isolation (cloacal and tracheal swabs, pools of internal organs, blood) were taken from dead, sick and healthy birds at the farm where the epizootic occurred, and from wild birds in the vicinity [55, 56]. Three strains were isolated from dead chickens, two strains from sick or dead ducks, and one strain from a healthy grebe. Sequence analysis of the HA gene of two strains [57], one isolated from a duck and one from a grebe, revealed a close relatedness to the hemagglutinin of the H5N1 strains isolated near Qinghai Lake, China. Sequencing of the other genes of the isolates confirmed their close relatedness to the H5N1 viruses from China, A/Great black-headed gull/Qinghai/1/05 and A/Bar-headed goose/Qinghai/65/05 [58]. Several features, such as Lys627 residue in PB2 and Glu92 residue in NS1, thought to be characteristic for mammalian H5N1 variants, correlated with the high pathogenicity of the Novosibirsk isolates. A deletion in the NA gene in amino acid positions 49–60 indicated that the strains belonged to the genotype Z, which dominated in Southeast Asia in 2004 [59]. The other group of strains was isolated by a team of researchers from The State Research Center for Virology and Biotechnology Vector, Koltsovo, Novosibirsk Region. Two strains were isolated from chickens and 1 strain from a turkey in Suzdalka village, Dovolnoe County, in July 2005. The viruses were isolated from the homogenates of turkey spleen and chicken kidneys. The isolated viruses belonged to subtype H5N1, and their HA gene was closely related to the HA gene of the viruses isolated near Qinghai Lake in China in 2003 [60]. The viruses were highly pathogenic for chickens in a laboratory test [61].

In November 2005, ten H5N1 strains were isolated from mute swans during an outbreak with high mortality in the estuary of the Volga River [62]. The viruses were isolated from cloacal and tracheal swabs and viscera of sick or dead swans. The sequencing of all genes of 6 isolates revealed a close relatedness to the Qinghai H5N1 strains. The HA genes of the strains isolated from mute swans formed a separate branch in the phylogenetic tree together with the HA genes of the strains isolated in Mongolia in 2005 from whooper swans, the strains isolated from a grebe and a chicken in the Novosibirsk Region [55, 56], and the strains isolated in 2005 from bar-headed geese in Qinghai [58]. Among other H5N1 strains the closest homology to the Qinghai-like viruses was observed in the HA gene of the strain A/Chicken/Shantou/4231/03 isolated in Southern China [63]. The HA genes of the other strains

isolated in Southern China at that time were much more remote. Most likely, the Qinghai strains and their descendants isolated in Mongolia and Russia originate from a minor variant of the H5N1 virus circulating in Southern China. In June 2006, a severe outbreak occurred in the southern Siberia in Sayan Mountains near Ubsu-Nur Lake (Tyva Republic) in wild birds. Seven H5N1 strains were isolated from tracheal and cloacal swabs taken from healthy, sick or recently dead great-crested grebes, a tern and a cormorant (both clinically healthy), and sick coot. The genes of all the isolates were sequenced, and the strains were shown to belong to the Qinghai-Siberian branch, like the H5N1 viruses isolated in Siberia and European Russia in 2005 [64].

Interestingly, the H5N2 and H5N3 strains isolated earlier in Eastern Siberia and the Russian Far East, as well as the highly pathogenic H5N1 viruses isolated in 2005–2006 in Siberia and in the Caspian Sea Basin, were sensitive to rimantadine [64–66], unlike most of the H5N1 viruses which affected birds and humans in Vietnam and Thailand in 2003–2005. The close relatedness of the HA gene of a low-virulent avian Siberian strain isolated in 1991 to the HA gene of the highly pathogenic 1997 Hong Kong virus [40] reminds of the relationship between the A/Duck/Ukraine/1/63 (H3N8) strain and the pandemic Hong Kong virus of 1968 [5, 6, 9–12]. The time interval and the degree of similarity between the precursor strain and the appearance of the dangerous virus variant (the pandemic virus in 1968 and the highly pathogenic H5N1 virus in 1997) are similar in both cases. The comparative analysis of the nucleotide sequences of the genes of probable precursor viruses circulating in wild birds in Russia in the 1990s, highly pathogenic H5N1 Asian human isolates and recent Russian H5N1 viruses isolated from poultry and wild birds provides a basis for a general hypothesis of the provenance of the potentially dangerous Asian strains regarded as a source of the future pandemic virus. It seems appropriate to suggest that low virulent strains circulating in Siberia and in Russian Far East in wild birds are transferred to Southeast Asia during the autumn migrations. Some of them there infect poultry, and highly pathogenic variants are selected. The latter return to the wild bird population, and are transferred back to Siberia and further to the Middle East and Europe, where they again are introduced into poultry farms. The role of migratory birds in the spread of H5N1 avian influenza was recently confirmed by a comprehensive analysis of the individual introduction events in Asia, Europe, and Africa [67]. The authors suggest that the virus may have been introduced by migratory birds into Russia at the peak of migration, 1–2 months earlier than the outbreak occurred. These considerations stress the necessity of constant monitoring of influenza virus circulation in birds in Russia and neighboring countries. Monitoring should include not only isolation of virus strains and subtype identification, but also phenotypic characterization (including the assessment of resistance to antiviral drugs), and genotypic characterization, i.e. sequencing of virus genes and evaluation of the genetic relatedness to earlier influenza A isolates. Such studies are being continued in Russia, and, hopefully, they will be extended.

References

1 Tsimokh PF: Hemagglutination reaction in infectious sinusitis of ducklings (in Russian). Veterinariia 1961;38:63–65.

2 Prokof'eva MT, Gurova EI, Tsimokh PF: Virus influenza of ducklings (in Russian). Veterinariia 1963;40:33–35.

3 Prokof'eva MT, Tsimokh PF: Virus influenza of ducks; in Poultry Diseases (in Russian). Moscow, Kolos, 1966, pp 95–106.

4 Smirnova GA, Stakhanova VM: Biological properties of animal influenza viruses (in Russian). Veterinariia 1964;41:7–12.

5 Zakstel'skaia LIa, Antonova IV, Evstigneeva NA, Isachenko VA, Shenderovich SF: A new variant of influenza A2 (Hong Kong) 1-68 virus and its connection with the outbreaks of influenza observed in the USSR in 1968 (in Russian). Vopr Virusol 1969; 14:320–326.

6 Zakstelskaja LJ, Evstigneeva NA, Isachenko VA, Shenderovich SPh, Efimova VA: Influenza in the USSR: new antigenic variant A2/Hong Kong/1/68 and its possible precursors. Am J Epidemiol 1969;90: 400–405.

7 Tumova B, Isačenko VA, Easterday BC: A new subtype of influenza virus: antigenic characteristics of envelope antigens. Bull World Health Organ 1972;47: 503–506.

8 Isachenko VA, Molibog EV, Zakstel'skaia LIa: Antigenic characteristics of the influenza viruses isolated from domestic animals and birds in the USSR (in Russian). Vopr Virusol 1973;18:700–705.

9 Laver VG, Webster RG: Studies on the origin of pandemic influenza. III. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. Virology 1973;51:383–391.

10 Nakajima K, Nakajima S, Sugiura A: The possible origin of H3N2 influenza virus. Virology 1982;120: 504–509.

11 Scholtissek C, Rohde W, von Hoyningen V, Rott R: On the origin of human influenza virus subtypes H2N2 and H3N2. Virology 1978;87:13–20.

12 Fang R, Min Jou W, Huelebroeck D, Devos R, Fiers W: Complete structure of A/Duck/Ukraine/63 influenza hemagglutinin gene: animal virus as progenitor of human Hong Kong 1968 influenza hemagglutinin. Cell 1981;25:315–323.

13 Webster RG, Laver WG: The origin of pandemic influenza. Bull World Health Organ 1972;47:449–452.

14 Osidze NG, Tkachenko AV, Bogautdinov ZF, Siurin VN: Study of some biological properties of influenza virus strains of poultry and horses isolated in the USSR (in Russian). Vopr Virusol 1973;18: 705–709.

15 Zakstel'skaja LJa, Isačenko VA, Osidze NG, Timofeeva CC, Slepushkin AN, Sokolova NN: Some observations on the circulation of influenza viruses in domestic and wild birds. Bull World Health Organ 1972;47:497–501.

16 Osidze NG, Lvov DK, Siurin VN, Smorzhevskaia LS, Lenchenko EV, Radchuk LA, Braude NA, Miasnikova IA, Podcherniaeva RIa, Kir'anova AI, Shaikhanova ZT: A new variety of chicken influenza virus (in Russian). Veterinaria 1979;56:29–31.

17 Lvov DK, Il'ichev VD: Migration of Birds and the Transfer of the Infectious Agents (in Russian). Moscow, Nauka, 1979.

18 Zhezmer VIu, Lvov DK, Isachenko VA, Zakstel'skaia LIa: Isolation of the Hong Kong variant of type A influenza virus from sick chickens in the Kamchatka region (in Russian). Vopr Virusol 1973;18:94–99.

19 Siurin VN, Osidze NG, Chistova ZIa, Rodin IuV: Epizootic potential of avian influenza virus (in Russian). Veterinariia 1972;49:41–43.

20 Lvov DK, Zhdanov VM: Persistence of the genes of epidemic influenza A viruses in natural populations (in Russian). Usp Sovrem Biol 1982;93:323–337.

21 Lvov DK: Influenza A viruses – a sum of populations with a common protected gene pool; in Zhdanov VM (ed): Soviet Medical Reviews, Section E, Virology Reviews. Glasgow, Bell & Bain Ltd, 1987, vol 2, pp 15–37.

22 Slepushkin AN, Pysina TV, Gonsovsky FK, Sazonov AA, Isačenko VA, Sokolova NN, Polivanov VM, Lvov DK, Zakstel'skaja LJa: Haemagglutination-inhibiting activity to type A influenza viruses in the sera of wild birds from the far east of the USSR. Bull World Health Organ 1972;47:527–530.

23 Lvov DK, Sazonov AA, Chernetsov YuV, Obrosova-Serova NP, Zakstel'skaia LIa, Isachenko VA: Comprehensive study of the ecology of the influenza viruses in Commander Islands (in Russian). Vopr Virusol 1973;18:747–750.

24 Zakstel'skaia LIa, Isachenko VA, Timofeeva SS, Molibog EV, Vasilenko VA, Peikre EA: Serological evidence of the circulation of various variants of the influenza type A viruses among migratory birds in northern and western zones of the USSR (in Russian). Vopr Virusol 1973;18:760–764.

25 Chernetsov IuV, Lvov DK, Obrosova-Serova NP, Nikiforov LP, Slepushkin AN, Grachev VA, Zakstel'skaia LIa: Serologic examination of birds for antibodies to influenza virus in Eastern Khazakhstan; in Lvov DK (ed): Ecology of Viruses (in Russian). Moscow, The D.I. Ivanovsky Institute of Virology, 1976, vol 4, pp 122–128.

26 Lvov DK, Sidorova GA, Eminov AE, Vladimirtseva EA, Braude NA, Miasnikova IA, Andreev VP: Properties of Hav6Neq2 and Hsw1(H0)Nav2 influenza viruses isolated from waterfowl in southern Turkmenia (in Russian). Vopr Virusol 1980;25: 415–419.
27 Saiatov MKh, Beisembaeva RU, Lvov DK, Daulbaeva KD, Miasnikova IA, Gavrilov EI, Dubova EA, Bagasheva SS, Iamnikova SS, Savin YuG, Khriukov VV: Influenza viruses isolated from wild birds (in Russian). Vopr Virusol 1981;26:466–471.
28 Lvov DK, Iamnikova SS, Shemiakin IG, Agafonova LV, Miasnikova IA, Vladimirtseva EA, Nimedava P, Dashtseren P, Bel-Ochir Zh, Zhdanov VM: Persistence of the genes of epidemic influenza viruses (H1N1) in natural populations (in Russian). Vopr Virusol 1982;27:401–405.
29 Pysina TV, Lvov DK, Braude NA, Sorochenko SA, Miasnikova IA, Blinova VK, Podcherniaeva RYa, Klimenko SM, Ronina MV: Influenza virus A/Anas Acuta/Primorie/695/76 isolated from wild ducks in the USSR (in Russian). Vopr Virusol 1978;23: 300–304.
30 Sazonov AA, Lvov DK, Webster RG, Sokolova TV, Braude NA, Portyanko NV: Isolation of an influenza virus, similar to A/Port Chalmers/1/73 (H3N2) from a common murre at Sakhalin Island in USSR (Strain A/common murre/Sakhalin/1/74). Arch Virol 1977;53:1–7.
31 Pysina TV, Lvov DK, Braude NA, Sorochenko SA, Podcherniaeva RIa, Miasnikova IA, Rovnova ZI: Influenza virus A/Anas acuta/Primorie/730/76 (H3N2) isolated from wild ducks in the Maritime Territory (in Russian). Vopr Virusol 1979;24:489–493.
32 Shablovskaia EA, Lvov DK, Sazonov AA, Webster RG, Vinograd IA, Braude NA, Stel'makh SG, Portianko NV, Koval'chuk-Ivaniuk, Sokolova NN: Isolation of influenza strains identical to influenza virus A/Anglia/42/72 from semisynanthropic bird species in Rovno Province, the Ukrainian SSR (in Russian). Vopr Virusol 1977;22:414–418.
33 Zhdanov VM, Yamnikova SS, Isachenko VA, Dotsenko GN, Sokolov BP, Zakstel'skaia LIa, Douglas AR, Skehel JJ, Lvov DK: Human and avian viruses of the Hong Kong series (in Russian). Vopr Virusol 1981;26:657–664.
34 Sidorenko EV, Ignatenko TA, Iamnikova SS, Smogorzhevskii LA, Zelenskaia TP, Lvov DK: Isolation of an influenza virus from a tree sparrow and the infection rate of the virus in wild birds in the mid-Dnieper Region (in Russian). Vopr Virusol 1985;30:657–661.
35 Webster RG, Isachenko VA, Carter M: A new avian influenza virus from feral birds in the USSR: recombination in nature? Bull World Health Organ 1974;51: 325–332.
36 Zakstel'skaia LIa, Timofeeva SS, Iakhno MA, Molibog EV, Isachenko VA: Isolation of influenza A viruses from wild migratory waterfowl in the north of European part of the USSR; in Lvov DK (ed): Ecology of Viruses (in Russian). Moscow, The D.I. Ivanovsky Institute of Virology, 1975, vol 3, pp 58–63.
37 Roslaia IG, Lvov DK, Iamnikova SS: The incidence of influenza virus infection in black-headed gulls (in Russian). Vopr Virusol 1984;29:155–157.
38 Lvov DK, Andreev VP, Braude NA, Shchukina NV, Podcherniaeva RYa, Raikova AP, Klimenko SM: Isolation of influenza virus with the antigenic formula Hav4 Nav2 and Hav5 Nav2 during epizootic infection among seagulls in the Astrakhan district in the summer of 1976 (in Russian). Vopr Virusol 1978;23:399–403.
39 Chernetsov IuV, Slepushkin AN, Lvov DK, Braude NA, Gavrilov AE, Berezovskii VG, Egorova AN, Manykin AA, Gavrilov EA: Isolation of influenza virus from *Chlidonias nigra* and serologic examination of the birds for antibodies to influenza virus (in Russian). Vopr Virusol 1980;25:35–40.
40 Lvov DK, Iamnikova SS, Fediakina IT, Aristova VA, Lvov DN, Lomakina NF, Petrova ES, Zlobin VI, Khasnatinov VA, Chepurgina EA, Kovtunov AI, Dzharkenov AF, Sankov MN, Leonova GN, Maslov DV, Shchelkanov MIu, Nepoklonov EA, Aliper TI: Ecology and evolution of influenza viruses in Russia, 1979–2002 (in Russian). Vopr Virusol 2004;49:17–24.
41 Hinshaw VS, Air GM, Gibbs AJ, Graves L, Prescott B, Karunakaran D: Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls. J Virol 1982;42: 865–872.
42 Iamnikova SS, Kovtun TO, Dmitriev GA, Aristova VA, Krivonosov GA, Rusanov GM, Konechnyi GA, Lvov DK: Circulation of the influenza A virus of H13 serosubtype among seagulls in the Northern Caspian, 1979–1985 (in Russian). Vopr Virusol 1989; 34:426–430.
43 Chambers TM, Yamnikova S, Lvov DK, Kawaoka Y, Webster RG: Antigenic and molecular characterization of subtype H13 hemagglutinin of influenza virus. Virology 1989;172:180–188.
44 Iamnikova SS, Gambaryan AS, Fediakina IT, Shilov AA, Petrova ES, Lvov DK: Monitoring of the circulation of influenza A viruses in the populations of wild birds of the North Caspian (in Russian). Vopr Virusol 2001;46:39–43.
45 Kawaoka Y, Yamnikova S, Chambers TM, Lvov DK, Webster RG: Molecular characterization of a new hemagglutinin, subtype H14, of influenza A virus. Virology 1990;179:759–767.

46 Okazaki K, Takada A, Ito T, Imai M, Takakuwa H, Hatta M, Ozaki H, Tanizaki T, Nagano T, Ninomiya A, Demenev VA, Tyaptirganov MM, Karatayeva TD, Yamnikova SS, Lvov DK, Kida H: Precursor genes of future pandemic influenza viruses are perpetuated in ducks nesting in Siberia. Arch Virol 2000;145:885–893.

47 Lvov DK, Gorin OZ, Iamnikova SS, Zlobin VI, Lvov ND, Khasnatinov MA, Fediakina IT, Chumakov VN, Nepoklonov VA, Aliper TI: Isolation of influenza A viruses from wild birds and a muskrat in the western part of the East Asia migration route (in Russian). Vopr Virusol 2001;46:35–39.

48 Lvov DK, Yamnikova SS, Fedyakina IT, Lomakina NF, Lvov DN, Synitsyn BV, Petrova ES, Gambaryan AS, Blinov VM, Suarez DL, Swayne DE: Evolution of H4, H5 influenza A viruses in natural ecosystems in Northern Eurasia (2000–2002); in Kawaoka Y (ed): Options for the Control of Influenza V, International Congress Series. Amsterdam, Elsevier, 2004, vol 1263, pp 169–183.

49 Razumova IuV, Shchelkanov MIu, Durymanova AA, Zolotykh SI, Ternovoi VA, Slavskii AA, Iurlov AK, Beklemishev AB, Shestopalov AM, Lvov DK: Genetic diversity of influenza A virus in the populations of wild birds in the south of Western Siberia (in Russian). Vopr Virusol 2005;50:31–35.

50 Razumova IuV, Shchelkanov MIu, Zolotykh SI, Durymanova AA, Ternovoi VA, Beklemishev AB, Slavskii AA, Iurlov AK, Shestopalov AM, Lvov DK, Netesov SV: The 2003 results of monitoring of influenza A virus in the populations of wild birds in the south of Western Siberia (in Russian). Vopr Virusol 2006;51:32–37.

51 Shestopalov AM, Tserennorov D, Shchelkanov MIu, Otgonbaatar D, Zolotykh SI, Khasnatinov MA, Abmed D, Batjav D, Myagmar J, Durymanova AA, Ikranbegin R, Lvov DK, Loktev VB: The study of circulation of influenza and West Nile viruses in the natural biocenoses of Mongolia (in Russian); in Current Topics of Virology. Abstract Book of Xth National Mongolian Conference, Ulaanbaatar, November 25, 2004, pp. 122–123.

52 Kaverin NV, Gambaryan AS, Bovin NV, Rudneva IA, Shilov AA, Khodova OM, Varich NL, Sinitsin BV, Makarova NV, Kropotkina EA: Post-reassortment changes in influenza A virus hemagglutinin restoring HA-NA functional match. Virology 1998;244:315–321.

53 Kaverin NV, Matrosovich MN, Gambaryan AS, Rudneva IA, Shilov AA, Varich NL, Makarova NV, Kropotkina EA, Sinitsin BV: Intergenic HA-NA interactions in influenza A virus: post-reassortment substitutions of charged amino acid in the hemagglutinin of different subtypes. Virus Res 2000;66: 123–129.

54 Zhang G, Shoham D, Gilichinsky D, Davydov S, Castello JD, Rogers SO: Evidence of influenza A virus RNA in Siberian lake ice. J Virol 2006;80: 12229–12235.

55 Lvov DK, Shchelkanov MYu, Vlasov NA, Deriabin PG, Grebennikova TV, Prilipov AG, Nepoklonov EA, Onishchenko GG, Aliper TI, Zaberezhnyi, AD, Kireev DE, Krasheninnikov OP, Kiriukhin ST, Burtseva EI, Fediakina IT, Slepushkin AI: Isolation of influenza A/H5N1 virus strains from wild birds and poultry during epizootics in Western Siberia (2005 July); in: Genodiagnostics of Infectious Diseases, CERIS, Novosibirsk, 2005, pp 133–136.

56 Lvov DK, Shchelkanov MIu, Deriabin PG, Grebennikova TV, Prilipov AG, Nepoklonov EA, Onishchenko GG, Vlasov NI, Aliper TI, Zaberezhnyi AD, Kireev DE, Krasheninnikov OP, Kiriukhin ST, Burtseva EI, Slepushkin AN: Isolation of influenza A/H5N1 virus strains from poultry and wild birds in West Siberia during epizooty (July 2005) and their depositing to the state collection of viruses (August 8, 2005) (in Russian). Vopr Virusol 2006;51:11–14.

57 Lvov DK, Prilipov AG, Shchelkanov MIu, Deriabin PG, Shilov AA, Grebennikova TV, Sadykova GK, Liapina OV: Molecular genetic analysis of the biological properties of highly pathogenic influenza A/H5N1 virus strains isolated from wild birds and poultry during epizooty in Western Siberia (July 2005) (in Russian). Vopr Virusol 2006;51:15–19.

58 Chen H, Smith GJD, Zhang SY, Qin K, Wang J, Li KS, Webster RG, Peiris JS, Guan Y: Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature 2005;436:191–192.

59 Lipatov AS, Govorkova EA, Webby RJ, Ozaki H, Peiris M, Guan Y, Poon L, Webster RG: Influenza: emergence and control. J Virol 2004;78:8951–8959.

60 Shestopalov AM, Durimanov AG, Evseenko VA, Ternovoi VA, Rassadkin YN, Razumova YV, Zaykovskaya AV, Zolotykh SI, Netesov SV: H5N1 influenza virus, domestic birds, Western Siberia, Russia. Emerg Infect Dis 2006;12:1167–1168.

61 Onishchenko GG, Shestopalov AM, Ternovoi VA, Evseenko VA, Durymanov AG, Rassadkin YN, Zaikovskaia AV, Zolotykh SI, Iurlov AK, Mikheev VN, Netesov SV, Drozdov IG: Study of highly pathogenic H5N1 influenza virus isolated from sick and dead birds in Western Siberia (in Russian). Zh Mikrobiol Epidemiol Immunobiol 2006(5):47–54.

62 Lvov DK, Shchelkanov MIu, Deriabin PG, Burtseva EI, Galkina IV, Grebennikova TV, Prilipov AG, Usachev EV, Liapina OV, Shliapnikova OV, Poglazov AB, Slavskii AA, Morozova TN, Vasil'ev AV, Zaberezhnyi AD, Dzarkenov AF, Gabbasov FB, Evdokimova MI, Aliper TI, Litvin KE, Gromashevskii VL, Vlasov NA, Iashkulov KB, Kovtunov AI, Onishchenko GG, Nepoklonov EA, Suarez DL: Highly pathogenic influenza A/H5N1 virus-caused epizooty among mute swans *(Cygnus olor)* in the lower estuary of the Volga River (November 2005) (in Russian). Vopr Virusol 2006; 51:10–16.

63 Li KS, Guan Y, Wang J, et al: Genesis of a highly pathogenic pandemic H5N1 virus in Eastern Asia. Nature 2004;430:209–213.

64 Lvov DK, Shchelkanov MIu, Deriabin PG, Fediakina IT, Burtseva EI, Prilipov AG, Kireev DE, Usachev EV, Aliper TI, Zaberezhnyi AD, Grebennikova TV, Galkina IV, Slavskii AA, Litvin KE, Dongur-ool AM, Medvedev BA, Dokper-ool MD, Mongush AA, Arapchor MSh, Kenden AO, Vlasov HA, Nepoklonov EA, Suarez D: Isolation of highly pathogenic influenza virus strains A/H5N1 from wild birds in an epizooty focus near Ubsu-Nur Lake in June 2006 and their depositing to State Virus Collection of the Russian Federation (July 3, 2006) (in Russian). Vopr Virusol 2006;51:14–18.

65 Fediakina IT, Iamnikova SS, Galegov GA, Lvov DK: The effect of antiviral preparations on the reproduction of the avian influenza A/H5 virus isolated in Russia (in Russian). Vopr Virusol 2005;50:35–37.

66 Lvov DK, Fediakina IT, Shchelkanov MIu, Prilipov AG, Deriabin PG, Galegov GA: In vitro effects of antiviral drugs on the reproduction of highly pathogenic influenza A/H5N1 virus strains that induce epizooty among poultry in the summer of 2005 (in Russian). Vopr Virusol 2006;51:20–22.

67 Kilpatrick AM, Chmura AA, Gibbons DW, Fleisher RC, Marra PP, Daszak P: Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci USA 2006;103:19368–19373.

Prof. D.K. Lvov, PhD, DSci, Acad. RAMS
The D.I. Ivanovsky Institute of Virology
Gamaleya Street 16, Moscow 123098 (Russia)
Tel. +7 499 190 2842, Fax +7 499 190 2867, E-Mail dk_Lvov@mail.ru

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 59–70

Avian Influenza in Italy 1997–2006

Ilaria Capua · Giovanni Cattoli · Calogero Terregino · Stefano Marangon

National OIE/FAO Reference Laboratory for Newcastle Disease and Avian Influenza,
Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro/Padova, Italy

Abstract

Between 1997 and 2005, Italy has been challenged by several introductions of avian influenza of the H5 and H7 subtypes. These include viruses of high pathogenicity that apparently emerged as such in domestic poultry or that were derived from viruses of low pathogenicty, following their circulation in poultry for several months. They also include viruses of low pathogenicity that did not mutate to the highly pathogenic form following extensive or limited spread in domestic poultry. Italy has been the first EU member state to apply an emergency and a prophylactic vaccination programme for avian influenza viruses of the H5 and H7 subtypes. Thus the Italian field experience with avian influenza, complemented with the generation of scientific data from our collection of isolates, is a source of information which can be used as a model to better comprehend the dynamics of other AI infections in animals.

HPAI H5N2 1997–1998

Towards the end of October 1997, episodes of sudden high mortality in backyard poultry flocks were recorded in North Eastern Italy. Even though there had been no record of outbreaks of HPAI in Italy for some 60 years [1], official veterinarians at field investigation centres suspected HPAI very early on, and all possible control and preventive measures were implemented quickly. The primary concern for the official and private veterinarians was to prevent the spread of disease into the densely populated industrial poultry areas of the Veneto and Friuli Venezia-Giulia regions.

Capua et al. [2] reported that between the 27th October 1997 and the 11th January 1998, eight outbreaks of HPAI were diagnosed, notified and stamped out according to EU Council Directive 92/40/EC [3]. A total of 6,505 birds (1,846 chickens, 1,503 turkeys, 2,270 ducks, 45 geese, 731 guineafowl, 98 quail and 12 pigeons) were involved in the outbreaks and died or were killed as part of the stamping-out control policy.

Common signs in galliform birds were weakness and ruffled feathers, incoordination, the comb and wattles appeared cyanotic and swollen, and most birds presented

rales and coughing. Death took place 24–48 h after the onset of clinical signs. Some birds exhibited atypical signs [2]. At necropsy, birds exhibited swelling and cyanosis of the head, comb and wattles. The trachea often had haemorrhagic mucosa and was oedematous. Air sacs appeared thickened and lined with a fibrinous exudate. Haemorrhages were present in the mucosa of the proventriculus and the caecal tonsils, and catarrhal haemorrhagic enteritis was also present. The spleen appeared enlarged, and in some cases fibrinous peritonitis was observed. Highly pathogenic H5N2 viruses with IVPI values of 2.98–3.0 and with a deduced amino acid sequence of PQRRRKKR*GLF at the HA0 cleavage site were isolated from all 8 outbreaks.

Given the fact that the outbreaks affected either backyard flocks or medium-small poultry traders, the outcome of the epidemiological investigation was limited by the lack of accurate and precise information concerning the number and species present in each group at any given time, including the dates and numbers of birds introduced to the flocks and the precise dates concerning the onset of clinical signs and mortality. However it was possible to identify several risk factors associated with the outbreaks, such as rearing of mixed species – particularly galliforms and waterfowl, contact with wild birds and introduction of birds originating from live bird traders – who, by definition, obtain birds from different sources and sell them to small farms.

1999–2001 H7N1

Emergence of H7N1 HPAI

The H7N1 HPAI virus that caused the 1999–2000 epidemic in Italy emerged from a LPAI precursor, which had circulated in the poultry population of North Eastern Italy for approximately 9 months [4, 5]. The LPAI virus had an IVPI of 0.0 in 6-week-old SPF chickens, and a deduced amino acid sequence for the HA0 cleavage site of the precursor of the haemagglutinin molecule of PEIPKGR*GLF, a typical motif of LPAI viruses. At the time of occurrence of the LPAI outbreaks there was no legislative basis to intervene with statutory measures to control the spread of the LPAI virus, although the possibility of viral mutation to a highly pathogenic form could not be ruled out. The disease occurred in the Veneto and Lombardia regions, which accounted for approximately 65% of the Italian industrially reared poultry, particularly in the provinces of Verona, Vicenza, Mantova, and Brescia (fig. 1). This area had developed into a multi-species densely-populated poultry area with the highest density of turkeys in Italy, and one of the greatest in Europe.

From a structural and functional point of view, biosecurity levels were generally below standard due to the absence of physical barriers between establishments and the common practice of sharing staff and equipment among farms. Moreover, the local poultry industry had developed and grown into one that intensively reared a number of different avian species such as chickens, turkeys, guineafowl, quail and ostriches. Production circuits of these different birds often overlapped, since the feed

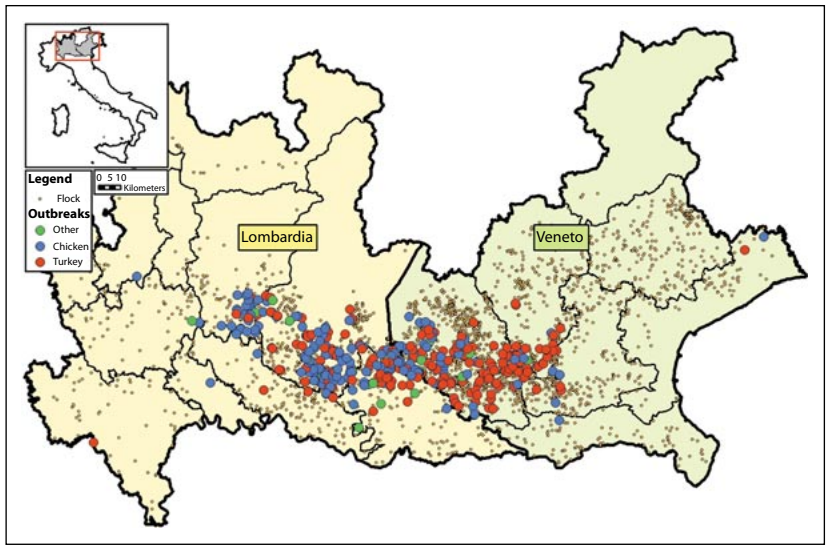


Fig. 1. Distribution of poultry flocks and flocks infected during the 1999–2000 H7N1 HPAI outbreaks in North Eastern Italy.

mills and slaughtering plants were owned by single companies that served a number of farms.

Despite the absence of legislative powers, because the LPAI viruses were causing real disease problems, often with high mortality in meat turkeys, voluntary restriction policies aimed primarily at avoiding the movement of infected birds were put in place. Although these initially appeared to have some success with a marked decline in outbreaks during the hot summer months, the number of H7N1 LPAI outbreaks rose again during the autumn and early winter of 1999.

In mid-December 1999, a field veterinarian submitted samples from a suspected influenza outbreak in a meat-turkey flock exhibiting high mortality. HPAI was diagnosed within 4 days of submission with the characterisation of an H7N1 isolate with an intravenous pathogenicity index of 3.0 and a deduced HA0 cleavage site amino acid sequence of PEIPKGSRVRR*GLF. This motif, although unusual, contained multiple basic amino acids, typical of HPAI viruses.

Spread of the H7N1 HPAI Virus

Because the isolation of an H7 virus from turkey flocks showing high mortality was not unusual at the time, the implementation of statutory control measures, which would normally be employed pre-emptively on suspicion, had been delayed until the laboratory confirmation of HPAI and this resulted in spread of infection. From the epidemiological follow-up of subsequent outbreaks, it appeared that at least 16 flocks

were already infected on the day restriction policies were implemented. The resulting loss of control of the infection culminated in 413 outbreaks notified between 17.12.1999 and 05.04.2000 [6].

Retrospective analysis of the outbreaks indicated that infection was detected more frequently in turkey ($\chi^2 = 118.37$, $p < 0.0001$) and layer farms ($\chi^2 = 373.04$, $p < 0.0001$) than in any other type of farmed category. Layers and turkeys accounted for 73% of the outbreaks. Other risk factors were the size of the flock as larger flocks were shown to be more at risk ($z = 5.895$, $p < 0.0001$), and location as poultry farms located in the plains (altitude ≤300 m) ($\chi^2 = 37.27$, $p < 0.0001$) were shown to be at higher risk. Tracing exercises carried out on affected premises allowed the identification of the possible origin of the infection in 66.3% of the outbreaks. In particular, the origin of infection could be attributed to: movement of animals (1.0%), indirect contacts at the time of loading for slaughter of female turkeys (8.5%), neighbourhood spread (within 1-km radius) (26.2%), lorries for the transport of feedstuff, litter and carcasses (21.3%), and other indirect contacts (i.e. exchange of manpower, machinery, equipment) (9.4%) [7].

Efforts to eradicate the HPAI virus were focused basically on the application of restriction to movements and stamping out of infected or suspected infected farms. HPAI was eradicated in just over 4.5 months.

Clinical and Pathological Findings

Although the clinical signs and pathological findings were similar to those reported from HPAI outbreaks preceding 1999 or reported subsequently, the diversity of species of poultry involved was probably unprecedented and several unusual observations were made.

HPAI affected a greater number of establishments than LPAI, and was often characterised by 100% morbidity and high mortality in the affected flocks. All intensively reared species were affected, flocks of turkeys, chickens and guineafowl often exhibited 100% mortality rates within a few days [6, 8], although as reported in other outbreaks the speed of spread within a flock was much quicker for birds reared on the ground compared to those in cages. In particular, in chicken flocks reared on litter 100% flock mortality was observed 48–96 h from the onset of the first clinical signs. In contrast, in caged layers, the onset of mortality and clinical signs was slower. In such flocks severe depression or mortality could be seen initially in only one bird per cage in a restricted area of the house, and then progressed to neighbouring cages, generally reaching the far end of the shed 10–14 days from the first clinical signs. This different behaviour in spread between caged and litter-reared chickens was probably related to the amount of infected faeces in direct contact with the birds.

Guineafowl (*Numida meleagris*) appeared to be particularly susceptible to HPAI with 100% flock mortality being observed 48–72 h from the onset of the first clinical signs.

Varying degrees of resistance to the clinical disease were recorded in waterfowl (especially ducks), quail and ostriches [6, 9].

The Italian H7N1 outbreak in ostriches appeared to be the first recorded natural outbreak of HPAI in ratites. Clinical signs were observed only in juvenile (7–9 months of age) birds. The first clinical signs observed were anorexia and depression in a limited number of the young birds. Feed consumption dropped, and the birds appeared sleepy and depressed. Within the next couple of days, the clinical condition affected a significant number of the young birds, although the adults appeared healthy throughout the episode. One notable sign was the brilliant green urine produced by affected ostriches [10].

In Japanese quail (*Coturnix coturnix japonica*) mortality rates were probably influenced by their confinement in cages and were of approximately 5% per day. Infection in these birds was characterised by a severe respiratory condition, which in a couple of days evolved into a clinical status characterised by prostration, somnolence, listlessness, production of a whitish diarrhoea and gasping prior to death. Nervous signs such as opistothonus and torticollis could also be seen prior to death.

Although waterfowl are often described as resistant to the clinical disease caused by HPAI, during the Italian H7N1 HPAI epidemic this appeared to apply only to ducks and clinical signs and mortality were recorded both in geese *(Anser anser* var. *domestica)* and in Muscovy ducks *(Chairina moschata)* reared in a backyard flock [6].

Re-Emergence of the H7N1 LPAI and Emergency Vaccination

In late summer 2000, the H7N1 LPAI virus re-emerged following the repopulation of poultry farms. From August to November 2000, the H7N1 LPAI strain infected 51 meat-type turkey farms, which housed 845,000 turkeys, and 1 quail farm, with a total of 429,000 quails, located in the southern part of the province of Verona. Another 3 quail farms, with a total of 405,000 quails, located in a contiguous province were also affected. The latter were functionally linked to the farm situated in the province of Verona. All infected farms were depopulated. The origin of the outbreak was subsequently traced back. From the epidemiological investigation it appeared that the source of infection was the large Japanese quail *(C. c. japonica)* farm located in the densely populated poultry area (DPPA). Serum samples collected from quail breeders analysed after the detection of the outbreak indicated that the virus could have persisted undetected in the inter-epidemic period.

In order to prevent a possible re-emergence of the H7N1 LPAI virus, a coordinated set of measures, including strict biosecurity, a serological monitoring programme and a 'DIVA' (Differentiating Infected from Vaccinated Animals) vaccination strategy were enforced [11]. The 'DIVA' strategy, which was implemented from December 2000, was based on the use of an inactivated oil emulsion vaccine containing the same H subtype as the field virus, but a different N subtype, in this case an H7N3 strain (A/Ck/Pakistan/ 95). The possibility of using the diverse N group, to differentiate between vaccinated and naturally infected birds, was achieved through the development of an ad-hoc serological test based on the detection of specific anti-N1 antibodies [12].

The test is based on the expression of the N1 gene in a baculovirus system and on the use of the recombinant baculovirus in infected cells as an antigen for an indirect immunofluorescent antibody test assay (iIFA) [13]. The test has been extensively validated using field and experimental sera [13, 14].

The control of the field situation was ensured through an intensive sero-surveillance programme aimed at the detection of the LPAI virus. This was achieved through the regular testing of sentinel birds in vaccinated flocks and the application of the anti-N1 antibody detection test. Serological monitoring was also enforced in unvaccinated flocks, located both inside and outside the vaccination area. In addition, the efficacy of the vaccination schemes were evaluated in the field through regular testing of selected flocks. The vaccination programme did not include broilers or quails, but only longer living birds such as meat turkeys and layers and a very limited number of chicken and turkey breeders.

Notwithstanding the depopulation of the infected premises, the serological monitoring programme revealed an additional incursion of the LPAI H7N1 virus shortly after the beginning of the vaccination programme (December 2000 to March 2001). The H7N1 LPAI virus infected 3 meat-type turkey farms in the vaccination area and 20 poultry holdings (19 turkey and 1 layer farms) located in a contiguous unvaccinated area. Only one vaccinated flock was affected, and the virus did not spread from this to other vaccinated farms. All infected flocks were culled, with the last H7N1 LPAI infected poultry flock stamped out on the 26th March 2001. The emergency vaccination programme was discontinued in May 2002.

2002–2003 H7N3 LPAI Epidemic

During 2002–2003 Italy once again experienced outbreaks of AI involving an H7N3 subtype influenza A virus of low pathogenicity. In the month of October 2002, an H7N3 LPAI strain was introduced from the wild reservoir into the domestic poultry population located in the DPPA which had previously been affected by the H7N1 epidemic in 1999–2001 [15]. The new H7N3 virus was unrelated genetically to the H7N3 seed strain contained in the vaccine used in the 2000–2002 vaccination campaign [16].

Since the infection spread rapidly among poultry flocks, the use of vaccination was foreseen. The vaccination programme designed was based once again on a 'DIVA' strategy and was carried out using an AI-inactivated heterologous vaccine (strain A/ck/IT/1999-H7N1) in layers, capons and meat turkeys only. The implementation of the DIVA vaccination campaign was delayed until the 31st December 2002, due to the unavailability of an appropriate vaccine. Extensive spread of field virus therefore occurred initially within the affected area with no bird having a protective immunity against the infection.

From the 10th October 2002 to 10th October 2003, the H7N3 LPAI virus was able to spread and infect a total of 388 poultry holdings, of these, 88 were vaccinated. All the

infected vaccinated flocks were meat turkeys mainly located in a limited area of the southern part of Verona province. Stamping out and controlled marketing were applied to the infected birds. The last farm infected with H7N3 was cleared on the 9th October 2003.

The spread of infection prior and during the implementation of the vaccination campaign was assessed. Comparing the number of outbreaks in the same time interval, before and after the implementation of emergency vaccination, the reproduction rate (R value) decreased from 2.9 (95% confidence interval: 2.3–3.9) to 0.6 (95% confidence interval: 0.5–0.7). The R reduction resulted in a sharp decrease of the weekly incidence rate of the infection [17]. The basis for this reduction of spread under field conditions most probably lies in the combination between increased resistance to infection and reduction of virus shedding which is achieved in vaccinated birds [18].

From Emergency to Prophylactic Vaccination

In February 2004, the monitoring activities carried out in the areas at risk of infection enabled the detection of a LPAI virus of the H5N3 subtype in domestic ducks and geese belonging to a free-range backyard flock located in Brescia province (Lombardy region). The flock was stamped out and no further LPAI outbreak was detected.

Given the evidence that the area was at risk both for H5 and H7 viruses, and that in previous experiences the infection spread in absence of vaccination, a bivalent H5/H7 prophylactic vaccination programme was designed. The rationale behind the use of prophylactic vaccination was to be able to generate a minimal level of protective immunity in the population at risk. The immune response would then be boosted if there was evidence of the introduction of a field virus.

In July 2004, the EU Commission authorised Italy to implement a prophylactic vaccination programme in the high-risk areas of Veneto and Lombardy regions [19]. Italy was authorised to enforce the programme from the 1st October 2004 onwards based on the availability of a bivalent (H5/H7)-inactivated vaccine containing seed strains supplied by the Italian National Reference Laboratory. Since the 7th October 2004, the vaccination programme was implemented only in categories of poultry considered at high risk of exposure, namely meat turkeys and layers. The bivalent vaccination programme substituted the monovalent (H7) vaccination programme from the 31st August 2004.

Recurrence of the H7N3 LPAI Virus and Introduction of LPAI H5N2

In September 2004, about 1 year after the depopulation of the last H7N3 LPAI affected flock, the AI virus of the H7N3 subtype re-emerged in the southern part of Verona province. Affected farms were mostly in close proximity of each other and functionally connected. This occurrence required the application of strict control

measures in association with the rapid implementation of a booster immunization of the susceptible population.

From the 15th September to the 1st December 2004, the H7N3 LPAI virus infected 27 meat turkey and 1 quail farms, housing a total of 744,000 birds, located in a cluster of 9 municipalities with one of the highest turkey population densities in Europe. All the affected meat turkey flocks had been vaccinated with the monovalent (H7N1)-inactivated vaccine, but the majority of these flocks had been vaccinated only once or twice. The label registration indication for this species is three administrations. The increased resistance to challenge of the vaccinated birds and the reduction of the shedding levels combined with restriction measures, biosecurity and appropriate surveillance resulted in a significant reduction of the infectious pressure in the environment, and aided the rapid eradication of the infection. Only 28 farms were affected, the same virus having caused 388 outbreaks the previous year.

A similar situation was also observed in 2005, when a novel introduction of a LPAI H5N2 occurred in vaccinated turkey flocks in Lombardy. Infection spread to 15 turkey farms. Of these, 2 were unvaccinated and 13 were vaccinated. The latter consisted of adult turkeys close to slaughter, with post-vaccinal HI titres in that age of bird ranging between 1:4 and 1:16. These findings demonstrated the difficulty in obtaining an adequate duration of immunity in the turkey (compared with previous experiences in chickens) and may explain the ability of the virus to enter the vaccinated population following a field challenge towards the end of the birds' production period. Field evidence thus indicated that vaccinated layers were resistant to field challenge, as no vaccinated holding developed infection. In addition, although it was not possible to avoid the introduction of AI viruses in vaccinated turkey flocks, the spread of the infection was limited and the containment of the outbreaks was successful in a shorter time compared to previous epidemics, with a marked reduction in economic losses.

Since LPAI H5N2 was eradicated there have been no additional introductions of AI in the vaccinated poultry population of the DPPA in Northern Italy. Thus, no virus of the H5 or H7 subtype was introduced into the vaccinated population between April 2005 and December 2006, notwithstanding 17 viral isolations of LPAI H5/H7 strains in wild birds and the 16 swans and 1 mallard found positive to the Asian HPAI H5N1 virus [20]. The bivalent vaccination programme was discontinued on the 31st December 2006.

Infections in Wild Birds

During the HPAI epidemic, testing of wild birds yielded positive results for HPAI H7N1 virus only in two sparrows (*Passer domesticus*) and in a collared dove (*Streptopelia decaocto*), which were found dead inside poultry houses or in their close proximity [21].

Extensive surveillance efforts carried out in wild fowl in Italy since 2002 have yielded several interesting results. The progenitor of the H7N3 epidemic has been identified in the wild bird reservoir and appeared to be circulating since 2001 [15].

Intensive surveillance efforts have been carried out in North Eastern Italy, as this is where AI epidemics have occurred in the past, and has a unique combination of DPPAs and wetlands. Over 3 years (2004–2006) 7,532 wild birds (mainly migratory *Anseriformes* and *Charadriiformes*) were sampled during the active surveillance programmes. Out of 7,532 wild birds tested, 466 were positive for influenza type A by RRT-PCR. Following virus isolation attempts, 70 AIVs belonging to 16 different subtypes (H1N1, H1N3, H3N8, H4N6, H5N1, H5N2, H5N3, H6N2, H7N4, H7N7, H9N2, H10N1, H10N4, H10N7, H10N8, H11N9) were obtained from migratory waterfowl belonging to the order *Anseriformes*. One AI virus (AIV) of the H10N7 subtype was isolated from a wintering common snipe (*Gallinago gallinago*) and one H6N2 subtype from a greater flamingo (*Phoenicopterus roseus*) [22].

The high number of positive birds confirms the important role of wild waterfowl in the perpetuation of LPAI viruses during the winter season in the Mediterranean countries such as Italy.

In February 2006, an incursion of the HP H5N1 virus occurred in Central and Southern Italy. Mortality and clinical disease occurred in mute swans (*Cygnus olor*) migrating south as a result of a particularly cold winter in Central Europe [20]. An infected mallard (*Anas platyrhynchos*) was also found dead in Lake Trasimeno in Central Italy. There was no spread to domestic birds.

Infections in Backyard Flocks

More recently, surveillance efforts in wild birds have been complemented with a monitoring programme in backyard flocks. The number and location of backyard flocks to be sampled were identified by regional veterinary epidemiological centres on the basis of risk factors such as proximity of the farm with wetlands or DPPAs and rearing of mixed species in the open. The surveillance programme was designed to allow monitoring of the same farm approximately every 45 days. This programme has yielded in 3 years (2004–2006) 41 LPAI viruses including some isolates of the H5 and H7 subtypes. Sequencing and phylogenetic analysis has shown that H7N7 viruses present in the wild bird reservoir could also be found in backyard flocks during the same sampling period [22]. The risk of introduction of AIVs into free-range farms in Italy becomes very high during the period in which the highest number of natural reservoirs is present in the wetlands (autumn and winter). This is supported by the high isolation rate of AI viruses (>70%) in backyard flocks during the colder months.

Infection of Mammals Including Humans

Despite the large number and widespread nature of the H7N1 outbreaks, no case of infection in mammals was recorded throughout the duration of the outbreak.

Active and passive surveillance, including an investigation in poultry workers, farmers and veterinarians [23], failed to demonstrate interspecies transmission to humans.

Following the generation of full-length sequence data on the H7N1 viruses, it appeared that certain avian origin isolates exhibited mutations that are associated with enhanced replication in the mammalian host, such as the PB2 627 E→K mutation. In vivo experiments demonstrated that a selection of Italian HPAI H7N1 isolates were able to infect and replicate in the nervous and respiratory systems of experimentally infected mice [24]. As expected, the strain exhibiting the PB2 627 E→K mutation was lethal for mice. One selected strain originally harbouring PB2 627E residue was able to mutate to the 627K variant following one passage in mice. This study indicates that the Italian H7N1 HPAI viruses had the potential of interspecies transmission to the mammalian host.

Puzelli et al. [23] generated serological evidence of seroconversion of humans occupationally exposed to the LP H7N3 virus in 3.8% of human sera collected during the period of circulation in 2003. These findings indicate the risk of bird-to-human transmission of LPAI viruses of the H7N3 subtype.

Phylogenetic and Genetic Studies

Full-length sequencing of the HA gene of Italian H7N1 LP and HP viruses generated evidence on the acquisition of specific mutations following circulation in the domestic host. Namely the acquisition of additional glycosylation sites in position 123 and 149, indicative of viral host adaptation [25, 26] and of the multi-basic cleavage site, the latter responsible of the increased pathogenicity of the HPAI viruses from the LP progenitors [27].

Longitudinal studies on the collection of isolates by sequence analysis, indicated that the *ns1 gene* of selected H7N1 isolates showed a progressive carboxy-terminal truncation, resulting in a 6 and 10 AA deletion in the NS1 protein over a 2-year period [28]. The implication and significance of such truncation are yet to be clarified although similar genetic modifications have been reported in similar studies performed on other H7 viruses circulating in domestic poultry [28].

Conclusions

The Italian H7N1 outbreak was the first of a series of outbreaks of significant magnitude that have occurred in recent times. The outbreaks of HPAI in such an area highlighted several aspects of AI crisis management that had not been fully pinpointed prior to this outbreak. Possibly the most important outcome of this epidemic was that it indicated to EU legislators that viruses of the H5 and H7 subtype, regardless of their

virulence for poultry, should be included in EU legislation and that therefore a legislative basis to manage LPAI infections caused by viruses of H5 and H7 subtypes was necessary. Current EU legislation has been revised to incorporate this and other changes (2005/94/EC).

Moreover, the Italian field experience in managing AI epidemics in poultry has generated evidence that vaccination against AI viruses using a 'DIVA' strategy is a useful tool to control the spread of avian influenza viruses in DPPAs provided it is complemented with direct measures such as movement restriction, biosecurity, stamping out and controlled marketing. This is also reflected in the revised EU legislation.

Phylogenetic and sequence analysis of a portion of the vast collection of isolates obtained between 1999 and 2004 have generated data on adaptive mutations acquired by H7 viruses following extensive circulation in poultry and on the interspecies transmission potential of these viruses, including pathogenicity for the mammalian host. Although the significance of some of these modifications remains to be established, the data gathered strongly support that H7 viruses of low and high pathogenicity are to be eradicated in a timely manner rather than contained.

References

1 Petek M: Current situation in Italy. Proc. 1st International Symposium on Avian Influenza, Beltsville, MD, USA, April 22–24, 1981, 1982, pp 31–34.

2 Capua I, Marangon S, Selli L, Alexander DJ, Swayne DE, Dalla Pozza M, Parenti E, Cancellotti FM: Outbreaks of highly pathogenic avian influenza (H5N2) in Italy during October 1997–January 1998. Avian Pathol 1999;28:455–460.

3 CEC: Community measures for the control of avian influenza. Off J Eur Comm 1992;L167:1–15.

4 Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, Capua I, Cordioli P, Fioretti A, Alexander DJ: Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 2001;146: 963–973.

5 Capua I, Marangon S: The avian influenza epidemic in Italy, 1999–2000. Avian Pathol 2000;29:289–294.

6 Capua I, Mutinelli F: Mortality in Muskovy ducks and domestic geese associated with natural infection with a highly pathogenic avian influenza virus of H7N1 subtype. Avian Pathol 2001;30:179–183.

7 Marangon S, Capua I: Control of avian influenza in Italy: from stamping out to emergency and prophylactic vaccination. Dev Biol (Basel) 2006;124:109–115.

8 Capua I, Mutinelli F, Marangon S, Alexander DJ: H7N1 Avian Influenza in Italy (1999–2000) in intensively reared chickens and turkeys. Avian Pathol 2000;29:537–543.

9 Capua I, Mutinelli F, Terregino C, Cattoli G, Manvell RJ, Burlini F: Highly pathogenic avian influenza (H7N1) in ostriches farmed in Italy. Vet Rec 2000;146:356.

10 Capua I, Mutinelli F, Bozza MA, Terregino C, Cattoli G: Highly pathogenic avian influenza (H7N1) in ostriches (*Struthio camelus*). Avian Pathol 2000;29: 643–646.

11 CEC: Commission decision 2001/847/CE of 30 November amending for the third time decision 2000/721/EC to modify the Italian avian influenza vaccination programme and current trade restrictions for fresh meat originating from vaccinated turkeys. Off J Eur Comm 2001;315:61–63.

12 Capua I, Terregino C, Cattoli G, Mutinelli F, Rodriguez JF: Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol 2003;32:47–55.

13 Cattoli G, Terregino C, Brasola V, Rodriguez JF, Capua I: Development and preliminary validation of an ad hoc N1-N3 discriminatory test for the control of avian influenza in Italy. Avian Dis 2003;47 (suppl):1060–1062.

14 Cattoli G, Milani A, Bettini F, Beato MS, Mancin M, Terregino C, Capua I: Development and validation of an anti-N3 indirect immunofluorescent antibody test to be used as a companion diagnostic test in the framework of a 'DIVA' vaccination strategy for avian influenza infections in poultry. Avian Path 2006;35:154–159.
15 Campitelli L, Mogavero E, De Marco MA, Delogu M, Puzelli S, Frezza F, Facchini M, Chiapponi C, Foni E, Cordioli P, Webby R, Barigazzi G, Webster RG, Donatelli I: Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. Virology 2004;323: 24–36.
16 Cattoli G, Terregino C, Bertoli E, Ortali G, Marangon S, Capua I: The H7N3 avian influenza outbreak in Northern Italy in 2002. Proc. 52nd Western Poultry Disease Conference, March 8–11, 2003, Sacramento, CA, USA, pp 23–24.
17 Toson M, Dalla Pozza M, Rossi E, Busani L, Marangon S: Efficacy of vaccination during the H7N3 low pathogenicity avian influenza epidemic in Italy in 2002–2003. Dev Biol (Basel) 2006;124: 24–36.
18 Capua I, Terregino C, Cattoli G, Toffan A: Increased resistance of vaccinated turkeys to experimental infection with an H7N3 low-pathogenicity avian influenza virus. Avian Pathol 2004;33:158–163.
19 CEC: Commission decision 2004/666/EC on introducing vaccination to supplement the measures to control infections with low pathogenic avian influenza in Italy and on specific movement control measures and repealing Decision 2002/975/EC. Off J Eur Comm 2004;L303:35–44.
20 Terregino C, Milani A, Capua I, Marino AM, Cavaliere N: Highly pathogenic avian influenza H5N1 subtype in mute swans in Italy. Vet Rec 2006;158: 491.
21 Capua I, Grossele B, Bertoli E, Cordioli P: Monitoring for highly pathogenic avian influenza in wild birds in Italy. Vet Rec 2000;147:640.
22 Terregino C, De Nardi R, Guberti V, Scremin M, Raffini E, Martin AM, Cattoli G, Capua I: Active surveillance for avian influenza viruses in wild birds and backyards flocks in Northern Italy during 2004–2006. Avian Pathol 2007;36:337–344.
23 Puzelli S, Di Trani L, Fabiani C, Campitelli L, De Marco MA, Capua I, Aguilera JF, Zambon M, Donatelli I: Serological analysis of serum samples from humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. J Infect Dis 2005; 192:1318–1322.
24 Rigoni M, Shinya K, Toffan A, Milani A, Bettini F, Kawaoka Y, Cattoli G, Capua I: Pneumo- and neurotropism of avian origin Italian highly pathogenic avian influenza H7N1 isolates in experimentally infected mice. Virology 2007;364:28–35.
25 Banks J, Plowright L: Additional glycosylation at the receptor binding site of the hemagglutinin for H5 and H7 viruses may be an adaptation to poultry hosts, but does it influence pathogenicity? Avian Dis 2003;47(suppl):942–950.
26 Matrosovich M, Zhou N, Kawaoka Y, Webster R: The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol 1999; 73:1146–1155.
27 Banks J, Speidel EC, McCauley JW, Alexander DJ: Phylogenetic analysis of H7 haemagglutinin subtype influenza A viruses. Arch Virol 2000;145: 1047–1058.
28 Dundon W, Maniero S, Toffan A, Capua I, Cattoli G: Appearance of serum antibodies against the avian influenza non-structural 1 protein in experimentally infected chickens and turkeys. Avian Dis 2007;51(suppl):209–212.

Ilaria Capua
OIE/FAO Reference Laboratory for Newcastle Disease and Avian Influenza, Virology Department
Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe)
Viale dell'Università 10, IT–35020 Legnaro/Padova (Italy)
Tel. +39 049 808 4369, Fax +39 049 808 4360, E-Mail icapua@izsvenezie.it

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 71–87

Avian Influenza Outbreaks in Germany – Development of New Avian Vaccines

Ortrud Werner · Timm Harder · Jutta Veits · Angela Römer-Oberdörfer · Walter Fuchs · Martin Beer · Franz J. Conraths · Thomas C. Mettenleiter

Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

Abstract

Germany was among the first countries to be hit by highly pathogenic avian influenza (HPAI) or fowl plague virus soon after it first surfaced in Italy at the end of the 19th century. Subsequently, only very few sporadic outbreaks occurred despite the continuous presence of low pathogenic avian influenza viruses in wild birds in Germany. However, fowl plague episodes in poultry occurred in 1979 derived from a low-pathogenic strain originating from wild birds, in 2003 in the context of the Dutch epidemic and again in 2006 and 2007 by the introduction of HPAI virus (HPAIV) subtype H5N1 of Asian origin. The latter was associated with the westward spread of HPAIV H5N1 Asia out of China into Europe. In total, 343 (2006) and 326 (2007) wild birds, respectively, as well as one bird kept in a zoo, three cats and one stone marten as well as one commercial poultry holding were found infected with HPAIV H5N1 Asia. Molecular epidemiological analyses demonstrated that two distinct introductions occurred into Germany in spring 2006 resulting in a Northern and Southern German lineage. The last demonstration of HPAIV H5N1 in 2006 in Germany occurred in August. However, in June 2007, HPAIV H5N1 resurfaced in wild birds and also affected several poultry holdings leading to the destruction of more than 350,000 animals. Molecular epidemiological analyses demonstrated that in 2006 two genetically distinct viruses circulated in Germany, whereas in 2007 a third genotype was detected. This indicated a total of three separate introductions of HPAI H5N1 into Germany in 2006 and 2007. The arrival of HPAIV H5N1 in Germany together with restrictions in keeping poultry outdoors renewed requests for vaccination. However, currently available inactivated vaccines suffer from several shortcomings including the necessity for individual application and lack of an easy and sensitive differentiation between vaccinated and infected animals. Thus, the development of novel vaccines against HPAIV has become of prime importance. The most promising approaches include the use of viral vectors expressing the protective influenza virus hemagglutinin. They are primarily based on recombinant adeno-, pox-, paramyxo- or herpesviruses. In particular, the use of recombinant Newcastle disease virus as carrier for influenza hemagglutinin offers the advantage to be able to protect simultaneously against two of the most important chicken pathogens.

Avian Influenza in Germany

History of AIV in Germany

In 1878, Perroncito [1] described for the first time a contagious disease of chickens spreading over Northern Italy which resulted in severe losses. Subsequently, Künnemann [2] claimed that this so-called 'Lombardian disease' or 'fowl plague' had been transmitted by a poultry salesman from the province of Padua to Austria and Southern Germany. A nationwide poultry show in Braunschweig in 1901 greatly accelerated the spread of the disease in Germany [3], thereafter various small outbreaks flared up. Another outbreak in 1925–1927 also originated from Italy which involved almost all of Europe. The import of infected chickens was reportedly the source of introduction into Germany [4]. No further reports of fowl plague outbreaks in Germany have been recorded for the next decades during which Newcastle disease (ND) became more important.

In 1979 an outbreak of a severe disease was detected in an industrial holding of layer chickens in Taucha, close to the city of Leipzig (Saxony). Approximately one-third of the birds in this holding succumbed to the disease. At first, velogenic ND was suspected, although the flocks had been vaccinated against ND. However, the isolation of an AIV of subtype H7N7 led to the final diagnosis 'classical fowl plague' (highly pathogenic avian influenza = HPAI) leading to the culling of 1.2 million animals within the holding. At the same time, central nervous signs and increased mortality were recognized in a large geese breeding holding (8,000 geese) at a distance of approximately 40 km to the affected chicken farm. These geese were held outside and had free access to surface water. This holding proved to be positive for HPAIV H7N7 as well and, consequently, the animals were culled. An epidemiological link via movement of staff between these two holdings was identified. No further outbreaks in poultry or an involvement of wild birds had been noticed. One virus isolate from the chicken holding and three from the geese flocks were subsequently analyzed [5]. The hemagglutinin (HA) genes of these four viruses were very similar, with only 10 nucleotide substitutions. However, they possessed varying numbers of basic amino acid residues (KKKKR, KRKKR, KKRKKR, KKKKKKR) at the proteolytic cleavage site. All four variants were highly pathogenic for chickens and shared recent ancestral HAs with A/Tern/Potsdam/342-6/79 (H7N7) and A/Swan/Potsdam/63-6/81 (H7N7). These viruses which had been isolated from wild birds, contained a monobasic cleavage site and, thus, were considered to be of low pathogenicity. In conclusion, the domestic geese may have been an important link in the evolution of the highly pathogenic H7N7 influenza strain A/Chicken/Leipzig/79.

In May 2003, in the context of the large epidemic of HPAIV H7N7 in the Netherlands and Belgium, an outbreak also involved a broiler holding in North Rhine-Westphalia close to the Dutch border. Birds of this holding and of two in-contact farms in the close vicinity were culled comprising a total of 81,000 chickens. The etiological agent was identical to that causing the Dutch and Belgian outbreaks (IVPI = 2.93, polybasic HA cleavage site KRRRR).

Table 1. Avian influenza viruses detected in poultry holdings in Germany since 1995

Year	Viruses detected, n	Subtype	Host species
1995	5	H9N2	turkey, chicken
1996	6	H9N2	turkey
1997	–	–	–
1998	4	H9N2, H6N5	chicken, turkey
1999	4	H6N2, H6N1	turkey
2000	–	–	–
2001	1	H7N7 (LPAI)	turkey
2002	15	H6N2	turkey
2003	2	H7N7 (HPAIV) H6N1	chicken, duck
2004	1	H6N2	goose
2005	3	H6N2, H3N8	duck, goose, turkey
2006	7	H5N3 (LPAIV), H11N1, H6Nx, H3N8, H6Nx, H5Nx H5N1 (HPAIV)	ostrich, duck, goose turkey

Low pathogenic avian influenza viruses (LPAIV) have occasionally been isolated from poultry in Germany during the last years (table 1), particularly viruses of subtypes H9 and H6 which caused clinical disease in turkeys [6, 7]. In ducks and geese flocks, LPAIV were detected by chance, i.e. in clinically healthy flocks, in the frame of routine monitoring activities [8]. In 2001, an LPAIV of subtype H7N7 was detected in a small mixed turkey holding with contact to aquatic wild birds. The flock was culled [9]. Further indications for occasional occurrences of H5/H7 LPAIV infections, particularly in geese flocks and free range poultry, were obtained by serosurveillance since 2004. The seemingly increasing number of LPAIV infections in poultry flocks in 2006 may, at least partly, be associated with an intensified monitoring using more sensitive diagnostic tools (see below).

Outbreak of H5N1 Asia in Germany in 2006 and 2007

Increased mortality among mute and whooper swans *(Cygnus olor, Cygnus cygnus)* wintering along the Northwestern shores of the Island of Rügen in the Baltic Sea had been observed since early February 2006. At that time the coastal sea was frozen except for a few areas which remained ice-free due to strong tidal currents. This area of open coastal landscape, shallow waters and inlets of the Baltic Sea is a wintering site for thousands of migratory aquatic birds, and substantial mortality among wintering birds, e.g. due to starvation, especially in strong winters like 2005/2006, is not uncommon. In February 2006, however, several moribund whooper and mute swans showed pronounced neurological signs of disease including torticollis, ataxia and

paralysis. Necropsy did not reveal conspicuous alterations, except a multifocal necrosis in the pancreas. Virological investigations carried out in the frame of a routine wild bird monitoring program revealed the presence of very high loads of HPAIV H5N1 of Asian lineage in diseased birds. Nucleotide sequencing of the HA and NA genes identified these viruses as representatives of the 'Qinghai' lineage of H5N1 viruses (clade 2.2) [10]. A similar situation was encountered at the same time in several other European countries along a line from Stockholm south to mainland Italy, Sicily and the Evro delta in Greece. This is suggestive of an almost simultaneous introduction of HPAIV H5N1, most likely by wild birds responding, by a westward move, to episodes of cold weather in Central and Southern Russia in late January 2006. Interestingly, HPAIV H5N1 of Asian lineage had first been detected in Europe in October 2005 in Romania and Croatia.

The finding of HPAIV H5N1 in Germany on February 14th sparked a nationwide intensified monitoring resulting in the sampling of more than 69,000 wild birds in Germany in 2006. A concerted action including regional laboratories in Germany led to the detection of HPAIV H5N1 in 343 wild birds, the majority being mute and whooper swans, several species of geese, tufted ducks *(Aythya fuligula)*, common pochards *(Aythya ferina)* and several species of birds of prey and gulls. Pathological and immunohistochemical investigations revealed disease patterns which were different in the various affected species. Swans and geese frequently showed signs of a rapid, endotheliotropic course with high virus titers also in the central nervous system [11]. A strong neurotropism leading to a massive virus-induced encephalomyelitis was also evident in birds of prey, which most likely became infected by scavenging on highly infectious carcasses of swans and geese. Cases were scattered in a semicircle from the Northern to the Southwestern part of Germany (fig. 1). Foci of infection existed around the Island of Rügen and at Lake Constance. The crowding of large numbers of highly susceptible birds like swans and geese in ice-free inlets may have led to accelerated viral transmission rates similar to the H5N1 outbreak among bar-headed geese at Lake Qinghai, where birds concentrated in high numbers at their breeding grounds. Detection of H5N1 ceased in early May. However, a single isolated appearance of the virus was noticed in a juvenile black swan *(Cygnus astratus)* kept in the zoo of the city of Dresden in South-East Germany in August 2006.

Until June 2007, no further cases of HPAIV H5N1 in wild birds had been detected despite continued nationwide monitoring. In late June 2007, however, H5N1 HPAIV was discovered in several dead mute swans found at lakes in the city center of Nuremberg. At the same time, the virus was reported from the Czech Republic in a turkey holding and a wild mute swan, and in the East of France in several mute swans. Further outbreaks in wild birds were subsequently detected in other locations scattered throughout Southeast Germany (fig. 1). One of these outbreaks involved more than 280 black-necked grebes (*Podiceps nigricollis*) which amounts to at least 70% of a local population of this otherwise rare species at Lake Kelbra, Thuringia. A total of

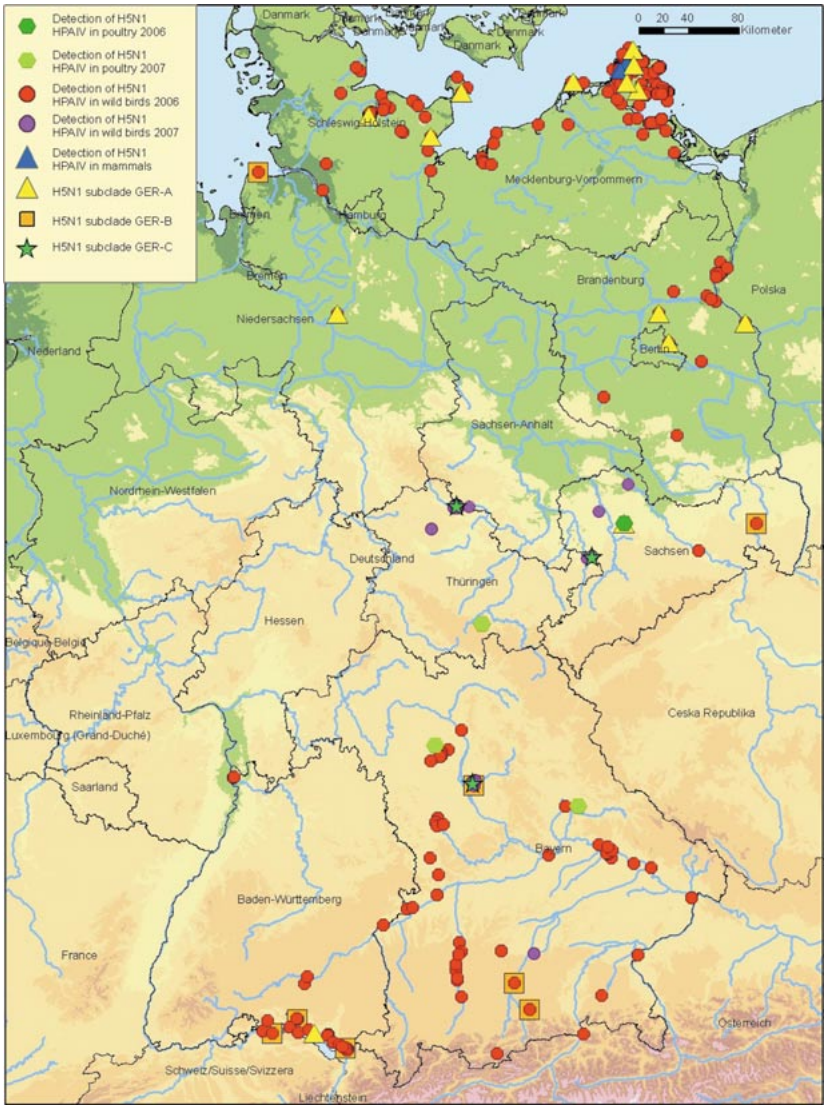


Fig. 1. Localization of wild birds and poultry holdings infected with HPAIV H5N1 in Germany in 2006 and 2007.

326 wild bird cases was recorded until mid-August in Southeast Germany when no further virus activity was detected.

In addition to infected birds, three H5N1-infected stray cats and a stone marten *(Martes foina)* were found on the Island of Rügen in 2006. All four mammals were found dead or moribund. Marked liver alterations detected in the cats suggested an oral route of infection, and scavenging on carcasses of infected wild birds is the most likely source [10, 12, 13]. The stone marten, in contrast, was diagnosed with an influenza-induced viral encephalitis [14]. The viruses isolated from these mammals

were very closely related to those from wild birds at this location, suggesting a direct transmission [10]. Intensive monitoring among feral and wild omni- and carnivores (e.g. wild boar, martens, stray cats) on the Island of Rügen in 2006 led to the detection of only five additional cats which were seropositive to H5 antigen.

As a prerequisite for an intensified wild bird monitoring, diagnostic methods had to be adapted to accommodate high-throughput screening. Therefore, real-time RT-PCR (rRT-PCR) was established in a cascade style to detect M-, H5/H7-, N1- and polybasic cleavage site-specific gene sequences [15, 16, and Hoffmann, pers. commun.]. Use of the newly developed clade- and pathotype-specific rRT-PCR allowed unambiguous diagnosis even from samples of decomposed carcasses from which virus isolation was not successful.

Phylogenetic analysis of the HA and NA genes of 24 Qinghai-like isolates from different regions of Germany gave evidence for the presence of two distinct, geographically restricted clusters [Starick, pers. commun.]. They differ by single mutations in the HA and NA genes. Towards the end of the epidemic in Germany the strict geographical distinction softened and some 'Northern' isolates were detected in the South and vice versa. Closest relatives of the Northern (GER-A, subclade 2.2.2) lineage were observed in 2005 in Central and Southern Russia while those of the Southern cluster (GER-B, subclade 2.2.1) seem to originate from the Black Sea area. Thus, two separate introductions into Germany of Qinghai-like H5N1 viruses of different origin have occurred at almost the same time in 2006. According to a recently proposed terminology these strains relate to the so-called subclades EMA-2 and -1 (Europe-Middle East-Africa) within clade 2.2 [17]. The viruses detected in Germany in 2007 together with those from the Czech Republic and France proved to cluster with yet another sublineage (GER-C, subclade 2.2.3 representing EMA clade 3). Therefore, a new introduction from an unknown source in 2007 rather than a continued presence of viruses from 2006 most likely accounted for the recurrence in 2007.

While HPAIV H5N1 was detected mainly in wild birds in Germany, several poultry holdings were also affected. In 2006, a medium-sized farm keeping geese parents and fattening turkeys, situated on a peninsula in a lake in Saxony, became involved when severe and rapidly increasing mortality among turkeys on that holding was reported. Although no affected wild birds were detected in the vicinity, introduction via wild birds is highly suggestive. Clinical disease was first noticed in a closed turkey section. Swift confirmative diagnosis followed by rapid culling and flanking restriction measures contained the virus at the index farm. The lack of further outbreaks in poultry despite wide geographical distribution of H5N1 HPAIV in wild birds in Germany in 2006 may, at least in part, be the result of the nationwide confinement of free-range poultry to stables which had been in force since autumn 2005. Few exceptions from this general confinement were approved by local authorities, e.g. for geese breeding holdings. The geese of the Saxonian index farm were allowed outdoors daily for a few hours. Interestingly, the same holding had been affected by the HPAIV H7N7 outbreak in 1979.

Since late June 2007, three more poultry holdings were involved. While the first case in poultry in 2007 was found in a very small backyard holding of geese, the other two outbreaks had a much bigger impact. Ducks in a large fattening unit in Northeastern Bavaria were found infected by HPAIV H5N1; however, only slightly increased mortality rates had been seen in young ducklings at this holding. Subsequently, intensified virological examinations finally led to the detection of HPAIV H5N1 infected ducks in another large duck fattening farm in the same region, an in-contact holding to the first farm. Stamping-out measures finally involved more than 365,000 ducks.

Development of Recombinant Vaccines against HPAIV

Vaccination has been considered as a tool to protect poultry during the period of overt presence of HPAIV H5N1 in wild birds in Germany. Two main reasons led to abstention from vaccination: (i) Syndrome surveillance is one of the most powerful tools to detect the presence of HPAIV in fully susceptible gallinaceous poultry. In an immunized population which is protected from clinical disease this option would no longer be valid. (ii) While vaccination with currently licensed inactivated full virus vaccines results in clinical protection, sterile immunity is not induced under field conditions and field-virus may circulate undetected in vaccinated flocks. A first system to differentiate animals which had been vaccinated with these vaccines from field-virus-infected poultry (DIVA) [18] is based on vaccination with viruses of the circulating HA subtype but containing a different neuraminidase subtype [19]. The different specificity of the anti-neuraminidase antibodies could then be used for differentiation. However, its application is limited, especially when multiple AIV subtypes circulate in the environment. Moreover, the differentiation of antibodies against different NA proteins is labor-intensive and insensitive. Thus, there is a continuous need for improvement of the existing vaccines.

Vaccines Based on Recombinant AIV

Plasmid-based reverse genetic systems for influenza virus were developed in the late 1990s [20–22] and became a powerful tool to generate reassortant influenza vaccine candidates which derive their HA gene from circulating influenza A viruses [23–26] with internal genes from vaccine donor strains like influenza A/Puerto Rico/8/34 (PR8) (H1N1) or A/WSN/33 (H1N1). Inactivated vaccine formulations of these reassortants conferred protection levels in terms of reduction of viral shedding after challenge infections comparable to the inactivated parental strains [23].

Reverse genetics have also been used to create influenza virus reassortants with HA and NA genes of current H5N1 HPAIV strains. An inactivated recombinant influenza virus with genes of an avian H5N1 isolate conferred protection against the

parental HPAIV A/Goose/Guangdong/96 (H5N1) and against heterologous HPAIV H5N1 challenge infection. The vaccine prevented mortality as well as morbidity and reduced virus shedding in chickens [27]. It was also effective in geese and ducks, although repeated doses of high antigen content had to be used for successful immunization in these species in field studies. In all these reassortants the polybasic HA cleavage site had been modified to overcome the requirement for high level biocontainment facilities and to obtain high virus yields in embryonated chicken eggs. Nevertheless, these vaccines were used in inactivated form, since live recombinant influenza virus may revert to virulence [28].

The NS1 gene is a virulence factor in chickens [29], and, thus, is a possible target for developing live vaccines [30]. Influenza virus recombinants with truncations of the NS1 gene are attenuated and protective [31]. Furthermore, NS1 deletion mutants can be used in a DIVA strategy based on a diagnostic test detecting anti-NS1 antibodies [32]. Another interesting approach which also allows the DIVA strategy is the generation of a neuraminidase-deficient virus [33]. An alternative candidate for a live vaccine could be an influenza virus with an atypical HA cleavage site that is resistant to activation during natural infection but can readily be activated under in vitro conditions [34].

Furthermore, influenza viruses can be used as vectors for foreign genes [33, 35]. A recombinant influenza virus expressing the ectodomain of NDV HN instead of the ectodomain of AIV NA would allow immunization against two important diseases in poultry, avian influenza and ND, but the resulting virus was too attenuated to induce a satisfactory immune response.

Since these replication-competent vaccines may still undergo reassortment, another approach is the development of live vaccines that are limited to a single cycle of replication. NS2-deleted influenza viruses are replication-deficient but protective in mice [36]. Administration of virus-like particles made by expression of viral proteins has also been shown to be effective [37–40]. Although protection against AIV has to be improved, an HA expressing alphavirus replicon may even be used for in ovo vaccination [41].

Another objective is the generation of a cross-protective vaccine against different influenza subtypes. Ernst et al. [42] demonstrated that immunizations with the conserved extracellular portion of the M2 protein protected mice from challenge with different influenza strains. However, all tested vaccines lacking the HA or NA and based solely on conserved internal proteins lack protective power in poultry.

DNA Vaccines and Adenovirus-Based Vector Vaccines

The application of plasmid-cloned genes encoding influenza virus NP and HA to mice was the first demonstration that DNA vaccines can induce specific antibodies and confer protective immunity [43, 44]. A single DNA vaccination of chickens with expression plasmids encoding H5 or H7 protected the animals from lethal challenge infection with HPAIV of corresponding subtypes, even if the HA of the

challenge virus differed by more than 10% from the plasmid-encoded protein [45, 46]. DNA vaccines have been prepared for most of the 16 known HA subtypes, and, since they were used for production of monospecific diagnostic antisera in chickens [47], they might be suitable for immunization of poultry against the corresponding low pathogenic AIV strains. In comparative studies using different vectors, HA expression under control of the human cytomegalovirus immediate-early (HCMV-IE) promoter induced the strongest immune responses, and efficacy could be improved by increased amounts of DNA, booster immunizations, several adjuvants, or by coexpression of HA with the conserved M protein of AIV [45, 48, 49]. In contrast, DNA vaccines encoding only the conserved NP did not confer satisfying protection [46]. Thus, like other immunization strategies, plasmid technology does not permit generation of a single vaccine against all avian influenza A viruses. However, immunization of chickens with a mixture of plasmids encoding H5 and H7 HA protected against HPAIV of both subtypes [46]. The dispensability of the immunogenic NP for protection renders this protein a useful marker for identification of AIV infected animals in populations vaccinated with HA expression plasmids. ELISA tests for the detection of NP-specific serum antibodies have been developed [50–53].

Despite positive results in laboratory experiments, practical use of DNA vaccines is still hampered. Most plasmids contain antibiotic resistance genes, which might be transferred to pathogenic bacteria. Furthermore, at least 10 μg of plasmid DNA bound to gold particles had to be used for successful vaccination of chickens [46] and, thus, for immunization of huge flocks considerable production costs would arise. Costs would be further increased by the necessity of individual administration, which also might be too time-consuming in cases of emergency. Several of these problems can be overcome with DNA vaccines suitable for oral or nasal spray delivery by the use of synthetic microparticles or non-pathogenic bacteria as carriers [54, 55], or bacteriophages as vectors [56]. Whereas the suitability of these systems for vaccination against AIV remains to be evaluated, non-replicating human adenovirus vectors are promising carriers for HA expression cassettes [57, 58]. These viral vectors possess deletions of essential early genes (E1 and E3), and foreign genes can be inserted by site-directed Cre-*lox* recombination [59], or by mutagenesis of the plasmid-cloned virus genome in *Escherichia coli* [60]. The obtained adenovirus recombinants represent safe vaccines, since they are defective, but, nevertheless, can replicate to very high titers in trans-complementing cell lines. A single intramuscular or subcutaneous application of adenovirus particles containing the HA gene of Asian H5N1 HPAIV under control of the HCMV-IE promoter induced both humoral and cellular immune responses in mice, and protected mice and chickens from a lethal challenge infection with homologous HPAIV [57]. However, only a very limited protection of chickens was achieved by intranasal application of the vaccine. Another defective adenovirus recombinant containing a H5 HA gene conferred protective immunity to chickens after in ovo vaccination [58]. However, efficacy of the vaccine appears to

depend on the degree of homology between the HA genes of challenge virus and vaccine, since only two-thirds of the vaccinated animals were protected against lethal infection with heterologous Asian H5N1 virus [58]. Thus, frequent 'updates' of the adenovirus-based vaccines might be required, but will be facilitated by the convenient mutagenesis techniques available.

Live Vector Vaccines against HPAIV

Replication-competent infectious agents are probably still the most economic vaccines, since their productive replication in the immunized organism makes them efficacious even at low doses. In addition, no infectious HPAIV is required, and, thus, vaccine production does not require high level biosecurity. Furthermore, since the recombinant vaccines contain only one or two major antigens of AIV, usually HA and/or NA, vaccinated and naturally infected animals can easily be differentiated by the absence or presence of serum antibodies against other immunogenic AIV proteins like NP.

During the last decades, not only AIV, but also other chicken viruses like fowlpox virus (FPV), infectious laryngotracheitis virus (ILTV), and Newcastle disease virus (NDV), have become accessible to recombinant DNA technology which enabled directed mutagenesis of the virus genomes [61–64]. Moreover, *Salmonella* spp. have been engineered to express AIV antigen [65]. These methods facilitated the generation of safe vaccines by identification and irreversible deletion of virulence factors, and allowed the insertion and expression of foreign genes. Considering the high mutation rates of influenza viruses, vectored vaccines based on genetically more stable viruses have been preferentially developed.

Poxviruses/Fowlpox Virus

Poxviruses were among the first viral vectors used for the expression of heterologous proteins [62]. Attenuated fowlpox viruses, which were already in use as vaccines for chickens and turkeys, have been further modified by insertion of AIV genes [66–69] into different non-essential regions of the genome, e.g. at the thymidine kinase gene locus [67]. The foreign genes were expressed under control of strong poxvirus promoters, e.g. the vaccinia virus H6 promoter [66]. The considerable size of the FPV genome of nearly 300 kbp allowed not only insertions of single genes, but also simultaneous expression of several genes, encoding e.g. HA and NA [69], or HA and NP [68].

Single vaccinations with ca. 10^5 infectious units of H5-, or H7-expressing FPV recombinants protected chickens against lethal challenge infections with HPAIV of the corresponding subtype [70–72]. Moreover, H5-expressing FPV protected chickens as efficiently as conventional inactivated AIV vaccines against a variety of different H5 HPAIV strains, including recent Asian H5N1 isolates. However, like

other AIV vaccines, HA-expressing FPV does not confer sterile immunity, as could be demonstrated by reisolation of the HPAIV challenge virus from tracheal and cloacal swabs [66, 71].

Since NP-expressing FPV was not protective in chickens, and coexpression of NP did also not enhance the efficacy of HA-expressing recombinants [68], NP proved to be dispensable for successful vaccination, and NP-specific antibodies can be used in a DIVA strategy. FPV-based vaccines have been licensed for emergency use in the USA, and are currently applied in Central America and in China.

FPV live virus vaccines can be produced economically on the chorioallantois membrane of chicken embryos or in primary chicken cell cultures, and can be administered already to 1-day-old animals [73]. However, individual subcutaneous vaccination, usually by the wing-web method, is required to obtain optimal protection. Another limitation for the use of FPV vectors is the presence of antibodies in chickens, which had been immunized against fowlpox. In these animals, replication of HA-FPV is inhibited, and, consequently, only insufficient protection ensued [74]. Since the natural host range of FPV is largely limited to chickens, it remains to be evaluated to which extent FPV vector vaccines are suitable for other species threatened by HPAIV. Although an HA-expressing FPV recombinant induced a specific immune response even in cats [72], protection of turkeys immunized with this virus was significantly less pronounced than observed in chickens [66]. Possibly, these problems can be overcome by the use of more recently developed vectors based on other poxviruses. Recently, modified vaccinia virus Ankara has been engineered to express AIV H5 HA and shown to protect mice from H5N1 HPAIV challenge [75].

Paramyxoviruses/Newcastle Disease Virus

In the past few years, several recombinant NDV vector vaccines expressing AIV HA have been described. Swayne et al. [76] reported the construction of a NDV recombinant expressing AIV H7 which provided partial protection in chickens (40% survival) after challenge infection by velogenic NDV or H7 HPAIV. The rather low protection rate was attributed to overattenuation of the non-fusogenic vector strain. Park et al. [35] generated a fusogenic NDV vector by alteration of the cleavage site of the NDV fusion (F) protein and expressed AIV H7 as chimeric protein with the cytoplasmic domain of the NDV F protein resulting in the location of chimeric HA in virus particles. This modified recombinant conferred complete protection against a lethal NDV challenge and increased the survival rate against HPAIV to 90%. However, NDV recombinants expressing authentic H5 also incorporated the heterologous protein into the virion envelope and conferred protection against lethal infections with NDV and homologous as well as heterologous H5 HPAIV [53, 77] to chickens with no evidence of viral shedding. These recombinants represent promising candidates for the control of avian influenza as they enable a simple DIVA strategy based on detection of antibodies against NP [53], and the use in a cost efficient mass application against two

devastating avian pathogens. Reportedly, one of these recombinants is already in widespread use to control AIV in China.

Herpesviruses/Infectious Laryngotracheitis Virus

Live virus vaccines against infectious laryngotracheitis (ILT) of chickens can be produced in sufficient quantity in embryonated chicken eggs or in primary chicken cell cultures. Moreover, the alphaherpesvirus ILTV can be successfully administered by eye drop, and is also suitable for mass application via spray or drinking water [78]. Furthermore, chickens are not generally vaccinated against ILT, and, therefore, in most cases preexisting immunity does not affect the efficacy of ILTV-based vector vaccines. Like poxviruses, ILTV possesses a double-stranded DNA genome that contains numerous genes which are dispensable for replication in cultured chicken cells, and which could be deleted or replaced by foreign DNA sequences. Most of these genetic manipulations led to a significant and irreversible reduction of virulence in chickens, and, therefore, provided vaccines which are safer than classically attenuated and genetically uncharacterized ILTV strains [reviewed in 61]. In the genomes of ILTV recombinants lacking the non-essential dUTPase (UL50) or UL0 genes, the coding sequences of HA subtypes H5 and H7 have been expressed under control of the HCMV-IE promoter [79–81]. A single ocular immunization of chickens with 10^3–10^5 plaque-forming units of the HA-expressing recombinants protected the animals against challenge with pathogenic ILTV, or lethal doses of HPAIV of the respective subtypes.

Antibody titers and degree of protection were dependent on vaccine doses, and only the highest doses prevented any clinical signs of subsequent HPAIV infections [79]. Homology between the HA genes of vaccine and challenge virus is also relevant, since equal doses of the H5-expressing ILTV recombinant completely protected chickens against the homologous Italian H5N2 HPAIV, whereas transient disease was observed in animals challenged with an older Scottish H5N1 virus possessing 94% of identical HA amino acids [80]. Remarkably, only 50% of immunized chickens survived challenge infection with an Asian H5N1 HPAIV, although sequence homology between the HA proteins was also 94% [Veits et al., unpubl. results]. This finding indicated that different virulence of challenge viruses might also influence the level of protection, and emphasizes the necessity to construct vector vaccines expressing the HA gene of currently relevant AIV isolates. Nevertheless, HA-expressing ILTV recombinants are promising candidates for emergency vaccines against fowl plague, since the protective effect of the described H5-expressing mutant against homologous H5N2 HPAIV was equivalent to that of inactivated AIV vaccines or HA-expressing NDV mutants [53, 80]. This also applied to the substantial reduction of challenge virus shedding which demonstrated the low risk of influenza virus transmission by healthy vaccinated individuals. Furthermore, like other subunit, DNA, or vectored AIV vaccines, HA-expressing ILTV mutants permit identification of infected animals by marker diagnostics, and even in previously

immunized chickens NP-specific serum antibodies could be detected after the AIV challenge by ELISA tests [79].

A limitation of ILTV-based vector vaccines might result from the narrow host range of this virus, which is restricted to chickens and pheasants, and only barely replicates in other avian species like turkeys [78]. Thus, for this important host of HPAIV, vaccines based on other viral vectors will be presumably more efficacious. A good candidate might be the apathogenic herpesvirus of turkeys (HVT), which has already been used as live virus vaccine against Marek's disease virus, and which was successfully tested as vector vaccine expressing immunogenic proteins of NDV and infectious bursal disease virus in chickens [82, 83]. However, HVT recombinants expressing influenza virus proteins have not been described up to now.

Acknowledgments

The authors wish to thank all colleagues who have contributed to the data as part of the 'FLI-Flu Team'. These include members of the FLI institutes for epidemiology (Matthias Kramer, Carsten Pötzsch, Christoph Staubach, Kathrin Teske, Fred Unger), diagnostic virology (Anja Globig, Bernd Hoffmann, Elke Starick), infectology (Robert Klopfleisch, Jens Teifke) and molecular biology (Dorothee Helferich, Dörte Lüschow, Egbert Mundt, Thomas Vahlenkamp, Siegfried Weber). We also appreciate the excellent cooperation with the regional veterinary laboratories and authorities.

References

1 Perroncito E: L'epizoozia tifoide die polli. Ann Accad Agricolt Turino 1878;21:87–126.

2 Künnemann D: Beobachtungen über die Vogelpest. Dtsch Tierärztl Wschr 1902;10:413–415.

3 Greve L: Beobachtungen über eine von der Braunschweiger Geflügelausstellung in die Stadt und das Amt Oldenburg eingeschleppte Hühnerseuche. Dtsch Tierärztl Wschr 1901;9:373.

4 Schmidt U: Die Geflügelpest; in Röhrer H (ed): Handbuch der Virusinfektionen bei Tieren. Jena, Fischer, 1968, pp 283–437.

5 Röhm C, Süss J, Pohle V, Webster RG: Different hemagglutinin cleavage site variants of H7N7 in an influenza outbreak in chickens in Leipzig, Germany. Virology 1996;218:253–257.

6 Hafez HM, Prusas C, Cortez de Jäckel S, Aldehoff D, Werner O: Investigations on avian influenza A in meat turkey flocks in Germany. Arch Geflügelkd 2003;67:11–15.

7 Sieverding E: Klinischer Verlauf von Influenza A H9N2 Infektionen bei Puten; in Proc. Fachgruppe Geflügelkrankheiten. Giessen, DVG, 1997, pp 69–78.

8 Werner O, Globig A: Zum Vorkommen von aviären Influenzaviren bei Geflügel in verschiedenen Haltungsformen in Deutschland; in Proc. Fachgruppe Geflügelkrankheiten. Giessen, DVG, 2004, pp 83–91.

9 Werner O, Starick E, Grund CH: Isolation and characterization of a low-pathogenicity H7N7 influenza virus from a turkey in a small mixed free-range poultry flock in Germany. Avian Dis 2003;47: 1104–1108.

10 Weber S, Harder T, Starick E, Beer M, Werner O, Hoffmann B, Mettenleiter TC, Mundt E: Molecular analysis of highly pathogenic avian influenza virus of subtype H5N1 isolated from wild birds and mammals in northern Germany. J Gen Virol 2007;88: 554–558.

11 Teifke JP, Klopfleisch R, Globig A, Starick E, Hoffmann B, Wolf PU, Beer M, Mettenleiter TC, Harder TC: Pathology of natural infections by H5N1 highly pathogenic avian influenza virus in mute *(Cygnus olor)* and whooper *(Cygnus cygnus)* swans. Vet Pathol 2007;44:137–143.

12 Wolf PU, Uhl W, Gerst S, Gerst K, Klopries M, Teifke JP, Klopfleisch R, Harder T, Werner O, Hoffmann B, Starick E, Mundt E, Beer M, Mettenleiter TC: Letal verlaufende Influenza bei Hauskatzen nach natürlicher Infektion mit H5N1/Asia in Deutschland. Dtsch Tierärztblatt 2006;54:426–431.
13 Klopfleisch R, Wolf PU, Uhl W, Gerst S, Harder T, Starick E, Vahlenkamp TW, Mettenleiter TC, Teifke JP: Distribution of lesions and antigen of highly pathogenic avian influenza virus A/Swan/ Germany/ R65/06 (H5N1) in domestic cats after presumptive infection by wild birds. Vet Pathol 2007;44:261–268.
14 Klopfleisch R, Wolf PU, Wolf C, Harder T, Starick E, Niebuhr M, Mettenleiter TC, Teifke JP: Encephalitis in a stone marten *(Martes foina)* after natural infection with highly pathogenic avian influenza virus subtype H5N1. J Comp Pathol 2007;137:155–159.
15 Spackman F, Senne DA, Bulaga LL, Myers TJ, Perdue ML, Garber LP, Lohmann K, Daum LT, Suarez DL: Development of real-time RT-PCR for the detection of avian influenza virus. Avian Dis 2003;47:1079–1082.
16 Hoffmann B, Harder T, Starick E, Depner K, Werner O, Beer M: Rapid and highly sensitive pathotyping of avian influenza A H5N1 virus by using real-time reverse transcription-PCR. J Clin Microbiol 2007;45:600–603.
17 Salzberg SL, Kingsford C, Cattoli G, Spiro DJ, Janies DA, et al: Genome analysis linking recent European and African influenza (H5N1) viruses. Emerg Infect Dis 2007;13:713–718.
18 Van Oirschot JT: Diva vaccines that reduce virus transmission. J. Biotechnol 1999;73:195–205.
19 Capua I, Terregino C, Cattoli G, Mutinelli F, Rodriguez JF: Development of a DIVA (differentiating infected from vaccinated animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza. Avian Pathol 2002;32:47–55.
20 Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A: Rescue of influenza A virus from recombinant DNA. J Virol 1999;73: 9679–9682.
21 Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG: A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA 2000;97:6108–6113.
22 Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y: Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci USA 1999;96:9345–9350.
23 Lee CW, Senne DA, Suarez DL: Generation of reassortant influenza vaccines by reverse genetics that allows utilization of a DIVA (differentiating infected from vaccinated animals) strategy for the control of avian influenza. Vaccine 2004;22:3175–3181.
24 Liu M, Wood JM, Ellis T, Krauss S, Seiler P, Johnson C, Hoffmann E, Humberd J, Hulse D, Zhang Y, Webster RG, Perez DR: Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics. Virology 2003;314:580–590.
25 Webster RG, Webby RJ, Hoffmann E, Rodenberg J, Kumar M, Chu HJ, Seiler P, Krauss S, Songserm T: The immunogenicity and efficacy against H5N1 challenge of reverse genetics-derived H5N3 influenza vaccine in ducks and chickens. Virology 2006;351:303–311.
26 Middleton D, Bingham J, Selleck P, Lowther S, Gleeson L, Lehrbach P, Robinson S, Rodenberg J, Kumar M, Andrew M: Efficacy of inactivated vaccines against H5N1 avian influenza infection in ducks. Virology 2007;359:66–71.
27 Tian G, Zhang S, Li Y, Bu Z, Liu P, Zhou J, Li C, Shi J, Yu K, Chen H: Protective efficacy in chickens, geese and ducks of an H5N1-inactivated vaccine developed by reverse genetics. Virology 2005;341:153–162.
28 Horimoto T, Takada A, Fujii K, Goto H, Hatta M, Watanabe S, Iwatsuki-Horimoto K, Ito M, Tagawa-Sakai Y, Yamada S, Ito H, Ito T, Imai M, Itamura S, Odagiri T, Tashiro M, Lim W, Guan Y, Peiris M, Kawaoka Y: The development and characterization of H5 influenza virus vaccines derived from a 2003 human isolate. Vaccine 2006;24:3669–3676.
29 Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H: The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol 2006;80:11115–11123.
30 Cauthen AN, Swayne DE, Sekellick MJ, Marcus PI, Suarez DL: Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. J Virol 2007;81:1838–1847.
31 Richt JA, Lekcharoensuk P, Lager KM, Vincent AL, Loiacono CM, Janke BH, Wu WH, Yoon KJ, Webby RJ, Solorzano A, Garcia-Sastre A: Vaccination of pigs against swine influenza viruses by using an NS1-truncated modified live-virus vaccine. J Virol 2006;80:11009–11018.
32 Tumpey TM, Alvarez R, Swayne DE, Suarez DL: Diagnostic approach for differentiating infected from vaccinated poultry on the basis of antibodies to NS1, the nonstructural protein of influenza A virus. J Clin Microbiol 2005;43:676–683.
33 Shinya K, Fujii Y, Ito H, Ito T, Kawaoka Y: Characterization of a neuraminidase-deficient influenza A virus as a potential gene delivery vector and a live vaccine. J Virol 2004;78:3083–3088.

34 Stech J, Garn H, Wegmann M, Wagner R, Klenk HD: A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin. Nat Med 2005;11:683–689.
35 Park MS, Steel J, Garcia-Sastre A, Swayne D, Palese P: Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. Proc Natl Acad Sci USA 2006;103:8203–8208.
36 Watanabe T, Watanabe S, Neumann G, Kida H, Kawaoka Y: Immunogenicity and protective efficacy of replication-incompetent influenza virus-like particles. J Virol 2002;76:767–773.
37 Galarza JM, Latham T, Cupo A: Virus-like particle vaccine conferred complete protection against a lethal influenza virus challenge. Viral Immunol 2005; 18:244–251.
38 Pushko P, Tumpey TM, Bu F, Knell J, Robinson R, Smith G: Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. Vaccine 2005;23:5751–5759.
39 Quan FS, Huang C, Compans RW, Kang SM: Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. J Virol 2007;81:3514–3524.
40 Sylte MJ, Hubby B, Suarez DL: Influenza neuraminidase antibodies provide partial protection for chickens against high pathogenic avian influenza infection. Vaccine 2007;25:3763–3772.
41 Schultz-Cherry S, Dybing JK, Davis NL, Williamson C, Suarez DL, Johnston R, Perdue ML: Influenza virus (A/HK/156/97) hemagglutinin expressed by an alphavirus replicon system protects chickens against lethal infection with Hong Kong-origin H5N1 viruses. Virology 2000;278:55–59.
42 Ernst WA, Kim HJ, Tumpey TM, Jansen AD, Tai W, Cramer DV, Adler-Moore JP, Fujii G: Protection against H1, H5, H6 and H9 influenza A infection with liposomal matrix 2 epitope vaccines. Vaccine 2006;24:5158–5168.
43 Fynan EF, Webster RG, Fuller DH, Haynes JR, Santoro JC, Robinson HL: DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc Natl Acad Sci USA 1993;90: 11478–11482.
44 Ulmer JB, Donnelly JJ, Parker SE, Rhodes GH, Felgner PL, Dwarki VJ, Gromkowski SH, Deck RR, DeWitt CM, Friedman A, Hawe LA, Leander KR, Martinez D, Perry HC, Shiver JW, Montgomery DL, Liu MA: Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993;259:1745–1749.
45 Kodihalli S, Goto H, Kobasa DL, Krauss S, Kawaoka Y, Webster RG: DNA vaccine encoding hemagglutinin provides protective immunity against H5N1 influenza virus infection in mice. J Virol 1999;73: 2094–2098.
46 Kodihalli S, Kobasa DL, Webster RG: Strategies for inducing protection against avian influenza A virus subtypes with DNA vaccines. Vaccine 2000;18: 2592–2599.
47 Lee CW, Senne DA, Suarez DL: Development and application of reference antisera against 15 hemagglutinin subtypes of influenza virus by DNA vaccination of chickens. Clin Vaccine Immunol 2006;13: 395–402.
48 Cherbonnel M, Rousset J, Jestin V: Strategies to improve protection against low-pathogenicity H7 avian influenza virus infection using DNA vaccines. Avian Dis 2003;47:1181–1186.
49 Suarez DL, Schultz-Cherry S: The effect of eukaryotic expression vectors and adjuvants on DNA vaccines in chickens using an avian influenza model. Avian Dis 2000;44:861–868.
50 De Boer GF, Back W, Osterhaus AD: An ELISA for detection of antibodies against influenza A nucleoprotein in humans and various animal species. Arch Virol 1990;115:47–61.
51 Shafer AL, Katz JB, Eernisse KA: Development and validation of a competitive enzyme-linked immunosorbent assay for detection of type A influenza antibodies in avian sera. Avian Dis 1998;42:28–34.
52 Starick E, Werner O, Schirrmeier H, Köllner B, Riebe R, Mundt E: Establishment of a competitive ELISA (cELISA) system for the detection of influenza A virus specific nucleoprotein antibodies and its application to field sera of different species. J Vet Med B 2006;53:370–375.
53 Veits J, Wiesner D, Fuchs W, Hoffmann B, Granzow H, Starick E, Mundt E, Schirrmeier H, Mebatsion T, Mettenleiter TC, Römer-Oberdörfer A: Newcastle disease virus expressing H5 gene protects chicken against Newcastle disease and avian influenza. Proc Natl Acad Sci USA 2006;103:8197–8202.
54 Chen SC, Jones DH, Fynan EF, Farrar GH, Clegg JC, Greenberg HB, Herrmann JE: Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles. J Virol 1998;72:5757–5761.
55 Darji A, zur Lage S, Garbe AI, Chakraborty T, Weiss S: Oral delivery of DNA vaccines using attenuated *Salmonella typhimurium* as carrier. FEMS Immunol Med Microbiol 2000;27:341–349.
56 Jepson CD, March JB: Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. Vaccine 2004;22:2413–2419.

57 Gao W, Soloff AC, Lu X, Montecalvo A, Nguyen DC, Matsuoka Y, Robbins PD, Swayne DE, Donis RO, Katz JM, Barratt-Boyes SM, Gambotto A: Protection of mice and poultry from lethal H5N1 avian influenza virus through adenovirus-based immunization. J Virol 2006;80:1959–1964.
58 Toro H, Tang DC, Suarez DL, Sylte MJ, Pfeiffer J, Van Kampen KR: Protective avian influenza in ovo vaccination with non-replicating human adenovirus vector. Vaccine 2007;25:2886–2891.
59 Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML: Construction of adenovirus vectors through Cre-lox recombination. J Virol 1997;71:1842–1849.
60 He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B: A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci USA 1998;95:2509–2514.
61 Fuchs W, Veits J, Helferich D, Granzow H, Teifke JP, Mettenleiter TC: Molecular biology of avian infectious laryngotracheitis virus. Vet Res 2007;38: 261–279.
62 Paoletti E: Poxvirus recombinant vaccines. Ann NY Acad Sci 1990;590:309–325.
63 Römer-Oberdörfer A, Mundt E, Mebatsion T, Buchholz UJ, Mettenleiter TC: Generation of recombinant lentogenic Newcastle disease virus from cDNA. J Gen Virol 1999;80:2987–2995.
64 Peeters BP, de Leeuw OS, Koch G, Gielkens AL: Rescue of Newcastle disease virus from cloned cDNA: evidence that cleavability of the fusion protein is a major determinant for virulence. J Virol 1999;73:5001–5009.
65 McEwen J, Levi R, Horwitz RJ, Arnon R: Synthetic recombinant vaccine expressing influenza haemagglutinin epitope in *Salmonella* flagellin leads to partial protection in mice. Vaccine; 1992;10:405–411.
66 Taylor J, Weinberg R, Kawaoka Y, Webster RG, Paoletti E: Protective immunity against avian influenza induced by a fowlpox virus recombinant. Vaccine 1988;6:504–508.
67 Tripathy DN, Schnitzlein WM: Expression of avian influenza virus hemagglutinin by recombinant fowlpox virus. Avian Dis 1991;35:186–191.
68 Webster RG, Kawaoka Y, Taylor J, Weinberg R, Paoletti E: Efficacy of nucleoprotein and haemagglutinin antigens expressed in fowlpox virus as vaccine for influenza in chickens. Vaccine 1991;9:303–308.
69 Qiao CL, Yu KZ, Jiang YP, Jia YQ, Tian GB, Liu M, Deng DH, Wang XR, Meng QW, Tang XY: Protection of chickens against highly lethal H5N1 and H7N1 avian influenza viruses with a recombinant fowlpox virus co-expressing H5 haemagglutinin and N1 neuraminidase genes. Avian Pathol 2003;32:25–32.
70 Boyle DB, Selleck P, Heine HG: Vaccinating chickens against avian influenza with fowlpox recombinants expressing the H7 haemagglutinin. Aust Vet J 2000;78:44–48.
71 Swayne DE, Garcia M, Beck JR, Kinney N, Suarez DL: Protection against diverse highly pathogenic H5 avian influenza viruses in chickens immunized with a recombinant fowlpox vaccine containing an H5 avian influenza hemagglutinin gene insert. Vaccine 2000;18:1088–1095.
72 Bublot M, Pritchard N, Swayne DE, Selleck P, Karaca K, Suarez DL, Audonnet JC, Mickle TR: Development and use of fowlpox vectored vaccines for avian influenza. Ann NY Acad Sci 2006;1081:193–201.
73 Tripathy DN, Reed WM: Pox; in Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE (eds): Diseases of Poultry. Ames, Iowa State Press, 2003, pp 253–270.
74 Swayne DE, Beck JR, Kinney N: Failure of a recombinant fowlpox virus vaccine containing an avian influenza hemagglutinin gene to provide consistent protection against influenza in chickens preimmunized with a fowlpox vaccine. Avian Dis 2000;44:132–137.
75 Kreijtz JH, Suezer Y, van Amerongen G, de Mutsert G, Schnierle BS, Wood JM, Kuiken T, Fouchier RA, Lower J, Osterhaus AD, Sutter G, Rimmelzwaan GF: Recombinant modified vaccinia virus Ankara-based vaccine induces protective immunity in mice against infection with influenza virus H5N1. J Infect Dis 2007;195:1598–1606.
76 Swayne DE, Suarez DL, Schultz-Cherry S, Tumpey TM, King DJ, Nakaya T, Palese P, Garcia-Sastre A: Recombinant paramyxovirus type 1-avian influenza-H7 virus as a vaccine for protection of chickens against influenza and Newcastle disease. Avian Dis 2003;47:1047–1050.
77 Ge J, Deng G, Wen Z, Tian G, Wang Y, Shi J, Wang X, Li Y, Hu S, Jiang Y, Yang C, Yu K, Bu Z, Chen H: Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. J Virol 2007;81: 150–158.
78 Guy JS, Bagust TJ: Laryngotracheitis; in Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE (eds): Diseases of Poultry. Ames, Iowa State Press, 2003, pp. 121–134.
79 Fuchs W, Veits J, Mettenleiter TC: Recombinant viruses of poultry as vector vaccines against fowl plague. Berl Münch Tierärztl Wochenschr 2006;119: 160–166.

80 Lüschow D, Werner O, Mettenleiter TC, Fuchs W: Protection of chickens from lethal avian influenza A virus infection by live-virus vaccination with infectious laryngotracheitis virus recombinants expressing the hemagglutinin (H5) gene. Vaccine 2001;19: 4249–4259.

81 Veits J, Lüschow D, Kindermann K, Werner O, Teifke JP, Mettenleiter TC, Fuchs W: Deletion of the non-essential UL0 gene of infectious laryngotracheitis (ILT) virus leads to attenuation in chickens, and UL0 mutants expressing influenza virus haemagglutinin (H7) protect against ILT and fowl plague. J Gen Virol 2003;84:3343–3352.

82 Morgan RW, Gelb J Jr, Schreurs CS, Lütticken D, Rosenberger JK, Sondermeijer PJ: Protection of chickens from Newcastle and Marek's diseases with a recombinant herpesvirus of turkeys vaccine expressing the Newcastle disease virus fusion protein. Avian Dis 1992;36:858–870.

83 Tsukamoto K, Saito S, Saeki S, Sato T, Tanimura N, Isobe T, Mase M, Imada T, Yuasa N, Yamaguchi S: Complete, long-lasting protection against lethal infectious bursal disease virus challenge by a single vaccination with an avian herpesvirus vector expressing VP2 antigens. J Virol 2002;76:5637–5645.

Thomas C. Mettenleiter
Friedrich-Loeffler-Institute, Südufer 10
DE–17493 Greifswald-Insel Riems (Germany)
Tel. +49 38351 7250, Fax +49 38351 7151, E-Mail thomas.mettenleiter@fli.bund.de

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 88–100

The Role of Pigs in Interspecies Transmission

Ian H. Brown

Veterinary Laboratories Agency-Weybridge, Addlestone, Surrey, UK

Abstract

Pigs are an important host in influenza virus ecology since they are susceptible to infection with both avian and human influenza A viruses, often being involved in interspecies transmission, facilitated by regular close contact with humans or birds. This cross-species transfer of viruses to pigs can lead to co-infections with subsequent opportunities for genetic reassortment of influenza A viruses, and as a result 'new' or previously unrecognised viruses can emerge. Pigs are known to be susceptible to influenza A viruses of most haemagglutinin subtypes although only three serotypes, H1N1, H3N2 and H1N2, predominantly infect pigs worldwide. The changing epidemiology of influenza in poultry and humans has direct implications for the circulation of viruses in pigs. Significantly H9N2 is increasingly being detected in pigs in Asia together with occasional spillover of H5N1 highly pathogenic avian influenza from avian species. The apparent natural selection and persistence of viruses containing mixtures of avian, swine and human influenza virus genes demonstrates the importance of optimal gene constellations that permit efficient replication and intra-species transmission. The genesis of new influenza virus strains principally through reassortment but also by host adaptation does present theoretical opportunities for the production of pandemic strains in pigs, so their potential role in interspecies transmission remains important.

Reservoirs of Influenza A Viruses

Influenza A viruses infect a large variety of animal species including humans, pigs, horses, sea mammals and birds. Given the worldwide interaction between humans, pigs, birds and other mammalian species there is a high potential for cross-species transmission of influenza viruses in nature. Phylogenetic studies of influenza A viruses have revealed species-specific lineages of viral genes and have demonstrated that the prevalence of interspecies transmission depends on the animal species [1], the virus and its genetic characteristics. Aquatic birds are known to be the source of all influenza viruses for other species. Pigs are an important host in influenza virus ecology since they are susceptible to infection with both avian and human influenza

A viruses, often being involved in interspecies transmission, facilitated by regular close contact with humans or birds. This cross-species transfer of viruses to pigs can lead to co-infections with subsequent opportunities for genetic reassortment of influenza A viruses, and as a result 'new' or previously unrecognised viruses can emerge. Pigs are known to be susceptible to influenza A viruses of most haemagglutinin (HA) serotypes [2] although only three serotypes, H1N1, H3N2 and H1N2, predominantly infect pigs worldwide.

Following the transmission to, and independent spread of avian or human influenza A viruses in pigs, these viruses are generally referred to as 'avian-like' swine or 'human-like' swine, reflecting their previous host, and following genetic reassortment with other influenza A viruses, some of the genes of these viruses may be maintained in the resulting progeny viruses. Therefore, the evolution of influenza genes in species-specific gene lineages is an invaluable characteristic in studying influenza virus epidemiology.

Early History of Swine Influenza

Swine influenza (SI) was first observed in 1918 in the USA, Hungary and China. It coincided with an influenza pandemic in humans, which was the most severe of modern times. Those who first noticed the disease in pigs, recognised similarities between the porcine and human disease and suggested they had a common aetiology. Later retrospective serological investigations confirmed that the disease in humans and pigs had been caused by closely related influenza A viruses in both cases. The causative agent was an H1N1 influenza A virus that had most probably spread from humans to pigs following its introduction direct from an avian species [3]. Descendants of these viruses continue to persist in pigs worldwide. This sequence of events in relation to pigs is supported by observations from veterinarians who did not describe the disease in pigs until just after its appearance in humans. Subsequently, influenza virus infections have become a common and important cause of respiratory disease for pigs throughout most of the world.

Infection Course

The dynamics of virus infection and maintenance within a pig population will be influenced by a number of factors including the virus strain, its level of adaptation to pigs, herd or population immune status, age of pig, husbandry and production methods including movement of animals and climatic factors. Virus strains that are well adapted to pigs (i.e. those that may be considered endemic) will often result in extended virus shedding and spread within a population possibly persisting for many months or even years. This presents a more continuous interface with other susceptible

animal populations especially those that come in close contact with pigs presenting increased risk for virus transmission. In contrast, some viruses are poorly adapted or possess restricted capability to replicate in pigs and therefore offer reduced risk for transmission to other hosts. However the species barrier to infection is complex and will depend on the host, environment and the virus itself. Therefore it is theoretically possible that a novel virus to pigs may replicate inefficiently in swine but may still transmit to an alternative host for which the virus has enhanced replicative potential.

Emergence and Re-Emergence of Influenza Viruses in Pigs

Central to the role of pigs in the transmission of influenza virus is the diversity of mechanisms for virus emergence or re-emergence that have been proven to occur on many occasions. Emergence of new strains or modifications to existing viruses can occur by three methods. Firstly, an influenza A virus can transmit in-toto to pigs from another host species. Secondly, an influenza virus may undergo antigenic change or drift as a result of accumulating mutations with time in the genes encoding the major viral antigens (genetic drift can also occur in the genes encoding internal proteins). Finally, co-infection of a pig with two unrelated influenza A viruses can result in the production of a new virus derived by genetic mixing of the progenitor strains leading to the potential emergence of a new virus with different antigenic and genetic characteristics. If this process involves a change in the HA and/or NA serotype it is referred to as antigenic shift and is the mechanism whereby human pandemic viruses emerge.

There is increasing evidence that all three mechanisms have occurred naturally in pigs at a global level but especially where pigs are reared in abundance such as Europe, South East Asia and North America. Transmission of influenza A viruses from pigs to humans and birds has been reported widely, contributing to the pig being considered as a potential intermediate host for the reassortment of influenza A viruses, which may lead to the generation of a pandemic strain for the human population. The potential for avian and human influenza viruses to infect pigs is well established since they appear to present a lower host barrier to infection although there is apparent restriction on the range of virus subtypes that can become well established in pigs, leading to endemnicity.

Epidemiology

Influenza A viruses of subtypes H1N1, H3N2 and more recently H1N2 have been reported widely in pigs, associated frequently with clinical disease. These include classical swine H1N1, 'avian-like' H1N1 and 'human-like' H3N2 viruses together with numerous combinations of H1N2. Viruses of H1N1 and H3N2 subtypes have remained

largely endemic in major pig-producing regions with some clearly distinguishable patterns in epidemiology especially at a continental level. Infection of pig populations with well-adapted swine viruses has been responsible for one of the most prevalent respiratory diseases in pigs. Although usually regarded as an endemic disease, epizootics may result when influenza infection occurs in an immunologically naive population (which can be linked to significant antigenic drift) or through exacerbation by a variety of factors such as poor husbandry, secondary bacterial or viral infections and cold weather. Serosurveillance results in Great Britain indicated that more than half of adult pigs in the national population had been infected with one or more influenza A viruses during their lifetime, including 14% of pigs which had been infected with influenza viruses of both human and swine origin [4]. This provides substantive evidence for the risk of genetic reassortment of influenza A viruses in pigs (see Genetic Reassortment).

Classical H1N1

Following the reported occurrence of influenza in pigs at the time of the 1918 pandemic, SI was for a long time apparently confined to North America where it remained the dominant subtype until the 1990s. These and viruses related closely are termed classical viruses and have also been isolated widely from pigs in South America, Europe, and Asia [5, 6]. In addition, the virus has also been reported in wild pigs presumably as a result of spillover from domestic populations. In Europe during the latter part of the last century the virus became endemic in pigs with a seroprevalence of 20–25% [5] but following the emergence of 'avian-like' H1N1 virus it apparently disappeared. In Asia, classical H1N1 viruses are apparently the predominant influenza viruses infecting pigs [7]. In North America these viruses remained antigenically and genetically highly conserved until the 1990s when significant variability has been reported although the underlying factors affecting such variability are largely unknown [8].

'Human-Like' Viruses

The close and frequent interaction between people and pigs provides significant opportunities for infection of pigs with the prevailing human subtypes under natural conditions. It was not until the isolation of Hong Kong H3N2 virus from pigs in Taiwan in 1970 that investigations began to examine the potential transmission of human strains to pigs. Although no disease was reported among infected pigs, in the next several years H3N2 viruses were isolated regularly from pigs, and/or antibody was demonstrated in swine populations throughout the world [5, 6]. H3N2 influenza A viruses related most closely to a human strain from 1973 continued to circulate in European pig populations long after their disappearance from the human population. Subsequently, these viruses became well adapted to pigs and were associated with outbreaks of clinical influenza in pigs where infections were frequently characterised by high seroprevalence indicative of efficient intraspecies transmission. Furthermore, there is evidence for genetic and antigenic drift among the H3N2 viruses that have persistently circulated among pigs in Europe, but the antigenic drift is much slower

than that of human H3N2 viruses [9].This apparently high level of H3N2 infections in European pigs was initially in contrast to the epizootiology in pigs in North America where infection occurred infrequently as a result of repeated introduction from infected humans [10]. However in the 1990s, multiple introductions of a human H3N2 influenza virus into the pig population were key factors in the emergence of the reassortant viruses (see also Genetic Reassortment) that significantly changed the epidemiology of SI in the pig population in North America.

Human H1N1 viruses can also infect pigs, but although pig-to-pig transmission has been demonstrated under experimental conditions, most strains are not readily transmitted among pigs in the field [5]. Serological surveillance studies worldwide suggest that the prevailing human H1N1 strains are readily transmitted to pigs [4] and have resulted occasionally in the isolation of virus but are not apparently maintained in pigs independently of the human population.

'Avian-Like' Viruses

Since 1979 the dominant H1N1 viruses in European pigs have been 'avian-like' H1N1 viruses which are antigenically and genetically distinguishable from North American classical swine H1N1 influenza viruses, but related closely to H1N1 viruses isolated from ducks [11]. All of the gene segments of the prototype viruses were of avian origin [12] indicating that transmission of a whole avian virus into pigs had occurred. These 'avian-like' viruses appear to have a selective advantage over classical swine H1N1 viruses that are related antigenically, since in Europe they have replaced classical SI virus [5, 13]. Within 2 years of the introduction of 'avian-like' viruses into pigs in Great Britain, classical swine H1N1 apparently disappeared as a clinical entity. More recently an independent introduction of H1N1 virus from birds to pigs has occurred in Southern China and these viruses have been detected in pigs in South East Asia since 1993 [7] where they are currently co-circulating with classical H1N1 viruses. Phylogenetic analysis of the genes of these viruses has revealed that they form an Asian sublineage of the Eurasian avian lineage. In addition, some of the H3N2 viruses isolated from pigs in Asia since the 1970s have been entirely 'avian-like' and have been introduced apparently from ducks, although their association with epizootics of respiratory disease and persistence in pigs is unproven.

H1N2 Viruses

There have been multiple events worldwide that have led to the introduction and persistence of H1N2 viruses in pig populations. The genotypic and antigenic characteristics of these viruses are diverse and differ between continents or regions reflecting different origins. The existence of this virus subtype in pigs was recognised before reports of human infection and the former derived from co-infections with endemic H1N1 and H3N2 viruses co-circulating in pig populations. Viruses derived from classical swine H1N1 and 'human-like' swine H3N2 viruses have been isolated in Japan [14], Korea [15] and France [16]. In Asia, these viruses appear to have spread widely

within pigs and are associated frequently with respiratory epizootics [14]. Subsequently an H1N2 influenza virus (see Genetic Reassortment) related antigenically to human and 'human-like' swine viruses has emerged and become endemic in pigs in Europe [4, 17] often in association with respiratory disease. In the UK, establishment of these viruses coincided with the disappearance of human-like swine H3N2 from pigs thereby significantly impacting epidemiology at local level although this pattern has not apparently occurred elsewhere in Europe despite widespread occurrence of H1N2. In North America since 1999, H1N2 viruses have emerged and subsequently spread throughout the swine population of the USA and Canada [10, 18, 19].

Factors Affecting Host Range and Adaptation to Pigs

Successful cross-species transmission of influenza virus is dependent on both host and virus genetic factors and subsequent spread within the new host population requires a period of adaptation of the virus to the new host [1, 5]. The results of serosurveillance studies plus occasional isolations of virus have indicated that the prevailing human viruses and avian influenza viruses of various subtypes are transmitted to pigs, but fail to persist [6]. The frequent close contact between humans or birds and pigs facilitates interspecies transmission. It is not clear why generally these viruses fail to persist in pigs, but strains with different genetic and antigenic characteristics may be disadvantaged compared to the 'highly-adapted' established viruses, which continually circulate within a large susceptible population. Host range is a polygenic trait with compatibility between gene segments in a given host cell important. Host specificity is determined in part by the binding preference of the HA protein to cellular sialic acids [20]. These binding specificities vary between human and avian viruses reflecting the predominant sialic acids expressed at the primary replication sites of the host. Swine respiratory epithelium expresses substantial quantities of receptors of both specificities which probably contribute to pigs being uniquely susceptible to productive infection with avian and human influenza viruses [21, 22] [see also contribution by Matrosovich, Gambaryan and Klenk, this volume] and is a theoretical basis for pigs serving as a mixing vessel for reassortment between these viruses [see also contribution by Scholtissek, this volume].

Successful transmission between species can follow genetic reassortment, with a progeny virus containing a specific gene constellation having the ability to replicate in the new host. Reassorted viruses with other gene constellations may have a relatively low fitness, and will not be able to perpetuate in the new host [1]. In addition, single amino acid mutations can influence intraspecies transmission involving pigs especially when located in the receptor-binding domain [23]. Following interspecies transmission and/or genetic reassortment, an influenza virus may undergo many pig-to-pig transmissions because of the continual availability of susceptible pigs. The mechanisms whereby an avian or human virus is able to establish a new lineage in

pigs remain unknown, although following the introduction of an avian virus into European pigs in 1979, the mutation rate of this virus did not subsequently increase. However, wholly human lineage viruses in all gene segments are restricted in their ability to infect and spread amongst pigs compared to viruses containing genes from human, avian and swine lineages that had emerged in pigs [24, 25]. This fitness is strongly influenced by balance and compatibility between the HA and neuraminidase controlling virus attachment to and release from the cell [26].

It would appear that the adaptive processes can take many years as occurred following natural transmission of both avian H1N1 and human H3N2 viruses to pigs [5]. It is possible that, following the transmission of an avian H1N1 virus to pigs in continental Europe in 1979, subsequent infection of pigs was usually subclinical since the virus was not well adapted to its new host. It would appear that the introduction from continental Europe of an 'avian-like' swine H1N1 virus well adapted to its new host [27], into an immunologically naive pig population, such as found in Great Britain in 1992, may partly explain the rapid spread of the virus and its widespread association with disease outbreaks [28] which was consistent with the epidemiology of the virus in pigs in Europe as a whole. Furthermore, human-like viruses of H3N2 subtype appear to have increased fitness in the natural setting when they contain genes encoding internal proteins of avian origin [6].

Interspecies Transmission of Virus between Pigs and Other Species

Naturally acquired infections of pigs with avian influenza viruses have been documented from multiple areas of the world. There have been at least three independent introductions of distinct wholly avian H1N1 viruses to pigs [5, 7, 11]. In Europe, avian H1N1 viruses were transmitted to pigs in the late 1970s, established a stable lineage retaining an entirely avian genetic composition throughout their maintenance in pigs, but have undergone genetic and antigenic drift [27] and have spread from pigs to domestic turkeys [29].

Elsewhere in the world, serum antibodies against avian H4, H5 and H9 viruses have been detected in pigs in China [30], avian H1N1, H3N2 and H9N2 viruses have been isolated from Asian pigs [7, 31], and avian H4N6, H3N3 and H1N1 viruses have been recovered from pigs in Canada [32]. Viruses of H1N1 and H3N2 subtypes have been widely documented to transmit between pigs and poultry. These infections have predominantly involved turkeys that appear more susceptible to swine viruses than other species of domestic poultry [5, 6].

Influenza viruses of subtype H3N2 are ubiquitous in animals and endemic in pig populations worldwide following the first transmissions from humans in the early 1970s. There is no apparent evidence of pigs being infected with this subtype prior to the pandemic in humans in 1968. Indeed the appearance of a H3N2 subtype variant strain in the pig population of a country often coincides with the epidemic strain

infecting the human population at that time [reviewed in 5]. The prevailing strains of H3N2 virus in the human population have been frequently transmitted to pigs since the early 1970s [4] but do not usually persist independently without genetic reassortment. Transmission of human H1N1 influenza viruses to pigs has also been demonstrated and although these viruses appear unable to persist independently they are able to donate genes through genetic reassortment with other influenza viruses that are well adapted to pigs. Despite failing to establish a stable lineage in pigs, human H1N1 virus was one of the progenitor strains for the newly established H1N2 viruses.

Zoonotic infections (including fatal infection) with influenza virus from pigs have been reported in the USA, Asia, and Europe. Most of these were classical H1N1 SI infections. However, the avian-like swine H1N1 viruses, human-like H3N2 viruses (with avian internal protein genes) and swine H1N1 virus (genes encoding internal proteins of mixed origin) have also been recovered from humans [reviewed in 10]. The majority of these infections have probably resulted from close contact with pigs. Serological studies have documented increased rates of human exposure to influenza viruses from pigs among persons in direct contact with pigs [reviewed in 6]. Nevertheless, some infections have occurred without apparent animal contact, but there is little evidence for spread of swine viruses from person to person.

Genetic Reassortment in Pigs

The segmented nature of the influenza genome permits two viruses that co-infect a single host to exchange RNA segments during viral replication. The pig has been the leading contender for the role of intermediate host for reassortment of influenza A viruses. Pigs are the only mammalian species which are domesticated, reared in abundance and are susceptible to and allow productive replication of avian [12, 33] and human influenza viruses. This susceptibility is due to the presence of both α2,3- and α2,6-galactose sialic acid linkages in cells lining the pig trachea which can result in modification of the receptor-binding specificities of avian influenza viruses from α2,3 to α2,6 linkage [21], which is the native linkage in humans, thereby providing a potential link from birds to humans [see also contribution by Matrosovich, Gambaryan and Klenk, this volume]. The unique co-circulation of influenza A viruses in pigs may lead to pigs serving as a mixing vessel for reassortment between influenza viruses from mammalian and avian hosts with unknown implications for both humans and pigs [see also contribution by Scholtissek, this volume]. Many different genotypes of reassortant viruses have been isolated from pigs around the world.

Reassortant viruses have been reported in pigs for over 30 years. Initially this related to reassortment of 'human-like' swine lineage H3N2 and classical swine H1N1 viruses that were co-circulating widely in pig populations following the 1968 human pandemic. The H1N2 virus apparently persisted in Japan [14], but was more transiently detected in France [16] and continues to circulate in East Asia including China [34] and Taiwan [35].

Evidence for the pig as a mixing vessel of influenza viruses of non-swine origin was first demonstrated in Europe [24] following the detection of reassortment of human and avian viruses in Italian pigs. Phylogenetic analyses of human H3N2 viruses circulating in Italian pigs revealed that genetic reassortment had been occurring between avian and 'human-like' viruses since 1983 [24]. It would appear that human H1 viruses are able to perpetuate in pigs following genetic reassortment. Furthermore, these viruses may be maintained in pigs long after one or both of the progenitor viruses have disappeared from their natural hosts. Reassortant viruses of H1N2 subtype derived from human and avian viruses through multiple events spread widely within pigs in Great Britain following the first detection in 1994 [36] and subsequently spread to the rest of Europe [17, 37]. These viruses typically contained human-lineage HA and NA genes and internal protein genes derived from the avian-like European H1N1 swine viruses, although some H1N2 viruses from Italian pigs have derived from further reassortment events containing an avian-like swine H1 HA gene [37].

Studies of influenza viruses isolated from pigs in North America and Southern China during the early 1990s failed to detect any reassortant viruses containing internal protein gene segments of non-swine origin. However since the mid 1990s coincident with much improved surveillance systems for influenza in pigs, numerous reassortant viruses with mixtures of human and classical swine virus genes have been isolated from pigs in Asia and the USA [reviewed in 6]. In addition, H3N2 viruses with similar characteristics to those detected in European pigs since 1983 have been isolated from pigs in Asia [31]. Finally, unique 'triple reassortant' H3N2 viruses have been isolated frequently from pigs throughout the USA since 1998. These viruses contain HA, NA, and PB1 polymerase genes of human influenza virus origin, NP, M, and NS genes of classical swine H1N1 virus origin, and PB2 and PA polymerase genes of North American avian virus origin [38, 39]. These viruses have spread to many countries including Canada [40] and have continued to evolve with further reassortment with prevailing human H3N2 viruses.

Subsequent to the initial isolations of the 'triple' reassortant H3N2 viruses, influenza-like illness and abortions in pigs in the USA was associated with an H1N2 virus that had the overall genotype of the triple reassortant H3N2 viruses, but had acquired an H1 HA gene through reassortment with a classical H1N1 swine virus [6]. Similar H1N2 viruses spread subsequently throughout the swine population of the USA [18, 19] and crossed the species barrier to domestic turkeys [40, 41] but perhaps more unusually to wild waterfowl [42].

Consequent to the establishment of H3N2 triple reassortant viruses in North American pigs was genotypic variability in classical H1N1 viruses. These viruses inherited the internal protein gene constellation from the H3N2 or H1N2 viruses and have become the dominant genotype of H1N1 viruses in pigs [40] together with isolation from a human with influenza-like illness in Wisconsin who had direct contact with pigs. This event further underlines the potential for novel influenza viruses to emerge in pigs that can then subsequently transmit to other hosts including humans.

It would appear that through increased genotypic diversity in SI viruses worldwide, stable lineages of H3N1 viruses have emerged. Following the first detections in Great Britain in 1993 [6] the virus that was a reassortant of human-like swine H3N2 and classical H1N1 did not become established presumably through lack of fitness compared to the contemporary viruses. However in North America since 2004, H3N1 viruses recovered from diseased pigs have derived from the co-circulating H3N2 and H1N1 viruses with an internal gene constellation from the high fitness H3N2 viruses [43]. Further diversity includes genotypes with contemporary human H3 in Korea [44] and human N1 in the USA [45]. In addition, conventional H3N1 viruses derived from co-circulating human-like swine H3N2 and classical H1N1 have been detected more recently in pigs in Taiwan [35].

Other subtype combinations resulting from genetic reassortment are very rare, typified by an H1N7 virus that was isolated in 1992 from pigs on a single farm in the UK. This virus contained A/Equine/Prague/1/56-like NA and M genes, with the remaining genes being of human influenza virus origin, and was of low pathogenicity in pigs infected experimentally [46]. However, the virus was presumably of low relative fitness and did not spread.

Future Perspectives and Conclusions

The changing epidemiology of influenza in both poultry and humans has direct implications for the circulation of viruses in pigs. Pigs are the only mammalian species that are reared in abundance and their production results in frequent contact and interaction with both poultry and humans. Furthermore, their relatively unique susceptibility to viruses of both avian and human origin provides opportunities for genetic exchange and interspecies transmission.

The increasing complexity of SI epidemiology in pigs has led to enhanced opportunities for genetic reassortment in pigs. The frequent transmission of avian and human viruses to pigs has resulted in the emergence of multiple genotypes where pigs are reared intensively. The apparent natural selection and persistence of viruses containing mixtures of avian, swine and human influenza virus genes demonstrates the importance of optimal gene constellations that permit efficient replication and intraspecies transmission. It is noteworthy that most viruses that emerge and establish endemic infections in pigs possess H1 or H3 HA genes implying host range restriction in a natural environment. Furthermore, extensive genetic diversity presents an increased risk that some viruses will possess the necessary characteristics that enable them to cross between species and ultimately transmit within the new host population. These populations at risk include domestic poultry and humans.

During the last 10 years there have been significant changes in the epidemiology of influenza in the avian reservoir. Firstly the successful introduction and spread of H9N2 to poultry has led to global dissemination of this virus including extensive spread in

wild birds. There have been numerous reports of H9N2 in pigs in South East Asia, particularly China [31, 47, 48]. These viruses have evolved in the poultry reservoir and acquired receptor specificity for α2,6-galactose sialic acid and therefore the potential for these viruses to become widely established in pigs is a real and significant possibility. Isolations of virus from pigs over several years in China possibly indicate that the virus is already transmitting successfully within the pig population. This presents a significant reservoir of virus that in particular is a novel subtype to humans, which may possess the replicative capacity to transmit within the human population and the inherent dangers that would present. Although there have been some sporadic cases of H9N2 in humans, to date this virus has failed to transmit successfully between humans.

The second major development involves the unprecedented panzootic of H5N1 highly pathogenic avian influenza. These viruses have undergone significant host adaptation and evolution in the surface glycoproteins but also in the genes encoding the internal proteins. Experimental studies showed that the viruses replicated in the swine respiratory tract but were not transmitted to contact pigs [49]. There have been also a number of reports of natural H5N1 infections in pigs predominantly from South East Asia, but again there is no definitive evidence at the present time that these viruses are capable of efficient replication and transmission within pigs. These incidents probably relate to spillover as a result of close exposure to infected poultry and contaminated products. Nevertheless, for viruses of wholly avian origin a period of adaptation may be required before the virus can successfully infect and spread within a pig population. It is therefore vital that surveillance programs in animal populations include particular focus on pigs in order to provide greater insights and early warning of changes in the epidemiology of such viruses.

Although pigs have been implicated as a significant host for the generation of pandemic strains, there is no substantive evidence supporting the appearance of a pandemic strain in pigs prior to the emergence in the human population. However, this needs to be balanced by the lack of appropriate surveillance in animal populations prior to the emergence of the three major pandemics in the last century. The genesis of new influenza virus strains principally through reassortment but also by host adaptation does represent theoretical opportunities for the production of pandemic strains in pigs and as such their potential role in interspecies transmission remains important.

References

1 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–179.

2 Kida H, Ito T, Yasuda J, Shimizu Y, Itakura C, Shortridge KF, Kawaoka Y, Webster RG: Potential for transmission of avian influenza viruses to pigs. J Gen Virol 1994;75:2183–2188.

3 Taubenberger JK, Reid AH, Janczewski TA, Fanning TG: 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 Lond B Biol Sci 2001;356:1829–1839.

4 Brown IH, Harris PA, Alexander DJ: Serological studies of influenza viruses in pigs in Great Britain 1991–2. Epidemiol Infect 1995;114:511–520.

5 Brown IH: The epidemiology and evolution of influenza viruses in pigs. Vet Microbiol 2000;74: 29–46.
6 Olsen CW, Brown IH, Easteday BC, Van Reeth K: Swine influenza; in Straw BE, Zimmerman JJ, D'Allaire S, Taylor DJ (eds): Diseases of Swine, ed 9. London, Blackwell, 2006, chapt 28, pp 469–482.
7 Guan Y, Shortridge KF, Krauss S, Li PH, Kawaoka Y, Webster RG: Emergence of avian H1N1 influenza viruses in pigs in China. J Virol 1996;70:8041–8046.
8 Olsen CW, Carey S, Hinshaw L, Karasin AI: Virologic and serologic surveillance for human, swine and avian influenza virus infections among pigs in the north-central United States. Arch Virol 2000;145:1399–1419.
9 De Jong JC, Smith DJ, Lapedes AS, Donatelli I, Campitelli L, Barigazzi G, Van Reeth K, Jones TC, Rimmelzwaan GF, Osterhaus ADME, Fouchier RAM: Antigenic and genetic evolution of swine influenza A (H3N2) viruses in Europe. J Virol 2007; 4315–4322.
10 Olsen CW: Emergence of novel strains of swine influenza virus in North America; in Morilla A, Yoon KJ, Zimmerman JJ (eds): Trends in Emerging Viral Infections of Swine. Ames, Iowa State University Press, 2002, pp 37–43.
11 Pensaert M, Ottis K, Vandeputte J, Kaplan MM, Bachmann PA: Evidence for the natural transmission of influenza A virus from wild ducks to swine and its potential importance for man. Bull World Health Organ 1981;59:75–78.
12 Schultz U, Fitch WM, Ludwig S, Mandler J, Scholtissek C: Evolution of pig influenza virus. Virology 1991;183:61–73.
13 Campitelli L, Donatelli I, Foni E: Continued evolution of H1N1 and H3N2 influenza viruses in pigs in Italy. Virology 1997;232:310–318.
14 Ouchi A, Nerome K, Kanegae Y, Ishida M, Nerome R, Hayashi K, Hashimoto T, Kaji M, Kaji Y, Inaba Y: Large outbreak of swine influenza in Southern Japan caused by reassortant (H1N2) influenza viruses: its epizootic background and characterization of the causative viruses. J Gen Virol 1996;77: 1751–1759.
15 Jung K, Chae C: Phylogenetic analysis of an H1N2 influenza A virus isolated from a pig in Korea. Arch Virol 2004;149:1415–1422.
16 Gourreau JM, Kaiser C, Valette M, Douglas AR, Labie J, Aymard M: Isolation of two H1N2 influenza viruses from swine in France. Arch Virol 1994;135: 365–382.
17 Van Reeth K, Brown IH, Pensaert M: Isolations of H1N2 influenza A virus from pigs in Belgium. Vet Rec 2000;146:588–589.
18 Choi YK, Goyal SM, Farnham MW, Joo HS: Phylogenetic analysis of H1N2 isolates of influenza A virus from pigs in the United States. Virus Res 2002;87:173–179.
19 Karasin AI, Landgraf JG, Swenson SL, Erickson G, Goyal SM, Woodruff M, Scherba G, Anderson GA, Olsen CW: Genetic characterization of H1N2 influenza A viruses isolated from pigs throughout the United States. J Clin Microbiol 2002;40: 1073–1079.
20 Ito T: Interspecies transmission and receptor recognition of influenza A viruses. Microbiol Immunol 2000;44:423–430.
21 Ito T, Couceiro JNSS, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y: Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 1998;72:7367–7373.
22 Gambaryan AS, Karasin AI, Tuzikov AB, Chinarev AA, Pazynina GV, Bovin NV, Matrosovich MN, Olsen CW, Klimov AI: Receptor-binding properties of swine influenza viruses isolated and propagated in MDCK cells. Virus Res 2005;114:15–22.
23 Yassine HM, Al-Natour MQ, Lee CW, Saif YM: Interspecies and intraspecies transmission of triple reassortant H3N2 influenza A viruses. J Virol 2007; 28:129.
24 Castrucci MR, Donatelli I, Sidoli L, Barigazzi G, Kawaoka Y, Webster RG: Genetic reassortment between avian and human influenza viruses in Italian pigs. Virology 1993;193:503–506.
25 Landolt GA, Karasin AI, Phillips L, Olsen CW: Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. J Clin Microbiol 2003;41:1936–1941.
26 Landolt GA, Karasin AI, Schutten MM, Olsen CW: Restricted infectivity of a human-lineage H3N2 influenza A virus in pigs is hameagglutinin and neuraminidase gene dependent. J Clin Microbiol 2006;44:297–301.
27 Brown IH, Ludwig S, Olsen CW, Hannoun C, Scholtissek C, Hinshaw VS, Harris PA, McCauley JW, Strong I, Alexander DJ: Antigenic and genetic analyses of H1N1 influenza A viruses from European pigs. J Gen Virol 1997;78:553–562.
28 Brown IH, Done SH, Spencer YI, Cooley WA, Harris PA, Alexander DJ: Pathogenicity of a swine influenza H1N1 virus antigenically distinguishable from classical and European strains. Vet Rec 1993; 132:598–602.
29 Ludwig S, Haustein A, Kaleta EF, Scholtissek C: Recent influenza A (H1N1) infections of pigs and turkeys in northern Europe. Virology 1994;202: 281–286.
30 Ninomiya A, Takada A, Okazaki K, Shortridge KF, Kida H: Seroepidemiological evidence of avian H4, H5, and H9 influenza A virus transmission to pigs in southeastern China. Vet Microbiol 2002;88: 107–114.

31 Peiris JSM, Guan Y, Ghose P, Markwell D, Krauss S, Webster RG, Shortridge KF: Co-circulation of avian H9N2 and human H3N2 viruses in pigs in Southern China; in Osterhaus A, Cox N, Hampson A (eds): Options for the Control of Influenza. IV. Excerpta Medica International Congress Series. Amsterdam, Excerpta Medica, 2001, No 1219, pp 195–200.
32 Karasin AI, Brown IH, Carman S, Olsen CW: Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J Virol 2000;74:9322–9327.
33 Hinshaw VS, Webster RG, Easterday BC, Bean WJ: Replication of avian influenza A viruses in mammals. Infect Immun 1981;34:354–361.
34 Qi X, Lu CP: Genetic characterization of novel reassortant H1N2 influenza A viruses isolated from pigs in southeastern China. Arch Virol 2006;151: 2289–2299.
35 Tsai C-P, Pan M-J: New H1N2 and H3N1 influenza viruses in Taiwanese pig herds. Vet Rec 2003;153:408.
36 Brown IH, Harris PA, McCauley JW, Alexander DJ: Multiple genetic reassortment of avian and human influenza A viruses in European pigs, resulting in emergence of an H1N2 virus of novel genotype. J Gen Virol 1998;79:2947–2955.
37 Marozin S, Gregory V, Cameron K, Bennett M, Valette M, Aymard M, Foni E, Barigazzi G, Lin Y, Hay A: Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. J Gen Virol 2002;83:735–745.
38 Karasin AI, Schutten MM, Cooper LA, Smith CB, Subbarao K, Anderson GA, Carman S, Olsen CW: Genetic characterization of H3N2 influenza viruses isolated from pigs in North America, 1977–1999: evidence for wholly human and reassortant virus genotypes. Virus Res 2000;68:71–85.
39 Webby RJ, Swenson S, Krauss SL, Gerrish PJ, Goyal SM, Webster RG: Evolution of swine H3N2 influenza viruses in the United States. J Virol 2000;74: 8243–8251.
40 Olsen CW, Karasin AI, Carmen S, Li Y, Bastien N, Ojkic D, Alves D, Charbonneau G, Henning BM, Low DE, Burton L, Broukhanski G: Triple reassortant H3N2 influenza A viruses, Canada 2005. Emerg Infect Dis 2006;12:1132–1135.
41 Suarez DL, Woolcock PR, Bermudez AJ, Senne DA: Isolation from turkey breeder hens of a reassortant H1N2 influenza virus with swine, human, and avian lineage genes. Avian Dis 2002;46:111–121.
42 Olsen CW, Karasin A, Erickson G: Characterization of a swine-like reassortant H1N2 influenza virus isolated from a wild duck in the United States. Virus Res 2003;93:115–121.
43 Ma W, Gramer M, Rossow K, Yoon KJ: Isolation and genetic characterization of new reassortant H3N1 swine influenza virus from pigs in the midwestern United States. J Virol 2006;5092–5096.
44 Shin JY, Song MS, Lee EH, Lee YM, Kim SY, Kim HK, Choi JK, Kim CJ, Webby RJ, Choi YK: Isolation and characterization of novel H3N1 swine influenza viruses from pigs with respiratory diseases in Korea. J Clin Microbiol 2006;3923–3927.
45 Lekcharoensuk P, Lager KM, Vemulapalli R, Woodruff M, Vincent AL, Richt JA: Novel swine influenza virus subtype H3N1, United States. Emerg Infect Dis 2006;12:787–794.
46 Brown IH, Alexander DJ, Chakraverty P, Harris PA, Manvell RJ: Isolation of an influenza A virus of unusual subtype (H1N7) from pigs in England, and the subsequent experimental transmission from pig to pig. Vet Microbiol 1994;39:125–134.
47 Xu C, Fan W, Wei R, Zhao H: Isolation and identification of swine influenza recombinant A/swine/Shandong/1/2003 (H9N2) virus. Microbes Infect 2004;6:919–925.
48 Cong YL, Pu J, Liu QF, Wang S, Zhang GZ, Zhang XL, Fan WX, Brown EG, Liu JH: Antigenic and genetic characterization of H9N2 swine influenza viruses in China. J Gen Virol 2007;88:2035–2041.
49 Choi YK, Nguyen TD, Ozaki H, Webby RJ, Puthavathana P, Buranthai C, Chaisingh A, Auewarakul P, Hanh NTH, Ma KS, Hui PY, Guan Y, Peiris JSM, Webster RG: Studies of H5N1 influenza virus infection of pigs by using viruses isolated in Vietnam and Thailand in 2004. J Virol 2005;79: 10821–10825.

Ian H. Brown
Veterinary Laboratories Agency, Weybridge
Addlestone, Surrey KT15 3NB (UK)
Tel. +44 1 932 357 339, Fax +44 1 932 357 239, E-Mail i.h.brown@vla.defra.gsi.gov.uk

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 101–117

History of Research on Avian Influenza

Christoph Scholtissek

Linden, Germany

Abstract

This chapter on the history of avian influenza viruses starts at the beginning of the 20th century with the description of early fundamental experiments on the characterization and replication of fowl plague virus before it was recognized as an influenza A virus. Virus replication is explained using molecular approaches, and the importance of the cleavability of the hemagglutinin for starting the infection, for pathogenicity, organ tropism, and outbreaks is a central theme. The role of host factors for specific modifications of viral components for understanding species specificity is discussed as well as virus genetics leading to the concept of a segmented genome which helps to explain reassortments and creation of pandemic viruses. Studies on the ecology revealed a huge reservoir of avian influenza viruses in waterfowl in evolutionary stasis, from which from time to time an avian virus arises to pass the species barrier to mammals, explaining well the disastrous pandemic of 1918–1919. Possible outbreaks of future pandemics and how they might be dealt with are also discussed.

Introduction

Influenza has a long recorded history, dating back to 412 BC at least when Hippocrates described a typical human influenza epidemic in Greece. Avian influenza, initially called fowl plague (Hühnerpest, peste aviaire), was discovered much later. In the second half of the 19th century there were severe outbreaks of fowl plague in Northern Italy exhibiting symptoms that were distinct from those of fowl cholera, leading Centanni [1] in 1901 to characterize the causative agent as a virus. The agent was found to pass bacterial filters and could be passaged 'indefinitely' through chicken. However, it was not until 1954 that Werner Schäfer [2] demonstrated that fowl plague virus (FPV) and human influenza A viruses were not distinguishable by physicochemical and serological means.

At that time, severe health regulations concerning bench work in the USA restricted research possibilities with such highly pathogenic and contagious viruses

and, accordingly, much of the early fundamental work on what came to be known as influenza viruses was done in Europe. Inevitably, those publications were written mainly in Italian, German, or French. For those who are not familiar with these languages consultation of the excellent summary by Mahy [3] is recommended on this matter. It is a pleasure to read these early original articles to learn how at that time scientists designed, executed, and discussed their experiments. Moreover, the papers are a treasure trove for those, who like to repeat old findings using the most modern techniques and to publish them as brand new results!

Early Studies with Fowl Plague Virus

Influenza virus research can be considered to have begun with the discovery by Centanni [1] in 1901 that the causative agent of fowl plague is a virus. At the end of the 19th century, FPV appeared to have been limited to Northern Italy, but thereafter it was isolated also in Austria, and after a fowl exhibition in Braunschweig it spread throughout Germany. In infected chicken a broad spectrum of symptoms was observed resulting from hemorrhagic lesions in many organs and tissues including the brain. 'I never saw a sick chicken which recovered', wrote Centanni. Moreover, in his physicochemical studies he clearly determined the heat stability of FPV in chicken blood; the virus withstood 30 min heating at 55°C, being inactivated only at 60°C and above. Centanni also investigated the species specificity of FPV. Mammals like rabbits, dogs, foxes, guinea pigs, rats, and mice could not be infected, while among infected chickens, turkeys, ducks, geese, and guinea-fowl many died after 2–3 days. Also of three sparrows, two died within 3 days after infection, while the third one 'was brisk on the 6th day, when it unfortunately escaped'. Pigeons were the least susceptible to infection among the bird species studied. Centanni discussed even at that early stage of study the possible role of feral birds in the dissemination of the disease. Moreover, he propagated FPV in embryonated eggs, a technique which was reintroduced 30 years later by Woodruff and Goodpasteur investigating fowl pox virus. Karl Landsteiner, before he received the Nobel Prize in 1930 for his studies on blood groups, also worked for a while on FPV. In 1906 he, together with Russ [4], published an observation indicating that FPV had an extremely high affinity to chicken erythrocytes, long before Hirst developed the hemagglutination test for quantifying influenza viruses. In 1912, Landsteiner, together with Berliner [5], followed up an observation by Marchoux, propagating FPV in an in vitro system, namely in defibrinated chicken blood. They were able to passage the virus 9 times with a dilution factor of about 200 at each stage. After the 9th passage a chicken was still killed with a dilution of 10^{-7}. They showed that living cells were necessary for the propagation of the virus and that, when the blood was heated for 20 min at 58°C or freeze-thawed 7 times, the virus could not be replicated. Growth of FPV in rabbit blood was not possible. Later, in 1931, Hallauer [6] was able to propagate FPV in primary embryonic

chicken fibroblast cultures. He also pioneered 'organ tropism' in that he was able to show that the virus replicated well in cell cultures of brain, in iris epithelial and skin cells, but not in cultured heart cells, osteoblasts, or blood monocytes. Furthermore, the virus grew in cultures of embryonic cells from pigeons, geese, and ducks, but not in cultures of mouse and rat cells. Following such studies, chick embryo fibroblast cultures were used by Waterson [7] to develop a perfect plaque test for an exact quantification of FPV.

In the early 1950s, Schäfer and colleagues [8] published a series of papers on the physicochemical properties of FPV. Adopting a method developed by Hoyle for human influenza viruses, they treated purified virus concentrates with ether and obtained split products of high purity. The so-called 'gebundene (g) Antigen' was located in the interior of the virus particle and contained the genetic material, single-stranded RNA. The hemagglutinin (HA), a glycoprotein, which clumped chicken red blood cells, was located outside the particle. They recognized already that the g-antigen sedimented heterogeneously in the ultracentrifuge, though without yet concluding that the viral genome might be segmented. In contrast to what had been found with other small RNA viruses, they never succeeded in obtaining an infectious RNA from FPV. By complement fixation and cross-immunization they showed that the g-antigen of FPV and human influenza A viruses cross-reacted [2]. Because of the great resemblance of the physicochemical properties and the serological cross-reactions, Schäfer [8] finally classified FPV in the group of influenza A viruses. There was no cross-reaction between the HAs of these viruses, and Schäfer noted already the great advantage of using the isolated HA as a split vaccine.

Studies on Avian Influenza Virus Replication

Elucidation of the structure of the virion was obtained mainly using human influenza viruses (for work done on human influenza viruses, see Lamb and Krug [9]). In this chapter only research done particularly on avian influenza viruses will be cited. Ten (with many but not all strains 11) proteins are translated from viral RNA: The two glycoproteins (hemagglutinin (HA) and neuraminidase (NA)) and the matrix (M2) protein, which is a minor component and exhibits ion channel activity, are embedded into the lipid bilayer which is derived from the host cell. Inside the lipid bilayer are the matrix (M1) protein and the nucleocapsid located. The nucleocapsid consists of the viral vRNA, the three polymerase proteins PB1, PB2, and PA, and as the major component the nucleoprotein (NP), which is identical with the g-antigen. In addition, there are two so-called non-structural proteins (NS1 and NS2) which are mainly found in infected cells. So far, 16 HA and 9 NA subtypes have been discovered in viruses obtained from waterfowl [10].

An important discovery came from Mahy's group in Cambridge, England: the smallest RNA segment of FPV contained an overlapping reading frame giving rise to

two mRNAs coding for the NS1 and NS2 proteins [11]. The same was found later for the M gene by Lamb and colleagues investigating a human influenza virus. The time course of synthesis and location within cells of these various viral proteins had been studied in tissue cultures. In 1957, Breitenfeld and Schäfer [12] have shown, using fluorescent antibodies, that the NP of FPV first accumulated in the cell nucleus of infected cells and later was found also in the cytoplasm, while HA was restricted exclusively to the cytoplasm. This indicated the involvement of a nuclear phase during virus replication. In the 1960s there was a debate as to whether influenza viral RNA was synthesized by a cellular DNA-dependent RNA polymerase, as suggested by a group in Cambridge, England [13, 14], or whether the virus produced its own RNA-dependent RNA-synthesizing enzyme. The question was finally answered by the finding by Scholtissek [15] in 1969 of a RNA polymerase in FPV-infected cells, which synthesized single-stranded RNA complementary to FPV vRNA as shown by hybridization and nearest-neighbor analysis. In contrast to the cellular RNA polymerase, the FPV enzyme could not be inhibited in vitro by actinomycin D, but by polyanions like polyvinyl sulfate and dextran sulfate, compounds which were normally added to cell extracts to inhibit ubiquitous RNases. The polymerase was the first viral activity detected after infection. The enzyme activity was not found when actinomycin D was added immediately after infection. However, when the antibiotic was added 2 h post-infection, viral RNA synthesis ceased completely at 6 h after infection, in spite of the fact that viral RNA polymerase activity could be recovered at this time at almost normal yields in cell extracts [16]. Subsequently, in 1971, Skehel [17] found that this polymerase was part of the virus particle. Then the structure of the viral RNA polymerase complex and the specific functions of its subunits PB1, PB2, and PA were elucidated mainly by Krug and colleagues in the USA and by Ishihama and colleagues in Japan using human virus strains.

Influenza virus infection starts with the attachment of the virion by its HA to the carbohydrate receptor at the cell surface. Receptor binding is followed by endocytosis and membrane fusion that is also exerted by HA. Proteolytic activation of the fusion activity, the enzymes involved, and its roles in the life cycle of the virus and in pathogenicity are described in detail in the contribution by Garten and Klenk to this volume. The uptake into the endosomes was shown not to commence before the HA molecule was cleaved into the subunits HA1 and HA2 by a protease [18]. At the relatively low pH within the endosomes a drastic conformational change occurs before fusion of the viral membrane with the endosomal membrane starts, as shown by Skehel and colleagues. Cleavage activation was elucidated mainly by Klenk and Rott in Giessen, Germany. In contrast to all other influenza A viruses, some avian strains belonging to the HA subtypes H5 and H7 were released from infected cells already with a cleaved HA. These strains were extremely pathogenic for chicken with a mortality rate close to 100% [19]. After Porter et al. [20] had sequenced as the first influenza virus gene the HA gene of FPV, the group in Giessen established that all highly pathogenic strains contained at the cleavage site a connecting peptide consisting of

several basic amino acids, while non-pathogenic strains had a single arginine at that site [21]. The cleavability could also be correlated with the spread of the virus in embryonated chicken eggs [22]. The HA of viruses with a single arginine at the cleavage site were activated after release from the infected cell by a trypsin-like protease, which was available only at certain sites in the body and therefore determined also the site of replication. In contrast, viruses with a multibasic cleavage site were activated by furin, a subtilisin-like endoprotease, which is ubiquitous [23]. This explained why the latter viruses replicated in many organs of the body including the brain, and therefore were so highly pathogenic for chicken.

However, this is not the whole story, since in April 1983 a H5N2 virus was isolated from chicken in Pennsylvania, which was avirulent but had a multibasic cleavage site in its HA. In October of the same year a severe outbreak of fowl plague occurred in the same locality caused by a H5N2 virus, which differed from the earlier isolate by only a few mutations. Kawaoka et al. [24] found that a carbohydrate side chain in the vicinity of the connecting peptide had protected the cleavage site against the action of the enzyme, which was later identified as furin. The virulent strain had lost that carbohydrate side chain by mutation. Then in 1994 another outbreak in chicken farms caused by a H5N2 virus occurred in Mexico, starting again with a non-pathogenic progenitor, which gathered basic amino acids at the cleavage site within a few months [25]. Similarly, during a severe outbreak of fowl plague in a chicken farm in British Columbia, Canada, in 2004, a H7N3 virus was isolated which had an insertion of 21 nucleotides from the M gene by non-homologous recombination leading to a cleavable HA [26]. A similar insertion of a piece of ribosomal RNA had been found before under experimental conditions [27]. Furthermore, Scholtissek et al. [28] isolated reassortants of FPV which were non-pathogenic for chicken although they had retained the cleavable HA of FPV. This indicated that in addition to cleavability of the HA the gene constellation played a decisive role in pathogenicity.

In other experiments, Webster et al. [29] studied the replication of influenza viruses in ducks. They were able to isolate virus from the respiratory tract as well as from cells lining the intestinal tract of infected animals. Infectious virus was never obtained from blood, kidney, spleen, or liver. Prior replication of the virus in the lung was not necessary for intestinal infection, which suggested that the duck influenza viruses reached the intestine directly by ingestion. Human and swine influenza viruses replicated exclusively in the respiratory tract of experimentally infected ducks. The question was how the duck viruses survived at the low pH in the gizzard (about pH 3.7), at which these viruses were normally rapidly inactivated. A possible explanation might come from an observation by Scholtissek [30], who discovered that influenza viruses with a non-cleaved HA are, in contrast to the same viruses with a cleaved HA, extremely stable at low pH. Thus, a duck virus with non-cleaved HA might pass the gizzard and might become activated by trypsin in the digestive tract.

For studies on replication of avian influenza viruses in animals other than chickens, mice were most commonly used, because they could be handled easily, and

because genetically well-defined mouse strains were available. Such studies showed that intranasal inoculation of these mice resulted in the spread of the virus through the olfactory and trigeminal pathway into the brain, and the mice died. This proved that highly neurotropic influenza viruses were able to invade the central nervous system after infection via the natural route [31].

Inhibitors of Virus Replication

Viral inhibitors such as the M2 ion channel inhibitor amantadine or neuraminidase inhibitors like oseltamivir have been developed to fight human influenza, and such compounds are stockpiled as a countermeasure against a possible pandemic. Inhibitors can also be very useful to study fundamental aspects in virus replication. Thus, glucosamine and 2-deoxyglucose have been found to interfere with the glycosylation of HA and NA of FPV. The protein backbones of both molecules were still synthesized in the presence of these inhibitors, but they were metabolically unstable. Although all non-glycosylated viral proteins were produced in normal yields, no infectious particles were formed [32].

Inhibitors which interfere with host cellular activities might be even useful for therapeutic application, since virus mutations leading to drug resistance will not occur [33]. In 1962, Barry et al. [13] discovered that the replication of FPV was inhibited by actinomycin D. The underlying mechanisms became clear when Krug and colleagues found that influenza viruses are unable to synthesize cap structures for their mRNAs and therefore capture them from mRNA of the host cell. Thus, if the host mRNA synthesis is inhibited by actinomycin D the virus cannot replicate. α-Amanitin has a similar effect [34].

Influenza viral nucleoproteins (NP) can be differentiated by strain-specific phosphorylation patterns. These patterns changed dramatically during virus replication, and they were shown to be influenced by phosphokinase inhibitors such as the isoquinoline sulfonamide H7. H7 inhibited the replication of FPV [35] by specifically retaining the mRNAs of the viral glycoproteins in the cell nucleus of the infected cells [36].

When chicken embryo fibroblasts infected with FPV were treated with the methyltransferase inhibitor 3-deazaadenosine, virus replication was also inhibited by specific interference with the synthesis of the late viral proteins (HA, NA, M1). There was no effect on the production of the HA mRNA or its in vitro translation. The replication of other RNA viruses with no nuclear phase like NDV, Semliki Forrest virus, or West Nile virus, was not affected. A different methyltransferase inhibitor, 3-deazaaristeromycin, had no effect on FPV replication [37]. Later it was shown that the mRNAs of the late proteins of FPV were retained in the cell nucleus in the presence of 3-deazaadenosine [36]. These results indicated that specific modifications of influenza virus components were essential for virus replication, and that interference with these modifications abolishes viral reproduction.

Genetics of Avian Influenza Viruses

Early studies of influenza virus genetics by Burnet and Hirst and their colleagues were concerned with human influenza A viruses. Since the rate of recombination between strains was unexpectedly high, it was suggested by Hirst that the genome might be segmented. Subsequently, Tumova and Pereira [38] introduced the plaque-forming FPV into their genetic experiments. They followed the technique by Simpson and Hirst in that they inactivated FPV with UV light and reactivated the virus with non-plaque-forming human strains by mixed infection of primary chick embryo cells. All plaque formers which they obtained carried the HA of the UV-treated FPV. Now we understand this result; the human strains did not contain a cleavable HA necessary for plaque formation [19] (see above). In 1961, Barry [39] described the phenomenon of multiplicity reactivation with FPV. This again was a hint that the viral genome might be segmented. In 1964, Scholtissek and Rott [40] demonstrated stepwise inactivation of FPV. A short treatment of the virus with a RNA-destroying agent abolished its capacity to produce infectious progeny, leaving the capacity to synthesize HA, NA, and g-antigen intact. Longer treatment abolished also the capacity to produce HA but not that of NA and g-antigen. In contrast, one hit into the genome of the parainfluenza virus NDV destroyed all those capacities at once. All of these observations support the concept that influenza viruses, in contrast to NDV, have a segmented genome.

In the late 1960s and the early 1970s, many temperature-sensitive (ts) mutants were isolated and characterized in studies carried out in the USA and Australia on human influenza A viruses and in Europe on FPV. This work that was comprehensively summarized by Mahy [41] indicated that the ts mutants could be placed into maximally 8 recombination/complementation groups, suggesting that the influenza A virus genome consists of 8 genes.

With the advent of polyacrylamide gel electrophoresis, three research groups independently succeeded in 1976 in separating influenza viral RNA into eight segments. Different methods were developed to assign viral proteins to corresponding RNA segments. Using human influenza A viruses, Palese and colleagues compared apparent differences in electrophoretic mobilities of RNA segments and proteins of different strains and recombinants (reassortants) thereof. In contrast, Scholtissek et al. [42] used ts mutants of FPV of known phenotypic defects to obtain specific reassortants with non-plaquing strains, which grew at the non-permissive temperature. Non-labeled complementary RNA was extracted from cells infected with these reassortants and parent viruses, and was used for molecular hybridization with ^{32}P-labeled vRNA segments of FPV. By comparing the ribonuclease-resistant fraction of the homologous hybrids with that of the heterologous hybrid, the parental origin of any segment could be defined [43]. The third method developed by Mahy and colleagues was based on the inability of mRNA/vRNA double-stranded hybrid molecules to be translated in vitro. Thus, viral mRNA was extracted from infected cells and

hybridized with the one or the other vRNA segment prior to in vitro translation. Each vRNA segment specifically prevented the translation of a specific viral protein for which it coded [44, 45].

Molecular hybridization techniques have also been used to place influenza A viruses into two groups according to the genetic relatedness of their NS genes [46], now called A and B alleles. So far, all mammalian viruses belong to the A allele [47]. Finally, hybridization studies made it necessary to revise the nomenclature system of influenza viruses [48, 49]. For example, according to their genetic relatedness, the former neuraminidases subgroups Nav2 and Nav3 had to be placed together into subgroup N3 and were shown to be different from subgroup Nav6, now subgroup N9 [50].

Specific reassortants were obtained by double infection with a ts mutant of a known defect and another influenza A virus from the same or a different species at the non-permissive temperature long before the technique of reverse genetics was available. In most cases only the gene carrying the ts defect of the mutant was replaced, though other genes were sometimes also transferred. When a double mutant was used, two genes were regularly replaced. The pathogenicity of these reassortants for chicken and other animals has been analyzed in numerous studies [43, 51–54]. With a few exceptions, a clear correlation between loss or gain of pathogenicity and gene constellation was not obvious in these studies [53–55].

Ecology of Avian Influenza Viruses

After Schäfer's initial discovery that FPV was an influenza A virus [2], such viruses were subsequently isolated from other avian species, including domestic poultry and feral birds, or were detected by demonstration of corresponding antibodies in sera of infected birds (for a review, see Easterday [56]). A dramatic epizootic occurred in 1961 in South Africa, during which Becker [57] isolated from afflicted terns an influenza virus which was later characterized as a H5N3 strain with a highly cleavable HA. This explains the high mortality of the virus in terns, which was never seen again in a feral bird population until the outbreak of H5N1 viruses in waterfowl in Hong Kong in 2002 [58].

In most cases, infection of waterfowl with avian influenza viruses proceeds without symptoms. In 1972, Slemons et al. [59] isolated in California from over 2,000 wild ducks 41, and from domestic ducks 7 influenza viruses belonging to different subtypes. They pointed out the possibility of dissemination of influenza A viruses by feral birds over long distances, leading to new hybrid viral strains by genetic interaction. In the years from 1976 to 1983, Hinshaw and Webster and their colleagues studied mainly influenza viruses in breading areas of waterfowl in Alberta, Canada, and compared them with isolates from New York. The virus samples were obtained from apparently healthy ducks, from mallards, pintails, blue-winged teals, and other waterfowl. In Alberta the rate of infection and subtype combinations varied from year to year. In a 3-year survey,

1,262 influenza viruses, including those of HA subtypes H5 and H7, were isolated from 4,827 ducks, the highest rate of infection was up to 60%, and altogether 27 different combinations of HA and NA subtypes were found. Only 6 of 27 subtype combinations were found every year. On their flight route along the Mississippi the yield of virus isolates was marginal, only two isolates were found in 1,350 ducks in Memphis, Tenn. [60]. When isolates from ducks in Alberta were compared with those from ducks in New York in the same year, the subtype combinations were completely different and again differed from year to year. Thus, ducks using different flyways carried viruses of different subtype combinations [61]. Similar surveillance of influenza viruses in migratory birds was performed by Sinnecker et al. [62] in Eastern Germany between 1977 and 1981 with comparable results. They recognized a relatively high percentage of double infections in Pekin ducks. Shortridge et al. [63] isolated influenza viruses from avian species in Hong Kong. Such studies have been extended to the analysis of the M genes of shorebirds, gulls, and other waterfowl [64]. The latest detailed summary of avian influenza surveillance over the last 26 years is from 2004 [65]. Meanwhile the largest collection of avian influenza viruses from many different bird species has been assembled in RG Webster's Division of Virology at the St. Jude Children's Research Hospital in Memphis, Tenn., USA, from which scientists can obtain strains for their research.

Transmission of Avian Influenza Viruses to Other Species

Mammals can be infected by large doses of avian influenza viruses, but the virus is usually not transmitted within the new host. Adaptation to the new host and hence formation of a new stable lineage occurs only very rarely. Crossing of the species barrier in this way occurred in 1979 in Northern Europe, when an avian H1N1 influenza virus entered the pig population. All genes of this new swine virus were closely related to avian virus genes [66]. A phylogenetic analysis indicated that this new lineage exhibited the highest mutational rate ever seen in influenza viruses indicating rapid adaptation for growth in the new species [67]. Similarly, in 1993, an avian-like H1N1 influenza virus was isolated from pigs from a restricted locality in Southern China [68]. According to a phylogenetic analysis the 11 isolates were different from the North European swine viruses, but it is not known whether they established a new stable lineage. From December 1979 onwards there were several severe outbreaks of influenza infections in seals in North America, which were caused by avian-like influenza viruses, as shown by competitive RNA-RNA hybridization of all eight genes. They belonged to different subtype combinations, but they were distinct from avian influenza viruses in that they replicated readily in mammals but poorly in avian species, and they were not enterotropic [69, 70].

Avian-like influenza viruses were also isolated from whales in the Pacific and Atlantic Oceans [70]. The comparison of the NP genes of these viruses to those from terns or gulls in the corresponding region established that the whale virus genes

resembled those of the corresponding birds from that region, but were significantly different to each other indicating that the whales might have obtained their viruses from different seabirds [71]. An avian-like influenza virus was also isolated from a severe epizootic in mink farms in the south of Sweden. The virus had a typical avian subtype combination, H10N7 [72], and the sequence of the NP gene was closely related to avian NP sequences but different from those of mammalian strains [73].

When the human influenza viruses causing the pandemics in 1957 and 1968 were analyzed, it turned out that they were reassortants between the prevailing human virus and avian strains replacing at least the HA of the human virus (antigenic shift). Using the peptide fingerprinting technique, Laver and Webster [74] demonstrated that the HA of the Hong Kong virus (H3N2) from 1968 was closely related to that of a duck and an equine isolate. They concluded that either an avian or equine H3 virus had donated their HA gene by reassortment. Using the hybridization technique, Scholtissek et al. [75] demonstrated that the pandemic strain from 1957 had obtained the HA as well as the NA and PB1 genes from an avian source, and the 1968 Hong Kong virus had replaced again the HA gene correspondingly. The HA gene of the human Hong Kong virus was most closely related to the HA gene of the duck virus and less so to that of the equine strain analyzed by Laver and Webster. Using the most precise technique of sequencing, Kawaoka et al. [76] finally showed that the PB1 gene of the pandemic strains was not only replaced in 1957 but again in 1968.

During recent discussions on pandemic planning, the question arose whether the highly pathogenic avian H5N1 virus from Southeast Asia may contribute its HA gene by reassortment to create the next pandemic strain, as happened in 1957 and 1968. Scholtissek et al. [77] have created a system to select for well-growing reassortants between a recent human, amantadine-resistant H3N2 strain (selecting for the species-determining M gene and avian HA) and avian influenza viruses, including a recent H5 virus. In contrast to earlier human H1N1 and H2N2 viruses, with the late human H3N2 virus the reassortants obtained grew only to low titers and formed only tiny plaques. The same was found recently for a late (amantadine-resistant) H1N1 isolate (A/New Caledonia/20/99 [C. Scholtissek and R.G. Webster, unpubl. data]). A similar result was obtained using the technique of reverse genetics [78]. These results indicate that the human influenza A viruses have evolved so far away from their original source, an avian influenza virus (see below), that reassortment with a recent avian H5N1 virus to produce a human reassortant with the avian H5 HA having the capacity to start a pandemic is extremely unlikely.

The most devastating influenza pandemic in history occurred in the winter of 1918–1919 with at least 40 million deaths. The first hints that the causative H1N1 virus was of avian origin came from phylogenetic studies. The groups around Scholtissek [79] and Webster [80] have compared the sequences of the NP gene of many strains from different years, from different regions of the world, and from different species and have established phylogenetic trees. They found that the avian strains were in evolutionary stasis, while the mammalian strains were under strong selection pressure.

Table 1. Relative synonymous codon usage of the PBI gene of influenza A viruses[1]

	Threonine			
	ACU	ACC	ACG	ACA
Group 1[2]	1.07 ± 0.06	1.13 ± 0.06	0.23 ± 0.08	1.56 ± 0.07
Group 2[3]	0.61 ± 0.07	0.72 ± 0.07	0.32 ± 0.09	2.34 ± 0.10
	Glycine			
	GGU	GGC	GGG	GGA
Group 1	0.73 ± 0.08	0.51 ± 0.08	1.13 ± 0.12	1.61 ± 0.11
Group 2	0.36 ± 0.09	0.28 ± 0.09	0.78 ± 0.13	2.57 ± 0.11
	Lysine			
			AAG	AAA
Group 1			0.81 ± 0.09	1.19 ± 0.09
Group 2			1.15 ± 0.05	0.85 ± 0.05

[1]The relative synonymous codon usage has been calculated according to Zhou et al. [89].
[2]Group 1 consists of one swine H1N1 and 20 human H1N1 viruses isolated in different years at different places.
[3]Group 2 consists of 61 avian strains, selected from 420 sequences, isolated from different clades and species, from different geographic regions in different years. It also includes 13 human strains of the subtype combinations H2N2 and H3N2, which contain an avian-like PB1 gene [75, 76].

When the nucleotide substitutions were plotted against the year of isolation, the regression line of the human and swine viruses crossed the time axis shortly after 1900 indicating that at about that time an avian virus might have crossed the species barrier, possibly first to swine and from there to humans [81, 82]. The same has been shown for the NS gene [47]. Taubenberger et al. [83, 84] have reconstructed the H1N1 virus from 1918 from fragments of RNA obtained either from old tissue slices or from a body buried in 1918 in a permafrost grave at the Brevig Mission in Alaska. In a phylogenetic analysis they established that all eight genes of this virus clustered with the early human and classical swine viruses and were most closely related to avian strains. From these data they concluded that the 1918 H1N1 virus was derived in toto from an avian source. However, this conclusion can be challenged by determining the relative synonymous codon usage (RSCU) patterns of threonine, glycine, and lysine. By this procedure the influenza A viruses can be divided into at least two groups in respect to the PB1 protein. One group comprised all human H1N1 strains and classical swine viruses, the other all avian viruses and human viruses belonging to the subtypes H2N2 and H3N2 (table 1) [C. Scholtissek, unpubl. data]. Concerning the human viruses, there was almost no drift within a time scale of about 60 years. In addition, when synonymous

Table 2. Last base of the four threonine codons at selected positions of the PB1 gene of influenza viruses[1]

	Strain									
	20	21	59	123	132	141	156	183	196	223
Sw/Iowa/1976/31	A	G	U	G	U	A	G	C	U	C
Brevig M./1/18	A	G	C	G	U	A	G	U	U	C
PR/8/34	A	U	C	A	U	A	G	U	U	C
FM/1/47	A	U	C	A	C	A	G	U	U	C
FW/50	A	U	C	A	U	A		C	U	C
Beijing/11/56	A	U	C	A	U	A		C	U	C
Texas/36/91	A	U	C	A	U	A		U	U	C
N Caledonia/20/99	A	U	C	A	U	A		U	U	C
NY/222/03	A	U	C	A	U	A		U	U	C
FPV/Rostock/34	U	A	G	C	A	U	A	A	C	A
Ty/Wisconsin/66	U	A	A	C	A	U	A	A	C	A
SWB/Austr/1/73	U	A	G	C	A	U	A	A	C	A
Mal/NY/6750/78	C	A	A	C	A	U	A	G	C	A
Af-starl/Eng/983/79	U	A	A	C	A	U	A	A	C	A
L gull/NY/75/85	C	G	A	C	A	U	G	G	C	A
Ty/Eng/50-92/91	C	A	A	C	A	U	A	A	A	A
Ck/Italy/1285/00	U	A	A	C	A	U	A	A	C	A
SB/DE/12/04	C	A	A	C	A	U	A	G	C	A
Vietnam/1203/04	C	C	A	C	A	U	A	A	C	A
Singapore/1/57	U	A	G	C	A	U	A	A	C	A
Albany/1/65	U	A	G	C	A	U	A	A	C	A
HK/43/75	C	A	A	C	A	U	A	A	C	A
Miyagi/29/95	C	A	A	U	A	U	A	A	C	A
NY/191/05	C	A	A	C	A	U	A	A	C	A

[1]Of 60 threonine positions of the PB1 gene, 23 have been selected of which group 1 strains use a different codon when compared with group 2 strains (see legend of table 1) with only rare exceptions. A blank field means an amino acid other than threonine at that position. Because of space limitations, only the most heterogeneous strains were selected.

codons were analyzed along the amino acid sequence, the two groups exhibited a significantly different pattern in respect to the PB1 protein. An extract of the data for threonine is shown in table 2 [C. Scholtissek, unpubl. data]. Of the 60 threonine positions, at more than a third of these positions (shown in table 2), the group 1 viruses used a codon different from that of group 2, with only a few exceptions. There is a slight heterogeneity when avian species belonging to different clades in the phylogenetic tree were included. Three isolates from gulls exhibited an almost identical pattern, which was slightly different from that of other avian strains. One gull example is included in table 2. The same holds true for shore bird and shearwater bird isolates.

Strain												
243	291	296	301	326	366	400	417	434	435	528	570	662
C	C	U	C	C	U	U	U	U		U	C	A
C	C	U	C	C	U	U	U	U	U	U	C	A
C	C	C	C	C	U	U	U	U	U	U	C	A
C	C	U	C	C	U	U	U	U	U	U	C	A
C	C	U	C	C	U	U	U	U	U	U	C	A
C	C	U	C	C	U	U	U	U	U	U	C	A
C	C	U	C	C	U	U	U	U	U	U	C	A
C	C	U	C	C	U	U	C	U	U	U	C	A
C	C	U	C	C	U	U	C	U	U	U	C	A
A	G	A	A	A	A	C	G	C	A	A	G	U
A	U	A	A	A	A	C	A	A	A	A	G	U
A	U	A	A	A	A	A	A	C	A	A	G	U
A	U	A	A	A	A	A	G	A	A	A	A	U
A	U	A	A	A	A	A	A	C	A	A	G	U
A	U	A	A	A	A	A	A	A	G	C	A	U
A	U	A	A	A	A	A	A	C	A	A	G	U
A	U	A	A	A	G	A	A	C	A	A	G	U
A	U	A	A	A	A	A	A	A	A	A	A	U
A	U	U	A	A	A	A	A	C	A	A	A	U
A	U	A	A	A	A	A	A	A	A	A	A	U
A	U	A	A	A	A	A	A	A	A	A	A	U
A	U	A	A	A	A	A	G	A	A	A	G	U
A	U	A	A	A	A	A	G	A	A	A	G	U
A	U	U	A	A	A	A	G	A	A	A	G	U

However, there was no avian isolate which resembled the group 1 pattern, and the same was found for other amino acids like glycine, arginine, and leucine. Altogether about 100 strains were analyzed for the PB1 gene. This observation does not hold true for the other seven genes. There were significant differences between the HA or NA subtypes, but not within a subtype. The absolute extremes were found between the A and B alleles of the NS gene. Since the Brevig Mission strain from 1918, in respect to the PB1 protein, exhibited a completely different RSCU pattern when compared to avian strains, it is doubted that the PB1 gene of the pandemic H1N1 virus from 1918 is derived from an avian source, indicating that this virus is a reassortant. It is known that the codon usage influences via a secondary RNA structure the local speed of protein synthesis, and in this way the secondary structure of a protein [for further literature, see 85, 86]. Thus, it should be possible to obtain two different PB1 proteins with almost

identical amino acid sequences but with different secondary structures. One can only speculate about the source of the PB1 gene in the Brevig Mission Strain. It is possible that there was a double infection of a pig with the prevailing human H3N8 and an avian virus leading to a corresponding reassortant containing the PB1 gene as the only human virus gene, giving rise to the new H1N1 human and swine virus lineages. Interestingly it has been shown recently that in US pig farms the classical H1N1 swine influenza virus is becoming replaced by reassortants containing genes from classical swine, human, and avian viruses [87, 88]. This situation would then resemble that shortly before the disastrous pandemic of 1918–1919. Therefore, we should consider the possibility that not a H5N1 but a reassortant pig virus from North America might start the next pandemic, as happened in 1918.

Acknowledgements

I thank J. Franks from the Department of Virology, St. Jude Children's Research Hospital, Memphis, Tenn., USA, and D. Anhlan from the Institit für Molekulare Virologie, Universität Münster, Germany, for providing me with sequences of the various influenza virus strains, M.F.G. Schmidt from the Freie Universität, Berlin, for helping me with the literature, E. Naylor from Menai Bridge, Wales, for improving the English, and S. Fischbach, Marburg, for typing the manuscript.

References

1 Centanni E: Die Vogelpest. Zentbl Bakt Parasitkd Abt I 1902;Orig 31:145–152; 182–201.
2 Schäfer W: Vergleichende seroimmunologische Untersuchungen über die Viren der Influenza und der klassischen Geflügelpest. Z Naturforsch 1954;10b: 81–91.
3 Mahy BW: Fowl plague and its contribution to the virus concept; in Mahy BW, Lvov DK (eds): Concepts in Virology, from Ivanovsky to the Present. Chur, Harwood Academic Publishers, 1993, pp 15–23.
4 Landsteiner K, Russ V: Beobachtungen über das Virus der Hühnerpest. Zentbl Bakt Parasitkd Abt I 1906;38:540–542.
5 Landsteiner K, Berliner M: Über die Kultivierung des Virus der Hühnerpest. Zentbl Bakt Parasitkd Abt I 1912;67:165–168.
6 Hallauer C: Über das Verhalten von Hühnerpestvirus in der Gewebekultur. Zentbl Bakt Parasitkde Abt I 1931;113:61–74.
7 Waterson AP: Some factors affecting the formation of plaques by fowl plague virus in chick embryo cells. Arch Gesamte Virusforsch 1958;8:113–122.
8 Schäfer W, Zillig W: Über den Aufbau des Virus-Elementarteilchens der klassischen Geflügelpest. Z Naturforsch 1954;9b:779–788.
9 Lamb R, Krug R: Orthomyxoviridae: the viruses and their replication; in Knipe B, Howley PM (eds): Fields Virology. Philadelphia, Lippincott-Raven, 2001, pp 1487–1531.
10 Fouchier RA, Munster V, Wallenstein A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus ADME: Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005;79: 2814–2822.
11 Inglis SC, Barrett T, Brown CM, Almond JW: The smallest genome RNA segment of influenza virus contains two genes that may overlap. Proc Natl Acad Sci USA 1979;76:3790–3794.
12 Breitenfeld PM, Schäfer W: The formation of fowl plague virus antigens in infected cells, as studied with fluorescent antibodies. Virology 1957;4:328–345.
13 Barry RD, Ives DR, Cruickshank JG: Participation of deoxyribonucleic acid in the multiplication of influenza virus. Nature 1962;194:1139–1140.
14 Borland R, Mahy BW: Deoxyribonucleic acid-dependent ribonucleic acid polymerase activity in cells infected with influenza virus. J Virol 1968;2: 33–39.

15 Scholtissek C: Synthesis in vitro of RNA complementary to parental viral RNA by RNA polymerase induced by influenza virus. Biochim Biophys Acta 1969;179:389–397.
16 Scholtissek C, Rott R: Ribonucleic acid nucleotidyl transferase induced in chick fibroblasts after infection with an influenza virus. J Gen Virol 1969;4:125–137.
17 Skehel JJ: RNA-dependent RNA polymerase activity of the influenza virus. Virology 1971;45:793–796.
18 Scholtissek C: Influenza A viruses with noncleaved hemagglutinin are not internalized after adsorption. Arch Virol 1986;90:159–163.
19 Bosch FX, Orlich M, Klenk HD, Rott R: The structure of the hemagglutinin, a determinant for the pathogenicity of influenza viruses. Virology 1979;95: 197–207.
20 Porter AG, Barber C, Carey NH, Hallewell RA, Threlfall G, Emtage JS: Complete nucleotide sequence of an influenza virus haemagglutinin gene from cloned DNA. Nature 1979;282:471–477.
21 Bosch FX, Garten W, Klenk HD, Rott R: Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology 1981;113:725–735.
22 Rott R, Reinacher M, Orlich M, Klenk HD: Cleavability of hemagglutinin determines spread of avian influenza viruses in the chorioallantoic membrane of chicken embryo. Arch Virol 1980;65:123–133.
23 Stieneke-Groeber A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W: Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J 1992;11:2407–2414.
24 Kawaoka Y, Naeve CW, Webster RG: Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology 1984;139:303–316.
25 Horimoto T, Rivera E, Pearson J, Senne D, Krauss S, Kawaoka Y, Webster RG: Origin and molecular changes associated with emergence of a highly pathogenic H5N2 influenza virus in Mexico. Virology 1995;213:223–230.
26 Pasick J, Handel K, Robinson J, Copps J, Ridd D, Hills K, Kehler H, Cottam-Birt C, Neufeld J, Berhane Y, Czub S: Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. J Gen Virol 2005;86:727–731.
27 Khatchikian D, Orlich M, Rott R: Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. Nature 1989;340:156–157.
28 Scholtissek C, Koennecke I, Rott R: Host range recombinants of fowl plague (influenza A) virus. Virology 1978;91:79–85.
29 Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG: Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology 1978; 84:268–278.
30 Scholtissek C: Stability of infectious influenza A viruses at low pH and at elevated temperature. Vaccine 1985;3:215–218.
31 Reinacher M, Bonin J, Narayan O, Scholtissek C: Pathogenesis of neurovirulent influenza A virus infection in mice. Route of entry of virus into brain determines infection of different populations of cells. Lab Invest 1983;49:686–692.
32 Scholtissek C: Inhibition of the multiplication of enveloped viruses by glucose derivatives. Curr Top Microbiol Immunol 1975;70:101–119.
33 Scholtissek C, Muller K: Failure to obtain drug-resistant variants of influenza virus after treatment with inhibiting doses of 3-deazaadenosine and H7. Arch Virol 1991;119:111–118.
34 Rott R, Scholtissek C: Specific inhibition of influenza replication by alpha-amanitin. Nature 1970; 228:56.
35 Kistner O, Mueller K, Scholtissek C: Differential phosphorylation of the nucleoprotein of influenza A viruses. J Gen Virol 1989;70:2421–2431.
36 Vogel U, Kunerl M, Scholtissek C: Influenza A virus late mRNAs are specifically retained in the nucleus in the presence of a methyltransferase or a protein kinase inhibitor. Virology 1994;198:227–233.
37 Fischer AA, Mueller K, Scholtissek C: Specific inhibition of the synthesis of influenza virus late proteins and stimulation of early, M2, and NS2 protein synthesis by 3-deazaadenosine. Virology 1990;177: 523–531.
38 Tumova B, Pereira HG: Genetic interaction between influenza A viruses of human and animal origin. Virology 1965;27:253–261.
39 Barry RD: The multiplication of influenza virus. II. Multiplicity reactivation of ultraviolet irradiated virus. Virology 1961;14:398–405.
40 Scholtissek C, Rott R: Behavior of virus-specific activities in tissue cultures infected with myxoviruses after chemical changes of the viral ribonucleic acid. Virology 1964;22:169–176.
41 Mahy BWJ: Mutants of influenza virus; in Palese P, Kingsbury D (eds): Genetics of Influenza Viruses. Wien, Springer, 1983, pp 192–254.
42 Scholtissek C, Bowles AL: Isolation and characterization of temperature-sensitive mutants of fowl plague virus. Virology 1975;67:576–587.

43 Scholtissek C, Harms E, Rohde W, Orlich M, Rott R: Correlation between RNA fragments of fowl plague virus and their corresponding gene functions. Virology 1976;74:332–344.
44 McGeoch D, Fellner P, Newton C: Influenza virus genome consists of eight distinct RNA species. Proc Natl Acad Sci USA 1976;73:3045–3049.
45 Inglis SC, McGeoch DJ, Mahy BWJ: Polypeptides specified by the influenza virus genome. 2. Assignment of protein coding functions to individual genome segments by in vitro translation. Virology 1977;78:522–536.
46 Scholtissek C, von Hoyningen-Huene V: Genetic relatedness of the gene which codes for the nonstructural (NS) protein of different influenza A strains. Virology 1980;102:13–20.
47 Ludwig S, Schultz U, Mandler J, Fitch WM, Scholtissek C: Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. Virology 1991;183:566–577.
48 WHO: Reconsideration of influenza A virus nomenclature: a WHO memorandum. Bull World Health Organ 1979;57:227–233.
49 WHO: A revision of the system of nomenclature for influenza viruses: a WHO memorandum. Bull World Health Organ 1980;58:585–591.
50 Scholtissek C, Rott R, Lvov DK, Myasnikova IA: Genetic relatedness of the neuramindase of influenza A strains Nav2, Nav3, and Nav6. Arch Virol 1980;65: 325–328.
51 Scholtissek C, Rott R, Orlich M, Harms E, Rohde W: Correlation of pathogenicity and gene constellation of an influenza A virus (fowl plague). I. Exchange of a single gene. Virology 1977;81:74–80.
52 Scholtissek C, Vallbracht A, Flehmig B, Rott R: Correlation of pathogenicity and gene constellation of influenza A viruses. II. Highly neurovirulent recombinants derived from non-neurovirulent or weakly neurovirulent parent virus strains. Virology 1979;95:492–500.
53 Rott R, Orlich M, Scholtissek C: Correlation of pathogenicity and gene constellation of influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. J Gen Virol 1979;44:471–477.
54 Bonin J, Scholtissek C: Mouse neurotropic recombinants of influenza A viruses. Arch Virol 1983;75: 255–268.
55 Hinshaw VS, Webster RG, Naeve CW, Murphy BR: Altered tissue tropism of human-avian reassortant influenza viruses. Virology 1983;128:260–263.
56 Easterday B: Animal influenza; in Kilbourne E (eds): The Influenza Virus and Influenza. New York, Academic Press, 1975, pp 449–481.
57 Becker WB: The isolation and classification of tern virus: influenza A/Tern/South Africa/1961. J Hyg (Lond) 1966;64:309–320.
58 Sturm-Ramirez KM, Ellis T, Bousfield B, Bissett L, Dyrting K, Regh JE, Poon L, Guan Y, Peiris M, Webster RG: Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. J Virol 2004;78:4892–4901.
59 Slemons RD, Johnson DC, Osborn JS, Hayes F: Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Dis 1974;18:119–124.
60 Hinshaw VS, Webster RG, Turner B: The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. Can J Microbiol 1980;26: 622–629.
61 Hinshaw VS, Wood JM, Webster RG, Deibel R, Turner B: Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America. Bull World Health Organ 1985;63:711–719.
62 Sinnecker R, Sinnecker H, Zilske E, Kohler D: Surveillance of pelagic birds for influenza A viruses. Acta Virol 1983;27:75–79.
63 Shortridge KF, Butterfield WK, Webster RG, Campbell CH: Isolation and characterization of influenza A viruses from avian species in Hong Kong. Bull World Health Organ 1977;55:15–20.
64 Widjaja L, Krauss SL, Webby RJ, Xie T, Webster RG: Matrix gene of influenza a viruses isolated from wild aquatic birds: ecology and emergence of influenza a viruses. J Virol 2004;78:8771–8779.
65 Krauss S, Walker D, Pryor SP, Niles L, Chenghong L, Hinshaw VS, Webster RG: Influenza A viruses of migrating wild aquatic birds in North America. Vector Borne Zoonotic Dis 2004;4:177–189.
66 Schultz U, Fitch WM, Ludwig S, Mandler J, Scholtissek C: Evolution of pig influenza viruses. Virology 1991;183:61–73.
67 Ludwig S, Stitz L, Planz O, Van H, Fitch WM, Scholtissek C: European swine virus as a possible source for the next influenza pandemic? Virology 1995;212:555–561.
68 Guan Y, Shortridge KF, Krauss S, Li PH, Kawaoka Y, Webster RG: Emergence of avian H1N1 influenza viruses in pigs in China. J Virol 1996;70:8041–8046.
69 Webster RG, Hinshaw VS, Bean WJ, Van Wyke KL, Geraci JR, St Aubin DJ, Petursson G: Characterization of an influenza A virus from seals. Virology 1981; 113:712–724.
70 Hinshaw VS: Influenza in other species (seal, whale and mink); in Nicholson K, Webster RG, Hay A (eds): Textbook of Influenza. Oxford, Blackwell Science, 1998, pp 163–167.

71 Mandler J, Gorman OT, Ludwig S, Schroeder E, Fitch WM, Webster RG, Scholtissek C: Derivation of the nucleoproteins (NP) of influenza A viruses isolated from marine mammals. Virology 1990;176: 255–261.
72 Klingeborn B, Englund L, Rott R, Juntti N, Rockborn G: An avian influenza A virus killing a mammalian species – the mink. Arch Virol 1985;86: 347–351.
73 Reinhardt U, Scholtissek C: Comparison of the nucleoprotein genes of a chicken and a mink influenza A H10 virus. Arch Virol 1988;103:139–145.
74 Laver WG, Webster RG: Studies on the origin of pandemic influenza. 3. Evidence implicating duck and equine influenza viruses as possible progenitors of the Hong Kong strain of human influenza. Virology 1973;51:383–391.
75 Scholtissek C, Rohde W, Von Hoyningen V, Rott R: On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology 1978;87:13–20.
76 Kawaoka Y, Krauss S, Webster RG: Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol 1989;63: 4603–4608.
77 Scholtissek C, Stech J, Krauss S, Webster RG: Cooperation between the hemagglutinin of avian viruses and the matrix protein of human influenza A viruses. J Virol 2002;76:1781–1786.
78 Maines TR, Chen LM, Matsuoka Y, Chen H, Rowe T, Ortin J, Falcon A, Hien NT, Mai LQ, Sedyaningsih ER, Harun S, Tumpey TM, Donis RO, Cox NJ, Subbarao K, Katz JM: Lack of transmission of H5N1 avian-human reassortant influenza viruses in a ferret model. Proc Natl Acad Sci USA 2006;103: 12121–12126.
79 Gammelin M, Altmueller A, Reinhardt U, Mandler J, Harley VR, Hudson PJ, Fitch WM, Scholtissek C: Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th-century avian ancestor. Mol Biol Evol 1990;7: 194–200.
80 Gorman OT, Bean WJ, Kawaoka Y, Webster RG: Evolution of the nucleoprotein gene of influenza A virus. J Virol 1990;64:1487–1497.
81 Gorman OT, Bean WJ, Kawaoka Y, Donatelli I, Guo Y, Webster RG: Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. J Virol 1991;65:3704–3714.
82 Scholtissek C, Ludwig S, Fitch WM: Analysis of influenza A virus nucleoproteins for the assessment of molecular genetic mechanisms leading to new phylogenetic virus lineages. Arch Virol 1993;131: 237–250.
83 Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG: Characterization of the 1918 influenza virus polymerase genes. Nature 2005;437: 889–893.
84 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A: Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 2005;310:77–80.
85 Oresic M, Shalloway D: Specific correlations between relative synonymous codon usage and protein secondary structure. J Mol Biol 1998;281: 31–48.
86 Komar AA, Lesnik T, Reiss C: Synonymous codon substitutions affect ribosome traffic and protein folding during in vitro translation. FEBS Lett 1999;462:387–391.
87 Zhou NN, Senne DA, Landgraf JS, Swenson SL, Erickson G, Rossow K, Liu L, Yoon KJ, Krauss S, Webster RG: Genetic reassortment of avian, swine, and human influenza A viruses in American pigs. J Virol 1999;73:8851–8856.
88 Webby RJ, Swenson SL, Krauss SL, Gerrish PJ, Goyal SM, Webster RG: Evolution of swine H3N2 influenza viruses in the United States. J Virol 2000; 74:8243–8251.
89 Zhou T, Gu W, Ma J, Sun X, Lu Z: Analysis of synonymous codon usage in H5N1 virus and other influenza A viruses. Biosystems 2005;81:77–86.

Dr. Christoph Scholtissek
Waldstrasse 53
DE–35440 Linden (Germany)
Tel. +49 6403 61246, Fax +49 6403 68824

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 118–133

Reverse Genetics of Influenza Viruses – Applications in Research and Vaccine Design

Gabriele Neumann[a] · Taisuke Horimoto[b] · Yoshihiro Kawaoka[a–c]

[a]Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisc., USA; [b]Division of Virology, Department of Microbiology and Immunology, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, Tokyo, and [c]CREST, Japan Science and Technology Agency, Saitama, Japan

Abstract

The artificial generation of influenza viruses from cloned cDNA was long considered an insurmountable obstacle. This changed in 1999 with the establishment of plasmid-based reverse genetics for influenza virus generation. Reverse genetics has now become a fundamental part of influenza virus research and the generation of influenza vaccines. Here, we describe the state of the art in influenza virus reverse genetics and discuss several major advancements in influenza virus research that would not have been possible without the ability to generate and modify influenza viruses. In addition, we describe the significance of reverse genetics for the generation and improvement of seasonal and pandemic influenza vaccines, and for the exploitation of influenza virus as a (vaccine) vector.

Eight years after its first description, the artificial generation of influenza viruses (referred to as 'reverse genetics') has become a standard tool in influenza virus research. This technology is based on the intracellular transcription of wild-type or mutant viral RNAs by a cellular enzyme, RNA polymerase I (fig. 1). This approach has proven to be extremely efficient, robust, and versatile, and has resulted in significant advances in both basic research and vaccine development. This chapter summarizes the influenza virus reverse genetics systems and landmark achievements based on reverse genetics technologies.

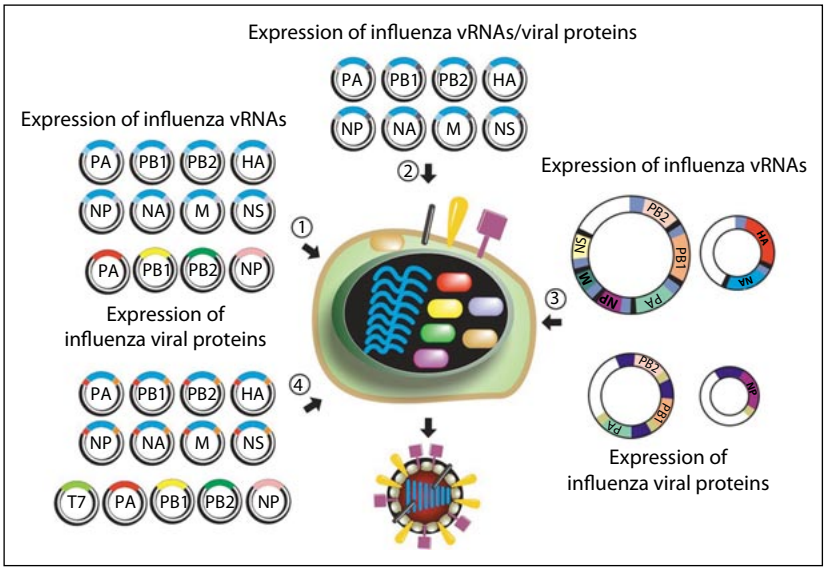


Fig. 1. Reverse genetics systems for the generation of influenza viruses from cloned cDNAs: (1) Transfection of cells with eight plasmids for the RNA polymerase I-dependent transcription of all eight influenza vRNAs, and four plasmids for the RNA polymerase II-dependent expression of PB2, PB1, PA, and NP proteins. In a modified approach (2), influenza viral cDNAs are flanked by RNA polymerase I terminator and promoter units. This cassette is then inserted between RNA polymerase II promoter and polyadenylation sequences, hence yielding both vRNA and mRNA from a single template. This approach thus eliminates the need for separate protein expression plasmids. (3) Further reduction in the number of plasmids required for influenza virus generation has been achieved by combining RNA polymerase I or II transcription units for vRNA or protein synthesis, respectively, on one plasmid. (4) Recently, influenza virus has also been generated from a T7 RNA polymerase-dependent transcription system, in which viral cDNAs are flanked by T7 RNA polymerase promoter and ribozyme sequences. T7 RNA polymerase was provided from an additional protein expression plasmid.

Influenza Virus

Influenza viruses cause a highly contagious respiratory disease [reviewed in 1]. Influenza A viruses infect humans and a variety of animal species. Outbreaks in poultry, pigs, or horses can have substantial economic impact. Wild waterfowl, in which influenza A viruses are typically asymptomatic, are the natural reservoir for viruses of all 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes.

Two surface glycoproteins (HA and NA) protrude from influenza virions [reviewed in 2]. Inside the virions are the membrane-associated matrix (M1) protein and eight viral ribonucleoprotein (vRNP) complexes, each composed of one of the eight single-stranded, negative-sense viral RNAs (vRNAs), the nucleoprotein NP, and the PB2, PB1, and PA polymerase proteins. In addition, influenza A viruses encode an interferon antagonist (NS1), a nuclear export protein (NEP, formerly called NS2),

an ion channel protein (M2) that executes functions early and late in infection (the latter for viruses with intracellularly cleaved HA), and the recently described PB1-F2 protein that has pro-apoptotic function.

Generating Influenza Virus – The Challenge

The artificial generation of influenza viruses presented several technical obstacles. First, influenza viruses contain segmented genomes of eight (for influenza A and B viruses) or seven (for influenza C virus) vRNA segments. The artificial generation of influenza viruses therefore requires that all segments be provided. Second, unlike most other RNA viruses, influenza viruses replicate in the nucleus of infected cells, requiring that artificially generated segments are synthesized in or delivered to the nucleus. Third, the influenza viral genome is of negative polarity, i.e., the vRNAs do not have messenger RNA quality. As a consequence, 'naked' influenza vRNAs are not infectious and viral replication is not initiated until the viral RNAs are replicated by the viral polymerase complex (i.e., the PB2, PB1, and PA proteins) and the nucleoprotein NP. These four proteins must therefore be co-produced with the eight influenza viral RNAs. Fourth, influenza vRNAs do not contain 5′-cap structures or 3′-polyA tails, hence demanding a system for the synthesis of transcripts with predetermined 5′- and 3′-ends that are not further modified by cellular enzymes. It was this combination of requirements that for many years posed a roadblock to the artificial generation of influenza viruses. In 1989, Palese and colleagues [3] established a system that allowed the introduction of a single, artificially generated vRNA into the viral genome. This system, however, did not allow the de novo synthesis of influenza viruses and suffered from low efficiencies and the need for helper viruses and selection systems.

Generating Influenza Virus – The Solution

The solution to the artificial generation of influenza viruses proved to be as simple as it was ingenious, and presented itself in the form of a cellular enzyme, RNA polymerase I. RNA polymerase I localizes to the nucleus where it transcribes ribosomal RNA that is neither capped nor polyadenylated. Early studies [4, 5] had shown RNA polymerase I transcription of 'foreign' cDNA that was inserted between RNA polymerase I promoter and terminator sequences. These studies also demonstrated the generation by RNA polymerase I of authentic influenza viral 5′- and 3′-ends [4, 5]. However, researchers were not able to take advantage of the full potential of the RNA polymerase I system until 1999, when the artificial generation of an influenza A virus was first documented [6]. Briefly, individual cDNAs encoding the eight viral RNA segments of an influenza A virus were inserted between RNA polymerase I promoter

and terminator sequences. The resulting eight plasmids were transfected into eukaryotic cells, resulting in the transcription of authentic influenza vRNAs in the nucleus of transfected cells (fig. 1). The polymerase and NP proteins needed to initiate the viral life cycle were provided from standard protein expression plasmids, bringing the number of plasmids required for the generation of influenza viruses to twelve. Although the transfection of cells with twelve different plasmids was thought to be impossible, this approach proved to be extremely efficient, resulting in the generation of $>10^7$ infectious viruses/ml of supernatant derived from transfected cells. The high efficiency of the RNA polymerase I system has proven to be a major asset since it allows the generation of highly attenuated influenza viruses.

Reverse Genetics of Influenza Viruses – State of the Art

Since the initial report, several modifications of the original approach have been described that include: (i) the use of a ribozyme instead of the RNA polymerase I terminator for the generation of 3′-ends of vRNAs [7]; (ii) the transcription of both negative-sense vRNA (required for replication) and positive-sense mRNA (required for protein synthesis) from one plasmid (fig. 1), thereby eliminating the need for separate protein expression plasmids encoding the polymerase and NP proteins [8] and reducing the number of plasmids required for influenza virus generation from twelve to eight; (iii) the combination of several RNA polymerase I transcription units (for vRNA synthesis), or several RNA polymerase II transcription units (for mRNA synthesis) on one plasmid (fig. 1), resulting in efficient virus generation from only three plasmids (encoding the eight vRNAs, the three polymerase proteins, and the NP protein) [9]. Although somewhat surprising, this approach also allowed efficient influenza virus generation from only one plasmid encoding all eight vRNAs [9].

RNA polymerase I systems for influenza virus generation are now available for an ever-increasing number of human, avian, swine, equine, and canine influenza virus isolates, covering all major groups of influenza A viruses. Moreover, reverse genetics systems have been established for influenza B [10, 11] and C [12, 13] viruses. The versatility of the RNA polymerase I system is further underscored by its use for the generation of lymphocytic choriomenengitis virus (LCMV) [14], an arenavirus that replicates in the cytoplasm of infected cells.

Recently, influenza A virus rescue has been also achieved with the use of T7 RNA polymerase [15] (fig. 1), an approach widely used for the generation of negative-sense RNA viruses that replicate in the cytoplasm of infected cells. For influenza A virus rescue, viral cDNAs were flanked by a T7 RNA polymerase promoter sequence, and ribozyme and T7 RNA polymerase terminator sequences. T7 RNA polymerase was provided from a protein expression plasmid. The comparison of T7 RNA polymerase variants that localize to the cytoplasm or nucleus revealed higher efficiencies of influenza vRNA synthesis for the latter version.

The ability to generate and modify influenza viruses has resulted in a tremendous number of studies that address the function of influenza viral proteins in the viral life cycle, the determinants of influenza virus host-range restriction, or the determinants of influenza virus pathogenicity. A number of studies that have had major impact on our understanding of influenza viruses as they infect cells and cause disease are described below.

Understanding the Pathogenicity of Highly Pathogenic Avian H5N1 Influenza Viruses
Since 1996, highly pathogenic avian influenza viruses of the H5N1 subtype have caused devastating outbreaks in poultry populations in Southeast Asia, posing enormous economic burdens on affected countries. The real threat to mankind, however, stems from the ability of these avian influenza viruses to transmit to humans [16–18] and cause respiratory disease with a greater than 50% case fatality rate.

Initial studies with viruses isolated from infected individuals in Hong Kong in 1997 demonstrated that these viruses fell into two groups of high and low pathogenicity in mice [19, 20]. Reverse genetics allowed the reconstruction of representative viruses of high or low pathogenicity [A/Hong Kong/483/97 (HK483), and A/Hong Kong/486/97 (HK486), respectively]; the reciprocal exchange of individual gene segments between these two viruses demonstrated a critical role for the PB2 gene in viral pathogenicity in mice [21]. Further analysis, based on amino acid differences between the HK483 and HK486 PB2 proteins, identified a single amino acid that determined H5N1 virus pathogenicity in mice: Lys at position 627 of PB2 rendered H5N1 viruses highly pathogenic, while Glu at this position resulted in viruses of low pathogenicity [21]. The nature of PB2-627 has since been widely recognized as a major determinant of H5N1 virus pathogenicity in mammalian species, and may also determine the pathogenicity of H7N7 influenza viruses [22, 23]. Together with several studies which indicated that PB2-627Lys confers a growth advantage in mammalian cells and efficient RNA transcription at the lower temperatures found in the upper respiratory tract [24–26], PB2-627 is now considered an important determinant of influenza virus pathogenicity in mammals. Collectively, these advances demonstrate the significant capacity of reverse genetics technology.

Other reverse genetics studies further suggested a role of the viral polymerase complex in pathogenicity. For example, the polymerase genes were shown to be responsible for pathogenicity of a human H5N1 virus in animal models [27]. Another study identified PB2-701 as a determinant of H5N1 virus pathogenicity: an Asp-to-Asn change enabled a duck H5N1 virus to kill mice [28], while the reverse change attenuated a highly pathogenic H5N1 virus in mice [28]. Hence, the polymerase proteins may be critical determinants of viral pathogenicity, an assumption that is further supported by the finding that the polymerase complex of an H7N7 influenza virus determines its pathogenicity [29].

Reverse genetics studies also identified additional determinants of H5N1 virus pathogenicity. Among those is glutamic acid at position 92 of the NS1 protein [30]. NS1 is an interferon antagonist [reviewed in 31, 32] that plays a critical role in inhibiting antiviral host cell responses. Glutamic acid at position 92 of NS1, but not aspartic acid as found for non-H5N1 viruses, confers resistance to the antiviral effects of interferon and tumor necrosis factor-α and is critical for virus pathogenicity in pigs [30]. This finding suggests that highly pathogenic influenza viruses may be better interferon antagonists than influenza viruses of low pathogenicity.

Highly Pathogenic Avian H5N1 Influenza Viruses – Why Do They Not Spread Among Humans?

Highly pathogenic avian H5N1 influenza viruses have not yet caused a human pandemic, because of their limited ability to spread among humans. Influenza virus host range restriction partially results from a mismatch between the receptor specificity of influenza viruses and the receptor distribution on host cells. Epithelial cells in the avian intestine (the major replication site of avian influenza viruses) contain on their surface sialic acid that is linked to galactose by α2,3-linkages (SAα2,3Gal); consequently, avian influenza viruses have evolved to preferentially recognize this kind of sialyloligosaccharide. By contrast, human influenza viruses bind more efficiently to sialyloligosaccharides with α2,6-linkages (SAα2,6Gal), which were shown to be expressed by epithelial cells in the human trachea [33].

Studies with in vitro differentiated human epithelial cells from tracheal/bronchial tissues, however, suggested a more complex pattern [34]: nonciliated epithelial cells (which account for a major population of epithelial cells) express SAα2,6Gal sialyloligosaccharides, whereas ciliated cells (which account for a minor population of epithelial cells) contain SAα2,3Gal sialyloligosaccharides. These findings are consistent with the infection of nonciliated cells by human viruses, but ciliated cells by avian influenza viruses [34]. Recent in vitro [34] and in vivo [35, 36] studies also revealed the presence of SAα2,3Gal (i.e., avian-type receptors) on alveolar cells in the human lung, and the infection of these cells by avian H5N1 viruses [36], which may explain human infections with avian influenza viruses. Another recent study [37] confirmed the presence of avian-type receptors on alveolar pneumocytes. Despite these somewhat controversial findings, which may be explained by differences among the experimental systems, most researchers assume that efficient influenza virus spread among humans will eventually require the ability to recognize human-type receptors.

Influenza virus receptor recognition is mediated by the HA protein and several key amino acids have been identified that mediate binding to SAα2,3Gal or SAα2,6Gal, respectively. Reverse genetics studies of recent H5N1 viruses identified two different amino acid changes in the HA protein that each converted the receptor-binding specificity from avian- to human-type [38]. Moreover, the combination of reverse genetics technologies and X-ray crystallographic analysis provided a powerful tool to

understand how single amino acid changes affect the receptor-binding specificity and thus pathogenicity of an influenza virus [38].

Deciphering the Deadly Secret of the Pandemic 1918 Influenza Virus

The most devastating influenza virus outbreak occurred in 1918/1919 when the so-called 'Spanish influenza' killed an estimated 40–50 million people worldwide. At the time, not even the infectious agent could be isolated, leaving future generations of researchers with seemingly no means to unlock the deadly secret of the virus. However, Taubenberger and colleagues [39–44] isolated influenza viral RNA from formalin-fixed, paraffin-embedded tissues of two 'Spanish influenza' victims, as well as from an Inuit woman who succumbed to the infection and whose body had been preserved in the permafrost of Alaska. RT-PCR amplification of the isolated RNA led to the re-creation of all eight viral RNA segments [39–44], and reverse genetics allowed the testing of 1918 virus genes in the background of heterologous viruses [39–47]. Two studies suggested a contribution of both the 1918 HA and NA genes to pathogenicity [45, 47], while one study demonstrated that the 1918 HA gene alone significantly increased the pathogenicity of recombinant viruses possessing this gene [46]. By contrast, the 1918 NS gene (which encodes the interferon antagonist NS1) did not significantly increase viral pathogenicity [39], which may, however, be explained by the heterologous genetic background it was tested in.

The effort to resurrect the 1918 'Spanish influenza' virus finally culminated in the re-creation of the authentic pandemic virus in 2005 [48]. Studies with the re-created 1918 virus established its pathogenicity in mice [48, 49] and non-human primates [50], and suggested that aberrant immune responses including an increased activation of host immune response genes may have accounted for the severe disease symptoms associated with 1918 virus infection [49, 50]. However, the exact mechanisms with which the 1918 'Spanish influenza' virus killed more humans than any other virus (including HIV) currently remain unknown.

The 1918 pandemic virus differed from highly pathogenic avian H5N1 influenza viruses by its ability to efficiently spread among humans. Among the few 1918 virus isolates recovered so far, at least two HA populations existed that preferentially bound SAα2,6Gal, or both SAα2,3Gal or SAα2,6Gal [51]. This finding again suggests that a shift from preferential recognition of SAα2,3Gal to SAα2,6Gal sialyloligosaccharides is a critical step in the generation of pandemic influenza viruses. A recent reverse genetics study demonstrated that only two amino acid changes in the HA protein, which affect the ability to bind to human-type receptors, abrogated 1918 virus transmissibility in ferrets [52].

The PB1-F2 Protein – A Long-Overlooked Determinant of Pathogenicity?

In addition to the PB1 protein of 757 amino acids, the PB1 segment encodes a second protein from the +1 reading frame, termed PB1-F2 [53]. This protein of 87–90 amino acids (depending on the virus strain) is encoded by most influenza viruses but

absent in some animal, particularly swine, virus isolates. Human H1N1 viruses encode a truncated version. PB1-F2 localizes to mitochondria [53, 54] and treatment of cells with a synthetic PB1-F2 peptide induced apoptosis [53]. Further studies demonstrated that PB1-F2 interacts with mitochondrial proteins that play a role in permeability changes in the mitochondrial membrane during apoptosis [55]. Reverse genetics allowed the generation of influenza viruses with a stop codon in the N-terminal region of PB1-F2 protein; these viruses induced less extensive apoptosis [53] and were attenuated in mice [56]. Collectively, the currently available data suggest PB1-F2 as a pro-apoptotic virulence factor.

How Do They Get In? – The Packaging of the Influenza Virus Segments

A long-standing question in the field was the mechanism by which the eight vRNA segments are assembled into budding virions. Two models had been discussed that assumed random incorporation of vRNAs into budding virions (consistent with the low ratio of infectious viruses to the total number of virions formed [57]), or a selective incorporation mechanism (consistent with data obtained with defective interfering particles that suggested segment-specific competition [58, 59]). The ability to generate virus-like particles (VLPs) that contain subsets of vRNAs demonstrated most efficient virion formation with all eight vRNAs, although particles with seven or six segments can be generated [60]. Virion incorporation studies with vRNAs expressing reporter proteins allowed the identification of segment-specific virion incorporation signals in the NA segment [60], and thereafter also in all other viral segments [61–64, and unpubl. data]. The incorporation signals of all eight vRNAs have in common that they are not limited to the noncoding regions but extend into the coding region, and that sequences at both ends of the vRNAs contribute to virion incorporation.

Reverse Genetics of Influenza Viruses – Applications in Vaccine Design

Vaccination is considered the most effective preventative measure to control influenza virus outbreaks. Below, we summarize the influenza vaccines that are currently in use and how reverse genetics technologies could contribute to efficient vaccine production and/or the development of improved vaccines. Moreover, we discuss the role of reverse genetics in the development of vaccines to pandemic influenza viruses.

Influenza Vaccines – State of the Art

Both inactivated and live attenuated vaccines have been developed for influenza viruses. The inactivated vaccine is typically generated by classical reassortment to combine the HA and NA genes of the circulating virus (or an antigenically related virus) with the remaining genes of a virus that grows efficiently in eggs (such as A/Puerto Rico/8/34;

PR8). The resulting reassortant, often referred to as '6 + 2 reassortant', is grown in embryonated chicken eggs, purified, and treated with formaldehyde or β-propiolactone for whole-virus formulations. For split or subunit formulations, purified viruses are treated with ether or detergents. Both formulations are delivered by intramuscular or subcutaneous injection.

Live attenuated vaccines elicit both humoral and cellular immune responses and may offer a wider protection against antigenic drift variants. A live attenuated vaccine (FluMist™ [reviewed in 65]) is now licensed in the USA. This vaccine was developed by serial passage of A/Ann Arbor/6/60 and B/Ann Arbor/1/66 viruses at low temperatures, resulting in type A and B virus master strains that are cold-adapted *(ca)*, temperature-sensitive *(ts)*, and attenuated *(att)*. These master strains contain a number of mutations in the six 'internal genes', but it was not until the era of reverse genetics that the amino acid changes that confer the *ca*, *ts*, and *att* phenotypes could be mapped [66–69]. For annual vaccine production, reassortants are created that contain the six 'internal' genes of the type A or B virus master strain in combination with the HA and NA genes of the circulating virus (or an antigenically related virus). The live attenuated vaccine is administered intranasally in the form of a spray.

Current Influenza Vaccines – The Need for Improvements

The production of the currently licensed vaccines presents several challenges. First and foremost, the selection of the desired reassortant vaccine virus from the background of reassortant viruses with unwanted properties (note that classical reassortment, i.e., the infection of cells with two different viruses, results in 256 different gene combinations from which the desired vaccine virus has to be isolated) can be cumbersome. Reverse genetics is the ideal tool to resolve this difficulty since it allows influenza virus generation from a predetermined set of plasmids. Hence, only one genotype is generated – the desired vaccine virus. In fact, reverse genetics has now been approved for the generation of FluMist™ [http://www.flu.org.cn/en/news-11930.html].

FluMist™ is currently approved for the 3- to 49-year age group, preventing its use for the two age groups that are at highest risk from influenza virus infections, i.e., young children and the elderly. Reverse genetics could be used to further attenuate the vaccine virus, thereby alleviating symptoms associated with its use and possibly expediting approval for the high-risk groups. Such attenuating mutations could, for example, include C-terminal deletions of the NS1 protein [70, 71] or deletions in the ion channel M2 protein [72].

Development of Pandemic Vaccines

Current vaccines are directed against human influenza A viruses of the H1N1 and H3N2 subtypes, and human influenza B viruses. Over the past decade, however, avian influenza A viruses of the H5N1 [16–18], H7N7 [22, 73], H9N2 [74], H7N3 [75], and H10N7 [http://www.paho.org/english/AD/DPC/CD/eid-eer-07-may-2004.htm#birdflu]

subtypes were transmitted to humans and caused mild illness to severe disease with significant mortality rates. The greatest challenge in influenza vaccine development may therefore lie in the development of safe and efficacious vaccines to these potentially pandemic viruses.

The current concept of vaccine virus generation through reassortment or reverse genetics, followed by amplification of the vaccine virus in embryonated chicken eggs, is not feasible in a pandemic situation. First, highly pathogenic avian H5N1 influenza viruses kill chicken embryos, resulting in limited virus growth. Secondly, the handling of highly pathogenic avian H5N1 influenza viruses for vaccine generation and amplification would pose a significant risk to production staff. Thirdly, the sudden surge in vaccine demand during a pandemic would likely leave embryonated chicken eggs in short demand. To overcome these problems, efforts have recently focused on two major areas – the development of low pathogenic H5 vaccine viruses, and the establishment of cell culture-based vaccines.

Cell Culture-Based Vaccines. Cell cultures are attractive alternatives to influenza virus amplification in embryonated chicken eggs since they are highly controllable systems that can be scaled up relatively quickly. Several studies indicated that the purity and immunogenicity of influenza vaccines produced in Madin-Darby canine kidney (MDCK) [76] or African green monkey kidney (Vero) [77–80] cells match that of vaccines produced in embryonated chicken eggs. In fact, both cell lines have now been approved for influenza virus vaccine production in the Netherlands.

The generation of recombinant vaccine viruses in MDCK or Vero cells was considered a difficulty due to their low transfection efficiencies (note that reverse genetics experiments are typically carried out in 293T human embryo kidney cells that can be transfected with high efficiencies). However, transfection of Vero cells with 12 plasmids produces virus, although at low efficiencies [9, 81]. Moreover, highly efficient virus generation in Vero cells was achieved with the improved reverse genetics system that uses concatemeric transcription units [9] (see above: 'Reverse Genetics of Influenza Viruses – State of the Art'). For vaccine production, a master set of three plasmids could be prepared that encode the three polymerase proteins, NP, and the six internal genes; the HA and NA genes which have to be updated frequently can be provided from a fourth plasmid.

Low Pathogenic H5 Vaccines. The development of H5 vaccines requires the safe handling of vaccine viruses outside of high biosafety level containment. Therefore, strategies were developed for the 'detoxification' of vaccine viruses possessing H5 HA proteins.

The pathogenicity of highly pathogenic avian influenza viruses is in part determined by their HA protein. The precursor HA protein is post-translationally processed into HA1 and HA2 subunits. This cleavage event exposes the fusogenic domain at the N-terminus of HA2 that mediates the fusion between the viral and the endosomal membranes and is therefore critical for viral infectivity. Highly pathogenic avian influenza viruses possess a series of basic amino acids at the HA cleavage

site [82]. This sequence is recognized by ubiquitous proteases, hence resulting in systematic virus spread. By contrast, avian influenza viruses of low pathogenicity cause localized infections in the respiratory or intestinal tract, owing to the limited distribution of proteases that recognize the single basic amino acid at the HA cleavage site of these viruses [83]. The knowledge that HA proteins of the highly-virulent type can be converted to an avirulent type by altering the sequence at the HA cleavage site [84] opened the door for the development of low pathogenic H5 vaccine candidates.

Reverse genetics technologies were used to replace the multibasic cleavage site of an HA protein derived from a highly pathogenic avian H5N1 virus with an avirulent-type HA cleavage site [85–88]. The avirulent-type H5 HA gene and the NA gene derived from the H5N1 virus were then combined with the internal genes of PR8 virus, again using reverse genetics. Using this strategy, three 6 + 2 reassortant vaccine candidates have been developed by St. Jude Children's Research Hospital, USA, the Centers for Disease Control and Prevention, USA, and the National Institute for Biological Standards and Control, UK, that have undergone preclinical testing [86–88]. Early results suggest low immunogenicity, probably necessitating the addition of adjuvants.

Using the same strategy, the NA and 'detoxified' HA genes of highly pathogenic avian H5N1 viruses could also be introduced into the backbone of the live attenuated type A master strain for the generation of live attenuated H5N1 vaccine virus [89]. Live attenuated H5N1 vaccine viruses would likely overcome the limited immunogenicity observed with inactivated H5N1 vaccines. However, to avoid the introduction of H5N1 HA and NA genes into human populations, live attenuated H5N1 vaccines should not be used until the avian H5 and N1 genes are already widespread in human populations.

Reverse Genetics of Influenza Viruses – Applications in Vaccine Vector Design

Reverse genetics has also been used to assess the use of influenza viruses as vaccine vectors. The availability of 16 different HA subtypes and 9 different NA subtypes would allow re-immunization, and the fact that the viral RNA is not integrated into the genome would contribute to the biosafety of the vaccine vector. In addition, influenza viruses can accommodate additional genetic material. Early studies suggested limited genetic stability of the additional genetic material [3], a challenge that was overcome by the identification and utilization of packaging signals [90]. The use of packaging signals allowed the generation of a live, genetically stable, bivalent influenza vaccine that expressed the ectodomain of a parainfluenza virus hemagglutinin-neuraminidase protein in the background of an influenza virus that lacked the NA gene [90]. This bivalent vaccine protected mice against lethal challenge with either virus [90], attesting to the feasibility of this approach.

Outlook: Reverse Genetics – Blessing or Curse?

With the ability to generate and modify influenza viruses at will, much has been learned, and much more will be learned about the viral life cycle and the mechanisms of influenza virus pathogenicity. This knowledge may in the future translate into the ability to predict the pathogenic and pandemic potential of newly emerging influenza viruses. Reverse genetics has already left its mark on influenza virus vaccine production and the development of vaccine candidates for pandemic influenza, an achievement that would not have been possible without reverse genetics technologies.

On the other hand, reverse genetics technologies and the increasing knowledge about the factors that determine influenza virus pathogenicity may one day culminate in the ability to tailor-make highly pathogenic, pandemic influenza viruses for biological warfare. Influenza viruses must be considered an attractive agent for bioterrorists and biological warfare since they can be maintained easily, grown to high titers, distributed easily, and *now* be manipulated easily. As an added advantage, vaccines can be prepared in advance to protect friendly troops and/or civilian populations. Despite this, the potential benefits of continued research on these viruses to global public health outweigh the possibility of surreptitious use.

Acknowledgements

We thank Krisna Wells for editing the manuscript. We also thank those in our laboratories who contributed to the data cited in this review. Our original research was supported by National Institute of Allergy and Infectious Diseases Public Health Service research grants; by CREST (Japan Science and Technology Agency), and by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1 Wright PF, Neumann G, Kawaoka Y: Orthomyxoviruses; in Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds): Fields Virology. Philadelphia, Wolters Kluwer, Lippincott Willams & Wilkins, 2007, pp 1691–1740.

2 Palese P, Shaw M: Orthomyxoviridae: The Viruses and Their Replication; in Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds): Fields Virology. Philadelphia, Wolters Kluwer, Lippincott Willams & Wilkins, 2007, pp 1647–1689.

3 Luytjes W, Krystal M, Enami M, Pavin JD, Palese P: Amplification, expression, and packaging of foreign gene by influenza virus. Cell 1989;59:1107–1113.

4 Neumann G, Zobel A, Hobom G: RNA polymerase I-mediated expression of influenza viral RNA molecules. Virology 1994;202:477–479.

5 Zobel A, Neumann G, Hobom G: RNA polymerase I catalysed transcription of insert viral cDNA. Nucleic Acids Res 1993;21:3607–3614.

6 Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y: Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci USA 1999;96:9345–9350.

7 Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A: Rescue of influenza A virus from recombinant DNA. J Virol 1999;73:9679–9682.

8 Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG: A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA 2000;97:6108–6113.

9 Neumann G, Fujii K, Kino Y, Kawaoka Y: An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. Proc Natl Acad Sci USA 2005;102:16825–16829.
10 Hoffmann E, Mahmood K, Yang CF, Webster RG, Greenberg HB, Kemble G: Rescue of influenza B virus from eight plasmids. Proc Natl Acad Sci USA 2002;99:11411–11416.
11 Jackson D, Cadman A, Zurcher T, Barclay WS: A reverse genetics approach for recovery of recombinant influenza B viruses entirely from cDNA. J Virol 2002;76:11744–11747.
12 Muraki Y: Reverse genetics study of influenza C virus. Abstracts of the XIII International Congress of Virology, San Francisco 2005.
13 Muraki Y, Murata T, Takashita E, Matsuzaki Y, Sugawara K, Hongo S: A mutation on influenza C virus M1 protein affects virion morphology by altering the membrane affinity of the protein. J Virol 2007;81:8766–8773.
14 Flatz L, Bergthaler A, de la Torre JC, Pinschewer DD: Recovery of an arenavirus entirely from RNA polymerase I/II-driven cDNA. Proc Natl Acad Sci USA 2006;103:4663–4668.
15 De Wit E, Spronken MI, Vervaet G, Rimmelzwaan GF, Osterhaus AD, Fouchier RA: A reverse-genetics system for Influenza A virus using T7 RNA polymerase. J Gen Virol 2007;88:1281–1287.
16 Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, Hemphill M, Rowe T, Shaw M, Xu X, Fukuda K, Cox N: Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 1998;279:393–396.
17 Claas EC, Osterhaus AD, Van Beek R, de Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG: Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351:472–477.
18 Claas EC, de Jong JC, Van Beek R, Rimmelzwaan GF, Osterhaus AD: Human influenza virus A/Hong Kong/156/97 (H5N1) infection. Vaccine 1998;16:977–978.
19 Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y: Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 1999;73:3184–3189.
20 Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM: A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol 1999;73:5903–5911.
21 Hatta M, Gao P, Halfmann P, Kawaoka Y: Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 2001;293:1840–1842.
22 Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD: Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci USA 2004;101:1356–1361.
23 Shinya K, Watanabe S, Ito T, Kasai N, Kawaoka Y: Adaptation of an H7N7 equine influenza A virus in mice. J Gen Virol 2007;88:547–553.
24 Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y: PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 2004;320:258–266.
25 Massin P, van der Werf S, Naffakh N: Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J Virol 2001;75:5398–5404.
26 Naffakh N, Massin P, Escriou N, Crescenzo-Chaigne B, van der Werf S: Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses. J Gen Virol 2000;5:1283–1291.
27 Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, Hulse-Post DJ, Humberd J, Trichet M, Rehg JE, Webby RJ, Webster RG, Hoffmann E: The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med 2006;203:689–697.
28 Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K: Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J Virol 2005;79:12058–12064.
29 Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J: The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci USA 2005;102:18590–18595.
30 Seo SH, Hoffmann E, Webster RG: Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 2002;8:950–954.
31 Krug RM, Yuan W, Noah DL, Latham AG: Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. Virology 2003;309:181–189.
32 Garcia-Sastre A: Antiviral response in pandemic influenza viruses. Emerg Infect Dis 2006;12:44–47.
33 Couceiro JN, Paulson JC, Baum LG: Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res 1993;29:155–165.

34 Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD: Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci USA 2004;101: 4620–4624.
35 Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y: Avian flu: influenza virus receptors in the human airway. Nature 2006;440:435–436.
36 Van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T: H5N1 Virus attachment to lower respiratory tract. Science 2006;312:399.
37 Nicholls JM, Chan MC, Chan WY, Wong HK, Cheung CY, Kwong DL, Wong MP, Chui WH, Poon LL, Tsao SW, Guan Y, Peiris JS: Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat Med 2007;13:147–149.
38 Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y: Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature 2006;444:378–382.
39 Basler CF, Reid AH, Dybing JK, Janczewski TA, Fanning TG, Zheng H, Salvatore M, Perdue ML, Swayne DE, Garcia-Sastre A, Palese P, Taubenberger JK: Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. Proc Natl Acad Sci USA 2001;98:2746–2751.
40 Reid AH, Fanning TG, Janczewski TA, Taubenberger JK: Characterization of the 1918 'Spanish' influenza virus neuraminidase gene. Proc Natl Acad Sci USA 2000;97:6785–6790.
41 Reid AH, Fanning TG, Hultin JV, Taubenberger JK: Origin and evolution of the 1918 'Spanish' influenza virus hemagglutinin gene. Proc Natl Acad Sci USA 1999;96:1651–1656.
42 Reid AH, Fanning TG, Janczewski TA, McCall S, Taubenberger JK: Characterization of the 1918 'Spanish' influenza virus matrix gene segment. J Virol 2002;76:10717–10723.
43 Reid AH, Fanning TG, Janczewski TA, Lourens RM, Taubenberger JK: Novel origin of the 1918 pandemic influenza virus nucleoprotein gene. J Virol 2004;78:12462–12470.
44 Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG: Characterization of the 1918 influenza virus polymerase genes. Nature 2005;437: 889–893.
45 Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Basler CF: Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. Proc Natl Acad Sci USA 2004;101:3166–3171.
46 Kobasa D, Takada A, Shinya K, Hatta M, Halfmann P, Theriault S, Suzuki H, Nishimura H, Mitamura K, Sugaya N, Usui T, Murata T, Maeda Y, Watanabe S, Suresh M, Suzuki T, Suzuki Y, Feldmann H, Kawaoka Y: Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature 2004;431:703–707.
47 Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Pantin-Jackwood MJ, Schultz-Cherry S, Solorzano A, Van Rooijen N, Katz JM, Basler CF: Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol 2005;79:14933–14944.
48 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A: Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 2005;310:77–80.
49 Kash JC, Tumpey TM, Proll SC, Carter V, Perwitasari O, Thomas MJ, Basler CF, Palese P, Taubenberger JK, Garcia-Sastre A, Swayne DE, Katze MG: Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. Nature 2006;443:578–581.
50 Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M, Feldmann F, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H, Kawaoka Y: Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 2007;445: 319–323.
51 Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, Tumpey TM, Basler CF, Taubenberger JK, Palese P: A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol 2005;79:11533–11536.
52 Tumpey TM, Maines TR, Van Rooijen N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A: A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 2007;315:655–659.
53 Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW: A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 2001;7:1306–1312.
54 Gibbs JS, Malide D, Hornung F, Bennink JR, Yewdell JW: The influenza A virus PB1-F2 protein targets the inner mitochondrial membrane via a predicted basic amphipathic helix that disrupts mitochondrial function. J Virol 2003;77:7214–7224.

55 Zamarin D, Garcia-Sastre A, Xiao X, Wang R, Palese P: Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS Pathog 2005;1:e4.
56 Zamarin D, Ortigoza MB, Palese P: Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. J Virol 2006;80:7976–7983.
57 Donald HB, Isaacs A: Counts of influenza virus particles. J Gen Microbiol 1954;10:457–464.
58 Duhaut SD, McCauley JW: Defective RNAs inhibit the assembly of influenza virus genome segments in a segment-specific manner. Virology 1996;216: 326–337.
59 Odagiri T, Tashiro M: Segment-specific noncoding sequences of the influenza virus genome RNA are involved in the specific competition between defective interfering RNA and its progenitor RNA segment at the virion assembly step. J Virol 1997;71: 2138–2145.
60 Fujii Y, Goto H, Watanabe T, Yoshida T, Kawaoka Y: Selective incorporation of influenza virus RNA segments into virions. Proc Natl Acad Sci USA 2003; 100:2002–2007.
61 Fujii K, Fujii Y, Noda T, Muramoto Y, Watanabe T, Takada A, Goto H, Horimoto T, Kawaoka Y: Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. J Virol 2005;79:3766–3774.
62 Muramoto Y, Takada A, Fujii K, Noda T, Iwatsuki-Horimoto K, Watanabe S, Horimoto T, Kida H, Kawaoka Y: Hierarchy among viral RNA (vRNA) segments in their role in vRNA incorporation into influenza A virions. J Virol 2006;80:2318–2325.
63 Watanabe T, Watanabe S, Noda T, Fujii Y, Kawaoka Y: Exploitation of nucleic acid packaging signals to generate a novel influenza virus-based vector stably expressing two foreign genes. J Virol 2003;77: 10575–10583.
64 Liang Y, Hong Y, Parslow TG: cis-Acting packaging signals in the influenza virus PB1, PB2, and PA genomic RNA segments. J Virol 2005;79: 10348–10355.
65 Belshe RB, Nichol KL, Black SB, Shinefield H, Cordova J, Walker R, Hessel C, Cho I, Mendelman PM: Safety, efficacy, and effectiveness of live, attenuated, cold-adapted influenza vaccine in an indicated population aged 5–49 years. Clin Infect Dis 2004; 39:920–927.
66 Chen Z, Aspelund A, Kemble G, Jin H: Genetic mapping of the cold-adapted phenotype of B/Ann Arbor/1/66, the master donor virus for live attenuated influenza vaccines (FluMist). Virology 2006;345: 416–423.
67 Hoffmann E, Mahmood K, Chen Z, Yang CF, Spaete J, Greenberg HB, Herlocher ML, Jin H, Kemble G: Multiple gene segments control the temperature sensitivity and attenuation phenotypes of ca B/Ann Arbor/1/66. J Virol 2005;79:11014–11021.
68 Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, Kemble G, Greenberg H: Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. Virology 2003;306:18–24.
69 Jin H, Zhou H, Lu B, Kemble G: Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. J Virol 2004; 78:995–998.
70 Quinlivan M, Zamarin D, Garcia-Sastre A, Cullinane A, Chambers T, Palese P: Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 2005;79:8431–8439.
71 Talon J, Salvatore M, O'Neill RE, Nakaya Y, Zheng H, Muster T, Garcia-Sastre A, Palese P: Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. Proc Natl Acad Sci USA 2000;97: 4309–4314.
72 Watanabe T, Watanabe S, Kida H, Kawaoka Y: Influenza A virus with defective M2 ion channel activity as a live vaccine. Virology 2002;299:266–270.
73 Koopmans M, Wilbrink B, Conyn M, Natrop G, van der Nat H, Vennema H, Meijer A, van Steenbergen J, Fouchier R, Osterhaus A, Bosman A: Transmission of H7N7 avian influenza A virus to human beings during a large outbreak in commercial poultry farms in the Netherlands. Lancet 2004;363:587–593.
74 Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, Orr WK, Shortridge KF: Human infection with influenza H9N2. Lancet 1999;354:916–917.
75 Tweed SA, Skowroski DM, David ST, Larder A, Petric M, Lees W, Li Y, Katz J, Krajden M, Tellier R, Halpert C, Hirst M, Astell C, Lawrence D, Mak A: Human illness from avian influenza H7N3, British Columbia. Emerg Infect Dis 2004;10:2196–2199.
76 Palker T, Kiseleva I, Johnston K, Su Q, Toner T, Szymkowiak C, Kwan WS, Rubin B, Petrukhin L, Wlochowski J, Monteiro J, Kraiouchkine N, DiStefano D, Rudenko L, Shaw A, Youil R: Protective efficacy of intranasal cold-adapted influenza A/New Caledonia/20/99 (H1N1) vaccines comprised of egg- or cell culture-derived reassortants. Virus Res 2004;105:183–194.
77 Bruhl P, Kerschbaum A, Kistner O, Barrett N, Dorner F, Gerencer M: Humoral and cell-mediated immunity to Vero cell-derived influenza vaccine. Vaccine 2000;19:1149–1158.

78 Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Dorner F: Development of a mammalian cell (Vero) derived candidate influenza virus vaccine. Vaccine 1998;16:960–968.
79 Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Eder G, Dorner F: Development of a Vero cell-derived influenza whole virus vaccine. Dev Biol Stand 1999;98:101–110.
80 Kistner O, Barrett PN, Mundt W, Reiter M, Schober-Bendixen S, Eder G, Dorner F: A novel mammalian cell (Vero) derived influenza virus vaccine: development, characterization and industrial scale production. Wien Klin Wochenschr 1999;111: 207–214.
81 Nicolson C, Major D, Wood JM, Robertson JS: Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 2005; 23:2943–2952.
82 Kawaoka Y, Webster RG: Sequence requirements for cleavage activation of influenza virus hemagglutinin expressed in mammalian cells. Proc Natl Acad Sci USA 1988;85:324–328.
83 Garten W, Klenk HD: Understanding influenza virus pathogenicity. Trends Microbiol 1999;7:99–100.
84 Horimoto T, Kawaoka Y: Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J Virol 1994;68:3120–3128.
85 Horimoto T, Takada A, Fujii K, Goto H, Hatta M, Watanabe S, Iwatsuki-Horimoto K, Ito M, Tagawa-Sakai Y, Yamada S, Ito H, Ito T, Imai M, Itamura S, Odagiri T, Tashiro M, Lim W, Guan Y, Peiris M, Kawaoka Y: The development and characterization of H5 influenza virus vaccines derived from a 2003 human isolate. Vaccine 2006;24:3669–3676.
86 Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, Gao Q, Zhang Z, Liu Y, Wang Z, Yang M, Sun R, Li C, Lin S, Ji M, Liu Y, Wang X, Wood J, Feng Z, Wang Y, Yin W: Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet 2006;368:991–997.
87 Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, Hoschler K, Zambon MC: Safety and immunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004 (H5N1) vaccine: phase I randomised trial. Lancet 2006;367:1657–1664.
88 Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M: Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med 2006;354:1343–1351.
89 Suguitan AL, McAuliffe J, Mills KA, Jin H, Duke G, Lu B, Luke CJ, Murphy B, Swayne DE, Kemble G, Subbarao K: Live, attenuated influenza A H5N1 candidate vaccines provide broad cross-protection in mice and ferrets. PLoS Med 2006;3:e360.
90 Maeda Y, Hatta M, Takada A, Watanabe T, Goto H, Neumann G, Kawaoka Y: Live bivalent vaccine for parainfluenza and influenza virus infections. J Virol 2005;79:6674–6679.

Yoshihiro Kawaoka
Department of Pathobiological Sciences, School of Veterinary Medicine, University or Wisconsin-Madison
2015 Linden Drive, Madison, WI 53706 (USA)
Tel. +1 608 265 4925, Fax +1 608 265 5622, E-Mail kawaokay@svm.vetmed.wisc.edu

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 134–155

Receptor Specificity of Influenza Viruses and Its Alteration during Interspecies Transmission

Mikhail N. Matrosovich[a] · Alexandra S. Gambaryan[b] · Hans-Dieter Klenk[a]

[a]Institute of Virology, Philipps University, Marburg, Germany, and
[b]M.P. Chumakov Institute of Poliomyelitis and Viral Encephalitides, Moscow, Russia

Abstract

Influenza infection is initiated by virus attachment to sialic acid-containing cell-surface molecules traditionally called viral receptors. The spectrum of sialylglycoconjugates varies substantially between viral host species as well as target tissues and cell types of the same species leading to variations in the receptor-binding specificity of viruses circulating in these hosts. It is believed that a poor fit of avian viruses to receptors in humans limits the emergence of new pandemic strains. Here we review current knowledge on receptors and receptor specificity of influenza viruses and on the role played by receptor specificity in the viral cell and tissue tropism, interspecies transmission and adaptation to a new host.

Sialic Acid Receptors of Influenza Viruses

Sialic acids (Sias) are a family of negatively charged 9-carbon sugars typically occurring at the terminal positions of glycoconjugates on the cell surface and secreted molecules of the deuterostome branch of the animal kingdom [1, 2]. Because of their highly exposed location, Sias often serve as recognition epitopes for endogenous lectins, such as selectins and siglecs, and as components of attachment sites utilized by microbial pathogens [2–5]. More than 40 known sialic acid (Sia) species differ from each other by substituents at the amino group (N5) and at four hydroxyl groups (O4, O7, O8, and O9). Influenza A and B viruses use as a receptor molecule the most common derivative and a biosynthetic precursor of other family members, non-O-acetylated N-acetylneuraminic acid (Neu5Ac) [reviewed in 6, 7].

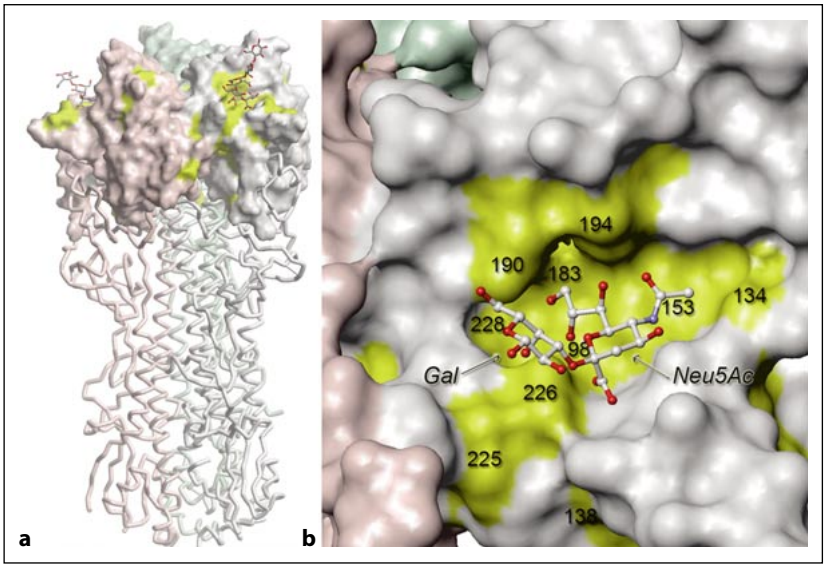


Fig. 1. Receptor-binding site of influenza virus HA. **a** Individual monomers of the HA trimer are coloured in white, pink and green. The HA globular head (amino acids 90–260) is shown as solvent-accessible surface; amino acids conserved among avian viruses are coloured yellow. Stick model illustrates position of bound sialyloligosaccharide LSTa (Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc). **b** Enlarged view of the RBS and Neu5Acα2-3Gal moiety of LSTa. Positions of some conserved amino acids of the avian RBS are indicated using the H3 numbering system. The figure is based on crystallographic data of Ha et al. [15] for the HA of A/Duck/Ukraine/1/63 (H3N8).

Sias are represented in vivo in the oligosaccharide chains of glycoproteins and glycolipids (gangliosides). It is often assumed that glycoproteins are more likely to serve for the initial influenza virus attachment, whereas subsequent binding to membrane-embedded gangliosides could facilitate the membrane fusion and entry of the viral genome into the cell [8, 9], however the relative roles of glycoproteins and gangliosides as influenza virus receptors remain unclear. Some studies suggested that gangliosides can mediate influenza virus entry into cells even in the absence of glycoprotein receptors [10]. At the same time, mutant laboratory cells lacking gangliosides could be infected by influenza viruses and produced infectious virus progeny demonstrating that glycoproteins alone support virus infection [11]. Possibly, the role of glycoproteins and gangliosides as receptors of influenza viruses vary among different viral host species and target tissues.

The structures of complexes of the viral receptor-binding protein haemagglutinin (HA) of influenza A and B viruses with natural and synthetic Sia compounds were determined by X-ray crystallography [12–17]. The Sia-binding site is a shallow pocket located on the globular head of HA (fig. 1). The interactions between the amino acid residues in the receptor-binding site (RBS) and the sialyloligosaccharide

moieties of the receptors are very weak (K_{diss} >0.1 mM); the high binding avidity of virus attachment to the cell is accomplished by cooperative binding of multiple HA spikes to multiple copies of the receptor. Virus binding depends not only on HA affinity for the terminal Sia residues, but also on the structure of the underlying oligosaccharide and protein or lipid moieties of the receptors, as well as on the abundance and accessibility of receptors on the cell surface. Because of this complex mode of binding, the receptor-binding properties of influenza viruses can be affected by amino acid substitutions inside the Sia-binding pocket, on the pocket rim, and by distant mutations resulting in altered glycosylation or altered electrostatic charge of the globular head of HA [reviewed in 7].

In natural glycoconjugates, Sias are α2-3- or α2-6-linked to Gal and GalNAc, α2-6-linked to GlcNAc, or α2-8-linked to the second Sia residue. Influenza viruses generally do not bind to α2-8-linked Neu5Ac moieties and can recognize only α2-3- or α2-6-linked Sia epitopes (Neu5Acα2-3/6Gal, Neu5Acα2-3/6GalNAc, and Neu5A cα2-6GlcNAc). These disaccharide moieties can be presented in highly variable microenvironments [1, 2, 5]. The structural diversity of Sia-containing natural molecules is increased by conformational flexibility of oligosaccharides, by variations in the position of oligosaccharide chains in the macromolecular receptors, and by their different accessibility in the context of other cell-surface molecules. The current knowledge on the receptor specificity of influenza viruses is mainly limited to virus recognition of the terminal Sia and one or two penultimate sugar residues.

Interspecies Transmission of Influenza Viruses and Selective Pressures on Viral Receptor Specificity

The primary natural reservoir of influenza A viruses are wild aquatic birds of the orders *Anseriformes* (ducks, geese and swans) and *Charadriiformes* (gulls, terns and waders) which harbour viruses of all currently known 16 HA and 9 NA antigenic subtypes [18–20, also see review by Osterhaus et al., this volume]. Dabbling ducks (*Anatinae*), such as mallards and teals, show particularly high virus isolation rates suggesting a unique role of these avian species in persistence of influenza viruses in nature. Occasionally, influenza viruses transmit from aquatic birds to other birds and to mammals and cause infections of various severities. Shortly after interspecies transfer, the viruses usually die out because of insufficient fitness in their new hosts. However, on rare occasions, they adapt to efficient replication and transmission in the new species and continue to circulate for prolonged periods of time, forming stable host-specific virus lineages. All presently known lineages of influenza A viruses in land-based poultry, pigs, horses and humans originated from the viruses of wild aquatic birds. Emergence of new host-specific lineages is often associated with changes in the mode of virus transmission and tissue tropism. Thus, duck influenza viruses replicate in the intestinal tissue of their natural hosts and transmit by the faecal-oral

route via contaminated water, whereas in mammals these viruses replicate in the respiratory tract and transmit by air.

During their life cycle, influenza viruses interact with a variety of Sia-containing molecules in the infected host tissues. The virus has to bind to specific receptors on target cell membranes to initiate infection. It can also bind to 'decoy receptors' on the cellular glycocalix and extracellular mucus, which interfere with infection [reviewed in 21]. The balance between these favourable and unfavourable interactions is essential for the fitness of influenza virus in its host. The spectrum of sialoglycoconjugates can vary substantially between different species even for closely related species, such as humans and great apes [5]. Furthermore, expression of glycoconjugates varies between tissues and cell types of the same species [2]. As a result, initial interspecies transmission of influenza viruses can be limited by a poor fit of the virus to receptors/inhibitors in a new host, and adaptation of the virus to a new species can involve selection of a virus variant with altered receptor-binding specificity.

Avian Influenza Viruses Preferentially Bind to α2-3-Linked Sialic Acids

Paulson and colleagues [reviewed in 8] found in the early 1980s that duck viruses with the H3 HA bound to terminal Neu5Acα2-3Gal-containing receptors significantly stronger than to Neu5Acα2-6Gal-containing ones, whereas human influenza viruses displayed the opposite binding preference. Later, the same binding pattern was observed in studies on larger panels of avian and human viruses with H1, H2, and H3 HA subtypes [22–24], and on avian virus strains of most other HA subtypes [25–27].

Studies on the binding of influenza viruses to free Neu5Ac and monovalent synthetic and natural sialosides demonstrated that in addition to binding to terminal Sia, influenza viruses can interact with the penultimate galactose residue [24, 26–30]. In particular, avian viruses displayed at least a 10-fold higher affinity for 3′-sialyllactose (3′SL, Neu5Acα2-3Galβ1-4Glc) than for free Neu5Ac, indicative of energetically favourable interactions between the avian virus HA and the 3-linked Gal moiety. By contrast, avian viruses bound to 6′-sialyllactose (6′SL, Neu5Acα2-6Galβ1-4Glc) and to 6′-sialyl(N-acetyllactosamine) (6′SLN, Neu5Acα2-6Galβ1-4GlcNAc) with a weaker affinity than they bound to free Neu5Ac. This result suggested that the 6-linked lactose/N-acetyllactosamine moiety does not fit into the avian RBS. The molecular mechanisms of these effects were explained by crystallographic data obtained from H3, H5 and H9 HA complexes with the 3- and 6-linked sialylpentasaccharides LSTa and LSTc [13–15]. The 3-linked sialyloligosaccharide LSTa was bound to avian H3 and H5 HAs with the minimum-energy *trans* conformation of the Neu5Ac-Gal linkage. In this conformation, the C3 methylene group of the α2-3 linkage and the whole 3-linked Gal are projected upward so that the axial 4-OH group of Gal and the glycosidic oxygen of the linkage form hydrogen bonds with the side chain amide and

carbonyl groups of the glutamine in HA position 226 (226Q) (fig. 1b, 2a). The 6-linked receptor LSTc binds to avian HAs in its own lowest energy *cis* conformation. In this case, the hydrophobic C6 methylene group of the α2-6 linkage projects downward toward the polar atoms of the side chain of 226Q (fig. 2b). The unfavorable polar-non-polar interactions between 6-linked Gal and 226Q reduce the binding affinity of avian viruses for 6-linked receptors. Thus, the receptor specificity of avian viruses is determined, at least partially, by the optimal fit of the RBS of HA to the most abundant minimum-energy *trans* conformer of the Neu5Acα2-3Gal moiety and by a poor fit to the major *cis* conformer of the Neu5Acα2-6Gal-containing receptor (fig. 2a, b).

The common preferential binding of different avian influenza virus subtypes to Neu5Acα2-3Gal correlates with the high conservation of the RBS [25, 27] (see fig. 1, 3). Interestingly, with a few distinct avian virus lineages (such as H13 and H16 gull viruses and H9N2 poultry viruses) and especially in swine and human viruses, several conserved amino acids of the RBS that either directly (190E, 194L, 225G, and 226Q) or indirectly (138A and 228G) participate in interactions with Neu5Acα2-3Gal moiety have been exchanged. Mutations in any of these positions were found to decrease the binding of avian virus to the 3-linked Gal moiety and to lower the virus affinity for the Neu5Acα2-3Gal-containing receptors [24]. Apparently the conservation of these amino acids in the HA of most avian viruses is required for the recognition of the *trans* conformer of the 3-linked sialyloligosaccharide moieties which is essential for efficient virus attachment to target cells in birds.

Viruses from Ducks (*Anatidae*), Gulls (*Laridae*) and Gallinaceous Domestic Birds Have Distinguishable Receptor Specificity

Based on the early experiments with limited numbers of viruses and receptor analogues, it was generally assumed that all avian viruses have similar receptor-binding specificity. The first evidence arguing against this concept was provided in a study on H5N1 avian influenza viruses that caused human infections in Hong Kong 1997 [29]. The H5N1 viruses isolated from poultry and humans were found to have a lower receptor-binding affinity and a lower neuraminidase activity than closely related viruses of aquatic birds. Furthermore, analysis of the HA and NA sequences of H5 and H7 viruses from various avian species revealed that poultry viruses differ from duck viruses by additional N-linked glycans at the top of HA and by large deletions in the stalk of NA [29]. These changes of HA and NA were detected in many independent lineages of poultry viruses [29, 31, 32] suggesting a functional role in the adaptation of viruses from aquatic birds to domestic gallinaceous birds. These findings led to the hypothesis that Sia receptors in different birds are not identical and that distinctions in receptors determine differences in the viral fine receptor specificity and neuraminidase activity. Studies performed in the following years confirmed this hypothesis.

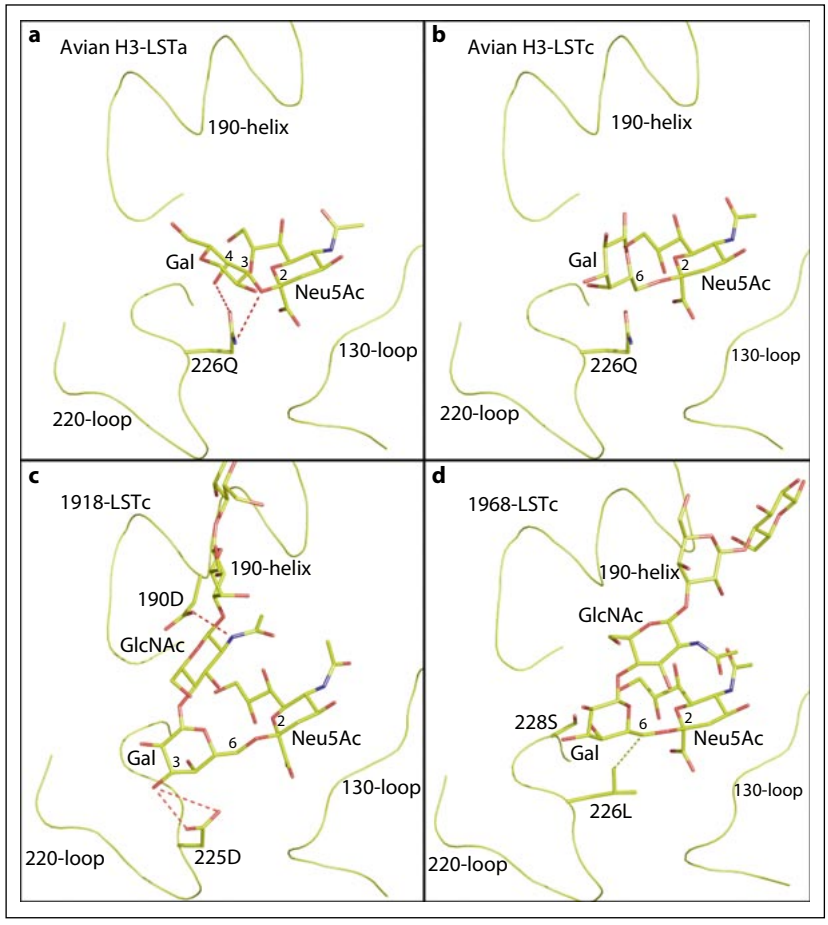


Fig. 2. Interactions of avian and human influenza virus HAs with asialic portions of Neu5Acα2-3Gal- and Neu5Acα2-6Gal-teminated pentasaccharides LSTa (**a**) and LSTc (**b–d**) [13–16]. **a** Receptor-binding site of A/Duck/Ukraine/63 (H3N8) with bound Neu5Aca2-3Gal moiety of LSTa. The galactose residue is bound in the minimum-energy *trans* conformation of the glycosidic linkage that allows hydrogen bonding (red dotted lines) of the glycosidic oxygen and the 4-OH group of Gal to the side chain atoms of 226Q. **b** Neu5Acα2-6Gal moiety of LSTc is bound to the RBS of A/Duck/Ukraine/63 in a *cis* conformation. The hydrophobic C6-methylene group of Gal projects towards the polar side chain of 226Q thus interfering with the avian HA binding to 6-linked receptors. **c** The model of the RBS of H1N1/1918 pandemic virus with bound LSTc. The model was made by introducing G225D substitution into the HA-LSTc complex of A/swine/Iowa/30 (H1N1) [16]. The mutations E190D and G225D in the RBS of the 1918 virus HA enables formation of additional hydrogen bonds (red dotted lines) with N-acetamido group of GlcNAc and 3-OH group of Gal within the *cis* conformer of the 6′SLN moiety. **d** RBS of H3N2/1968 pandemic virus with bound LSTc. Mutations G228S and Q226L in the HA make the RBS wider and improve sterical fit of the 6-linked Gal moiety. Non-polar side chain of 226L participates in energetically favourable van-der-Waals and hydrophobic interactions (yellow dotted line) with the C6-methylene group of Gal. The models were generated using DeLano Scientific PyMOL (DeLano, W.L. The PyMOL Molecular Graphics System, http://pymol.sourceforge.net).

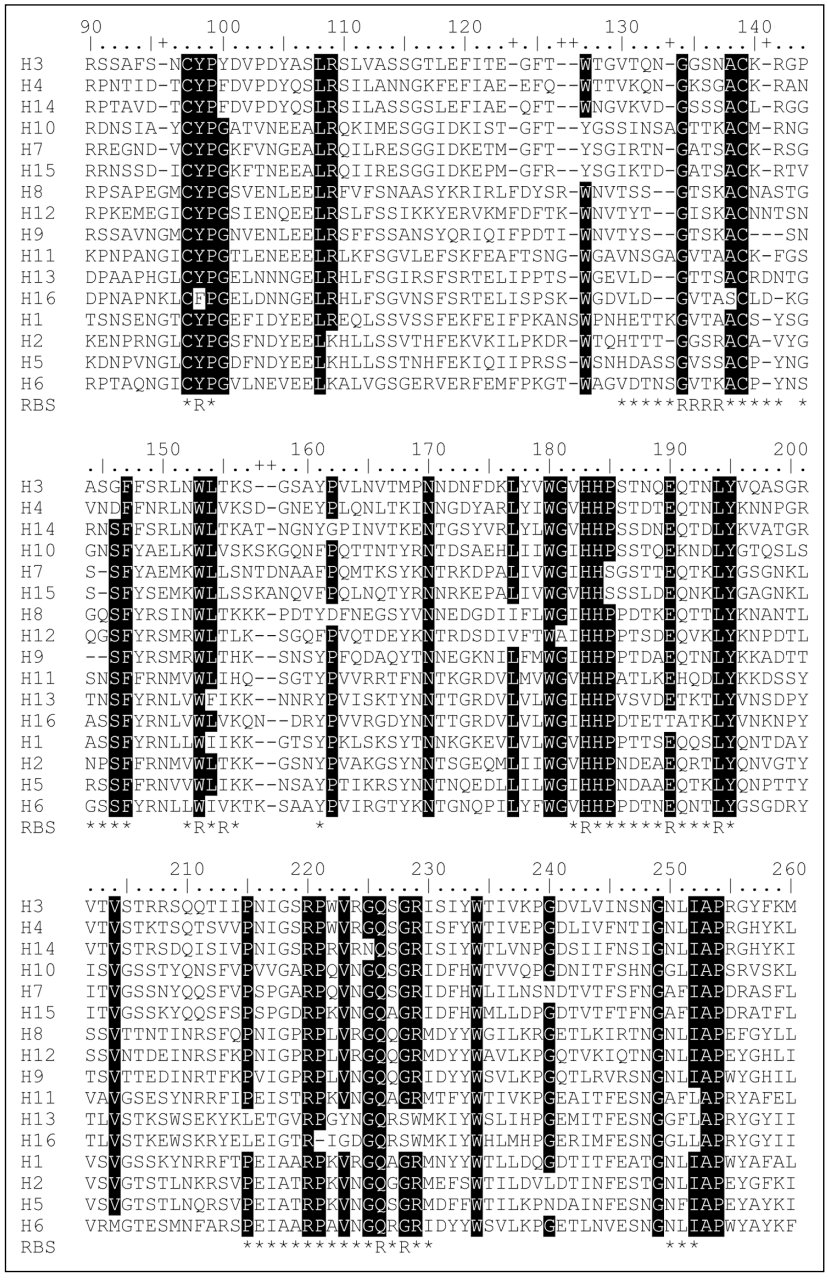


Fig. 3. Conserved amino acids in the HA globular head of avian influenza A viruses of 16 known HA subtypes. Top line shows the H3 numbering; amino acid positions that contain insertions with respect to H3 HA are indicated by '+'. RBS line: star indicates that at least one atom of the amino acid is within 10 Å distance from the Neu5Acα2-3Gal moiety in the HA receptor complex of Duck/Ukraine/63 (H3N8), 1MQM [15]. 'R' indicates that amino acid contacts sialic acid. The sequences are listed according to their homology, amino acids conserved among at least 13 of 16 sequences are shaded (see figure 1 for the location of conserved amino acids on the HA crystallographic model).

In 1999, several humans were infected by H9N2 poultry viruses in China and Hong Kong, both human and closely related poultry viruses carried mutations in the conserved positions of the RBS, such as positions 190 and 226 [33]. Furthermore, these H9N2 poultry viruses differed from H9 viruses of other known lineages by their binding to 6-linked Sias [34, 35] and by mutations that destroyed the hemadsorption site of their neuraminidases [34]. The Asian H9N2 viruses represented the first example of an avian influenza virus lineage with human-virus-like receptor specificity and an ability to infect different species of poultry (quail, chicken, pheasant, pigeon, etc.). This finding suggested that a strict preference for 3-linked Sia receptors, which is maintained by influenza viruses in wild aquatic birds, may not be essential for influenza virus perpetuation in other avian species.

A significant advance in the knowledge on the receptor specificity of the avian influenza viruses came with the use of synthetic sialylglycopolymers (SGPs), monospecific macromolecular probes which comprised multiple copies of sialyloligosaccharide moieties attached to soluble hydrophilic polymeric carrier [36]. By comparing the viral binding to a panel of SGPs that harboured the same Neu5Acα2-3Gal moiety in a context of different oligosaccharide core sequences, Gambaryan, Bovin and colleagues [37–41] were able to specify the role of the more distant parts of sialyloligosaccharides in receptor recognition. These studies revealed significant distinctions between duck, gull and chicken viruses (table 1).

Duck viruses of various HA subtypes bound to Neu5Acα2-3Galβ1-3GalNAc- and Neu5Acα2-3Galβ1-3GlcNAc-containing SGPs with a higher affinity than they bound to Neu5Acα2-3Galβ1-4GlcNAc-containing analogues. A fucose substituent at the third saccharide residue reduced viral binding and a sulpho substituent at the 6-OH group of GlcNAc had little or no effect. These findings indicated that duck viruses prefer the β1-3 bond between the terminal Neu5Acα2-3Gal moiety and the next sugar residue, and that biologically relevant receptor epitopes in ducks are likely neither fucosylated nor sulphated. This specificity of duck viruses correlated with their strong binding to ganglioside GD1a (Neu5Acα2-3Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ-Cer) and to gangliosides from duck intestine [27, 42]. It is therefore possible that binding to gangliosides plays an important role for the viral infection of duck intestinal cells in vivo.

Only some gull viruses can infect ducks under experimental conditions suggesting a degree of host-range restriction between these species [43]. In particular, the viruses with H13 and H16 HAs appear to be endemic in gulls and are very rarely isolated from ducks [18–20, 44]. These viruses differ from all other avian viruses by mutations of the conserved amino acids in the RBS (H13: positions 228, 229; H16: positions 98,138,190,228,229; see fig. 3) suggesting that gull viruses are adapted to a different receptor. Indeed, a typical feature of the H4, H6, H13, H14 and H16 subtype viruses isolated from gulls was high-affinity binding to sialylglycopolymers bearing fucosylated sialyloligosaccharide moieties Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc (SLe^x) [38, 40]. All tested H13 and H16 viruses with Lys in position 193 of HA had maximal

Table 1. Receptor specificity of influenza viruses in different species

Virus host species	HA subtype	High-affinity binding sialyloligosaccharide		Ref.
Ducks	H1, H2, H3, H4, H5,	Neu5Acα2-3Galβ1-3GalNac Neu5Acα2-3Galβ1-3GlcNAc	STF SLe^c	39–41
Gulls	H4, H5, H6, H14, H13, H16	Neu5Acα2-3Galβ1-3(Fucα1-4)GlcNAc Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc Neu5Acα2-3Galβ1-4(Fucα1-3)(6-O-HSO$_3$)GlcNAc	SLe^a, SLe^x Su-SLe^x	38, 40[1]
Chickens	H5, H7	Neu5Acα2-3Galβ1-4(6-O-HSO$_3$)GlcNAc Neu5Acα2-3Galβ1-4(Fucα1-3)(6-O-HSO$_3$)GlcNAc	Su-3′SLN Su-SLe^x	39–41[1]
Poultry[2]	H9N2	Neu5Acα2-6Galβ1-4GlcNAc Neu5Acα2-3Galβ1-4(6-O-HSO$_3$)GlcNAc Neu5Acα2-3Galβ1-4(Fucα1-3)(6-O-HSO$_3$)GlcNAc	6′SLN Su-3′SLN Su-SLe^x	34, 45[1]
Pigs	H1, H3, H4	Neu5Acα2-6Galβ1-4GlcNAc	6′SLN	24, 50, 56, 65
Humans	H1, H2, H3, type B	Neu5Acα2-6Galβ1-4GlcNAc	6′SLN	24, 26–28, 30, 57

[1]Gambaryan, Tuzikov, Pazynina, Bovin, Klimov, unpublished data.
[2]Various species of terrestrial poultry from Eurasia.

affinity for the sulphated analogue Neu5Acα2-3Galβ1-4(Fucα1-3)(6-O-HSO$_3$) GlcNAc (Su-SLe^x) [Gambaryan et al., unpubl. data]. Thus, adaptation for the recognition of fucosylated sialyloligosaccharide receptors seems to be required for the efficient replication of influenza viruses in gulls.

In contrast to duck viruses, highly pathogenic H5N1 human and chicken viruses isolated in Hong Kong in 1997 preferred receptors with a β1-4 bond between Neu5Acα2-3Gal and the next sugar residue, for example, Neu5Acα2-3Galβ1-4GlcNAc (3′SLN). In addition, these H5N1 viruses showed particularly high affinity for a sulphated analogue Neu5Acα2-3Galβ1-4(6-O-HSO$_3$)GlcNAc (Su-3′SLN) [39]. Subsequent detailed analysis of H5 subtype viruses from different species confirmed these observations and revealed evolutionary changes in the receptor specificity of H5 viruses that accompanied their circulation in poultry [41]. In particular, Asian H5N1 isolates from chickens and humans in 2003 and 2004, similar to HK/97 viruses, displayed a very strong binding to Su-3′SLN and additionally acquired high affinity

for the fucosylated analogue Su-SLe^x (table 1). Importantly, many tested H5 virus isolates from North American poultry also showed increased affinity for Su-3′SLN, suggesting that acquisition of this property occurred independently in two lineages of H5 poultry viruses. The ability of these viruses to bind to sulphated analogues of 3′SLN correlated with the presence of a positively charged amino acid (R or K) in HA position 193; molecular modelling predicted potential ionic interactions between the sulphate group of the receptor and the side chain of 193R/K [39, 41]. H7 viruses with Lys193 also have increased affinity for sulphated oligosaccharides [39, 40, 45]. Furthermore, Asian H9N2 viruses that recognize 6-linked Sias were found to bind strongly to Su-3′SLN and Su-SLe^x [45, and Gambaryan et al., unpubl. data].

Recently, a glycan microarray was developed by the Consortium for Functional Glycomics which comprises a library of more than 260 structurally defined sugars printed on glass slides [46]. First reports demonstrate the capacity of the array to provide highly detailed profiles of influenza virus binding to sialyloligosaccharides [47–49]. This powerful assay will greatly facilitate studies on the fine receptor specificity of avian and mammalian influenza viruses.

Sialic Acid-Containing Glycoconjugates in Avian Tissues

The first study on Sia receptors in bird tissues was performed by Ito et al. [50]. Using two linkage-specific Sia-binding lectins, *Maackia amurensis* agglutinin (MAA) and *Sambucus nigra* agglutinin (SNA), they found that duck intestinal epithelial cells mainly express Siaα2-3Gal moieties recognized by MAA and that they lack expression of 6-linked Sias recognized by SNA. This pattern correlated with the preference of avian influenza viruses for 3-linked Sias.

Gambaryan et al. [42] compared binding of duck, chicken and human influenza viruses to cell membranes and gangliosides from epithelial tissues of ducks, chickens and African green monkeys. Human viruses bound to monkey and chicken cell membranes but not to those of ducks, suggesting that chicken cells similar to monkey cells express Siaα2-6Gal-containing receptors. Two other research groups demonstrated that quails, similar to chickens, express both types of Sia epitopes on the surface of tracheal and intestinal epithelium [51, 52]. These studies revealed the molecular basis for the existence of avian H9N2 viruses with human-virus-like receptor specificity and emphasized the roles of quails and chickens as potential intermediate hosts for the transmission of viruses from aquatic birds to humans.

SNA and MAL-I lectins together with a panel of human and avian influenza viruses with well-characterized receptor specificity were used to compare expression of influenza virus receptors in intestinal tissues of about 20 different species from 6 orders of birds, including *Anseriformes*, *Charadriiformes*, and *Galliformes* [53]. Ducks were found to differ from other birds by a reduced expression of sialyloligosaccharides recognized by both SNA and MAL-1 in duck intestinal cells, whereas duck viruses

efficiently bound to these cells. This pattern demonstrated lack of Neu5Acα2-6Gal-containing moieties recognized by SNA and of Neu5Acα2-3Galβ1-4GlcNAc-moieties recognized by MAL-I and presence of Neu5Acα2-3Galβ1-3Glc/GalNAc-moieties, which are preferentially recognized by duck viruses [27, 39–41], but not by MAL-I [54]. The cells from intestinal epithelium of gulls bound MAL-I and avian influenza viruses, but did not bind SNA and human viruses. These data showed a deficiency of 6-linked Sias and an abundant expression of Siaα2-3Galβ1-4GlcNAc-moieties in the epithelial tissues of gulls. Intestinal cells of several *Galliformes* were found to bind SNA, MAL-I and both avian and human influenza viruses indicating that these cells contain both Siaα2-3Galβ1-4GlcNAc- and Siaα2-6Galβ1-4GlcNAc. Using viruses with high binding affinity for fucosylated sialyloligosaccharides as molecular probes, the authors demonstrated expression of fucosylated receptors on the surface of chicken cells but not on the surface of duck cells. These findings revealed that Sia receptors vary substantially among different avian species, and that distinctive receptor specificity of influenza viruses circulating in ducks, gulls and chickens correlates with the availability of specific receptor moieties on the target cells.

Swine Viruses Have Human-Virus-Like Receptor Specificity

Because pigs support replication of both avian and human viruses, they were considered to be a plausible intermediate host for the generation of human pandemic strains by gene reassortment [reviewed in 55, see also reviews by Scholtissek and Brown, this volume]. This theory was further supported by the finding that both 3- and 6-linked Sia moieties were detected by lectin staining on the histological sections of pig tracheal epithelium [50].

Gambaryan et al. [56] studied a few H1N1 and H3N3 avian-like viruses that were isolated from pigs but apparently were not yet fully adapted to this host. These viruses retained an avian-virus-like preferential binding to Neu5Acα2-3Gal-containing receptors but differed from typical duck viruses by a higher binding to Su-SLe^x and by an ability to bind to 6′SLN.

Two known stable swine virus lineages originated from independent introductions of H1N1 avian viruses either directly from birds or via some intermediate host species [see review by Brown, this volume]. 'Classical' swine H1N1 virus emerged in the beginning of the 20th century and shared its immediate ancestor with human 1918 pandemic virus; 'avian-like' H1N1 virus was established in pigs in Europe at the end of 1970s. Both classical and avian-like swine viruses preferentially bind to 6-linked Sias; substitutions at HA positions 190 and 225 were primarily responsible for the acquisition of this human-virus-like receptor specificity [see 7, 24, and references therein]. The earliest available isolates from the avian-like European lineage displayed an enhanced affinity for Neu5Acα2-6Gal-containing receptors relative to the closely related H1N1 avian viruses. However, unlike pandemic human H2 and H3 viruses,

the early avian-like swine viruses retained the ability of their avian predecessors to bind to Neu5Acα2-3Gal. Virus binding to 3-linked receptors gradually decreased during its circulation in pigs, but did not disappear completely. As a result, the binding specificity of the avian-like swine viruses isolated after 1985 was similar to that of classical swine viruses [24, 50].

All early studies on swine influenza viruses were done using viruses that were grown in embryonated hen's eggs. However, similar to human influenza viruses, swine viruses appear to change their receptor specificity in eggs. Indeed, non-egg-adapted classical swine influenza viruses that were isolated and propagated solely in MDCK cells displayed a strict preference for 6′SLN-containing receptors and did not bind to 3-linked receptors [56]. This binding pattern is typical for non-egg-adapted human influenza viruses, and it differs from the previously described receptor specificity of egg-adapted swine influenza viruses [7, 24, 50]. Thus, the receptor specificity of pig viruses may be even closer to that of human viruses than it was believed before. This notion emphasizes the importance of swine as an intermediate host in transmission of avian viruses to humans.

Mutations in the HA Receptor-Binding Site and Receptor Specificity of Pandemic Viruses

Three antigenic subtypes of human influenza A viruses (H1N1, H2N2, H3N2) evolved from pandemic viruses of 1918, 1957, and 1968, respectively. These pandemic strains originated from avian influenza viruses that were transmitted to humans either as a whole or as reassortants with contemporary human viruses. Type B viruses are also believed to be descendants of avian influenza A viruses [18]. Despite their independent evolution in humans, all epidemic human influenza A and B viruses share a high affinity for 6′SLN (Neu5Acα2-6Galβ1-4GlcNAc) and do not bind to Neu5Acα2-3Gal-terminated receptors [24, 26, 27, 30, 57]. However, detailed molecular interactions with the 6′SLN moiety vary among different virus types, subtypes and strains. For example, H2N2 viruses and H3N2 viruses that circulated until the middle 1990s bind to both Neu5Ac and penultimate Gal residues of 6′SLN, whereas H1N1 viruses and currently circulating H3N2 viruses bind to Neu5Ac and GlcNAc [for reviews on receptor specificity of human epidemic viruses, see 7, 21].

The acquisition of preferential binding to 6′SLN occurred soon after the interspecies transmission of the avian HA, as the earliest available influenza A virus strains from the three pandemics harboured mutations in the conserved positions of the HA RBS and displayed a human-virus-like receptor specificity [23, 24, 48, 58, 59].

Mutation E190D was found in all five sequenced HAs of the 1918 pandemic H1N1 viruses [60]. Three of the sequences contained a second mutation G225D in the conserved RBS position, whereas two other sequences preserved the avian-virus-like 225G. The virus with the single HA mutation E190D differed from avian viruses by

its ability to bind to 6-linked receptors and by its decreased affinity for 3-linked receptors; a double mutant (E190D, G225D) mainly bound to 6-linked receptors [48, 59, also see Steel and Palese, this volume]. Based on the available biochemical [24, 26–28, 30] and crystallographic [16, 61] data, it is believed that the mutation E190D enhanced virus binding to human type receptors by improving sterical fit of the 6′SLN-containing receptor to the H1 HA and by allowing hydrogen bonding between the carbonyl group of 190D and the N-acetamido group of GlcNAc within the *cis* conformer of the 6′SLN moiety (fig. 2c). The effects of mutation G225D appear to involve the hydrogen bond formation between side chain of 225D and the Gal residue of 6′SLN (fig. 2c) and concomitant ablation of hydrogen bonding between 226Q and 3-linked Gal of the Neu5Acα2-3Gal-terminated receptors.

In the case of H2 and H3 pandemic viruses, substitution Q226L was essential for the acquisition of the human-virus-like specificity, whereas an additional mutation G228S had a marginal effect [23, 24, 62, 63]. In particular, some H2N2 human viruses isolated in 1957 had 226L but still maintained the avian-virus-like 228G. Similar to the 1918 viruses with a single HA mutation, the H2N2 viruses with a single Q226L substitution might represent the earliest step of adaptation of the avian HA to humans. The mutation Q226L makes the RBS wider [13, 14], thus improving accommodation of the 6-linked Gal moiety. Furthermore, the non-polar side chain of 226L participates in energetically favourable van-der-Waals and hydrophobic interactions with C6 methylene group of Gal [14, 28] (fig. 2d). At the same time, mutation Q226L reduces HA binding to avian-type receptors by preventing the formation of hydrogen bonds between the HA and the 3-linked Gal moiety of the receptor. The 226 mutation could be a rather general mechanism of avian virus adaptation to recognition of 6-linked receptors. Thus, mutations Q226L and G228S were identified in the HA of a H4N6 swine virus isolate [64]. The receptor specificity of this virus was identical to that of H3N2 pandemic viruses [65]. H9N2 Asian poultry viruses that carry mutation Q226L recognize both 6-linked and sulphated 3-linked receptors [34, 35, 45].

Receptor Distribution, Cell and Tissue Tropism of Influenza Viruses in Humans

The first studies on distribution of influenza virus receptors in men were performed by Paulson's group [66, 67] using fixed paraffin-embedded tissue sections of human trachea. They found that α2-6 linkage-specific lectin SNA and human influenza virus mainly bound to the apical surface of the epithelium, whereas α2-3 linkage-specific lectin MAA and avian-like virus bound to the intracellular secretory granules of goblet cells. The authors suggested that receptor-mediated restriction on avian virus replication in humans can be determined by two simultaneous selective pressures that result from the predominant expression of 6-linked Sias on cells and from the predominant expression of 3-linked Sias on extracellular inhibitors. Over the next years,

other researchers often overinterpreted these data and assumed that avian viruses cannot infect humans due to a lack of cellular receptors for such viruses in humans.

Studies on human and avian virus infection in differentiated cultures of human airway epithelial cells (HAE) suggested that some cells in the human airway epithelium express sufficient amounts of receptors to allow infection with avian viruses and that receptor specificity determines the viral cell tropism in the epithelium [68, 69]. Early in infection, human viruses preferentially infected non-ciliated cells, whereas avian viruses mainly infected ciliated cells. This pattern correlated with the lectin binding to both live and fixed cultures, i.e., with strong binding of SNA to non-ciliated cells and preferential binding of MAA to ciliated cells. Neither receptor distribution nor cell tropism was absolute; for example, human viruses infected all types of cells later in infection, whereas avian viruses could infect non-ciliated cells. These results were generally well reproduced in the studies by different groups [70–72]; some quantitative differences in the cell tropism could be explained by the differences in experimental conditions and virus strains used. Interestingly, the H3N2 virus strains that were isolated in the first years of the pandemic displayed less prominent tropism to non-ciliated cells than epidemic H3N2 and H1N1 human viruses [69, 70], this feature was related to the less strict receptor specificity of the pandemic virus [24, 69]. Studies on virus infection in HAE cultures highlighted the fact that differences in replication and pathogenicity of human and avian influenza viruses may be partially related to their receptor-mediated differential cellular tropism in humans.

Several groups studied expression of viral receptors in human biopsies and archival tissues using lectins SNA, MAL-I and/or MAL-II and human and avian influenza viruses as molecular probes [73–77]. Although the binding data in these studies are mainly qualitative and are not directly comparable, all authors agree with each other and with the previous reports [66, 67] on the high expression level of receptors for human viruses in the human nasal, tracheal and bronchial epithelium and on a low expression of receptors for avian viruses in these regions of the airways. These results are consistent with the common theory that paucity of receptors for avian viruses in the upper respiratory tract may be one of the factors preventing efficient human-to-human transmission. Compared to airways, the concentration of avian-type receptors was higher and concentration of the human-type receptors was lower in the terminal bronchioles and alveoli, so that the relative amounts of two receptor types were similar in the low respiratory tract [73–77]. Interestingly, avian viruses preferentially bound to type II pneumocytes in the alveoli, whereas human viruses bound to type I pneumocytes [75]. The binding studies on fixed tissue sections cannot differentiate between functional and non-functional viral receptors. Nevertheless, the new findings about virus binding sites in the lung parenchyma [73–77] correlate with the features of H5N1 virus infection in humans, such as severe pneumonia with diffuse alveolar damage and viral replication in type II pneumocytes [78, 79]. This correlation raised the theory that avian-virus-like receptor specificity of

H5N1 influenza viruses can be responsible for their alveolar tropism in humans [73–75]. However, the formal proof of this theory is still missing. In particular, there is no consensus on the tissue tropism of avian influenza viruses in experimental infections of ex vivo explants of human respiratory tissues [73, 76] and no full picture of the H5N1 virus replication sites in infected patients [78–80].

Based on the presence of 3-linked Sias in the ocular epithelial cells and on the high incidence of conjunctivitis during the H7N7 avian influenza in the Netherlands in 2003, Olofsson et al. [81] suggested that the eye can serve as a port of entry for avian influenza viruses in humans. Further studies are needed to test this interesting hypothesis.

Receptor Specificity and Transmissibility of Influenza Viruses

Preferential binding of human pandemic viruses to 6-linked receptors suggested that a human-virus-like receptor specificity is a prerequisite for the efficient replication and human-to-human transmission of influenza viruses [7, 82, 83]. However, this assumption was based on circumstantial evidence. Indeed, Paulson and colleagues [62] had pointed that the shift in the receptor specificity of the avian virus HA in humans might occur over many passages and that receptor specificity may not necessarily represent a major barrier restricting the host range of a virus.

This question was directly addressed in two recent studies that used differentiated cultures of HAE as a model of human respiratory epithelium. Matrosovich et al. [69] used reverse genetics to prepare a pair of viruses that differed solely by two amino acids in the HA RBS. The first virus harboured the HA of the pandemic human virus A/Hong Kong/1/68 (H3N2), whereas the second virus was the L226Q/S228G HA mutant with avian-virus-like receptor specificity. The virus with an avian-like HA grew to 2- to 10-fold lower titres than did its counterpart with the human virus HA. Wan and Perez [72] assessed replication in HAE cultures of the natural and recombinant H9N2 poultry viruses that had either glutamine or leucine at HA position 226 and differed by receptor specificity (avian- or human-virus-like, respectively). The 226L-containing viruses reached approximately 100-fold-higher peak titres than those containing 226Q. These studies for the first time formally demonstrated significance of the receptor-dependent restriction for the avian influenza virus replication in human airway epithelium.

Transmission experiments in ferrets indicated that receptor specificity also plays a critical role in influenza virus transmissibility [84, also see Steel and Palese, this volume]. The authors compared respiratory droplet transmission of recombinant 1918 viruses with either original or mutated HA. The recombinant virus A/South Carolina/1/18 harboured HA with 190D and 225D. It bound mainly to 6-linked receptors and efficiently transmitted in ferrets. Virus with reversions at these HA positions towards the avian HA consensus sequence (190E and 225G) had a typical

avian-virus-like receptor specificity and did not transmit. A single-point HA mutant (225G) bound to both 3- and 6-linked Sia receptors and displayed substantially lower transmission efficiency than the parent virus. Notably, all three recombinant viruses replicated to similar titres in the upper respiratory tract of inoculated ferrets and were highly virulent. However, only the A/South Carolina/1/18 caused sneezing in infected animals. These results indicated that a low affinity for 3-linked Sias and/or strong binding to 6-linked Sias is essential for droplet transmission of influenza viruses in ferrets. This study also highlighted the fact that the mechanisms of receptor-mediated host range restriction are still not fully understood and that the extent of virus replication in the respiratory tract may only be one factor among others.

Mutations in the HA that Could Allow Adaptation of H5N1 Avian Influenza Viruses to Receptors in Humans

Avian H5N1 viruses that were isolated from infected humans usually had no mutations in the conserved positions of the Sia binding pocket and displayed preferential binding to 3-linked Sias typical for avian viruses [29, 41, 47, 85]. However, on a few occasions, viruses with mutations inside or near the RBS were isolated either as predominant variants or as minor variants in mixed virus populations. Thus, viruses A/Hong Kong/212/03 and A/Hong Kong/213/03 harboured HA mutation S227N and displayed a decreased affinity for Siaα2-3Gal-containing receptors and increased affinity for 6′SLN [41, 86]. A clonal variant of A/Vietnam/302811/04 had HA substitutions S227N and Q196R, whereas a clone of A/Thailand/KAN-1/04 carried mutations G143R and N186K [85]. Either virus was able to bind to 6-linked receptors. The role of these mutations in receptor specificity was confirmed by introducing them individually and in combinations into the HA of A/Vietnam/1194/04 using reverse genetics [85, also see review by Neumann et al., this volume]. In a more recent study, virus A/Thailand/676/05 isolated from a fatal human case was shown to bind equally well to both Siaα2-3Gal and Siaα2-6Gal due to a double mutation L133V and A138V [87]. In all these cases, the viral affinity for 6-linked receptor analogues did not exceed its affinity for the 3-linked analogues, suggesting that the viruses did not yet acquire the receptor-binding phenotype typical for pandemic viruses, i.e., preferential binding to 6-linked Sia receptors and weak if any binding to 3-linked ones [24, 59]. Furthermore, with the exception of substitution A138V, the described H5 mutations did not involve any of the six key positions of the avian RBS known to be associated with viral interspecies transmission [24, 27]. It seems likely therefore that the receptor-binding mutants of H5N1 viruses identified so far are not yet sufficiently adapted to receptors in humans to allow pandemic spread.

Potential effects of HA mutations responsible for the emergence of previous pandemic viruses in the context of H5N1 viruses were studied using H5 HA mutants

expressed from cDNA [47, 88]. Harvey et al. [88] showed that mutations Q226L and G228S introduced simultaneously into the HA of A/Hong Kong/156/97 resulted in a mixed 2-3/2-6 binding pattern. Stevens et al. [47] analyzed baculovirus-expressed H5 HA of the A/Vietnam/1203/04 virus using glycan microarray. They found that neither single nor combined mutations E190D and G225D allowed HA binding to Siaα2-6Gal-containing glycans. Mutation Q226L alone was also inefficient. A combination of this mutation with substitution G228S resulted in HA binding to both 3- and 6-linked receptors. However, the complete switch to the α2-6 specificity was not observed. These studies suggested that mutations that were responsible for the altered receptor specificity of the previous pandemic viruses may not have the same effect in H5 viruses.

Concluding Remarks

The widespread circulation of H5N1 avian influenza viruses and their occasional transmission to humans greatly stimulate research on the molecular mechanisms of host range restriction and pathogenicity of these viruses. The role of the viral receptor-binding specificity attracts particular attention. Recent progress in this field has been substantial due to the development of new receptor-binding assays, X-ray analyses of avian HA complexes with sialyloligosaccharides, generation of defined receptor-binding virus mutants using reverse genetics, and utilization of in vitro and ex vivo models of human airway epithelium.

It was found, in particular, that viruses of different avian species differ in their fine receptor specificity and that this variation corresponds to distinct patterns of expression of Sia receptors in the target tissues in different birds. It seems important to determine whether viruses of some avian species, such as domestic poultry, fit better to receptors in humans than other avian viruses. Studies in human airway epithelial cultures demonstrated that receptor specificity determines cell tropism of influenza viruses in the respiratory epithelium. These data emphasize the necessity of cell-level-oriented studies on the replication and pathogenicity of influenza viruses. Lectin- and virus-binding studies using human tissue sections suggest that amount and specificity of virus receptors vary in different regions of the respiratory tract. Further studies are needed to understand how these variations affect susceptibility of airway and alveolar epithelium to infection in vivo. Experiments with recombinant influenza viruses in a ferret contact model demonstrated that non-optimal receptor specificity may prevent airborne virus transmission. This finding further supports the concept that alterations of the receptor specificity of the HA is essential for the emergence of pandemic viral strains.

Despite significant recent advances, we still do not fully understand mechanisms of receptor-mediated host range restriction on virus transmission, have no solid data on the role of receptor specificity in viral tissue tropism, pathogenicity, innate and

specific immune responses, and cannot predict specific mutations that would allow H5N1 viruses to initiate a pandemic. Thus, we are left with many attractive targets for future studies.

Acknowledgements

Our own work was supported by the Deutsche Forschungsgemeinschaft (SFB 286 and SFB 593); the Fonds der Chemischen Industrie; the European Commission projects VIRGIL, ESNIP-2, FLUPATH, FLUINNATE and FLUPOL and the Russian Foundation for Basic Research (research grant 05-04-48934).

References

1 Schauer R, Kamerling JP: Chemistry, biochemistry and biology of sialic acids; in Montreuil J, Vliegenthart JFG, Schachter H (eds): Glycoproteins II. New Comprehensive Biochemistry. Amsterdam, Elsevier Science, 1997, vol 29b, pp 243–402.

2 Schauer R: Sialic acids: fascinating sugars in higher animals and man. Zoology (Jena) 2004;107:49–64.

3 Kelm S, Schauer R: Sialic acids in molecular and cellular interactions. Intern Rev Cytol 1997;175:137–240.

4 Lehmann F, Tiralongo E, Tiralongo J: Sialic acid-specific lectins: occurrence, specificity and function. Cell Mol Life Sci 2006;63:1331–1354.

5 Varki A: Glycan-based interactions involving vertebrate sialic-acid-recognizing proteins. Nature 2007; 446:1023–1029.

6 Suzuki Y: Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. Biol Pharm Bull 2005;28:399–408.

7 Matrosovich MN, Klenk H-D, Kawaoka Y: Receptor specificity, host range and pathogenicity of influenza viruses; in Kawaoka Y (ed): Influenza Virology: Current Topics. Wymondham, Caister Academic Press, 2006, pp 95–137.

8 Paulson JC: Interactions of animal viruses with cell surface receptors; in Conn M (ed): The Receptors. Orlando, Academic Press, 1985, vol 2, pp 131–219.

9 Herrler G, Hausmann J, Klenk H-D: Sialic acid as receptor determinant of ortho- and paramixoviruses; in Rosenberg A (ed): Biology of the Sialic Acids. New York, Plenum, 1995, pp 315–336.

10 Bergelson LD, Bukrinskaya AG, Prokazova NV, Shaposhnikova GI, Kocharov SL, Shevchenko VP, Kornilaeva GV, Fomina-Ageeva EV: Role of gangliosides in reception of influenza virus. Eur J Biochem 1982;128:467–474.

11 Matrosovich M, Suzuki T, Hirabayashi Y, Garten W, Webster RG, Klenk HD: Gangliosides are not essential for influenza virus infection. Glycoconj J 2006; 23:107–113.

12 Skehel JJ, Wiley DC: Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 2000;69:531–569.

13 Eisen MB, Sabesan S, Skehel JJ, Wiley DC: Binding of the influenza A virus to cell-surface receptors: structures of five hemagglutinin-sialyloligosaccharide complexes determined by X-ray crystallography. Virology 1997;232:19–31.

14 Ha Y, Stevens DJ, Skehel JJ, Wiley DC: X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. Proc Natl Acad Sci USA 2001;98: 11181–11186.

15 Ha Y, Stevens DJ, Skehel JJ, Wiley DC: X-ray structure of the hemagglutinin of a potential H3 avian progenitor of the 1968 Hong Kong pandemic influenza virus. Virology 2003;309:209–218.

16 Gamblin SJ, Haire LF, Russell RJ, Stevens DJ, Xiao B, Ha Y, Vasisht N, Steinhauer DA, Daniels RS, Elliot A, Wiley DC, Skehel JJ: The structure and receptor-binding properties of the 1918 influenza hemagglutinin. Science 2004;303:1838–1842.

17 Wang Q, Tian X, Chen X, Ma J: Structural basis for receptor specificity of influenza B virus hemagglutinin. Proc Natl Acad Sci USA 2007;104: 16874–16879.

18 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–179.

19 Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA: Global patterns of influenza a virus in wild birds. Science 2006;312: 384–388.

20 Munster VJ, Baas C, Lexmond P, Waldenstrom J, Wallensten A, Fransson T, Rimmelzwaan GF, Beyer WE, Schutten M, Olsen B, Osterhaus AD, Fouchier RA: Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog 2007;3:e61.
21 Matrosovich M, Klenk HD: Natural and synthetic sialic acid-containing inhibitors of influenza virus receptor binding. Rev Med Virol 2003;13:85–97.
22 Rogers GN, D'Souza BL: Receptor binding properties of human and animal H1 influenza virus isolates. Virology 1989;173:317–322.
23 Connor RJ, Kawaoka Y, Webster RG, Paulson JC: Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolates. Virology 1994;205: 17–23.
24 Matrosovich M, Tuzikov A, Bovin N, Gambaryan A, Klimov A, Castrucci MR, Donatelli I, Kawaoka Y: Early alterations of receptor-binding properties of H1, H2 and H3 avian influenza virus hemagglutinins after their introduction into mammals. J Virol 2000;74:8502–8512.
25 Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, Nakajima K: Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. Virology 1991;182:475–485.
26 Gambaryan AS, Tuzikov AB, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Bovin NV, Matrosovich MN: Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6′-sialyl(N-acetyllactosamine). Virology 1997;232:345–350.
27 Matrosovich MN, Gambaryan AS, Teneberg S, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Karlsson KA: Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. Virology 1997;233: 224–234.
28 Matrosovich MN, Gambaryan AS, Tuzikov AB, Byramova NE, Mochalova LV, Golbraikh AA, Shenderovich MD, Finne J, Bovin NV: Probing of the receptor-binding sites of the H1 and H3 influenza A and influenza B virus hemagglutinins by synthetic and natural sialosides. Virology 1993;196: 111–121.
29 Matrosovich M, Zhou N, Kawaoka Y, Webster R: The surface glycoproteins of H5 influenza viruses isolated from humans, chickens, and wild aquatic birds have distinguishable properties. J Virol 1999;73: 1146–1155.
30 Gambaryan AS, Robertson JS, Matrosovich MN: Effects of egg adaptation on the receptor-binding properties of human influenza viruses. Virology 1999;258:232–239.
31 Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, Capua I, Cordioli P, Fioretti A, Alexander DJ: Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 2001;146:963–973.
32 Campitelli L, Mogavero E, De Marco MA, Delogu M, Puzelli S, Frezza F, Facchini M, Chiapponi C, Foni E, Cordioli P, Webby R, Barigazzi G, Webster RG, Donatelli I: Interspecies transmission of an H7N3 influenza virus from wild birds to intensively reared domestic poultry in Italy. Virology 2004;323: 24–36.
33 Lin YP, Shaw M, Gregory V, Cameron K, Lim W, Klimov A, Subbarao K, Guan Y, Krauss S, Shortridge K, Webster R, Cox N, Hay A: Avian-to-human transmission of H9N2 subtype influenza A viruses: relationship between H9N2 and H5N1 human isolates. Proc Natl Acad Sci USA 2000;97: 9654–9658.
34 Matrosovich MN, Krauss S, Webster RG: H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology 2001;281: 156–162.
35 Saito T, Lim W, Suzuki T, Suzuki Y, Kida H, Nishimura SI, Tashiro M: Characterization of a human H9N2 influenza virus isolated in Hong Kong. Vaccine 2001;20:125–133.
36 Bovin NV: Polyacrylamide-based glycoconjugates as tools in glycobiology. Glycoconj J 1998;15:431–446.
37 Gambaryan AS, Tuzikov AB, Bovin NV, Yamnikova SS, Lvov DK, Webster RG, Matrosovich MN: Differences between influenza virus receptors on target cells of duck and chicken and receptor specificity of the 1997 H5N1 chicken and human influenza viruses from Hong Kong. Avian Dis 2003;47(suppl): 1154–1160.
38 Yamnikova SS, Gambaryan AS, Tuzikov AB, Bovin NV, Matrosovich MN, Fedyakina IT, Grinev AA, Blinov VM, Lvov DK, Suarez DL, Swayne DE: Differences between HA receptor-binding sites of avian influenza viruses isolated from Laridae and Anatidae. Avian Dis 2003;47(suppl):1164–1168.
39 Gambaryan AS, Tuzikov AB, Pazynina GV, Webster RG, Matrosovich MN, Bovin NV: H5N1 chicken influenza viruses display a high binding affinity for Neu5Acα2-3Galβ1-4(6-HSO3)GlcNAc-containing receptors. Virology 2004;326:310–316.

40 Gambaryan A, Yamnikova S, Lvov D, Tuzikov A, Chinarev A, Pazynina G, Webster R, Matrosovich M, Bovin N: Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain. Virology 2005;334:276–283.
41 Gambaryan A, Tuzikov A, Pazynina G, Bovin N, Balish A, Klimov A: Evolution of the receptor binding phenotype of influenza A (H5) viruses. Virology 2006;344:432–438.
42 Gambaryan A, Webster R, Matrosovich M: Differences between influenza virus receptors on target cells of duck and chicken. Arch Virol 2002;147: 1197–1208.
43 Kawaoka Y, Chambers TM, Sladen WL, Webster RG: Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology 1988;163:247–250.
44 Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, Rimmelzwaan GF, Olsen B, Osterhaus AD: Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls. J Virol 2005;79:2814–2822.
45 Gambarian A, Tuzikov A, Chinarev A, Pazynina G, Yamnikova S, Lvov D, Bovin N, Klimov A: Receptor specificity of influenza viruses from wild ducks, gulls, poultry, and mammals. Abstracts of The European Influenza Congress II, The European Scientific Group on Influenza, Malta, October 2005, P-S06-1a.
46 Paulson JC, Blixt O, Collins BE: Sweet spots in functional glycomics. Nat Chem Biol 2006;2:238–248.
47 Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA: Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science 2006;312:404–410.
48 Stevens J, Blixt O, Glaser L, Taubenberger JK, Palese P, Paulson JC, Wilson IA: Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J Mol Biol 2006;355:1143–1155.
49 Stevens J, Blixt O, Paulson JC, Wilson IA: Glycan microarray technologies: tools to survey host specificity of influenza viruses. Nat Rev Microbiol 2006; 4:857–864.
50 Ito T, Couceiro JNSS, Kelm S, Baum LG, Krauss S, Castrucci MR, Donatelli I, Kida H, Paulson JC, Webster RG, Kawaoka Y: Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol 1998;72:7367–7373.
51 Wan H, Perez DR: Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology 2006;346:278–286.
52 Guo CT, Takahashi N, Yagi H, Kato K, Takahashi T, Yi SQ, Chen Y, Ito T, Otsuki K, Kida H, Kawaoka Y, Hidari KI, Miyamoto D, Suzuki T, Suzuki Y: The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors. Glycobiology 2007;17:713–724.
53 Gambaryan AS, Marinina VP, Solodar' TA, Bovin NV, Tuzikov AB, Pazynina GV, Iamnikova SS, L'vov DK, Klimov AI, Matrosovich MN: Different receptor specificity of influence viruses from ducks and chickens and its reflection in the composition of sialosides on host cells and mucins. Vopr Virusol 2006;51:24–32.
54 Knibbs RN, Goldstein IJ, Ratcliffe RM, Shibuya N: Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from *Maackia amurensis*. Comparison with other sialic acid-specific lectins. J Biol Chem 1991;266:83–88.
55 Scholtissek C, Hinshaw VS, Olsen CW: Influenza in pigs and their role as the intermediate host; in Nicholson KG, Webster RG, Hay AJ (eds): Textbook of Influenza. London, Blackwell Science, 1998, pp 137–145.
56 Gambaryan AS, Karasin AI, Tuzikov AB, Chinarev AA, Pazynina GV, Bovin NV, Matrosovich MN, Olsen CW, Klimov AI: Receptor-binding properties of swine influenza viruses isolated and propagated in MDCK cells. Virus Res 2005;114:15–22.
57 Mochalova L, Gambaryan A, Romanova J, Tuzikov A, Chinarev A, Katinger D, Katinger H, Egorov A, Bovin N: Receptor-binding properties of modern human influenza viruses primarily isolated in Vero and MDCK cells and chicken embryonated eggs. Virology 2003;313:473–480.
58 Kobasa D, Takada A, Shinya K, Hatta M, Halfmann P, Theriault S, Suzuki H, Nishimura H, Mitamura K, Sugaya N, Usui T, Murata T, Maeda Y, Watanabe S, Suresh M, Suzuki T, Suzuki Y, Feldmann H, Kawaoka Y: Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature 2004;431:703–707.
59 Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, Tumpey TM, Basler CF, Taubenberger JK, Palese P: A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol 2005;79:11533–11536.
60 Reid AH, Janczewski TA, Lourens RM, Elliot AJ, Daniels RS, Berry CL, Oxford JS, Taubenberger JK: 1918 influenza pandemic caused by highly conserved viruses with two receptor-binding variants. Emerg Infect Dis 2003;9:1249–1253.
61 Stevens J, Corper AL, Basler CF, Taubenberger JK, Palese P, Wilson IA: Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. Science 2004;303:1866–1870.

62 Rogers GN, Paulson JC: Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 1983;127: 361–373.
63 Vines A, Wells K, Matrosovich M, Castrucci MR, Ito T, Kawaoka Y: The role of influenza A virus hemagglutinin residues 226 and 228 in receptor specificity and host range restriction. J Virol 1998;72: 7626–7631.
64 Karasin AI, Brown IH, Carman S, Olsen CW: Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. J Virol 2000;74:9322–9327.
65 Bateman AC, Landolt GA, Karasin AI, Busch MG, Olsen CW: Amino acids 223 and 225 of H4N6 influenza virus HA determine receptor specificity and swine respiratory epithelial cell infectivity. Options for the Control of Influenza VI, Toronto, Canada, 2007, abstract P202.
66 Baum LG, Paulson JC: Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. Acta Histochem Suppl 1990;40:S35–S38.
67 Couceiro JN, Paulson JC, Baum LG: Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. Virus Res 1993;29:155–165.
68 Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD: Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci USA 2004;101: 4620–4624.
69 Matrosovich M, Matrosovich T, Uhlendorff J, Garten W, Klenk HD: Avian-virus-like receptor specificity of the hemagglutinin impedes influenza virus replication in cultures of human airway epithelium. Virology 2007;361:384–390.
70 Thompson CI, Barclay WS, Zambon MC, Pickles RJ: Infection of human airway epithelium by human and avian strains of influenza a virus. J Virol 2006;80: 8060–8068.
71 Ibricevic A, Pekosz A, Walter MJ, Newby C, Battaile JT, Brown EG, Holtzman MJ, Brody SL: Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. J Virol 2006;80: 7469–7480.
72 Wan H, Perez DR: Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. J Virol 2007;81:5181–5191.
73 Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y: Avian flu: influenza virus receptors in the human airway. Nature 2006;440:435–436.
74 Van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T: H5N1 virus attachment to lower respiratory tract. Science 2006;312:399.
75 Van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T: Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol 2007;171:1215–1223.
76 Nicholls JM, Chan MC, Chan WY, Wong HK, Cheung CY, Kwong DL, Wong MP, Chui WH, Poon LL, Tsao SW, Guan Y, Peiris JS: Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat Med 2007;13:147–149.
77 Yao L, Korteweg C, Hsueh W, Gu J: Avian influenza receptor expression in H5N1-infected and noninfected human tissues. FASEB J 2007 DOI: 10.1096/fj.06-7880com.
78 Uiprasertkul M, Puthavathana P, Sangsiriwut K, Pooruk P, Srisook K, Peiris M, Nicholls JM, Chokephaibulkit K, Vanprapar N, Auewarakul P: Influenza A H5N1 replication sites in humans. Emerg Infect Dis 2005;11:1036–1041.
79 Gu J, Xie Z, Gao Z, Liu J, Korteweg C, Ye J, Lau LT, Lu J, Gao Z, Zhang B, McNutt MA, Lu M, Anderson VM, Gong E, Yu AC, Lipkin WI: H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 2007;370:1137–1145.
80 De Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha dQ, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J: Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 2006;12:1203–1207.
81 Olofsson S, Kumlin U, Dimock K, Arnberg N: Avian influenza and sialic acid receptors: more than meets the eye? Lancet Infect Dis 2005;5:184–188.
82 Horimoto T, Kawaoka Y: Pandemic threat posed by avian influenza A viruses. Clin Microbiol Rev 2001;14: 129–149.
83 Baigent SJ, McCauley JW: Influenza type A in humans, mammals and birds: determinants of virus virulence, host-range and interspecies transmission. Bioessays 2003;25:657–671.
84 Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A: A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 2007;315: 655–659.

85 Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y: Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. Nature 2006;444:378–382.

86 Shinya K, Hatta M, Yamada S, Takada A, Watanabe S, Halfmann P, Horimoto T, Neumann G, Kim JH, Lim W, Guan Y, Peiris M, Kiso M, Suzuki T, Suzuki Y, Kawaoka Y: Characterization of a human H5N1 influenza A virus isolated in 2003. J Virol 2005;79: 9926–9932.

87 Auewarakul P, Suptawiwat O, Kongchanagul A, Sangma C, Suzuki Y, Ungchusak K, Louisirirotch-anakul S, Lerdsamran H, Pooruk P, Thitithanyanont A, Pittayawonganon C, Guo CT, Hiramatsu H, Jampangern W, Chunsutthiwat S, Puthavathana P: An avian influenza H5N1 virus that binds to a human-type receptor. J Virol 2007;81:9950–9955.

88 Harvey R, Martin AC, Zambon M, Barclay WS: Restrictions to the adaptation of influenza a virus H5 hemagglutinin to the human host. J Virol 2004; 78:502–507.

Dr. Mikhail N. Matrosovich
Institute of Virology, Philipps University
Hans-Meerwein-Strasse 3, DE–35043 Marburg (Germany)
Tel. +49 6421 286 5166, Fax +49 6421 286 8962, E-Mail M.Matrosovich@gmail.com

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 156–167

Cleavage Activation of the Influenza Virus Hemagglutinin and Its Role in Pathogenesis

Wolfgang Garten · Hans-Dieter Klenk

Institute of Virology, Philipps University, Marburg, Germany

Abstract

The prime determinant of the pathogenicity of avian influenza viruses is proteolytic activation of the fusion capacity of HA. Cleavage is regulated by the structure of the cleavage site and the availability of appropriate host proteases. Most low-pathogenic avian influenza (LPAI) viruses have a single arginine at the cleavage site, whereas highly pathogenic (HPAI) viruses usually exhibit a multibasic cleavage site. LPAI viruses are activated by trypsin-like proteases restricted to enteric or respiratory epithelia. These viruses cause therefore localized infection. In contrast, HPAI viruses are activated by furin and related ubiquitous subtilases and allow therefore rapid dissemination of the virus throughout the organism. There is increasing evidence that HPAI viruses are derived from LPAI viruses by insertion of an appropriate cleavage site resulting from RNA recombination or by other insertion mechanisms that are less well understood. It is also not clear why such insertion events and, thus, the emergence of HPAI viruses have so far only been observed with subtypes H5 and H7. Attenuated mutants with altered cleavage sites have been obtained from HPAI viruses by reversed genetic methods. This approach has been successfully applied for the generation of pandemic H5 vaccines. The use of protease inhibitors for antiviral therapy appears to be promising, but this concept has so far not received much attention.

Introduction

Avian influenza viruses vary widely in virulence. The spectrum ranges form low-pathogenic avian influenza (LPAI) viruses that cause no or only mild disease symptoms to highly pathogenic (HPAI) strains that cause fowl plague or fowl plague-like disease. HPAI viruses are defined as H5 and H7 viruses with a polybasic hemagglutinin cleavage site that cause 75% or higher mortality after experimental infection of chickens. All viruses that do not meet these criteria are classified as LPAI viruses [1, 2]. LPAI virus replication is confined to the intestinal and respiratory tract. Virus is

shed in the feces. A common route of dissemination among aquatic birds is therefore through contaminated water. HPAI viruses are also shed in feces but they are more readily transmitted by the nasal and oral routes. In contrast to the local LPAI virus infection of the intestinal or respiratory tract, HPAI viruses cause systemic infection. As a result, virus can be recovered from many organs of infected animals. Large hemorrhages distributed all over the body, edema, and cutaneous ischemia are major symptoms of the disease. The final stage of the infection can be characterized by the emergence of neurological signs, such as photobia and dullness [3, 4]. HPAI viruses have been found to specifically target lymphocytes and lymphoid tissues [5, 6], myocytes in the heart muscle [7], and endothelial cells [8, 9], and these cell tropisms may play an important pathogenic role in systemic virus dissemination and in the vascular leakage underlying hemorrhages and edema.

The natural hosts of all LPAI viruses are wild aquatic birds of the orders *Anseriformes* (waterfowl) and *Charadriiformes* (shorebirds). They are the reservoir from where influenza A viruses cross the species barrier to birds and mammals [10, 11]. HPAI viruses are believed to arise, in general, by introduction of LPAI viruses of serotypes H5 and H7 into domestic poultry with subsequent mutations in HA as described below.

Although it has been known for a long time that the outcome of influenza virus infection is a multifactorial trait [12], there is increasing evidence that some viral proteins play a particularly prominent role. These are the hemagglutinin, the polymerase, and the NS1 protein. The pathogenetic functions of the polymerase proteins and of NS1 will be reviewed elsewhere in this volume. We will concentrate here on cleavage activation of the hemagglutinin as a determinant of pathogenicity.

Structure, Maturation and Function of HA

The major surface glycoprotein HA is integrated in the virus envelope as a type I membrane protein. More than 7,500 nucleotide sequences of HA of various virus isolates comprising serotypes H1–H16 have been determined, among them more than 900 HA sequences of H5N1 virus isolates alone [13]. The three-dimensional structure of several serotypes has been analyzed by X-ray crystallography: H1 [14, 15], H3 [16, 17], H5 [18–20], H7 [15] and H9 [20]. The ectodomain of HA represents 90% of the polypeptide chain. The residual 10% of the HA sequence account for the transmembrane domain and the cytosolic domain. HA is synthesized as a precursor molecule HA0 (75 kDa) which assembles to homotrimers. HA0 is N-glycosylated, palmitoylated, and proteolytically cleaved by host proteases. Crystallographic analysis of the uncleaved precursor has shown that the cleavage site is located in a loop formed by 19 amino acids of which 8 amino acids protrude from the surface of the membrane-proximal third of the HA trimer [17]. There are differences in the orientation of the

loop with serotypes H1 and H3 [14] both of which contain a monobasic cleavage site. Structural data are not available for the loop of HPAI viruses, but it can be assumed that it is extended by the insertion of the multibasic cleavage site (see below). The amino-terminal cleavage fragment HA1 (50 kDa) contains the receptor binding site, the carboxy-terminal fragment HA2 (25 kDa) is membrane anchored and responsible for fusion. Cleavage of HA is necessary for membrane fusion which plays a central role in the initiation of infection [21, 22]. The following steps can be discriminated in this process: virions are internalized by receptor mediated endocytosis, and the low pH within endosomes induces successive conformational changes of HA. (i) The cytoplasmic domain of HA2 dissociates from the matrix protein M1 after proton influx into the virion via M2 ion channels. (ii) HA1 is folded back, and the N-terminal hydrophobic peptide (fusion peptide) of HA2 is released from a cavity in the stem region behind the cleavage site and is then immersed into the endosomal target membrane. (iii) HA2 undergoes a drastic conformational change to a hairpin-like structure that merges lipids of virus and endosome. (iv). After formation of fusion pores, viral ribonucleoprotein complexes (RNPs) are delivered into the cytosol. RNPs are then transported into the nucleus, where transcription and replication occurs [23–25].

Differential Cleavage Activation of LPAI and HPAI Viruses

Proteolytic activation of HA is a prime determinant for the pathogenicity of avian influenza viruses. This concept was first derived from comparisons of naturally occurring strains [26–29] and was later further corroborated in numerous other studies. This earlier literature has previously been reviewed in detail by us [30] and others [24]. We will therefore give here only a brief outline of the concept and focus on more recent studies.

The hemagglutinin of LPAI viruses, that is usually an arginine but sometimes a lysine [31, 32], is activated at the monobasic cleavage site by proteases secreted form epithelial cells that are present only in respiratory or intestinal tissues. Infection is therefore restricted to these organs (fig. 1). Although a protease homologous to blood clotting factor Xa which was isolated from the chorioallantoic membrane of chicken embryos [33, 34] and a number of other trypsin-like proteases, such as plasmin and tryptase Clara, have been found to activate hemagglutinin with a monobasic cleavage site in vitro [for references, see 30, 35, 36], little is known about the enzymes that activate these viruses in their natural setting. Recently, two serine proteases (TMPRSS2 and HAT) from human airway epithelium have been found to activate human influenza A viruses as well as a LPAI virus [37]. However, these enzymes have not been identified yet in avian tissues. Bacterial proteases may also activate HAs of restricted cleavability and promote the development of pneumonia in mice after combined viral-bacterial infection [38]. Among the viruses activated by bacterial

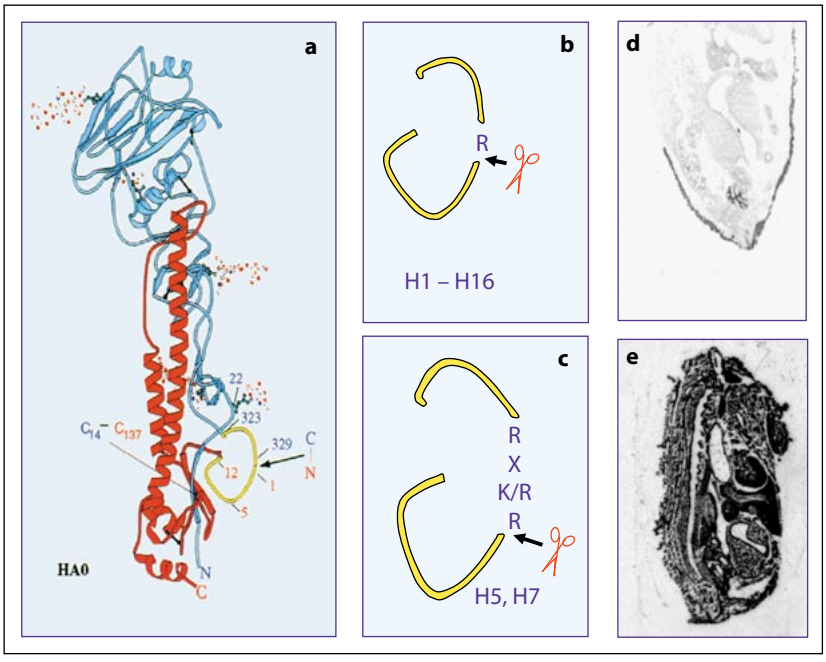


Fig. 1. The cleavage site of HA determines the pathogenicity of avian influenza viruses. HA is cleaved into subunits HA1 (blue) and HA2 (red). The cleavage site is located in a loop projecting from the surface of the molecule [17] (**a**). LPAI viruses (serotypes H1–H16) have a single arginine at the cleavage site that is recognized by trypsin-like proteases that are present only in specific tissues, such as intestinal epithelia (**b**). HPAI viruses (serotypes H5 and H7) are activated at a multibasic cleavage site R-X-K/R-R by the ubiquitous protease furin and furin-like proteases (**c**). Spread of the LPAI virus A/Chick(Germany/N/49 (H10N9) (**d**) and of the HPAI virus A/FPV/Rostock/34 (H7N1) (**e**) in chicken embryos. Thin sections were subjected to in situ hybridization with ^{35}UTP-labeled riboprobes specific for viral mRNA [8].

proteases are LPAI strains [39], but it is not known whether this type of activation plays a pathogenetic role in avian influenza.

HPAI viruses are activated by a different cleavage mechanism. Their hemagglutinins are activated at multibasic cleavage sites by furin, a member of the proprotein convertase family of eukaryotic subtilisin-like serine endoproteases [40]. The ubiquity of this enzyme accounts for the systemic infection typical for these viruses (fig. 1). Furin is a type I membrane protein of the constitutive secretory pathway. It is partially released at the plasma membrane as soluble enzyme and partially retrieved into the trans-Golgi network where it accumulates and co-localizes with HA [41–44]. Furin has a broad substrate spectrum of biologically important proteins, including nerve growth factor, insulin receptor, anthrax and shigella toxins, and many others [44, 45]. Besides HA of HPAI viruses, furin cleaves also a great number of glycoproteins of other enveloped viruses [for references, see 30, 46–48]. Other proprotein convertases

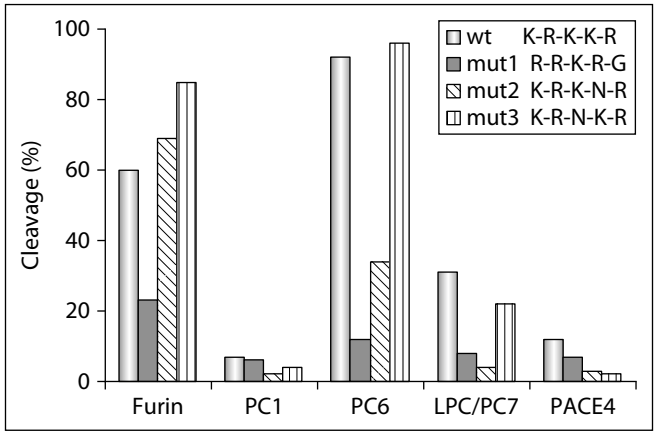


Fig. 2. Cleavage of FPV HA by subtilases as depending on the multibasic motif at the cleavage site. Wild-type and cleave site mutants of HA of influenza virus A/FPV/Rostock/34 (H7N1) were co-expressed with various subtilases in furin deficient Lovo cells from vaccinia virus vectors. 20 h p.i. cells were pulse-labeled with ^{35}S-methionine, and HA was analyzed by immunoprecipitation, SDS-PAGE, and autoradiography. The results show that HA is cleaved only by furin and PC6 and that cleavage requires the motif R-X-X-R. Positions -1 to -5 at the cleavage site are indicated [W. Garten, S. Hallenberger, S. Weigel, unpubl. results].

which resemble furin in structure and substrate specificity are PC1, PACE4, PC5/6, and LPC/PC7 [49]. Like furin, PC5/6 activates HAs with multibasic cleavage sites, whereas PC1, PACE and LPC/PC7 do not [50, 51] (fig. 2). All five proteases have been cloned from chicken tissues, and chicken furin which has 77% amino acid sequence homology with human furin was shown to activate FPV HA [52]. The HAs of most HPAI viruses have the consensus sequences R-X-K/R-R [for references, see 30] or R-X-X-R [for references, see 53] at the cleavage site, motifs that are both recognized by furin. Among the few exceptions to these rules is the HA of A/Chick/Pennsylvania/83 (H5N2) which contains the unusual tetrapeptide K-K-K-R [54]. Likewise, the HPAI virus A/Chicken/Leipzig/79 (H7N7) has a highly cleavable HA with the cleavage site K-K-K-K-R [55].

A multibasic cleavage site is not the only requirement for high cleavability. Another important determinant is a carbohydrate side chain close to the cleavage site that interferes with protease accessibility. Loss of this carbohydrate resulted in enhanced HA cleavability and pathogenicity [56]. However, masking of the cleavage site by this oligosaccharide was also overcome when the number of basic amino acids was increased [54, 57]. Finally, it was shown that HA can acquire high cleavability only if the basic sequence was introduced by insertion upstream of the cleavage site, but not by amino acid exchanges in the carboxy-terminus of HA1 [58]. Taken together, these observations indicate that the high cleavability of the HPAI virus HA

Virus		Cleavage site	Insertion mechanism	Reference
tur/Ore/71 (H7N3)	L	PENPKT----------R↓G---LF	Recombination with ribosomal 28S RNA	60
tur/Ore/TC1 (H7N3)	H	PENPKTSLSPLYPGRTTDLHV**RTAR**↓G---LF		
chi/Chi/176822/02 (H7N3)	L	PEKPK----------TR↓G---LF	Recombination with heterologous NP segment	62
chi/Chi/4957/02 (H7N3)	H	PEKPK--------TCSPLSRC**RKTR**↓G---LF		
chi/CN/6/04 (H7N3)	L	PENPK----------TR↓G---LF	Recombination with homologous M segment	63
chi/BC/NS1337-1/04 (H7N3)	H	PENPK----------QAYQK**RMTR**↓G---LF		
chi/Mex/31381-7/94 (H5N2)	L	PQ----------**RETR**↓G---LF	Polymerase slippage (?)	64
chi/Que/4588-19/95 (H5N2)	H	PQ----------RK**RKTR**↓G---LF		
tur/Ita/99 (H7N1)	L	PEIPKG----------R↓G---LF	unknown	66
tur/Ita/99 (H7N1)	H	PEIPKG----------SRVR**R**↓G---LF		
sea/Mass/1/80 (H7N7)	L	PENPKT----------R↓G---LF	unknown	59
sea/Mass/SC32 (H7N7)	H	PENPKT----------**RGRR**↓GLF		

Fig. 3. Emergence of HPAI from LPAI viruses by insertion of the motif R-X-K/R-R at the cleavage site of HA. The amino acid sequences at the cleavage site (arrow) of HPAI viruses (H) and their LPAI precursor viruses (L) are shown. Inserted amino acids are shaded, and the multibasic motifs are indicated by bold letters. Inserts are usually upstream of the original cleavage site, but there is also an example of a downstream insertion with restoration of the correct amino terminus of HA2 (A/Seal/Mass/SC32 (H7N7)). Note that A/Chicken/Mex/31381–7/94 (H5N2) contains a multibasic motif, although it is a LPAI virus. This observation underlines that high cleavability requires also an upstream insertion as present in the corresponding HPAI virus.

depends on the multibasic amino acid motif, an extended cleavage site loop, and the absence of a masking carbohydrate.

HPAI Viruses Are Derived from LPAI Viruses by Acquisition of a Multibasic Cleavage Site

Increased pathogenicity as a consequence of insertions at the cleavage site is a phenomenon that has been seen under experimental conditions as well as in natural outbreaks (fig. 3). It has first been observed in laboratory studies involving sequential cell culture passages of strains A/Seal/Massachusetts/1/80 (H7N7) [59] and A/turkey/Oregon/71 (H7N3). In the latter case, the acquisition of the furin recognition motif resulted from recombination of the HA gene with 28S ribosomal RNA [60, 61]. The HA gene may not only recombine with cellular RNA but also with other viral gene segments, as has been observed recently when new HPAI viruses emerging in the field have been analyzed. Thus, comparison of A/Chicken/Chile/02 (H7N3) isolates revealed that the HA genes of the highly pathogenic strains had an insertion of 30 nucleotides at the cleavage site that was presumably derived from the nucleoprotein gene of the unrelated A/Gull/Maryland/704/77 (H13N6) virus [62]. Recombination between HA and matrix protein genes of the same virus generated the highly pathogenic

A/Chicken/BC/04 (H7N3) viruses [63]. Polymerase slippage has been suggested as an alternative strategy by which a multibasic cleavage site is generated [64, 65]. However, there are other examples where the mechanism of insertion is not clear [66].

In fact, it is generally not understood why the cleavage site of H5 and H7 hemagglutinin is a hot spot for insertions. Possibly, there are specific interactions between the HA RNA and the nucleoprotein that facilitate insertions at this site. The exterior of the NP oligomers exposes a deep groove, in which the viral RNA is coiled up and accessible to the viral polymerase complex during cRNA synthesis [67, 68]. The vRNA of HA might have an unusual feature at the cleavage site and hence a disturbed interaction of the RNA with the viral polymerase complex may interfere with correct transcription. Several deviations seem possible: (i) Slippage of the viral transcriptional complex on the RNA template may lead to insertions of consecutive adenosines resembling the mechanisms by which editing of the glycoprotein gene of Ebola virus occurs [69, 70] or by which polyadenylation of mRNA is accomplished [71, 72]. (ii) Repeated transcription of the same RNA turn may result in a short amino acid sequence duplication. (iii) The polymerase complex together with the nascent cRNA may cross back to the HA-RNP. The result would be a non-homologous recombination with another influenza virus-specific segment. The recombining RNP may originate from the same or a different influenza virus. Non-homologous recombination with cellular RNA may be explained by a transient crossover to a single stranded RNA (ribosomal RNA).

Are HPAI Viruses Confined to Subtypes H5 and H7?

It is also not clear why the acquisition of a multibasic cleavage site and therefore the generation of HPAI viruses occurs in nature apparently only with subtypes H5 and H7. Interestingly however, high cleavability was also observed with a subtype H3 HA after in vitro insertion of a multibasic cleavage site and removal of an adjacent oligosaccharide by recombinant DNA technology [58]. Thus it appears that confinement of HPAI viruses to subtypes H5 and H7 cannot be attributed to structural restrictions of the HA protein, but that the responsible mechanisms are at the level of RNA replication with the RNA structure or, less likely, the polymerase structure as crucial determinants.

It has to be pointed out that amino acid sequences resembling the multibasic cleavage sites of HPAI viruses have also been observed in HAs of LPAI viruses that have retained restricted cleavability. Thus, H9N2 viruses with peptide motifs R-S-S-R and R-S-K-R at the cleavage site have been isolated on several occasions [73, 74]. It appears that these multibasic motifs have been generated only by substitutions of non-basic amino acids, but not by insertions. Since the size of the cleavage site loop which has to be extended for high cleavability was not affected, these H9N2 viruses exhibit low pathogenicity in birds. However, an amino acid insertion upstream of the

multibasic motif R-S-K-R would most likely convert these strains into HPAI viruses. Since H9N2 viruses have been transmitted to humans they belong to the avian strains with pandemic potential [75, 76]. The emergence of a highly pathogenic avian H9 virus would therefore be a matter of particular concern.

Use of Protease Activation Mutants as Vaccines

In contrast to natural evolution where HPAI viruses generally appear to be derived from LPAI viruses, recombinant viruses with reduced pathogenicity can be generated by in vitro mutation at the cleavage site. Thus, replacement of the multibasic cleavage site of a highly pathogenic H5N1 virus by a single arginine resulted in an attenuated virus that is used for mass production of an inactivated pandemic vaccine [77]. Protease activation mutants that are cleaved by elastase, an activating enzyme not occurring in a natural setting, are even less pathogenic and have the potential to be used as live vaccines. This strategy was first tested on a human strain [78] and has now been applied to a HPAI virus [79].

Use of Protease Inhibitors as Antiviral Agents

Replication of human influenza virus in mice was suppressed by administration of ε-aminocaproic acid and aprotinin [80]. Aprotinin (6 kDa), leupeptin (0.45 kDa) and *Cucurbita maxima* trypsin inhibitor (3 kDa) have also been used [81, 82]. Our preliminary data show that HAT and TMPRSS2 are sensitive to aprotinin and some other inhibitors. It remains to be seen whether these inhibitors effectively prevent propagation of LPAI viruses.

Inhibition of furin by peptidyl-chloromethylketons interferes with growth of HPAI viruses. We demonstrated that decanoylated R-V-K-R-cmk inhibits cleavage of FPV-HA, cell-cell fusion induced by cleaved HA, and virus replication in cell culture [40, 83, 84]. There are various other inhibitors specific for furin and closely related proprotein convertases, such as bioengineered serpins and short polybasic peptides (e.g. hexa-D-arginine) [85–88]. Moreover, small synthetic inhibitors were designed on the basis of the 3D structure of furin [89]. One of the most promising compounds appears to be a 2,5-dideoxystreptamine derivative that inhibits furin in the nanomolar range [90]. However, none of these substances has been tested for its ability to interfere with influenza virus replication.

Since these inhibitors target cellular enzymes they will not elicit the generation of drug-resistant viruses. This should be a major advantage when compared to conventional influenza antivirals, such as neuraminidase inhibitors. A short application time as required for a highly acute HPAI infection might compensate for the possible toxicity of the compounds. Thus, there is clearly a need to investigate their therapeutic potential.

References

1 Alexander DJ: Control strategies of the International Office of Epizooties, the European Union and the harmonization of international standards for the diagnosis of avian influenza; in Swayne DE, Slemons RD (eds): Fourth International Symposium on Avian Influenza. Richmond/VA, United States Animal Health Association, 1998, pp 353–357.

2 OIE: Manual Standards for Diagnostic Tests and Vaccines, 2000. Paris, Office International de Épizooties, 2003.

3 Gratzl E, Koehler H: Geflügelpest; in Spezielle Pathologie und Therapie der Geflügelkrankheiten. Stuttgart, Enke, 1968.

4 Narayan O, Thorsen J, Hulland TJ, Ankeli G, Joseph PG: Pathogenesis of lethal influenza virus infection in turkeys. I. Extraneural phase of infection. J Comp Pathol 1972;82:129–137.

5 Van Campen H, Easterday BC, Hinshaw VS: Virulent avian influenza A viruses: their effect on avian lymphocytes and macrophages in vivo and in vitro. J Gen Virol 1989;70:2887–2895.

6 Van Campen H, Easterday BC, Hinshaw VS: Destruction of lymphocytes by a virulent avian influenza A virus. J Gen Virol 1989;70:467–472.

7 Kobayashi Y, Horimoto T, Kawaoka Y, Alexander DJ, Itakura C: Pathological studies of chickens experimentally infected with two highly pathogenic avian influenza viruses. Avian Pathol 1996;25:285–304.

8 Feldmann A, Schafer MK, Garten W, Klenk HD: Targeted infection of endothelial cells by avian influenza virus A/FPV/Rostock/34 (H7N1) in chicken embryos. J Virol 2000;74:8018–8027.

9 Suarez DL, Perdue ML, Cox N, Rowe T, Bender C, Huang J, Swayne DE: Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. J Virol 1998;72: 6678–6688.

10 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–179.

11 Olsen B, Munster VJ, Wallensten A, Waldenstrom J, Osterhaus AD, Fouchier RA: Global patterns of influenza a virus in wild birds. Science 2006;312: 384–388.

12 Klenk HD, Rott R: The molecular biology of influenza virus pathogenicity. Adv Virus Res 1988;34: 247–281.

13 Chang S, Zhang J, Liao X, Zhu X, Wang D, Zhu J, Feng T, Zhu B, Gao GF, Wang J, Yang H, Yu J: Influenza Virus Database (IVDB): an integrated information resource and analysis platform for influenza virus research. Nucleic Acids Res 2007;35: D376–D380.

14 Stevens J, Corper AL, Basler CF, Taubenberger JK, Palese P, Wilson IA: Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. Science 2004;303:1866–1870.

15 Russell RJ, Gamblin SJ, Haire LF, Stevens DJ, Xiao B, Ha Y, Skehel JJ: H1 and H7 influenza haemagglutinin structures extend a structural classification of haemagglutinin subtypes. Virology 2004;325: 287–296.

16 Wilson IA, Skehel JJ, Wiley DC: Structure of the haemagglutinin membrane glycoprotein of influenza virus at 3 A resolution. Nature 1981;289: 366–373.

17 Chen J, Lee KH, Steinhauer DA, Stevens DJ, Skehel JJ, Wiley DC: Structure of the hemagglutinin precursor cleavage site, a determinant of influenza pathogenicity and the origin of the labile conformation. Cell 1998;95:409–417.

18 Garten W, Vey M, Ohuchi R, Ohuchi M, Klenk HD: Modification of the cleavage activation of the influenza virus hemagglutinin by site-specific mutagenesis. Behring Inst Mitt 1991:12–22.

19 Stevens J, Blixt O, Tumpey TM, Taubenberger JK, Paulson JC, Wilson IA: Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. Science 2006;312:404–410.

20 Ha Y, Stevens DJ, Skehel JJ, Wiley DC: H5 avian and H9 swine influenza virus haemagglutinin structures: possible origin of influenza subtypes. EMBO J 2002;21:865–875.

21 Klenk HD, Rott R, Orlich M, Blodorn J: Activation of influenza A viruses by trypsin treatment. Virology 1975;68:426–439.

22 Lazarowitz SG, Choppin PW: Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology 1975;68:440–454.

23 Skehel JJ, Wiley DC: Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem 2000;69:531–569.

24 Steinhauer DA: Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology 1999;258:1–20.

25 Weissenhorn W, Dessen A, Calder LJ, Harrison SC, Skehel JJ, Wiley DC: Structural basis for membrane fusion by enveloped viruses. Mol Membr Biol 1999;16:3–9.

26 Bosch FX, Garten W, Klenk HD, Rott R: Proteolytic cleavage of influenza virus hemagglutinins: primary structure of the connecting peptide between HA1 and HA2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology 1981;113:725–735.

27 Garten W, Bosch FX, Linder D, Rott R, Klenk HD: Proteolytic activation of the influenza virus hemagglutinin: the structure of the cleavage site and the enzymes involved in cleavage. Virology 1981;115: 361–374.
28 Garten W, Linder D, Rott R, Klenk HD: The cleavage site of the hemagglutinin of fowl plague virus. Virology 1982;122:186–190.
29 Garten W, Klenk HD: Characterization of the carboxypeptidase involved in the proteolytic cleavage of the influenza haemagglutinin. J Gen Virol 1983;64: 2127–2137.
30 Klenk HD, Garten W: Host cell proteases controlling virus pathogenicity. Trends Microbiol 1994;2: 39–43.
31 Guenther I, Glatthaar B, Doller G, Garten W: A H1 hemagglutinin of a human influenza A virus with a carbohydrate-modulated receptor binding site and an unusual cleavage site. Virus Res 1993;27:147–160.
32 Kawaoka Y, Yamnikova S, Chambers TM, Lvov DK, Webster RG: Molecular characterization of a new hemagglutinin, subtype H14, of influenza A virus. Virology 1990;179:759–767.
33 Gotoh B, Ogasawara T, Toyoda T, Inocencio NM, Hamaguchi M, Nagai Y: An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. EMBO J 1990; 9:4189–4195.
34 Suzuki H, Harada A, Hayashi Y, Wada K, Asaka J, Gotoh B, Ogasawara T, Nagai Y: Primary structure of the virus activating protease from chick embryo. Its identity with the blood clotting factor Xa. FEBS Lett 1991;283:281–285.
35 Kido H, Murakami M, Oba K, Chen Y, Towatari T: Cellular proteinases trigger the infectivity of the influenza A and Sendai viruses. Mol Cells 1999;9: 235–244.
36 Quang Lea T, Kawachia M, Yamada H, Shiota M, Okumura Y, Kido H: Identification of trypsin I as a candidate for influenza A virus and Sendai virus envelope glycoprotein processing protease in rat brain. Biol Chem 2006;387:1305.
37 Boettcher E, Matrosovich T, Beyerle M, Klenk HD, Garten W, Matrosovich M: Proteolytic activation of influenza viruses by serine proteases TMPRSS2 and HAT from human airway epithelium. J Virol 2006;80: 9896–9898.
38 Tashiro M, Ciborowski P, Klenk HD, Pulverer G, Rott R: Role of *Staphylococcus* protease in the development of influenza pneumonia. Nature 1987;325: 536–537.
39 Tashiro M, Ciborowski P, Reinacher M, Pulverer G, Klenk HD, Rott R: Synergistic role of staphylococcal proteases in the induction of influenza virus pathogenicity. Virology 1987;157:421–430.
40 Stieneke-Groeber A, Vey M, Angliker H, Shaw E, Thomas G, Roberts C, Klenk HD, Garten W: Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J 1992;11:2407–2414.
41 Schaefer W, Stroh A, Berghoefer S, Seiler J, Vey M, Kruse ML, Kern HF, Klenk HD, Garten W: Two independent targeting signals in the cytoplasmic domain determine trans-Golgi network localization and endosomal trafficking of the proprotein convertase furin. EMBO J 1995;14:2424–2435.
42 Stroh A, Schafer W, Berghofer S, Eickmann M, Teuchert M, Burger I, Klenk HD, Garten W: A mono phenylalanine-based motif (F790) and a leucine-dependent motif (LI760) mediate internalization of furin. Eur J Cell Biol 1999;78:151–160.
43 Teuchert M, Berghofer S, Klenk HD, Garten W: Recycling of furin from the plasma membrane. Functional importance of the cytoplasmic tail sorting signals and interaction with the AP-2 adaptor medium chain subunit. J Biol Chem 1999;274: 36781–36789.
44 Thomas G: Furin at the cutting edge: from protein traffic to embryogenesis and disease. Nat Rev Mol Cell Biol 2002;3:753–766.
45 Steiner DF: The proprotein convertases. Curr Opin Chem Biol 1998;2:31–39.
46 Richt JA, Furbringer T, Koch A, Pfeuffer I, Herden C, Bause-Niedrig I, Garten W: Processing of the Borna disease virus glycoprotein gp94 by the subtilisin-like endoprotease furin. J Virol 1998;72: 4528–4533.
47 Vey M, Schafer W, Reis B, Ohuchi R, Britt W, Garten W, Klenk HD, Radsak K: Proteolytic processing of human cytomegalovirus glycoprotein B (gpUL55) is mediated by the human endoprotease furin. Virology 1995;206:746–749.
48 Volchkov VE, Volchkova VA, Stroher U, Becker S, Dolnik O, Cieplik M, Garten W, Klenk HD, Feldmann H: Proteolytic processing of Marburg virus glycoprotein. Virology 2000;268:1–6.
49 Seidah NG, Chretien M: Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res 1999;848:45–62.
50 Horimoto T, Nakayama K, Smeekens SP, Kawaoka Y: Proprotein-processing endoproteases PC6 and furin both activate hemagglutinin of virulent avian influenza viruses. J Virol 1994;68:6074–6078.
51 Hallenberger S, Moulard M, Sordel M, Klenk HD, Garten W: The role of eukaryotic subtilisin-like endoproteases for the activation of human immunodeficiency virus glycoproteins in natural host cells. J Virol 1997;71:1036–1045.

52 Feldmann A: Gezielte Infektion von Endothelzellen durch das Virus der klassischen Geflügelpest A/FPV/Rostock/34 (H7N1) im Hühnerembryo. Marburg, Philipps University, 1998.
53 Neumann G, Kawaoka Y: Host range restriction and pathogenicity in the context of influenza pandemic. Emerg Infect Dis 2006;12:881–886.
54 Kawaoka Y, Webster RG: Interplay between carbohydrate in the stalk and the length of the connecting peptide determines the cleavability of influenza virus hemagglutinin. J Virol 1989;63:3296–3300.
55 Roehm C, Suss J, Pohle V, Webster RG: Different hemagglutinin cleavage site variants of H7N7 in an influenza outbreak in chickens in Leipzig, Germany. Virology 1996;218:253–257.
56 Kawaoka Y, Naeve CW, Webster RG: Is virulence of H5N2 influenza viruses in chickens associated with loss of carbohydrate from the hemagglutinin? Virology 1984;139:303–316.
57 Ohuchi M, Orlich M, Ohuchi R, Simpson BE, Garten W, Klenk HD, Rott R: Mutations at the cleavage site of the hemagglutinin after the pathogenicity of influenza virus A/Chick/Penn/83 (H5N2). Virology 1989;168:274–280.
58 Ohuchi R, Ohuchi M, Garten W, Klenk HD: Human influenza virus hemagglutinin with high sensitivity to proteolytic activation. J Virol 1991;65:3530–3537.
59 Li SQ, Orlich M, Rott R: Generation of seal influenza virus variants pathogenic for chickens, because of hemagglutinin cleavage site changes. J Virol 1990; 64:3297–3303.
60 Khatchikian D, Orlich M, Rott R: Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. Nature 1989;340:156–157.
61 Morsy J, Garten W, Rott R: Activation of an influenza virus A/turkey/Oregon/71 HA insertion variant by the subtilisin-like endoprotease furin. Virology 1994;202:988–991.
62 Suarez DL, Senne DA, Banks J, Brown IH, Essen SC, Lee CW, Manvell RJ, Mathieu-Benson C, Moreno V, Pedersen JC, Panigrahy B, Rojas H, Spackman E, Alexander DJ: Recombination resulting in virulence shift in avian influenza outbreak, Chile. Emerg Infect Dis 2004;10:693–699.
63 Pasick J, Handel K, Robinson J, Copps J, Ridd D, Hills K, Kehler H, Cottam-Birt C, Neufeld J, Berhane Y, Czub S: Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. J Gen Virol 2005;86:727–731.
64 Garcia M, Crawford JM, Latimer JW, Rivera-Cruz E, Perdue ML: Heterogeneity in the haemagglutinin gene and emergence of the highly pathogenic phenotype among recent H5N2 avian influenza viruses from Mexico. J Gen Virol 1996;77:1493–1504.
65 Perdue ML, Garcia M, Senne D, Fraire M: Virulence-associated sequence duplication at the hemagglutinin cleavage site of avian influenza viruses. Virus Res 1997;49:173–186.
66 Banks J, Speidel ES, Moore E, Plowright L, Piccirillo A, Capua I, Cordioli P, Fioretti A, Alexander DJ: Changes in the haemagglutinin and the neuraminidase genes prior to the emergence of highly pathogenic H7N1 avian influenza viruses in Italy. Arch Virol 2001;146:963–973.
67 Martin-Benito J, Area E, Ortega J, Llorca O, Valpuesta JM, Carrascosa JL, Ortin J: Three-dimensional reconstruction of a recombinant influenza virus ribonucleoprotein particle. 2001;2:313–317.
68 Ye Q, Krug RM, Tao YJ: The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. Nature 2006;444:1078–1082.
69 Volchkov VE, Becker S, Volchkova VA, Ternovoj VA, Kotov AN, Netesov SV, Klenk HD: GP mRNA of Ebola virus is edited by the Ebola virus polymerase and by T7 and vaccinia virus polymerases. Virology 1995;214:421–430.
70 Volchkov VE, Volchkova VA, Muhlberger E, Kolesnikova LV, Weik M, Dolnik O, Klenk HD: Recovery of infectious Ebola virus from complementary DNA: RNA editing of the GP gene and viral cytotoxicity. Science 2001;291:1965–1969.
71 Wahle E, Ruegsegger U: 3′-End processing of pre-mRNA in eukaryotes. FEMS Microbiol Rev 1999;23: 277–295.
72 Neumann G, Brownlee GG, Fodor E, Kawaoka Y: Orthomyxovirus replication, transcription, and polyadenylation. Curr Top Microbiol Immunol 2004;283: 121–143.
73 Perk S, Banet-Noach C, Shihmanter E, Pokamunski S, Pirak M, Lipkind M, Panshin A: Genetic characterization of the H9N2 influenza viruses circulated in the poultry population in Israel. Comp Immunol Microbiol Infect Dis 2006;29:207–223.
74 Xu KM, Li KS, Smith GJ, Li JW, Tai H, Zhang JX, Webster RG, Peiris JS, Chen H, Guan Y: Evolution and molecular epidemiology of H9N2 influenza A viruses from quail in Southern China, 2000–2005. J Virol 2007;81:2635–2645.
75 Guo CT, Takahashi N, Yagi H, Kato K, Takahashi T, Yi SQ, Chen Y, Ito T, Otsuki K, Kida H, Kawaoka Y, Hidari KI, Miyamoto D, Suzuki T, Suzuki Y: The quail and chicken intestine have sialyl-galactose sugar chains responsible for the binding of influenza A viruses to human type receptors. Glycobiology 2007;17:713–724.

76 Wan H, Perez DR: Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. J Virol 2007;81:5181–5191.
77 Subbarao K, Chen H, Swayne D, Mingay L, Fodor E, Brownlee G, Xu X, Lu X, Katz J, Cox N, Matsuoka Y: Evaluation of a genetically modified reassortant H5N1 influenza A virus vaccine candidate generated by plasmid-based reverse genetics. Virology 2003;305:192–200.
78 Stech J, Garn H, Wegmann M, Wagner R, Klenk HD: A new approach to an influenza live vaccine: modification of the cleavage site of hemagglutinin. Nat Med 2005;11:683–689.
79 Gabriel G, Garn H, Wegmann M, Renz H, Herwig A, Klenk HD, Stech J: The potential of a protease activation mutant of a highly pathogenic avian influenza virus for a pandemic live vaccine. Vaccine 2008;26:956–965.
80 Zhirnov OP, Ovcharenko AV, Bukrinskaya AG: Suppression of influenza virus replication in infected mice by protease inhibitors. J Gen Virol 1984;65:191–196.
81 Zhirnov OP, Ovcharenko AV, Bukrinskaya AG: Myxovirus replication in chicken embryos can be suppressed by aprotinin due to the blockage of viral glycoprotein cleavage. J Gen Virol 1985;66:1633–1638.
82 Zhirnov OP, Ikizler MR, Wright PF: Cleavage of influenza a virus hemagglutinin in human respiratory epithelium is cell associated and sensitive to exogenous antiproteases. J Virol 2002;76:8682–8689.
83 Garten W, Stieneke A, Shaw E, Wikstrom P, Klenk HD: Inhibition of proteolytic activation of influenza virus hemagglutinin by specific peptidyl chloroalkyl ketones. Virology 1989;172:25–31.
84 Garten W, Hallenberger S, Ortmann D, Schafer W, Vey M, Angliker H, Shaw E, Klenk HD: Processing of viral glycoproteins by the subtilisin-like endoprotease furin and its inhibition by specific peptidylchloroalkylketones. Biochimie 1994;76:217–225.
85 Jean F, Thomas L, Molloy SS, Liu G, Jarvis MA, Nelson JA, Thomas G: A protein-based therapeutic for human cytomegalovirus infection. Proc Natl Acad Sci U S A 2000;97:2864–2869.
86 Lu W, Zhang W, Molloy SS, Thomas G, Ryan K, Chiang Y, Anderson S, Laskowski M Jr: Arg15-Lys17-Arg18 turkey ovomucoid third domain inhibits human furin. J Biol Chem 1993;268:14583–14585.
87 Sarac MS, Cameron A, Lindberg I: The furin inhibitor hexa-D-arginine blocks the activation of *Pseudomonas aeruginosa* exotoxin A in vivo. Infect Immun 2002;70:7136–7139.
88 Fugere M, Appel J, Houghten RA, Lindberg I, Day R: Short polybasic peptide sequences are potent inhibitors of PC5/6 and PC7: use of positional scanning-synthetic peptide combinatorial libraries as a tool for the optimization of inhibitory sequences. Mol Pharmacol 2007;71:323–332.
89 Henrich S, Cameron A, Bourenkov GP, Kiefersauer R, Huber R, Lindberg I, Bode W, Than ME: The crystal structure of the proprotein processing proteinase furin explains its stringent specificity. Nat Struct Biol 2003;10:520–526.
90 Jiao GS, Cregar L, Wang J, Millis SZ, Tang C, O'Malley S, Johnson AT, Sareth S, Larson J, Thomas G: Synthetic small molecule furin inhibitors derived from 2,5-dideoxystreptamine. Proc Natl Acad Sci USA 2006;103:19707–19712.

Wolfgang Garten
Institute of Virology, Philipps University Marburg
Hans-Meerwein-Strasse 2, DE–35043 Marburg (Germany)
Tel. +49 6421 286 5145, Fax +49 6421 286 8962, E-Mail garten@staff.uni-marburg.de

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 168–186

Structure and Function of the Influenza A Virus Ribonucleoprotein: Transcription and Replication

Juan Ortín

Centro Nacional de Biotecnología (CSIC), Darwin 3, Cantoblanco, Madrid, Spain

Abstract

Influenza A viruses normally infect aquatic and terrestrial avian species without causing disease. From this reservoir, the viruses are occasionally transmitted to human or other mammalian species and establish new virus lineages that are the cause of pandemics and annual epidemics of respiratory disease. The transfer of avian influenza viruses to humans can take place by reassortment with an already existing human virus or by direct adaptation. These processes involve the fixation of mutations in many viral genes, among which those involved in viral replication in the new host cell are very important. In this article, the mechanisms of virus RNA transcription and replication are reviewed with special emphasis on the structural studies and on the interaction with cellular host factors, as improving our knowledge about these aspects will be essential for understanding the adaptation process.

Introduction

The genome of type A influenza viruses consists of a set of ribonucleoprotein particles (RNPs), each one containing a single-stranded RNA molecule of negative polarity [for a review, see 1]. As a whole, these RNA segments contain the information for 11 proteins, all of which except NS1 and PB1-F2 are components of the viral particle. The sequence of the virus genomic segments is almost fully conserved at their 5′- and 3′-terminus and show partial complementarity [2]. Thus, they can interact with each other to generate a closed conformation of the RNP [3]. The association of the viral RNA termini is maintained by interaction with the polymerase complex, while the rest of each RNA segment is bound to a number of nucleoprotein (NP) monomers [4]. The polymerase complex contains two basic (PB1 and PB2) and one acidic subunit (PA), whose roles are described in detail below.

Contrary to most viruses containing RNA as a genome, the influenza virus RNPs are independently transcribed and replicated in the nucleus of the infected cells [5]. These processes give rise to three types of viral RNA for each RNP, namely mRNA, cRNA and vRNA. The mRNAs are viral transcripts generated by cap snatching of cell pre-mRNAs [6]. They are incomplete transcripts, since their 3′-end poly(A) tail is generated by polymerase stuttering at an oligo-U polyadenylation signal located around 17 nt from the vRNA 5′-terminus [7, 8]. On the other hand, viral cRNAs are complete copies of the genomic vRNAs and are not capped [9]. In addition, these cRNAs are encapsidated with NP monomers and the polymerase complex, in a way similar to the parental virus RNPs (vRNPs) and serve as templates for the synthesis of progeny vRNPs in the nucleus.

Viral Ribonucleoprotein as RNA Synthesis Machine

The viral RNPs are ribbon-like structures which adopt a supercoiled conformation [10, 11]. Each ribbon unit represents a NP monomer and each RNP contains a single polymerase complex. Thus, in spite of not being directly visible by regular electron microscopy, only a polymerase entity can be detected by immunoelectron microscopy at one end of the supercoiled structure [12]. The general helical conformation of the RNPs is generated by the structure of the NP, as complexes formed by purified NP and unrelated RNA adopt helical structures [13]. Furthermore, purified NP can also form RNP-like helical particles in the absence of RNA [14]. The superhelical conformation of natural virus RNPs is determined by interaction among the viral RNA termini and association with the polymerase complex [4].

Structural Components of the RNP

The virion RNPs contains all essential components for viral RNA synthesis, namely the polymerase and the NP, in association with the virus RNA template. Transfection of these elements in normal cells is sufficient for the replication and transcription processes to occur [15–17].

Polymerase

The virus polymerase complex is a heterotrimer with an aggregate molecular mass of about 250 kDa, made up by the PB1, PB2 and PA subunits. All subunits are necessary to carry out transcription and replication [18–21] and their roles in these processes have been partially unraveled.

PB1 as Catalytic Subunit

The PB1 subunit is central in the polymerase complex, both because it is the core of the trimer [22] and because it is responsible for RNA synthesis itself. Thus, it contains

the sequence landmarks present in other RNA polymerases [23] and mutations of these conserved motifs abolish the capacity of the polymerase complex to synthesize RNA [24]. Consistent with its role as polymerase, a NTP-binding site has been mapped to this subunit [25, 26]. Furthermore, elongation of polymerase cross-linked ATP has allowed to locate the initiator nucleotide-binding site within the PB1 subunit [27]. However, the PB1 subunit may not only be responsible for RNA synthesis but may also act as endonuclease during the generation of capped primers for transcription initiation (see below). Such a hypothesis is supported by polymerase cross-linking experiments using thio-U-labeled capped oligonucleotides, in which PB1 subunit is specifically labeled [28]. The polymerase and endonuclease activities require binding to the virus RNA template [29, 30] and PB1 is a central but not exclusive actor in promoter recognition [31]. Hence, the isolated PB1 subunit can specifically recognize the vRNA promoter sequence with an affinity close to that shown by the polymerase complex [32]. However, contradictory results were obtained when mapping the PB1 protein regions involved in vRNA promoter recognition [32–34]. Moreover, the protein regions required for binding to the cRNA promoter were partly distinct from those used for recognition of the vRNA promoter [32, 35]. These mapping results are compatible with the recently reported differential thermolability of vRNA-polymerase and cRNA-polymerase interactions [36].

PB2 Subunit Is Involved in Cap Recognition

A wealth of experimental data support the contention that PB2 subunit is involved in cap recognition during the initial steps for viral mRNA transcription: (i) Temperature-sensitive virus mutants in PB2 are affected in virus mRNA synthesis, sometimes without alterations in virus RNA replication [37], and show altered cap recognition [38]. Likewise, directed PB2 mutations affecting the RNP activity in a recombinant system show defects in cap recognition [39]. (ii) Cross-linking studies using capped RNAs or cap analogs have identified PB2 as the cap-binding subunit within the polymerase [40, 41]. However, contradictory results have been reported on the localization of the cap-binding site within the subunit: cross-linking mapping identified various protein regions [28, 42] whereas mutations altering aromatic residues potentially involved in cap recognition showed the importance of Phe363 and Phe404 in such a function [43]. As indicated above, the endonuclease activity has not been mapped to the PB2, but to the PB1 subunit [28].

The implication of PB2 in virus RNA replication is a matter of discussion. Thus, whereas some reports suggested that it is dispensable [44, 45], others pointed to the need of all three polymerase subunits for full activity [18, 20] and specific mutations within PB2 showed normal transcription but reduced replication capacity [19].

PA Subunit Induces Proteolytic Degradation

The role that the PA protein plays within the polymerase is less clear. The classical genetic data indicated that it is important for viral RNA replication [37] but a number

of site-directed mutations generated over the years relate the PA protein with cRNA synthesis [46, 47], cap snatching [48], and cap binding and vRNA promoter recognition [49]. The only biochemical activity reported for PA protein relates to protein degradation. Thus, when expressed from cDNA it induces a generalized proteolytic degradation [50]. Although this activity was mapped to the N-terminal region of the protein [51], a serine-protease activity was also described whose active site would be located close to the C-terminus [52]. However, a virus point mutant defective in the N-terminal activity showed reduced RNA replication and was attenuated in mice [46] whereas mutation of the serine protease-active site led to viruses with near wt phenotype [53]. The presence of PA protein in the polymerase complex is essential for transcriptional and replicative activities [15, 16, 18, 20, 21], but some reports have suggested the possible role of a PB1-PB2 heterodimer in virus RNA transcription [45].

Nucleoprotein

The NP is an essential factor for virus RNA transcription and replication [15–17], since the naked virus RNA does not serve as an efficient template. Hence, mutations in the NP have been shown to impede viral RNA synthesis [54–57]. The NP serves two functions during viral transcription and replication: as an essential structural element in the template RNP and as packaging factor during encapsidation of progeny RNA into a new RNP structure. Central for these roles are the abilities of NP to interact with RNA, to oligomerize and to interact with the virus polymerase complex and cellular factors.

The NP binds single-stranded RNA without sequence specificity, the sugar-phosphate moieties being protected from chemical modification but the bases exposed to the solvent [58]. Protein sequences involved in RNA binding have been identified by in vitro binding after deletion mapping or site-directed mutagenesis [56, 59, 60] but their relevance to NP-RNA interaction must await for detailed structural analysis of NP-RNA complexes (see below). In spite of the lack of sequence specificity for RNA binding, the NP is only associated to viral RNA in infected cells. The basis for such specificity is not known but may be related to an exclusive binding of NP to RNA in the active RNP replicating complex. Such a model predicts that newly synthesized NP would be unable to interact with RNA until it is incorporated into progeny RNPs. Its interaction with the cellular splicing factor UAP56 has been proposed to serve such a function [61] (see below).

RNA Template

All influenza A virus vRNAs contain almost conserved terminal sequences (13 nt at the 5′-end and 12 nt at the 3′-end) and segment-specific conserved sequences up to the positions complementary to the ATG and termination codons. A run of 5–8 consecutive U residues are located close to the conserved 3′-terminal sequence and constitute the mRNA polyadenylation signal [7]. These conserved terminal sequences constitute the promoter for viral transcription and replication. Thus, a recombinant

vRNA comprising a non-viral ORF flanked by viral UTRs can replicate, be transcribed and become encapsidated into virions [62]. The viral vRNA promoter can form a panhandle conformation by RNA-RNA interaction [3, 58] that is stabilized by interaction with the polymerase [4]. Essential for this conformation are RNA-RNA interactions that form a proximal clamp structure, located close to the center of the virus RNA in the panhandle and formed also by segment-specific complementary sequences [63–65].

Structure of the Promoter

The structure of the virus RNA promoter is not completely clear at this point in time. Data collected using various experimental approaches, including NMR studies on model virus RNAs, RNA probing experiments in vitro, interaction studies and structure-function relationships of RNA point mutants analyzed in vitro and in vivo have led to several non-exclusive proposals: the panhandle, fork and corkscrew models.

The panhandle model includes a proximal stem, corresponding to the clamp region referred to above, a less organized area or loop and a terminal stem in which the 5′- and 3′-termini are held together. This model is supported by RNA probing data [58] and NMR studies of model RNAs [66, 67] and may represent the most stable structure of a short naked RNA. On the contrary, the fork and corkscrew models [63, 68] propose that RNA sequences terminal to the clamp structure do not interact to each other and remain available for binding to the polymerase. The corkscrew model further suggests that specific intra-strand interactions exist that would be essential for polymerase recognition and RNA synthesis (the handles of the corkscrew). These models are supported by the in vitro and in vivo phenotypes of point mutants and compensatory double mutants in the promoter RNA [63, 68–70]. These data indicate that such a promoter structure is important for viral RNA synthesis at some point in the process. In summary, although the NMR structure of the promoter may be the most accurate for a naked RNA, it is possible that some of its details are modulated by interaction of the template with the polymerase complex and NP within the RNP or during the transcription and replication processes.

Much less is known about the structure of the cRNA promoter, but the data available indicate that it is quite different from the vRNA promoter. The terminal stem does not exist in the NMR model and the internal loop is inverted [71, 72].

Three-Dimensional Structure of the Ribonucleoprotein

Ribonucleoprotein Complex

The virus RNPs are closed, supercoiled structures in which both RNA ends interact to each other and to the polymerase complex [reviewed in 73]. These structures are highly flexible and hence difficult to analyze in detail, but recombinant RNPs generated in vivo with short model vRNAs could be studied by electron microscopy and

image processing, leading to some general conclusions [74]: (i) For templates with a length below 350 nt the conformation of the RNP is circular or elliptical, whereas for longer templates superhelical structures are observed. (ii) A single polymerase complex is visualized that interacts differently with the adjacent NP monomers, and (iii) the ratio of the template RNA length and the number of NP monomers visualized indicates that each one associates to about 24 nt [74].

A three-dimensional model has been reported for one such recombinant mini-RNP [75], in which the NP-NP and NP-polymerase interactions are apparent and an improved model with enhanced resolution has been recently obtained for the same RNP [R. Coloma et al., unpubl. data]. The structure contains 9 NP monomers in a circular configuration and a polymerase complex. The NP monomers show a vorticity that could explain the superhelical conformation of recombinant RNPs with longer template RNAs or normal viral RNPs. Two protein domains can be distinguished in each NP monomer and the NP-NP interactions are established mainly through the bottom, larger domains. The interactions of the polymerase with the adjacent NP monomers in the RNP are distinct and involve either the top or the bottom domains in NP. The most intense protein-protein contacts appear to be established between the polymerase PB2 subunit and the bottom domain of one NP monomer, whereas lighter interaction seems to occur between the top domain of the other adjacent NP and the PB1 subunit of the polymerase. Although the position of the template RNA in the structure cannot be directly visualized, it is tempting to speculate that most of the molecule is positioned at the bottom of the particle, where a tighter interaction between NP monomers is apparent (but see below).

Polymerase

The structure of the polymerase complex present in the RNP has also been determined by electron microscopy and three-dimensional reconstruction (fig. 1) [76]. The model obtained shows a compact complex with no apparent delineation of their subunits. The position of specific subunit domains within the complex was determined by three-dimensional reconstruction of tagged polymerase-containing RNPs or RNP-monoclonal antibody complexes [76]. The N-terminal regions of both PB1 and PB2 appear close to the location of the adjacent NP monomers, in agreement with the biochemical NP-polymerase interaction data determined by biochemical assays [57, 77, 78]. More recently, the three-dimensional structure of the recombinant polymerase complex, not associated to the RNP, has been obtained by the same techniques [79]. The model obtained is a globular, hollow structure that contains an internal cavity and several channels connecting with the outer space. Its structure is very similar to that previously reported for the polymerase complex associated to viral RNPs [76] but shows a more open conformation. A detailed comparison and computational docking of both models indicated that specific areas in the complex show important conformational changes, especially in regions of the polymerase, which contact the adjacent NP monomers in the RNP. Thus, it is tempting to speculate that

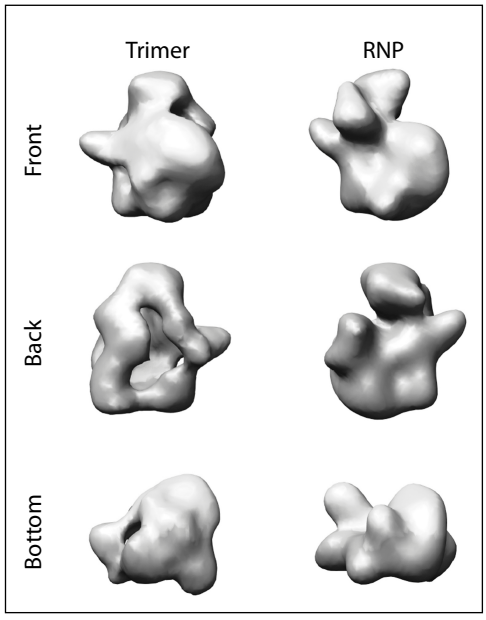


Fig. 1. Three-dimensional model for the soluble polymerase heterotrimer. The structural model for the soluble influenza polymerase heterotrimer (Trimer) is shown in comparison to the structure described for the polymerase complex present in the RNP (RNP) [76]. The various regions of the complex have been colored differently to help in structural comparison [adapted from 79].

polymerase-template and polymerase-NP interactions modulate the structure of the complex in a significant way.

The structural information of the polymerase at the atomic level is scanty at the present time. Only the structure of a C-terminal portion of the PB2 subunit is known, that encompasses the nuclear localization signal (NLS) for this subunit [80]. This region of the protein contains amino acid residues known to be involved in virus host range and hence its structure may help in understanding the mechanisms relevant for virus polymerase-host interactions (see below). Indeed, the structure of the PB2 C-terminus bound to importin 5 has allowed the redefinition of the previously mapped NLS [80].

Nucleoprotein

Electron microscopy analysis show that each NP monomer has a banana-like elongated structure, both when expressed as a RNA-free single protein [14] and when it is a component of a virus RNP [74, 75, and R. Coloma et al., unpubl. data]. Recent data on the atomic structure of isolated NP monomers [81] indicate that it contains two domains with a similar appearance to those described for NPs of *Mononegavirales* [82–84] but with a different protein topology. Interaction among the NP monomers occurs through a protein-flexible loop in the bottom large domain, in agreement with previous electron microscopy results [75]. Within the NP monomer, RNA binding has been proposed to occur in a channel between the two protein domains. Interaction with RNA could take place by contacts with amino acid residues located

throughout the protein sequence, as suggested by mutation analyses [56, 59, 60]. The distance between two consecutive RNA-binding sites is about 70 Å [81], consistent with the stoichiometry of 24 nt per NP monomer, although the length of RNA fitting within the predicted binding site may be shorter.

Mechanisms for Viral Transcription and Replication

Promoter Recognition

As indicated above, the complex between the 5′- and 3′-terminal conserved sequences in the viral vRNAs constitute the core promoter for RNA synthesis. Recognition of the promoter depends almost exclusively on the 5′-arm of the promoter, as corresponding oligonucleotides can be effectively bound by the polymerase [20, 29, 32]. Binding to the 3′-arm is weak but its recruitment to the polymerase is highly improved by the presence of the 5′-arm [29, 32], in agreement with the requirement for RNA synthesis of the proximal clamp indicated above. The recognition of the 5′-arm is structure-dependent. Thus, mutations at some positions abolish polymerase interaction [31] but these mutations would affect formation of the internal intra-strand stem loop proposed in the corkscrew model and the phenotypes can be partly rescued by compensatory mutations [69]. Although the 5′-arm of the promoter is sufficient for high-affinity binding to the polymerase, interaction to the preformed 5′-3′ core promoter is most effective [32] and the cap binding and endonuclease activities of such initiation complexes are improved as compared to sequentially formed complexes [85].

As indicated above, the cRNA and vRNA promoter structures differ substantially. This is reflected in a distinct recognition of the cRNA promoter by the polymerase, as shown by the in vitro binding of the PB1 subunit with the 5′- and 3′-arms of the cRNA promoter [35]. In contrast to the situation for vRNA promoter, the 3′-arm is efficiently recognized by the polymerase. These structural differences may also account for the preferential encapsidation of vRNPs versus cRNPs. The former are exported from the nucleus whereas the latter are not [86] and the 5′-bulged structure of the vRNA core promoter seems to play a role in the selection process [87].

Transcription versus Replication

In addition to mutations affecting template-polymerase interaction, that should affect all downstream-related activities, some sequence alterations have been shown to alter viral transcription without influencing the promoter-polymerase binding [63, 88]. Particularly interesting is the bulged A at position 10, that can be mutated but not deleted and seems to function as a flexible link between the two sections of the panhandle/corkscrew structure [63, 68, 70].

Binding of the 5′-terminal arm of the promoter is essential for cap recognition [30]. However, the requirements for cap snatching are not clear at present. It was first proposed that the entry into the complex of the 3′-terminal arm of the promoter would activate the endonuclease activity [30, 33, 89], but such a sequential model was not supported by subsequent experiments [85]. Furthermore, no requirement for 3′-arm interaction with the polymerase was observed for cleavage of host mRNAs that would generate a CA-terminal sequence in the cap primer [90], which are the most abundant ones among those observed in viral mRNAs [91].

Is There a Switch from Transcription to Replication?

The first products of viral RNA replication, the cRNAs, can be distinguished from the viral mRNAs because (i) they are initiated de novo instead of by cap snatching, (ii) therefore they contain a triphosphate at their 5′-ends, (iii) they are complete copies of vRNAs and are not polyadenylated, and (iv) they are encapsidated with NP monomers and polymerase as the vRNPs. The prevalent model to account for these differences implies the existence of a switch in the activity of parental RNPs early in the infection, from a transcription to a replication mode. Several hypotheses have been put forward to explain such transcription-to-replication switch, including the presence of newly synthesized NP monomers, that could alter the structure of the template RNP or the polymerase [4, 55, 77, 78, 92], the proteolytic activity of PA protein [46, 47] or the interactions with host cell factors (see below). However, recent evidence has challenged these views: the previous expression of polymerase and NP in the cell allows the detection of both mRNA and cRNA early in the infection, under conditions of complete inhibition of protein synthesis [93]. These results suggest that the parental RNPs are able to both transcribe and replicate upon entry in the infected cell nucleus and the presence of newly synthesized polymerase and NP is only required for stabilization of the cRNA product. Furthermore, virion-derived RNPs have been shown to synthesize cRNA in vitro in the absence of added polymerase or NP [94], suggesting that no other viral or cellular factors are strictly required to induce replication by the parental RNPs.

RNA Chain Initiation

The mechanism for transcription initiation in the influenza viruses has become a paradigm since it was first described by Krug's group [6]. The viral RNPs recognize nascent cellular mRNAs via PB2 cap binding [38] and cleave them some 10–15 nt downstream by the endonuclease activity located in PB1 [33]. Such capped oligonucleotides are then used as primers to copy the vRNA template within the RNP. Since most primers have CA 3′-terminal sequence, it is presumed that priming occurs by annealing to the U residue in the template and addition of a G residue opposite to the penultimate C. Thus, the first nucleotide would not be transcribed but provided by the primer. A transition in the kinetics of synthesis occurs at position 4 [95] suggesting that the change to the elongation mode takes place at this position. In contradistinction to the initiation of mRNA synthesis, vRNA and cRNA initiation takes place

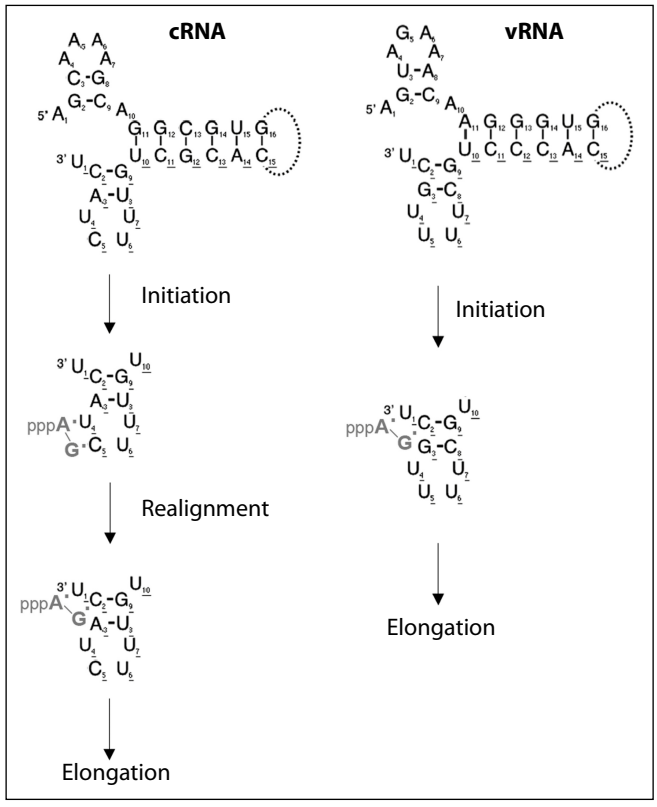


Fig. 2. Model for the mechanisms of initiation of vRNA and cRNA synthesis. The corkscrew models for the cRNA and vRNA promoters are shown at the top. De novo initiation takes place at positions 4 and 5 from the 3′-end of the cRNA promoter and the pppApG dinucleotide is transferred to positions 1 and 2 for elongation. In contrast, initiation on the vRNA promoter takes place directly on positions 1 and 2 [adapted from 97].

de novo, as a 5′-triphosphate can be found in these viral RNA species [9, 96] and the underlying mechanism is less understood. A widely used in vitro system for influenza RNA synthesis utilize the dinucleotide ApG as primer, but such activity does not reflect real initiation of viral RNA replication. Recently, in vitro systems have been developed for the analysis of de novo initiation of RNA replication (fig. 2) [27, 97]. The analysis of mutant cRNA and vRNA promoters with such systems has allowed proposing different mechanisms for the initiation of vRNA and cRNA synthesis. Initiation of the latter would be carried out using the 3′-terminal UG bases of vRNA as template. On the contrary, initiation of vRNA synthesis would use internal positions (U4 and G5) near the 3′-end of the cRNA template for the synthesis of an ApG dinucleotide that would then be transposed opposite to the 3′-terminal UG for further elongation, by means of a prime-and-realign mechanism [97]. Such a situation has precedents in the initiation of replication of DNA viruses [98], positive-stranded

RNA viruses [99] and negative-stranded RNA viruses [100], and may play a role in the maintenance of the genetic information at the regulatory sequences, which are critical for viral replication.

Chain Termination versus Polyadenylation

During transcription of virus RNPs the polymerase is unable to copy the 5′-terminal region of the vRNA template. Instead, it copies several times a short run of U residues located around 17 nt from the 5′-end. Several reports have indicated that this process in dependent on the association of the polymerase to the 5′-terminus of the vRNA template [8, 70, 101, 102], suggesting that the 5′-bound polymerase would constitute a steric barrier for the copy of the final sequence in the template and would induce the stuttering at the polyadenylation site. A consequence of this model is that the release of the polymerase from its 5′-terminal binding site would be required for replication to take place and the mechanism for such a change is not yet understood.

RNA Encapsidation

Another clear distinction exists between mRNA and cRNA synthesis: the latter is coupled to encapsidation of the RNA product into a cRNP structure while the former is not. As the initiation mechanisms for either one are distinct (see above) it is tempting to assume that the initiation and encapsidation (or lack of encapsidation) have to be coupled as well, in order to avoid the generation of cap-containing cRNPs or encapsidated mRNAs. The mechanism for such a potential coupling is unclear, but may be related to RNP interactions with other viral or cellular factors (see below).

Cellular Factors Involved in Viral RNA Replication and Gene Expression

Polymerase Complex Formation

The regions of the polymerase subunits involved in complex formation have been determined by a combination of biochemical and genetic approaches [39, 78, 103–107]. The PB1 protein is the core of the complex and makes contacts with the C-terminal sequences of PA by means of its N-terminal region, whereas several areas in PB1 protein interact with the PB2 subunit. No interaction between PB2 and PA proteins has been described.

Nuclear localization signals for each polymerase subunit have been identified [108–110] and the structure of the C-terminal NLS in PB2 has been re-evaluated recently by co-crystal formation with importin α5 [80]. However, the pathway and the intracellular site for complex formation are not clear at present. Nuclear transport of PB1 expressed as a GFP fusion protein from recombinant DNA is inefficient and can be enhanced by co-expression of PA subunit [111]. This fact together with the results of in vitro assembly of active polymerase from a PB1-PA heterodimer and singly expressed PB2 [112] suggest a pathway for in vivo polymerase complex formation in which a

PB1-PA dimer would be formed in the cytoplasm and transported to the nucleus independently of the PB2 subunit. The complete heterotrimer would then be formed in the nucleus. In agreement with these results, the PB1 subunit has been shown to interact with RanBP5 (importin β3) and silencing of this transport factor abolished nuclear import of PB1-PA dimer and reduced viral RNA synthesis [113]. However, these data contrast with the kinetics of nuclear import of polymerase subunits during a virus infection, which shows a delayed import of PA protein as compared with PB1 or PB2 [114]. Furthermore, a recent report describing the interaction of Hsp90 with PB1 and PB2 subunits suggest the possible formation of PB1-PB2 or PB1-PA heterodimers, their association with Hsp90 and co-transport into the nucleus [115, 116] and open up alternative pathways for polymerase complex formation in infected cells.

Transcription and Viral mRNA Export

Early studies indicated that viral transcription depends on active cellular mRNA synthesis, as the latter is also inhibited by actinomycin D or α-amanitin [117], in line with the cap-snatching mechanism for initiation of virus mRNA synthesis. Recent reports extended these observations and showed that there is a physical association of the viral polymerase and the cellular RNA polymerase II complex, particularly with those forms of the complex phosphorylated at Ser5, an association that is mediated by the CTD of RNA Pol-II [118]. The interaction of both viral and cellular polymerase complexes may be direct or may take place by other cellular factors, as the hCLE protein [119], a positive regulator of cellular mRNA synthesis that associates to the RNA Pol-II complex [120]. The association of viral RNA polymerase to the cell mRNA transcription machine might be essential for virus transcription and has relevant consequences for cellular mRNA synthesis, as the elongation process is inhibited [121] and the non-phosphorylated forms of RNA Pol-II are degraded during infection [122].

Little is known about the mechanisms for influenza virus mRNA export. First of all, various types of viral mRNAs are produced in the infected cell: Most of them do not contain introns, some are normally spliced (M2 and NEP) and yet others contain introns that are not removed before nucleocytoplasmic export (M1 and NS1). Hence, not all virus mRNAs can be exported by the most relevant, EJC/TAP-p15-dependent, cell mRNA export pathway [123]. In addition, it has been recently reported that influenza virus NS1 protein interacts with TAP-p15 export complex and Nup98 to inhibit normal cellular mRNA export [124], suggesting that most viral mRNA export should utilize alternative ways. One option would be the SR protein-dependent pathway [125], a possibility that is supported by recent results that link viral mRNA export to the association of virus transcription to the RNA Pol-II machinery [126] and the NS1-dependent inhibition of export of unspliced virus mRNA [127]. In this regard it is worth mentioning that the cellular cap-binding complex (CBC) is an important factor in the export and gene expression of cellular mRNAs [128] and at present it is not known whether CBC displaces the viral polymerase from the cap structure of viral mRNAs and plays a similar role for the expression of viral proteins.

Replication

A search for cellular factors important for viral RNA replication has been carried out by testing in vitro the stimulation of viral RNA synthesis with cellular extracts. In this way, two cellular fractions were purified – RAF-1 and RAF-2 [129]. The factor included in RAF-1 fraction was identified as Hsp90 and a role for it was proposed as remodeling factor for the polymerase complex during its activity on the RNP template [116] (but see Polymerase Complex Formation above). The RAF-2 fraction was characterized as a heterodimer with subunits of 36 and 48 kDa, the larger of which was identified as UAP56 [61], a potential RNA helicase involved in cellular mRNA splicing and export [130, 131]. It has been reported that UAP56 can bind RNA-free but not RNA-bound NP molecules and promotes the association of NP to RNA [61], suggesting that it may serve as a chaperone to modulate the interaction of influenza NP with the template RNA. According to this proposal, newly synthesized NP molecules would associate to UAP56 and thus be prevented to bind unspecific RNA. Such NP-UAP56 complexes would deliver new NP subunits to encapsidate progeny RNA during replication.

In addition to these directed searches for cellular factors involved in virus RNA replication, recent general proteomic approaches have been carried out. Recombinant virus RNPs or polymerase complexes have been prepared using strep-labeled NP or TAP-tagged polymerase subunits and purified by affinity chromatography. The cellular factors thus co-purified have been analyzed by mass spectrometry and a large number of protein factors have been identified [132, and N. Jorba, unpubl. results]. These included various nuclear proteins involved in transcription and splicing, RNA helicases and hnRNP proteins, but also cytoplasmic proteins and proteins from the cytoskeleton. One of the identified protein factors, nucleophosmin (NPM), was shown to re-localize during the infection from the nucleolus to the nucleoplasm and to enhance the activity of recombinant virus RNPs in vivo [132]. Much work will be necessary to determine the relevance of these polymerase/RNP-host associations and their role during the infection.

Some of the interactions between the viral replication machine and the host cell may play a role in the recruitment of the necessary elements to specific sites of viral RNA replication. Indeed, early reports indicated that viral RNA synthesis is associated to the nuclear cage and take place at fixed sites in the nucleus of infected cells [133] and synthesis of vRNA in vitro was found associated to nuclear matrix preparations [134], although no specific host factor has yet been identified as responsible for such intracellular localization.

Concluding Remarks

The recent outbreaks of highly pathogenic H5N1 avian influenza viruses, their spread into wild avian populations, and the appearance of occasional infections in humans

have opened the possibilities that a new pandemic might occur by either reassortment with normal human influenza strains or by direct adaptation to the human host. Although adaptation would imply changes in many viral genes, it has been shown that alterations in the viral transcription and replication machine are essential for efficient replication and pathogenesis of avian influenza in humans [135, 136]. Hence it is important to be prepared for a potential new pandemic by improving our understanding of the transcription and replication mechanisms of influenza virus and the implications and roles of host cell factors in these processes. Particularly relevant for this purpose will be the elucidation of detailed structures of the polymerase subunits and their interactions within the virus RNP, as well as the identification and the roles of the host factors involved in these processes. Recent advances in these topics suggest that new developments may be in sight.

Acknowledgments

Work in the author's laboratory was supported by grants BMC2001-1223 and BFU2004-491 from Ministerio de Educación y Ciencia, the VIRHOST Programme from Comunidad de Madrid, the European Vigilance Network for the Management of Antiviral Drug Resistance (VIRGIL) and the FLUPOL Strep Project (SP5B-CT-2007-044263) from the European Union.

References

1 Palese P, Shaw M: Orthomyxoviridae: the viruses and their replication; in Knipe DM, Howley PM (eds): Fields Virology, ed 5. Philadelphia, Lippincott Williams & Wilkins, 2006, vol 1, pp 1647–1689.
2 Skehel JJ, Hay AJ: Nucleotide sequences at the 5′-termini of influenza virus RNAs and their transcripts. Nucleic Acid Res 1978;5:1207–1219.
3 Hsu MT, Parvin JD, Gupta S, Krystal M, Palese P: Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc Natl Acad Sci USA 1987;84:8140–8144.
4 Klumpp K, Ruigrok RW, Baudin F: Roles of the influenza virus polymerase and nucleoprotein in forming a functional RNP structure. EMBO J 1997;16:1248–1257.
5 Herz C, Stavnezer E, Krug RM, Gurney T: Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. Cell 1981;26:391–400.
6 Krug RM, Broni BA, Bouloy M: Are the 5′-ends of influenza viral mRNAs synthesized in vivo donated by host mRNAs? Cell 1979;18:329–334.
7 Robertson JS, Schubert M, Lazzarini RA: Polyadenylation sites for influenza mRNA. J Virol 1981;38:157–163.
8 Poon LLM, Pritlove DC, Fodor E, Brownlee GG: Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template. J Virol 1999;73:3473–3476.
9 Hay AJ, Skehel JJ, McCauley J: Characterization of influenza virus RNA complete transcripts. Virology 1982;116:517–522.
10 Compans RW, Content J, Duesberg PH: Structure of the ribonucleoprotein of influenza virus. J Virol 1972;4:795–800.
11 Pons MW, Schulze IT, Hirst GK: Isolation and characterization of the ribonucleoprotein of influenza virus. Virology 1969;39:250–259.
12 Murti KG, Webster RG, Jones IM: Localization of RNA polymerases of influenza viral ribonucleoproteins by immunogold labeling. Virology 1988;164:562–566.
13 Yamanaka K, Ishihama A, Nagata K: Reconstitution of influenza virus RNA-nucleoprotein complexes structurally resembling native viral ribonucleoprotein cores. J Biol Chem 1990;265:11151–11155.

14 Ruigrok RW, Baudin F: Structure of influenza virus ribonucleoprotein particles. II. Purified RNA-free influenza virus ribonucleoprotein forms structures that are indistinguishable from the intact influenza virus ribonucleoprotein particles. J Gen Virol 1995; 76:1009–1014.
15 Huang TS, Palese P, Krystal M: Determination of influenza virus proteins required for genome replication. J Virol 1990;64:5669–5673.
16 De la Luna S, Martín J, Portela A, Ortín J: Influenza virus naked RNA can be expressed upon transfection into cells co-expressing the three subunits of the polymerase and the nucleoprotein from SV40 recombinant viruses. J Gen Virol 1993;74:535–539.
17 Mena I, de la Luna S, Martín J, Albó C, Perales B, Nieto A, Portela A, Ortín J: Systems to express recombinant RNA molecules by the influenza A virus polymerase in vivo; in Adolph KW (ed): Methods in Molecular Genetics. Molecular Virology Techniques, Part B. Orlando, Academic Press, 1995, pp 329–342.
18 Perales B, Ortín J: The influenza A virus PB2 polymerase subunit is required for the replication of viral RNA. J Virol 1997;71:1381–1385.
19 Gastaminza P, Perales B, Falcón AM, Ortín J: Influenza virus mutants in the N-terminal region of PB2 protein are affected in virus RNA replication but not transcription. J Virol 2003;76:5098–5108.
20 Lee MT, Bishop K, Medcalf L, Elton D, Digard P, Tiley L: Definition of the minimal viral components required for the initiation of unprimed RNA synthesis by influenza virus RNA polymerase. Nucleic Acids Res 2002;30:429–438.
21 Brownlee GG, Sharps JL: The RNA polymerase of influenza a virus is stabilized by interaction with its viral RNA promoter. J Virol 2002;76:7103–7113.
22 Digard P, Blok VC, Inglis SC: Complex formation between influenza virus polymerase proteins expressed in *Xenopus* oocytes. Virology 1989;171:162–169.
23 Poch O, Sauvaget I, Delarue M, Tordo N: Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. EMBO J 1990;8:3867–3874.
24 Biswas SK, Nayak DP: Mutational analysis of the conserved motifs of influenza A virus polymerase basic protein 1. J Virol 1994;68:1819–1826.
25 Romanos MA, Hay AJ: Identification of the influenza virus transcriptase by affinity-labeling with pyridoxal 5′-phosphate. Virology 1984;132:110–117.
26 Asano Y, Mizumoto K, Maruyama T, Ishihama A: Photoaffinity labeling of influenza virus RNA polymerase PB1 subunit with 8-azido GTP. J Biochem Tokyo 1995;117:677–682.
27 Kolpashchikov DM, Honda A, Ishihama A: Structure-function relationship of the influenza virus RNA polymerase: primer-binding site on the PB1 subunit. Biochemistry 2004;43:5882–5887.
28 Li ML, Rao P, Krug RM: The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. EMBO J 2001;20: 2078–2086.
29 Tiley LS, Hagen M, Mathews JT, Krystal M: Sequence-specific binding of the influenza virus RNA polymerase to sequences located at the 5′-end of the viral RNAs. J Virol 1994;68:5108–5116.
30 Cianci C, Tiley L, Krystal M: Differential activation of the influenza virus polymerase via template RNA binding. J Virol 1995;69:3995–3999.
31 Fodor E, Pritlove DC, Brownlee GG: The influenza virus panhandle is involved in the initiation of transcription. J Virol 1994;68:4092–4096.
32 González S, Ortín J: Characterization of the influenza virus PB1 protein binding to vRNA: two separate regions of the protein contribute to the interaction domain. J Virol 1999;73:631–637.
33 Li ML, Ramirez BC, Krug RM: RNA-dependent activation of primer RNA production by influenza virus polymerase: different regions of the same protein subunit constitute the two required RNA-binding sites. EMBO J 1998;17:5844–5852.
34 Jung TE, Brownlee GG: A new promoter-binding site in the PB1 subunit of the influenza A virus polymerase. J Gen Virol 2006;87:679–688.
35 González S, Ortín J: Distinct regions of influenza virus PB1 polymerase subunit recognize vRNA and cRNA templates. EMBO J 1999;18:3767–3775.
36 Dalton RM, Mullin AE, Amorim MJ, Medcalf E, Tiley LS, Digard P: Temperature-sensitive influenza A virus genome replication results from low thermal stability of polymerase-cRNA complexes. Virol J 2006;3:58.
37 Mahy BWJ: Mutants of influenza virus; in Palese P, Kingsbury DW (eds): Genetics of Influenza Viruses. Vienna, Springer, 1983, pp 192–253.
38 Ulmanen I, Broni BA, Krug RM: The role of two of the influenza virus core P proteins in recognizing cap 1 structures (m7GpppNm) on RNAs and in initiating viral RNA transcription. Proc Natl Acad Sci USA 1981;78:7355–7359.
39 Perales B, de la Luna S, Palacios I, Ortín J: Mutational analysis identifies functional domains in the Influenza A PB2 polymerase subunit. J Virol 1996;70:1678–1686.
40 Ulmanen I, Broni BA, Krug RM: Influenza virus temperature-sensitive cap (m7GpppNm)-dependent endonuclease. J Virol 1983;45:27–35.
41 Blaas D, Patzelt E, Keuchler E: Identification of the cap binding protein of influenza virus. Nucleic Acids Res 1982;10:4803–4812.
42 Honda A, Mizumoto K, Ishihama A: Two separate sequences of PB2 subunit constitute the RNA cap-binding site of influenza virus RNA polymerase. Genes Cells 1999;4:475–485.

43 Fechter P, Mingay L, Sharps J, Chambers A, Fodor E, Brownlee GG: Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. J Biol Chem 2003;278:20381–20388.
44 Nakagawa Y, Kimura N, Toyoda T, Mizumoto K, Ishihama A, Oda K, Nakada S: The RNA polymerase PB2 subunit is not required for replication of the influenza virus genome but is involved in capped mRNA synthesis. J Virol 1995;69:728–733.
45 Honda A, Mizumoto K, Ishihama A: Minimum molecular architectures for transcription and replication of the influenza virus. Proc Natl Acad Sci USA 2002;99:13166–13171.
46 Huarte M, Falcon A, Nakaya Y, Ortin J, Garcia-Sastre A, Nieto A: Threonine 157 of influenza virus PA polymerase subunit modulates RNA replication in infectious viruses. J Virol 2003;77:6007–6013.
47 Perales B, Sanz-Ezquerro JJ, Gastaminza P, Ortega J, Fernández-Santarén J, Ortín J, Nieto A: The replication activity of influenza virus polymerase is linked to the capacity of the PA subunit to induce proteolysis. J Virol 2000;74:1307–1312.
48 Fodor E, Crow M, Mingay LJ, Deng T, Sharps J, Fechter P, Brownlee GG: A single amino acid mutation in the PA subunit of the influenza virus RNA polymerase inhibits endonucleolytic cleavage of capped RNAs. J Virol 2002;76:8989–9001.
49 Hara K, Schmidt FI, Crow M, Brownlee GG: Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. J Virol 2006;80:7789–7798.
50 Sanz-Ezquerro JJ, de la Luna S, Ortín J, Nieto A: Individual expression of influenza virus PA protein induces degradation of co-expressed proteins. J Virol 1995;69:2420–2426.
51 Sanz-Ezquerro JJ, Zürcher T, de la Luna S, Ortin J, Nieto A: The amino-terminal one-third of the influenza virus PA protein is responsible for the induction of proteolysis. J Virol 1996;70:1905–1911.
52 Hara K, Shiota M, Kido H, Ohtsu Y, Kashiwagi T, Iwahashi J, Hamada N, Mizoue K, Tsumura N, Kato H, Toyoda T: Influenza virus RNA polymerase PA subunit is a novel serine protease with Ser624 at the active site. Genes Cells 2001;6:87–97.
53 Toyoda T, Hara K, Imamura Y: Ser624 of the PA subunit of influenza A virus is not essential for viral growth in cells and mice, but required for the maximal viral growth. Arch Virol 2003;148:1687–1696.
54 Mandler J, Muller K, Scholtissek C: Mutants and revertants of an avian influenza A virus with temperature-sensitive defects in the nucleoprotein and PB2. Virology 1991;181:512–519.
55 Mena I, Jambrina E, Albo C, Perales B, Ortin J, Arrese M, Vallejo D, Portela A: Mutational analysis of influenza A virus nucleoprotein: identification of mutations that affect RNA replication. J Virol 1999; 73:1186–1194.
56 Elton D, Medcalf L, Bishop K, Harrison D, Digard P: Identification of amino acid residues of influenza virus nucleoprotein essential for RNA binding. J Virol 1999;73:7357–7367.
57 Medcalf L, Poole E, Elton D, Digard P: Temperature-sensitive lesions in two influenza A viruses defective for replicative transcription disrupt RNA binding by the nucleoprotein. J Virol 1999;73:7349–7356.
58 Baudin F, Bach C, Cusack S, Ruigrok RW: Structure of influenza virus RNP. I. Influenza virus nucleoprotein melts secondary structure in panhandle RNA and exposes the bases to the solvent. EMBO J 1994;13:3158–3165.
59 Albo C, Valencia A, Portela A: Identification of an RNA binding region within the N-terminal third of the influenza A virus NP polypeptide. J Virol 1995;69: 3799–3806.
60 Kobayashi M, Toyoda T, Adyshev DM, Azuma Y, Ishihama A: Molecular dissection of influenza virus nucleoprotein: deletion mapping of the RNA binding domain. J Virol 1994;68:8433–8436.
61 Momose F, Basler CF, O'Neill RE, Iwamatsu A, Palese P, Nagata K: Cellular splicing factor RAF-2p48/NPI-5/BAT1/UAP56 interacts with the influenza virus nucleoprotein and enhances viral RNA synthesis. J Virol 2001;75:1899–1908.
62 Luytjes W, Krystal, M, Enami, M, Parvin J D, Palese P: Amplification, expression and packaging of a foreign gene by influenza virus. Cell 1989;59:1107–1113.
63 Fodor E, Pritlove DC, Brownlee GG: Characterization of the RNA fork model of virion RNA in the initiation of transcription in influenza A virus. J Virol 1995;69:4012–4019.
64 Odagiri T, Tashiro M: Segment-specific noncoding sequences of the influenza virus genome RNA are involved in the specific competition between defective interfering RNA and its progenitor RNA segment at the virion assembly step. J Virol 1997;71: 2138–2145.
65 Zheng H, Palese P, Garcia Sastre A: Nonconserved nucleotides at the 3′- and 5′-ends of an influenza A virus RNA play an important role in viral RNA replication. Virology 1996;217:242–251.
66 Lee MK, Bae SH, Park CJ, Cheong HK, Cheong C, Choi BS: A single-nucleotide natural variation (U4 to C4) in an influenza A virus promoter exhibits a large structural change: implications for differential viral RNA synthesis by RNA-dependent RNA polymerase. Nucleic Acids Res 2003;31:1216–1223.

67 Bae SH, Cheong HK, Lee JH, Cheong C, Kainosho M, Choi BS: Structural features of an influenza virus promoter and their implications for viral RNA synthesis. Proc Natl Acad Sci USA 2001;98:10602–10607.
68 Flick R, Neumann G, Hoffmann E, Neumeier E, Hobom G: Promoter elements in the influenza vRNA terminal structure. RNA 1996;2:1046–1057.
69 Flick R, Hobom G: Interaction of influenza virus polymerase with viral RNA in the 'corkscrew' conformation. J Gen Virol 1999;80:2565–2572.
70 Pritlove DC, Poon LLM, Devenish LJ, Mike B, Leahy MB, Brownlee GG: A hairpin loop at the 5′-end of influenza A virus virion RNA is required for synthesis of poly(A)$^+$ mRNA in vitro. J Virol 1999; 73:2109–2114.
71 Li X, Palese P: Mutational analysis of the promoter required for influenza virus virion RNA synthesis. J Virol 1992;66:4331–4338.
72 Park CJ, Bae SH, Lee MK, Varani G, Choi BS: Solution structure of the influenza A virus cRNA promoter: implications for differential recognition of viral promoter structures by RNA-dependent RNA polymerase. Nucleic Acids Res 2003;31:2824–2832.
73 Elton D, Digard P, Tiley L, Ortín J: Structure and function of the influenza virus RNA; in Kawaoka Y (ed): Current Topics in Influenza Virology. Norfolk, Horizon Scientific Press, 2005, pp 1–92.
74 Ortega J, Martín-Benito J, Zürcher T, Valpuesta JM, Carrascosa JL, Ortín J: Ultrastructural and functional analyses of recombinant influenza virus ribonucleoproteins suggest dimerization of nucleoprotein during virus amplification. J Virol 2000;74: 156–163.
75 Martín-Benito J, Area E, Ortega J, Llorca O, Valpuesta JM, Carrascosa JL, Ortín J: Three-dimensional reconstruction of a recombinant influenza virus ribonucleoprotein particle. EMBO Rep 2001;2: 313–317.
76 Area E, Martín-Benito J, Gastaminza P, Torreira E, Valpuesta JM, Carrascosa JL, Ortín J: Three-dimensional structure of the influenza virus RNA polymerase: localization of subunit domains. Proc Natl Acad Sci USA 2004;101:308–313.
77 Biswas SK, Boutz PL, Nayak DP: Influenza virus nucleoprotein interacts with influenza virus polymerase proteins. J Virol 1998;72:5493–5501.
78 Poole E, Elton D, Medcalf L, Digard P: Functional domains of the influenza A virus PB2 protein: identification of NP- and PB1-binding sites. Virology 2004;321:120–133.
79 Torreira E, Schoehn G, Fernández Y, Jorba N, Ruigrok RW, Cusack S, Ortin J, Llorca O: Three-dimensional model for the isolated influenza virus polymerase heterotrimer. Nucleic Acids Res 2007;35: 3774–3783.
80 Tarendeau F, Boudet J, Guilligay D, Mas PJ, Bougault CM, Boulo S, Baudin F, Ruigrok RW, Daigle N, Ellenberg J, Cusack S, Simorre JP, Hart DJ: Structure and nuclear import function of the C-terminal domain of influenza virus polymerase PB2 subunit. Nat Struct Mol Biol 2007;14:229–233.
81 Ye Q, Krug RM, Tao YJ: The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. Nature 2006;444:1078–1082.
82 Albertini AA, Wernimont AK, Muziol T, Ravelli RB, Clapier CR, Schoehn G, Weissenhorn W, Ruigrok RW: Crystal structure of the rabies virus nucleoprotein-RNA complex. Science 2006;313:360–363.
83 Green TJ, Zhang X, Wertz GW, Luo M: Structure of the vesicular stomatitis virus nucleoprotein-RNA complex. Science 2006;313:357–360.
84 Rudolph MG, Kraus I, Dickmanns A, Eickmann M, Garten W, Ficner R: Crystal structure of the borna disease virus nucleoprotein. Structure 2003;11: 1219–2126.
85 Lee MT, Klumpp K, Digard P, Tiley L: Activation of influenza virus RNA polymerase by the 5′- and 3′-terminal duplex of genomic RNA. Nucleic Acids Res 2003;31:1624–1632.
86 Shapiro GI, Gurney TJ, Krug RM: Influenza virus gene expression: control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. J Virol 1987;61:764–773.
87 Tchatalbachev S, Flick R, Hobom G: The packaging signal of influenza viral RNA molecules. RNA 2001;7:979–989.
88 Poon LL, Pritlove DC, Sharps J, Brownlee GG: The RNA polymerase of influenza virus, bound to the 5′-end of virion RNA, acts in cis to polyadenylate mRNA. J Virol 1998;72:8214–8219.
89 Hagen M, Chung TD, Butcher JA, Krystal M: Recombinant influenza virus polymerase: requirement of both 5′- and 3′-viral ends for endonuclease activity. J Virol 1994;68:1509–1515.
90 Rao P, Yuan W, Krug RM: Crucial role of CA cleavage sites in the cap-snatching mechanism for initiating viral mRNA synthesis. EMBO J 2003;22:1188–1198.
91 Shaw MW, Lamb RA: A specific sub-set of host-cell mRNAs prime influenza virus mRNA synthesis. Virus Res 1984;1:455–467.
92 Shapiro GI, Krug RM: Influenza virus RNA replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer. J Virol 1988;62:2285–2290.
93 Vreede FT, Jung TE, Brownlee GG: Model suggesting that replication of influenza virus is regulated by stabilization of replicative intermediates. J Virol 2004;78:9568–9572.
94 Vreede FT, Brownlee GG: Influenza virion-derived viral ribonucleoproteins synthesize both mRNA and cRNA in vitro. J Virol 2007;81:2196–2204.

95 Klumpp K, Ford MJ, Ruigrok RW: Variation in ATP requirement during influenza virus transcription. J Gen Virol 1998;79:1033–1045.
96 Honda A, Mizumoto K, Ishihama A: Identification of the 5′-terminal structure of influenza virus genome RNA by a newly developed enzymatic method. Virus Res 1998;55:199–206.
97 Deng T, Vreede FT, Brownlee GG: Different de novo initiation strategies are used by influenza virus RNA polymerase on its cRNA and viral RNA promoters during viral RNA replication. J Virol 2006;80: 2337–2348.
98 Mendez J, Blanco L, Esteban JA, Bernad A, Salas M: Initiation of phi 29 DNA replication occurs at the second 3′-nucleotide of the linear template: a sliding-back mechanism for protein-primed DNA replication. Proc Natl Acad Sci USA 1992;89:9579–9583.
99 Paul AV, Yin J, Mugavero J, Rieder E, Liu Y, Wimmer E: A 'slide-back' mechanism for the initiation of protein-primed RNA synthesis by the RNA polymerase of poliovirus. J Biol Chem 2003;278:43951–43960.
100 Garcin D, Kolakofsky D: Tacaribe arenavirus RNA synthesis in vitro is primer dependent and suggests an unusual model for the initiation of genome replication. J Virol 1992;66:1370–1376.
101 Pritlove DC, Poon LL, Fodor E, Sharps J, Brownlee GG: Polyadenylation of influenza virus mRNA transcribed in vitro from model virion RNA templates: requirement for 5′-conserved sequences. J Virol 1998;72:1280–1286.
102 Poon LL, Fodor E, Brownlee GG: Polyuridylated mRNA synthesized by a recombinant influenza virus is defective in nuclear export. J Virol 2000;74: 418–427.
103 Biswas SK, Nayak DP: Influenza virus polymerase basic protein 1 interacts with influenza virus polymerase basic protein 2 at multiple sites. J Virol 1996;70: 6716–6722.
104 González S, Zürcher T, Ortín J: Identification of two separate domains in the influenza virus PB1 protein responsible for interaction with the PB2 and PA subunits: a model for the viral RNA polymerase structure. Nucleic Acids Res 1996;24:4456–4463.
105 Toyoda T, Adyshev DM, Kobayashi M, Iwata A, Ishihama A: Molecular assembly of the influenza virus RNA polymerase: determination of the subunit-subunit contact sites. J Gen Virol 1996;77:2149–2157.
106 Zürcher T, de la Luna S, Sanz-Ezquerro JJ, Nieto A, Ortín J: Mutational analysis of the influenza virus A/Victoria/3/75 PA protein: studies of interaction with PB1 protein and identification of a dominant negative mutant. J Gen Virol 1996;77:1745–1749.
107 Pérez DR, Donis RO: A 48-amino-acid region of influenza A virus PB1 protein is sufficient for complex formation with PA. J Virol 1995;69:6932–6939.
108 Mukaigawa J, Nayak DP: Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. J Virol 1991;65:245–253.
109 Nieto A, de la Luna S, Bárcena J, Portela A, Ortín J: Complex structure of the nuclear translocation signal of the influenza virus polymerase PA subunit. J Gen Virol 1994;75:29–36.
110 Nath ST, Nayak DP: Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1N1). Mol Cell Biol 1990;10:4139–4145.
111 Fodor E, Smith M: The PA subunit is required for efficient nuclear accumulation of the PB1 subunit of the influenza A virus RNA polymerase complex. J Virol 2004;78:9144–9153.
112 Deng T, Sharps J, Fodor E, Brownlee GG: In vitro assembly of PB2 with a PB1-PA dimer supports a new model of assembly of influenza A virus polymerase subunits into a functional trimeric complex. J Virol 2005;79:8669–8674.
113 Deng T, Engelhardt OG, Thomas B, Akoulitchev AV, Brownlee GG, Fodor E: The role of ran binding protein 5 (RanBP5) in the nuclear import and assembly of the influenza virus RNA polymerase complex. J Virol 2006;80:11911–11919.
114 Akkina RK, Chambers TM, Londo DR, Nayak DP: Intracellular localization of the viral polymerase proteins in cells infected with influenza virus and cells expressing PB1 protein from cloned cDNA. J Virol 1987;61:2217–2224.
115 Naito T, Momose F, Kawaguchi A, Nagata K: Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. J Virol 2007;81:1339–1349.
116 Momose F, Naito T, Yano K, Sugimoto S, Morikawa Y, Nagata K: Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. J Biol Chem 2002;277:45306–45314.
117 Mark GE, Taylor JM, Broni B, Krug RM: Nuclear accumulation of influenza viral RNA transcripts and the effects of cycloheximide, actinomycin D, and α-amanitin. J Virol 1979;29:744–752.
118 Engelhardt OG, Smith M, Fodor E: Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. J Virol 2005;79:5812–5818.
119 Huarte M, Sanz-Ezquerro JJ, Roncal F, Ortin J, Nieto A: PA subunit from influenza virus polymerase complex interacts with a cellular protein with homology to a family of transcriptional activators. J Virol 2001;75:8597–8604.
120 Perez-Gonzalez A, Rodriguez A, Huarte M, Salanueva IJ, Nieto A: hCLE/CGI-99, a human protein that interacts with the influenza virus polymerase, is a mRNA transcription modulator. J Mol Biol 2006;362:887–900.

121 Chan AY, Vreede FT, Smith M, Engelhardt OG, Fodor E: Influenza virus inhibits RNA polymerase II elongation. Virology 2006;351:210–217.
122 Rodriguez A, Perez-Gonzalez A, Nieto A: Influenza virus infection causes the specific degradation of the largest subunit of cellular RNA polymerase II. J Virol 2007;81:5315–5324.
123 Stutz F, Izaurralde E: The interplay of nuclear mRNP assembly, mRNA surveillance and export. Trends Cell Biol 2003;13:319–327.
124 Satterly N, Tsai PL, van Deursen J, Nussenzveig DR, Wang Y, Faria PA, Levay A, Levy DE, Fontoura BM: Influenza virus targets the mRNA export machinery and the nuclear pore complex. Proc Natl Acad Sci USA 2007;104:1853–1858.
125 Huang Y, Steitz JA: SRprises along a messenger's journey. Mol Cell 2005;17:613–615.
126 Amorim MJ, Read EK, Dalton RM, Medcalf L, Digard P: Nuclear export of influenza A virus MRNAS requires ongoing RNA polymerase II activity. Traffic 2007;8:1–11.
127 Garaigorta U, Ortín J: Mutation analysis of a recombinant NS replicon shows that influenza virus NS1 protein blocks the splicing and nucleocytoplasmic transport of its own viral mRNA. Nucleic Acids Res 2007;35:4573–4582.
128 Fortes P, Inada T, Preiss T, Hentze MW, Mattaj IW, Sachs AB: The yeast nuclear cap binding complex can interact with translation factor eIF4G and mediate translation initiation. Mol Cell 2000;6:191–196.
129 Momose F, Handa H, Nagata K: Identification of host factors that regulate the influenza virus RNA polymerase activity. Biochimie 1996;78:1103–1108.
130 Fleckner J, Zhang M, Valcarcel J, Green MR: U2AF65 recruits a novel human DEAD box protein required for the U2 snRNP-branchpoint interaction. Genes Dev 1997;11:1864–1872.
131 Gatfield D, Le Hir H, Schmitt C, Braun IC, Kocher T, Wilm M, Izaurralde E: The DExH/D box protein HEL/UAP56 is essential for mRNA nuclear export in *Drosophila*. Curr Biol 2001;11:1716–1721.
132 Mayer D, Molawi K, Martinez-Sobrido L, Ghanem A, Thomas S, Baginsky S, Grossmann J, Garcia-Sastre A, Schwemmle M: Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches. J Proteome Res 2007;6:672–682.
133 Jackson DA, Caton AJ, McCready SJ, Cook PR: Influenza virus RNA is synthesized at fixed sites in the nucleus. Nature 1982;296:366–368.
134 López-Turiso JA, Martinez C, Tanaka T, Ortin J: The synthesis of influenza virus negative-strand RNA takes place in insoluble complexes present in the nuclear matrix fraction. Virus Res 1990;16:325–337.
135 Subbarao EK, London W, Murphy BR: A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 1993;67:1761–1764.
136 Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J: The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci USA 2005;102:18590–18595.

Juan Ortín
Centro Nacional de Biotecnología (CSIC)
Darwin 3, Cantoblanco, ES–28049 Madrid (Spain)
Tel. +34 91 585 4557, Fax +34 91 585 4506, E-Mail jortin@cnb.uam.es

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 187–194

Influenza A Virus Polymerase: A Determinant of Host Range and Pathogenicity

Jürgen Stech

Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health,
Institute of Molecular Biology, Greifswald-Insel Riems, Germany

Abstract

The influenza A polymerase catalyzes within the nucleus of the cell both the genomic replication of the negative-stranded vRNA via cRNA as intermediate template and transcription of vRNA into mRNA. There is accumulating evidence that enhanced polymerase activities facilitate species transmission and increase virulence in mammals. Remarkably, this enhancement is dependent on species origin of host cells: improved replication in mammalian cells is accompanied with diminished replication in avian cells in the case of a mammalian-adapted strain and vice versa in the case of a highly pathogenic avian strain. Several host factors have been demonstrated to interact with the influenza A virus polymerase. These findings suggest that the molecular correlates of host range and virulence are optimized interactions of the polymerase proteins with cellular proteins.

Structure and Regulation of the Influenza A Polymerase

The influenza A virus polymerase has an RNA-dependent RNA polymerase activity [1–6]. This enzyme consists of three subunits, the PB2, PB1, and PA proteins forming a heterotrimeric complex [7–12]. The polymerase is active in the nucleus and nuclear localization sequences have been identified on each subunit: PB1 [13], PB2 [14], and PA [15]. Recent results suggest that cytoplasmically expressed PB1 and PA may be imported as a sub-complex, which then assembles with separately imported PB2 [16]. Several nuclear and cytoplasmic host proteins have been shown to be cofactors required by the polymerase [17–20].

The viral polymerase catalyzes both the transcription of genomic RNA, i.e. viral RNA (vRNA), to mRNA and the replication of vRNA to complementary RNA (cRNA), the intermediate template which is necessary for copying new vRNA molecules [21].

Transcription is initiated by binding m^7GpppGm cap structures at the 5′ ends of cellular mRNAs by the PB2 and PB1 subunits [22–26]. Capped 5′ mRNA ends are then cut off by an endonuclease activity of the PB1 and PA subunits [27–29] and used as primers for further elongation which is eventually terminated due to stuttering [30, 31] at the uridine stretch adjacent to the 5′ end of the vRNA [32, 33] and due to the bound polymerase complex at the 5′ end [34] leading to polyadenylation. In contrast, the replication of vRNA is primer-independent and involves the de novo initiation from nascent RNA chains requiring first the synthesis of cRNA, which then serves as template for new vRNA [21, 35]. Two different initiation mechanisms have been proposed for replication of vRNA and cRNA. For the vRNA promoter, the primer-independent initiation occurs at the 3′ terminus of the cRNA followed by elongation [36] whereas for the cRNA promoter an internal initiation (nucleotides 4 and 5) is followed by realignment at the 3′ terminal nucleotides 1 and 2 and then by elongation. The promoters are composed of both the 5′ and 3′ ends of vRNA (genomic promoter) or cRNA (antigenomic promoter) which are highly conserved in all segments of all influenza A strains [37, 38]. Three promoter structures have been proposed: the panhandle model [39], the fork model [40, 41] and the corkscrew model [42]. According to the panhandle model, the entire promoter region is partially double-stranded whereas the fork model suggests the double-stranded configuration only at the distal promoter element. The corkscrew model predicts base-pairing at each 5′ or 3′ end rather than of the 5′ with the 3′ end and is considered the current model of the secondary structures of both the vRNA and cRNA promoters [43–45].

Earlier studies suggested that the polymerase is first engaged in transcription, and then switches to replication after sufficient amounts of nucleoprotein had been synthesized [21, 46–48]. However, the molecular mechanism of such a switch has remained uncertain. Remarkably, cRNA synthesis early in infection could be demonstrated [49]. Therefore, a stabilization model was proposed which does not assume different synthesis modes of the viral polymerase. The vRNPs of the infecting virions were demonstrated to synthesize both mRNA and cRNA. However, the mRNA is protected from degradation due to its 5′ cap and 3′ poly A tail whereas the naked cRNA is presumably subjected to degradation by cellular nucleases. Later, the cRNA becomes protected by the newly synthesized polymerase proteins and is then encapsidated by the nucleoprotein. Thus, although the newly synthesized polymerase proteins and the NP are necessary for replication, they do not induce a switch to another synthesis mode but their later abundance facilitates the replication indirectly by stabilizing replicative intermediates [49, 50].

Enhanced Polymerase Activity Leads to Increased Virulence

Animal studies based on comparison of reassortants demonstrated that virulence may be decreased or increased by the different constellations of polymerase and nucleoprotein genes. Two HPAI strains can reassort to a virus low pathogenic in chickens [51]

whereas conversely the double infection with two LPAI strains yielded a virus highly neuro- and pneumovirulent in mice [52, 53]. In addition, reassortant viruses with a mixed constellation of polymerase genes from an avian and a human strain (or carrying the nucleoprotein from the avian virus) were attenuated in squirrel monkeys in contrast to their parental viruses. These observations indicated that an optimized interaction of the polymerase complex with host factors is involved in efficient replication in vivo [54]. Taken together, these early reassortment studies demonstrated that circulating strains with a low pathogenic phenotype may carry viral genes with highly pathogenic potential and, thus, are able to give rise to highly virulent strains upon reassortment.

The advent of reverse genetics [55–57] made studies possible which could assign increased virulence to single point mutations within the viral genome. Several pathogenicity studies in mice and in ferrets found specific polymerase mutations leading both to increased virulence and enhanced replication. A single amino acid substitution in the PB2 protein, Glu-Lys at position 627, enables a single gene reassortant with a PB2 gene from an avian virus to replicate in mammalian cells. Strikingly, whereas in every avian influenza A virus PB2 protein, the amino acid at position 627 is glutamic acid, in every human influenza A strain, lysine is prevalent [58]. This mutation increases the polymerase activity in mammalian cells. Moreover, polymerase complexes derived from avian viruses exhibited cold sensitivity in mammalian cells mostly due to the residue 627 in PB2 [59]. The amino acid substitution PB2 627Lys was found to contribute to enhanced virulence of several highly pathogenic avian influenza viruses of the H5N1 subtype from Southeast Asia both in mice [60–63] and in ferrets [64]. However, several but not all H5N1 viruses isolated from humans carry this substitution [65, 66]. The relevance of this mutation in the field was underlined by its occurrence in another unrelated group of HPAIV of the H7N7 subtype. This mutation was found in an isolate from a deceased veterinarian during the fowl plague outbreak in the Netherlands in 2003 [67]. The characterization of the reconstructed PB2 sequence of the 1918 virus revealed the presence of 627Lys as well [68]. Moreover, several studies demonstrated the substitution PB2 701 Glu-Lys to increase the virulence of H7N7 and H5N1 HPAIV in mice [69–71]. Other residues correlating with high pathogenicity are PB2 714Arg, PA 615Arg, and NP 319Lys [69]. All these virulence enhancing mutations found in H7N7 and H5N1 viruses were then shown by minireplicon assays to increase polymerase activity in mammalian cells [62, 69, 72]. Moreover, this enhanced activity correlated with increased virulence in mice and ferrets [62, 69]. Remarkably, it appears that an optimum of polymerase activity exists because virus variants with extreme polymerase activity were not of highest virulence; highest levels of replication/transcription might be detrimental in the field [62, 69].

Convergent Evolution of the Influenza Polymerase Complex in Nature

The mutations PB2 627Lys, PB2 701Asn, PB2 714Arg, PA 615Asn, and NP 319Lys shown to enhance the polymerase activity and to increase virulence in mice occurred

independently in unrelated groups of mammalian isolates [62, 66, 69, 72, 73]. All other viruses including avian strains carry PB2 627Glu, PB2 701Asp, PB2 714Ser, PA 615Lys, and NP 319Asn [58, 69]. The substitution PB2 627Lys is well known in human strains [58] including the 1918 virus [68] and in H5N1 as well as in an H7N7 HPAIV isolated from humans [63, 67, 74]. Similarly, PB2 701Asn was found in several phylogenetic clades of H1N1, H1N2, and H3N2 swine isolates, in H3N8 and H7N7 equine isolates, and in several H5N1 HPAIV from Southeast Asia including human isolates such as A/Hk/488/97, A/HK/97/98, and A/Vietnam/3046/2004 [69]. Remarkably, among these strains is the avian-like swine H1N1 virus A/Swine/Germany/2/81 [75] which belongs to a lineage originating from an avian precursor that was transmitted as a whole to swine [76].

PB2 714Arg was found in four mammalian strains, whereas 16 avian H5N1 isolates (2001) highly pathogenic for chicken and quails carry isoleucine at this position. These strains replicate well in mouse lung in contrast to their precursors, the Goose/Guangdong-like strains [77]. One of these strains, A/Goose/Guangdong/1/96 [78], carries Ser instead of Ile at position 714 in PB2. In contrast to the Goose/Guangdong-like viruses, the H5N1 isolates appear to have an increased pathogenicity for mammals [77] suggesting that PB2 714Ile is a host range marker [69].

Instead of Lys at position 615 within the PA protein, Arg was found in several phylogenetic clades of human H1N1, H5N1, and H9N2 isolates including H5N1 HPAIV isolates of known pathogenicity like A/HK/483/97, A/HK/485/97, and A/HK/491/97 [60]. The avian strains A/Teal/HK/W312/97 (H6N1) and A/Quail/HK/G1/97 (H9N2), proposed donors of internal genes of H5N1 viruses [79–81], were found as well, emphasizing the relevance of PA 615Arg for host change [69].

NP 319Lys was found in human and equine strains as well as in several H5N1 HPAIV. Remarkably, some human H5N1 HPAIV (A/HK/156/97, A/HK/482/97, A/HK/486/97, and A/HK/538/97) isolates harbor both NP 319Lys and PA 615Arg [69]. A recent study in 18 individuals infected with H5N1 HPAIV indicated that high viral load and the resulting intense inflammatory response are central to pathogenesis. In 5 of 8 isolates from fatal cases and in 3 of 4 isolates from surviving patients, the PB2 627Lys substitution was found. There was no association between the presence of Lys627 and lethal outcome. Notably, 3 of 4 viruses containing PB2 627Glu common to avian strains, but none carrying PB2 627Lys, contained PB2 701Asn. This finding suggests that this substitution may compensate for the absence of PB2 627Lys in conferring enhanced polymerase activity. In addition, all viruses contained PA 615Arg [66].

Several polymerase mutations were identified and shown to increase the activity and virulence in mice. These substitutions occurred in several independent phylogenetic clades of influenza virus strains transmitted recently from birds and still undergoing adaptation. These findings indicate convergent evolution of the polymerase complex in nature [69].

Cell-Type Dependence of Polymerase Activity Suggests Optimized Interactions with Host Factors

Several studies reviewed in the previous sections demonstrated that the increase in virulence of HPAIV correlates with enhanced polymerase activity in mammalian cells raising the question on host dependence of polymerase activity. The comparison of polymerase activities of the mouse-adapted SC35M versus that of the HPAIV SC35 in mammalian and avian cells by primer extension revealed [82] that SC35M replicated more efficiently in mammalian cells but less efficiently in avian cells, contrary to SC35 which replicated better in avian cells. Remarkably, single point mutants of SC35 replicated in mammalian cells to some extent better than SC35 but were not diminished in their replication in avian cells. This finding might correspond with the observation that the HPAIV of the H5N1 subtype in the field carry rather only one polymerase-enhancing mutation [66, 69]. Such a single mutation would allow an avian virus to spread to mammals but still preserve its ability to circulate in poultry or wild birds. Therefore, the evolution of H5N1 viruses might still be in a transitional phase of species transmission.

To date, several cellular interaction partners of the viral polymerase could be identified using proteomics-based approaches. These host cell proteins fall into different functional categories: components of the ubiquitination machinery (ubiquitin carboxyl-terminal hydrolase 10 or the DNA-damage-binding protein 1), proteins involved in translation (such as the ribosomal proteins of the 60S and 40S subunits, elongation factor 1α, tRNA synthetases), chaperones, transcription factors (interleukin binding factor 3 or transcription intermediary factor 1β), and cellular cytoskeleton proteins (actin or various isoforms of tubulin) [17]. These studies are based on the laboratory strain A/WSN/33 (H1N1). It will be therefore of great relevance to compare the host cell interactions of natural influenza A virus strains.

References

1 Page MG, Ruck BJ, Brammer KW: Characterization of the ribonucleic acid product synthesized by on influenza A2 virus-induced ribonucleic acid polymerase in vitro. Biochem J 1968;109:43P.

2 Bishop DH, Obijeski JF, Simpson RW: Transcription of the influenza ribonucleic acid genome by a virion polymerase. II. Nature of the in vitro polymerase product. J Virol 1971;8:74–80.

3 Bishop DH, Obijeski JF, Simpson RW: Transcription of the influenza ribonucleic acid genome by a virion polymerase. I. Optimal conditions for in vitro activity of the ribonucleic acid-dependent ribonucleic acid polymerase. J Virol 1971;8:66–73.

4 Bishop DH, Roy P, Bean WJ Jr, Simpson RW: Transcription of the influenza ribonucleic acid genome by a virion polymerase. 3. Completeness of the transcription process. J Virol 1972;10:689–697.

5 Compans RW, Caliguiri LA: Isolation and properties of an RNA polymerase from influenza virus-infected cells. J Virol 1973;11:441–448.

6 Skehel JJ: RNA-dependent RNA polymerase activity of the influenza virus. Virology 1971;45:793–796.

7 Area E, Martin-Benito J, Gastaminza P, Torreira E, Valpuesta JM, Carrascosa JL, Ortin J: 3D structure of the influenza virus polymerase complex: localization of subunit domains. Proc Natl Acad Sci USA 2004;101:308–313.

8 Detjen BM, St Angelo C, Katze MG, Krug RM: The three influenza virus polymerase (P) proteins not associated with viral nucleocapsids in the infected cell are in the form of a complex. J Virol 1987;61: 16–22.
9 Digard P, Blok VC, Inglis SC: Complex formation between influenza virus polymerase proteins expressed in *Xenopus* oocytes. Virology 1989;171:162–169.
10 Inglis SC, Carroll AR, Lamb RA, Mahy BW: Polypeptides specified by the influenza virus genome I. Evidence for eight distinct gene products specified by fowl plague virus. Virology 1976;74: 489–503.
11 Inglis SC, McGeoch DJ, Mahy BW: Polypeptides specified by the influenza virus genoma. 2. Assignment of protein coding functions to individual genome segments by in vitro translation. Virology 1977;78:522–536.
12 Palese P, Ritchey MB, Schulman JL: Mapping of the influenza virus genome. II. Identification of the P1, P2, and P3 genes. Virology 1977;76:114–121.
13 Nath ST, Nayak DP: Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1 N1). Mol Cell Biol 1990;10:4139–4145.
14 Mukaigawa J, Nayak DP: Two signals mediate nuclear localization of influenza virus (A/WSN/33) polymerase basic protein 2. J Virol 1991;65:245–253.
15 Nieto A, de la Luna S, Barcena J, Portela A, Valcarcel J, Melero JA, Ortin J: Nuclear transport of influenza virus polymerase PA protein. Virus Res 1992;24: 65–75.
16 Fodor E, Smith M: The PA subunit is required for efficient nuclear accumulation of the PB1 subunit of the influenza A virus RNA polymerase complex. J Virol 2004;78:9144–9153.
17 Mayer D, Molawi K, Martinez-Sobrido L, Ghanem A, Thomas S, Baginsky S, Grossmann J, Garcia-Sastre A, Schwemmle M: Identification of cellular interaction partners of the influenza virus ribonucleoprotein complex and polymerase complex using proteomic-based approaches. J Proteome Res 2007;6: 672–682.
18 Engelhardt OG, Smith M, Fodor E: Association of the influenza A virus RNA-dependent RNA polymerase with cellular RNA polymerase II. J Virol 2005;79:5812–5818.
19 Deng T, Engelhardt OG, Thomas B, Akoulitchev AV, Brownlee GG, Fodor E: Role of ran binding protein 5 in nuclear import and assembly of the influenza virus RNA polymerase complex. J Virol 2006;80:11911–11919.
20 Ye Q, Krug RM, Tao YJ: The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. Nature 2006;444:1078–1082.
21 Hay AJ, Lomniczi B, Bellamy AR, Skehel JJ: Transcription of the influenza virus genome. Virology 1977;83:337–355.
22 Blaas D, Patzelt E, Kuechler E: Identification of the cap binding protein of influenza virus. Nucleic Acids Res 1982;10:4803–4812.
23 Blaas D, Patzelt E, Kuechler E: Cap-recognizing protein of influenza virus. Virology 1982;116: 339–348.
24 Fechter P, Mingay L, Sharps J, Chambers A, Fodor E, Brownlee GG: Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. J Biol Chem 2003;278:20381–20388.
25 Li ML, Ramirez BC, Krug RM: RNA-dependent activation of primer RNA production by influenza virus polymerase: different regions of the same protein subunit constitute the two required RNA-binding sites. EMBO J 1998;17:5844–5852.
26 Ulmanen I, Broni BA, Krug RM: Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m^7GpppNm) on RNAs and in initiating viral RNA transcription. Proc Natl Acad Sci USA 1981;78:7355–7359.
27 Hara K, Schmidt FI, Crow M, Brownlee GG: Amino acid residues in the N-terminal region of the PA subunit of influenza A virus RNA polymerase play a critical role in protein stability, endonuclease activity, cap binding, and virion RNA promoter binding. J Virol 2006;80:7789–7798.
28 Li ML, Rao P, Krug RM: The active sites of the influenza cap-dependent endonuclease are on different polymerase subunits. EMBO J 2001;20: 2078–2086.
29 Shi L, Summers DF, Peng Q, Galarz JM: Influenza A virus RNA polymerase subunit PB2 is the endonuclease which cleaves host cell mRNA and functions only as the trimeric enzyme. Virology 1995;208:38–47.
30 Poon LL, Pritlove DC, Fodor E, Brownlee GG: Direct evidence that the poly(A) tail of influenza A virus mRNA is synthesized by reiterative copying of a U track in the virion RNA template. J Virol 1999; 73: 3473–3476.
31 Zheng H, Lee HA, Palese P, Garcia-Sastre A: Influenza A virus RNA polymerase has the ability to stutter at the polyadenylation site of a viral RNA template during RNA replication. J Virol 1999;73: 5240–5243.
32 Li X, Palese P: Characterization of the polyadenylation signal of influenza virus RNA. J Virol 1994;68: 1245–1249.
33 Luo GX, Luytjes W, Enami M, Palese P: The polyadenylation signal of influenza virus RNA involves a stretch of uridines followed by the RNA duplex of the panhandle structure. J Virol 1991;65: 2861–2867.
34 Poon LL, Pritlove DC, Sharps J, Brownlee GG: The RNA polymerase of influenza virus, bound to the 5′ end of virion RNA, acts in cis to polyadenylate mRNA. J Virol 1998;72:8214–8219.
35 Hay AJ, Skehel JJ, McCauley J: Characterization of influenza virus RNA complete transcripts. Virology 1982;116:517–522.

36 Deng T, Vreede FT, Brownlee GG: Different de novo initiation strategies are used by influenza virus RNA polymerase on its cRNA and viral RNA promoters during viral RNA replication. J Virol 2006;80: 2337–2348.
37 Skehel JJ, Hay AJ: Nucleotide sequences at the 5′ termini of influenza virus RNAs and their transcripts. Nucleic Acids Res 1978;5:1207–1219.
38 Desselberger U, Racaniello VR, Zazra JJ, Palese P: The 3′- and 5′-terminal sequences of influenza A, B and C virus RNA segments are highly conserved and show partial inverted complementarity. Gene 1980;8:315–328.
39 Hsu MT, Parvin JD, Gupta S, Krystal M, Palese P: Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc Natl Acad Sci USA 1987;84:8140–8144.
40 Kim HJ, Fodor E, Brownlee GG, Seong BL: Mutational analysis of the RNA-fork model of the influenza A virus vRNA promoter in vivo. J Gen Virol 1997;78:353–357.
41 Fodor E, Seong BL, Brownlee GG: Photochemical cross-linking of influenza A polymerase to its virion RNA promoter defines a polymerase binding site at residues 9 to 12 of the promoter. J Gen Virol 1993; 74:1327–1333.
42 Flick R, Hobom G: Interaction of influenza virus polymerase with viral RNA in the 'corkscrew' conformation. J Gen Virol 1999;80:2565–2572.
43 Azzeh M, Flick R, Hobom G: Functional analysis of the influenza A virus cRNA promoter and construction of an ambisense transcription system. Virology 2001;289:400–410.
44 Crow M, Deng T, Addley M, Brownlee GG: Mutational analysis of the influenza virus cRNA promoter and identification of nucleotides critical for replication. J Virol 2004;78:6263–6270.
45 Flick R, Neumann G, Hoffmann E, Neumeier E, Hobom G: Promoter elements in the influenza vRNA terminal structure. RNA 1996;2:1046–1057.
46 Barrett T, Wolstenholme AJ, Mahy BW: Transcription and replication of influenza virus RNA. Virology 1979;98:211–225.
47 Hatada E, Hasegawa M, Mukaigawa J, Shimizu K, Fukuda R: Control of influenza virus gene expression: quantitative analysis of each viral RNA species in infected cells. J Biochem 1989;105:537–546.
48 Taylor JM, Illmensee R, Litwin S, Herring L, Broni B, Krug RM: Use of specific radioactive probes to study transcription and replication of the influenza virus genome. J Virol 1977;21:530–540.
49 Vreede FT, Jung TE, Brownlee GG: Model suggesting that replication of influenza virus is regulated by stabilization of replicative intermediates. J Virol 2004;78:9568–9572.
50 Vreede FT, Brownlee GG: Influenza virion-derived viral ribonucleoproteins synthesize both mRNA and cRNA in vitro. J Virol 2007;81:2196–2204.
51 Rott R, Orlich M, Scholtissek C: Correlation of pathogenicity and gene constellation of influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. J Gen Virol 1979;44:471–477.
52 Scholtissek C, Vallbracht A, Flehmig B, Rott R: Correlation of pathogenicity and gene constellation of influenza A viruses. II. Highly neurovirulent recombinants derived from non-neurovirulent or weakly neurovirulent parent virus strains. Virology 1979;95:492–500.
53 Vallbracht A, Scholtissek C, Flehmig B, Gerth HJ: Recombination of influenza A strains with fowl plague virus can change pneumotropism for mice to a generalized infection with involvement of the central nervous system. Virology 1980;107:452–460.
54 Snyder MH, Buckler-White AJ, London WT, Tierney EL, Murphy BR: The avian influenza virus nucleoprotein gene and a specific constellation of avian and human virus polymerase genes each specify attenuation of avian-human influenza A/Pintail/ 79 reassortant viruses for monkeys. J Virol 1987;61: 2857–2863.
55 Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG: A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci USA 2000;97:6108–6113.
56 Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y: Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci USA 1999;96:9345–9350.
57 Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, Garcia-Sastre A: Rescue of influenza A virus from recombinant DNA. J Virol 1999;73:9679–9682.
58 Subbarao EK, London W, Murphy BR: A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol 1993;67:1761–1764.
59 Massin P, van der WS, Naffakh N: Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J Virol 2001; 75:5398–5404.
60 Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y: Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 1999;73:3184–189.
61 Hatta M, Gao P, Halfmann P, Kawaoka Y: Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science 2001;293:1840–1842.
62 Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, Hulse-Post DJ, Humberd J, Trichet M, Rehg JE, Webby RJ, Webster RG, Hoffmann E: The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J Exp Med 2006;203:689–697.

63 Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y: PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology 2004;320: 258–266.
64 Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen TD, Hanh TH, Puthavathana P, Long HT, Buranathai C, Lim W, Webster RG, Hoffmann E: Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol 2005;79:2191–2198.
65 Puthavathana P, Auewarakul P, Charoenying PC, Sangsiriwut K, Pooruk P, Boonnak K, Khanyok R, Thawachsupa P, Kijphati R, Sawanpanyalert P: Molecular characterization of the complete genome of human influenza H5N1 virus isolates from Thailand. J Gen Virol 2005;86:423–433.
66 De Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha do Q, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J: Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 2006;12:1203–1207.
67 Fouchier RA, Schneeberger PM, Rozendaal FW, Broekman JM, Kemink SA, Munster V, Kuiken T, Rimmelzwaan GF, Schutten M, Van Doornum GJ, Koch G, Bosman A, Koopmans M, Osterhaus AD: Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome. Proc Natl Acad Sci USA 2004;101:1356–1361.
68 Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG: Characterization of the 1918 influenza virus polymerase genes. Nature 2005;437: 889–893.
69 Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J: The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci USA 2005;102:18590–18595.
70 Brown EG, Liu H, Kit LC, Baird S, Nesrallah M: Pattern of mutation in the genome of influenza A virus on adaptation to increased virulence in the mouse lung: identification of functional themes. Proc Natl Acad Sci USA 2001;98:6883–6888.
71 Hiromoto Y, Saito T, Lindstrom S, Nerome K: Characterization of low virulent strains of highly pathogenic A/Hong Kong/156/97 (H5N1) virus in mice after passage in embryonated hens' eggs. Virology 2000;272:429–437.
72 Naffakh N, Massin P, Escriou N, Crescenzo-Chaigne B, van der Werf S: Genetic analysis of the compatibility between polymerase proteins from human and avian strains of influenza A viruses. J Gen Virol 2000;81:1283–1291.
73 Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K: Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J Virol 2005;79:12058–12064.
74 Hatta M, Asano Y, Masunaga K, Ito T, Okazaki K, Toyoda T, Kawaoka Y, Ishihama A, Kida H: Mapping of functional domains on the influenza A virus RNA polymerase PB2 molecule using monoclonal antibodies. Arch Virol 2000;145:1947–1961.
75 Ludwig S, Stitz L, Planz O, Van H, Fitch WM, Scholtissek C: European swine virus as a possible source for the next influenza pandemic? Virology 1995;212:555–561.
76 Schultz U, Fitch WM, Ludwig S, Mandler J, Scholtissek C: Evolution of pig influenza viruses. Virology 1991;183:61–73.
77 Guan Y, Peiris JS, Lipatov AS, Ellis TM, Dyrting KC, Krauss S, Zhang LJ, Webster RG, Shortridge KF: Emergence of multiple genotypes of H5N1 avian influenza viruses in Hong Kong SAR. Proc Natl Acad Sci USA 2002;99:8950–8955.
78 Xu X, Subbarao, Cox NJ, Guo Y: Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology 1999; 261:15–19.
79 Choi YK, Ozaki H, Webby RJ, Webster RG, Peiris JS, Poon L, Butt C, Leung YH, Guan Y: Continuing evolution of H9N2 influenza viruses in Southeastern China. J Virol 2004;78:8609–8614.
80 Guan Y, Shortridge KF, Krauss S, Webster RG: Molecular characterization of H9N2 influenza viruses: were they the donors of the 'internal' genes of H5N1 viruses in Hong Kong? Proc Natl Acad Sci USA 1999;96:9363–9367.
81 Hoffmann E, Stech J, Leneva I, Krauss S, Scholtissek C, Chin PS, Peiris M, Shortridge KF, Webster RG: Characterization of the influenza A virus gene pool in avian species in southern China: was H6N1 a derivative or a precursor of H5N1? J Virol 2000;74: 6309–6315.
82 Gabriel G, Abram M, Keiner B, Wagner R, Klenk HD, Stech J: Differential polymerase activity in avian and mammalian cells determines host range of influenza virus. J Virol 2007;81:9601–9604.

Jürgen Stech
Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health
Institute of Molecular Biology, Südufer 10
DE–17493 Greifswald-Insel Riems (Germany)
Tel. 149 38351 7237, Fax 149 38351 7275, E-Mail juergen.stech@fli.bund.de

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 195–209

Influenza A Virus Virulence and Innate Immunity: Recent Insights from New Mouse Models

Otto Haller · Georg Kochs · Peter Staeheli

Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene,
Universität Freiburg, Freiburg, Germany

Abstract

Influenza virus infections continue to represent a major public health threat. Influenza A virus virulence is multifactorial and requires an optimal constellation of viral genes in the context of a susceptible host. Interferons exert powerful antiviral effects against influenza viruses and are critical for host survival. Here we summarize recent progress in identifying viral and host factors which determine the outcome of infection, using highly pathogenic Asian H5N1 viruses or the pandemic 1918 'Spanish flu' strain. Our infection models show that the full potential of type I and type III interferons against influenza viruses can only be appreciated in mice with a functional *Mx1* gene being the main mediator of interferon-induced protection. Notably, common inbred mouse strains have defective *Mx1* genes and, therefore, crippled innate defenses. Mx1 mouse models further revealed that IFN-α, IFN-β and IFN-λ contribute to resistance against influenza virus, and that animals lacking type I and type III IFN receptors are virtually defenseless. Influenza A viruses, in turn, evolved non-structural protein NS1 as an IFN antagonist and display diverse evasion mechanisms to escape the interferon system. Interestingly, a high virus multiplication speed may simply outrun the time-consuming establishment of the antiviral responses in immunocompetent Mx1-positive hosts.

Introduction

Influenza virus infections continue to represent a major public health threat. Epidemics caused by influenza A viruses (FLUAV) occur regularly, often leading to excess mortality in susceptible populations, and may result in devastating pandemics in humans [1]. An avian FLUAV originating from Asia and currently circulating among domestic birds in Europe and neighboring countries has the potential to infect and kill people. If further adaptation to humans occurred, this virus strain might become the origin of a future pandemic [2]. Although influenza viruses belong to the best studied viruses, the molecular

determinants governing increased virulence of emerging virus strains in humans are presently not well understood. Likewise, the properties required for host-to-host transmission remain to be elucidated. Unfortunately, efficient control of influenza virus infections is still far from being optimal. Immunization regimes are continually being confronted with the extreme antigenic variability of FLUAV brought about by antigenic drift and shift. The usefulness of currently available antiviral agents is limited by the requirement of very early medication and the emergence of resistant virus strains. New approaches and reagents to control influenza are therefore urgently needed. Future concepts may arise from new insights into the workings of intrinsic and innate immune mechanisms.

Cell-autonomous and innate immunity represent the first and foremost barriers against establishment of an influenza virus infection. Of particular importance are the interferons (IFNs), the pro-inflammatory cytokines and the chemokines which exert powerful antiviral effects against FLUAV in lung epithelial cells and other tissues. A major task is to identify the viral and host factors which govern the innate immune response and largely determine the outcome of infection. Likewise, it will be important to elucidate the mechanisms by which influenza viruses manage to escape the innate immune response of the mammalian host. Defining cellular restriction factors and how viruses deal with them will not only further our understanding of influenza viral host transmission and pathogenesis but also be essential for designing new preventive and therapeutic measures.

In this review we highlight recent advances in our understanding of the intricate interplay between influenza and other orthomyxoviruses and the host's innate immune system. The advent of reverse genetics allows generating mutant viruses at will which can be investigated in tissue culture and experimental animals. Although not being a natural host, the mouse is an increasingly important animal model due to the fact that it can be genetically manipulated. A wide variety of genetically well-defined mouse strains with interesting mutations are available. Appropriate crosses can be generated and used to dissect the genetics of the IFN and innate immune system, using genetically engineered influenza viruses. Here, we describe new insights into viral and host factors that determine the innate antiviral cytokine response and modulate virulence and pathogenicity in infection. The emphasis is on mouse strains which carry the wild-type *Mx1* gene as part of the full innate immune response system. Targeted mutations in specific genes have been introduced into these animals and we will describe their role in host defense against FLUAV in an otherwise fully immunocompetent genetic background. The present review is not comprehensive and we will refer to recent review articles which cover relevant aspects in more detail.

Influenza Virus and Type I Interferons

Type I IFNs (which comprise the various IFN-α and the single IFN-β subtypes) play a key role in host defense against FLUAV. Notably, type I IFNs were discovered by

Isaacs and Lindenmann [3] in 1957 as a cytokine induced by and interfering with influenza virus. They routinely used heat-inactivated virus for induction and measured the antiviral effect with live virus. However, when Lindenmann once used live virus for induction, he observed almost no IFN production in cell culture. Moreover, infection of cells with live FLUAV inhibited the subsequent induction of IFN by inactivated virus [4]. The reason for this phenomenon (called 'inverse interference') was unclear at the time but is now appreciated as the first notion of the IFN-antagonistic function mediated by the viral NS1 protein (see below) [5].

In the past 50 years, much progress has been made in demonstrating how type I IFNs are induced and how they act. It has become clear that type I IFNs are secreted by virus-infected cells, whereby specialized IFN-producing cells play a prominent role. Secreted IFNs circulate in the body and induce an antiviral program in susceptible cells which causes a degree of cell-intrinsic resistance against subsequent infection. In this way, further viral growth and spread in the infected organism is slowed down and eventually blocked. The whole process is time-consuming, as it involves not only IFN production and subsequent circulation in the body but also IFN action on new cells. These have to respond to the IFN signal by expression of a select set of genes and the synthesis of cellular factors which build up the antiviral state. Viruses, in turn, have evolved various means to escape the IFN response in order to guarantee host-to-host transmission and survival as a population. Viral escape mechanisms involve general shut-off of host cell metabolism or specific elimination of key components of the type I IFN system. Highly pathogenic FLUAV strains appear to use a combination of strategies. A simple but demanding way is to out-compete the antiviral IFN response by high virus multiplication speed, as illustrated below.

Type I IFNs are known to activate the expression of several hundred so-called IFN-stimulated genes (ISGs) which have antiviral, antiproliferative, and immunomodulatory functions. Among the induced proteins are the Mx GTPases which have antiviral activity against a range of viruses [6, 7]. Of note, sensitivity to the inhibitory action of Mx GTPases is a characteristic feature of all orthomyxoviruses, including Thogoto and Dhori viruses [8]. In the mouse, the Mx1 protein is the main factor mediating inhibition of influenza viruses by IFNs [9]. Unexpectedly, most inbred strains of mice carry defective non-functional Mx1 alleles and are therefore not fully immunocompetent [10]. The full potential of IFNs against infections with orthomyxoviruses can only be appreciated in Mx1-positive mice whereas common inbred mouse strains are not adequate. Below we summarize recent findings in wild-type and gene targeted Mx1-positive mice, revealing the relative contributions of type I and type III IFN in recovery from FLUAV infection.

Type III (Lambda) Interferons

IFNs are classified into type I IFNs (IFN-α/β), type II IFNs (IFN-γ), and type III IFNs (IFN-λ) on the basis of amino acid sequence, mode of induction, receptor usage, and

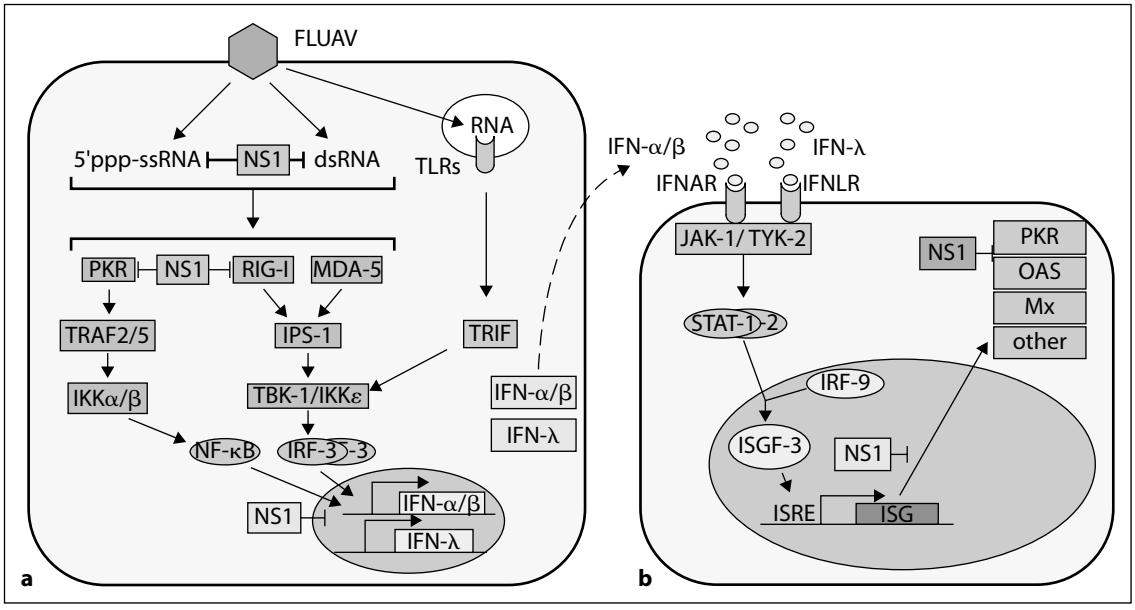


Fig. 1. Induction and suppression of the IFN system by pathogenic FLUAV. **a** IFN induction. Upon infection, the incoming genomic 5′-triphosphorylated ssRNA of FLUAV is recognized by the intracellular receptor RIG-I which starts the IFN induction pathway. In addition, dsRNA molecules generated during infection are sensed by RIG-I, MDA5 and PKR which, in turn, trigger the activation of the transcription factors IRF-3 and NF-κB via several intermediate signaling factors. IPS-1 (MAVS) is an essential adaptor protein located on mitochondria. The kinases TBK-1 and IKKε phosphorylate and activate IRF-3. NF-κB is mainly activated by the PKR pathway. The IFN antagonist NS1 of FLUAV targets dsRNA as well as RIG-I and PKR and suppresses the signaling pathway required for IFN gene expression. Furthermore, NS1 inhibits host cell pre-mRNA processing and blocks nuclear export of polyadenylated cellular mRNAs (see text for details). **b** IFN action. Type I and type III IFNs bind to their cognate receptors (IFNAR or IFNLR, respectively) and activate the expression of numerous IFN-stimulated genes (ISGs) via the JAK/STAT pathway. NS1 of FLUAV binds and sequesters dsRNA and prevents activation of the 2′-5′ oligoadenylate/RNase L system. It directly binds to PKR and blocks PKR activation. Moreover, it inhibits host cell mRNA processing and nuclear export (see text for details).

biological activity. The recently discovered lambda interferons (IFN-λ1, IFN-λ2 and IFN-λ3, also called IL-29, IL-28A and IL-28B, respectively) are functionally similar to the type I IFNs but are structurally and genetically distinct and engage a unique receptor to mediate their activities [11, 12]. It appears, however, that viruses use a common mechanism involving identical signaling components to transcriptionally activate type I and type III IFN genes (fig. 1) [13]. The receptor for lambda IFNs is composed of the interleukin (IL)-10 receptor-β chain and the IL-28 receptor-α chain. Binding of type III IFNs to their cognate receptor initiates a signaling cascade which is again shared by type I and type III IFNs and activates a comparable set of ISGs, including Mx (fig. 1). Lambda IFNs were shown to have antiviral activity against a range of DNA and RNA viruses, but their antiviral activity and potential role in

FLUAV infections was unknown. We used genetically altered Mx1-positive mice as a new animal model to demonstrate their contribution to influenza virus resistance, as outlined below.

Type I/Type III IFN Systems

Although type I (α/β) and type III (λ) IFNs are only distantly related, they use similar intracellular signaling cascades to get induced by viruses and to exert their antiviral activities (fig. 1a). These signaling pathways are described here only very briefly in as far as they are relevant for FLUAV virulence. The reader is referred to excellent recent reviews for details [14–17].

Conserved molecular signatures of viruses are recognized by specialized receptors of the host cell. These 'danger signals' appear to be viral single-stranded RNA genomes containing a 5′-triphosphate group [18–20]. Alternatively, double-stranded RNA (dsRNA) molecules formed as intermediates during virus replication represent viral signatures [21]. The sensing molecules are either the RNA helicase RIG-I or MDA5 which are both located in the cytoplasm of infected cells [22, 23]. Toll-like receptors (TLR) in endosomes play an additional role [24]. RIG-I senses genomes of negative-strand RNA viruses, including influenza viruses, whereas MDA5 is involved in recognition of positive strand RNA viruses. Upon RNA binding, the sensing helicase undergoes a conformational switch and associates with the adaptor molecule IPS-1 (also called MAVS, VISA or CARDIF) found inserted in the mitochondrial outer membrane. Interestingly, two non-redundant signaling pathways converge to the single IPS-1/MAVS adaptor molecule, which assumes a central role in triggering the innate immune response [25]. Clearly, IPS-1/MAVS is a master adaptor and indispensable for downstream signaling, whereby the mitochondrial location appears to be crucial for yet unknown reasons. Presumably the membrane topography helps to form a large and stable complex in which the IKK-like kinases IKK-ε and TBK-1 are recruited and phosphorylate interferon regulatory factor 3 (IRF-3). Phosphorylated IRF-3 homo-dimerizes and moves into the nucleus where it recruits the transcriptional co-activator, CREB-binding protein (CBP), to initiate IFN mRNA synthesis. In addition, NF-κB and ATF-2/cJUN (AP-1) are activated as a more general stress response. Together these transcription factors strongly up-regulate type I and type III IFN expression. A second IRF family member, IRF-7, is part of a positive feedback loop leading to amplification of IFN gene expression. Activated IRF-7 cooperates with IRF-3 and stimulates expression of the numerous IFN-α genes leading to a broad IFN-α response, especially in IFN-producing plasmacytoid dendritic cells in which IRF-7 is constitutively present at high levels.

Once secreted from the producer cells, the various type I IFN subspecies bind to and activate a common type I IFN receptor (IFNAR), whereas type III (λ) IFNs activate their cognate type III receptor (IFNLR, also designated IL-28R) (fig. 1b). Both

receptors signal to the nucleus through the so-called JAK-STAT pathway which is well characterized [26–29] and will not be described in detail here.

Type I and type III IFNs activate the expression of an overlapping set of more than 300 ISGs some of which have antiviral activity (fig. 1b). Three enzyme systems represent major antiviral pathways and have been extensively studied. These include protein kinase R (PKR) [30], the 2′-5′ oligoadenylate synthetase (OAS)/RNase L system [31] and the Mx GTPases [6, 7]. Mice lacking one or several of these pathways show dramatically increased virus susceptibilities [32]. Additional proteins with potentially important antiviral activities are ISG20, P56, guanylate-binding protein-1 (GBP-1), promyelocytic leukemia protein (PML) and ISG15. Overexpression of PML has been found to suppress replication of FLUAV [33], but cells from wild-type and PML knockout mice proved to be equally permissive [34], indicating that PML itself is not antiviral but may somehow influence IFN sensitivity of cells, as suggested by recent findings [35].

Role of Human and Mouse Mx GTPases in Host Defense against FLUAV

A single IFN-regulated gene, *Mx1* (for orthomyxovirus resistance gene 1), confers resistance to infection with FLUAV in mice [7, 36, 37]. Numerous experiments with Mx1-congenic or Mx1-transgenic mice demonstrated that the Mx1 system is indispensable for recovery from infection with otherwise deadly influenza viruses [38]. Moreover, it became clear that the course of disease observed in Mx1-positive mice reflects quite well the characteristics of an uncomplicated acute influenza virus infection in man, indicating that these animals better mimic the innate immune system of humans than standard laboratory mice. The human orthologue, called MxA, has a broad antiviral activity against a range of different viruses. MxA-sensitive viruses include members of the bunyaviruses, orthomyxoviruses, paramyxoviruses, rhabdoviruses, togaviruses, picornaviruses, reoviruses and hepatitis B virus [6, 7]. The mechanism of Mx action has been studied for only a few viruses so far and is still incompletely understood. In general, Mx proteins were found to bind to viral nucleocapsid components and to block their function. For example, the human MxA protein accumulates in the cytoplasm of IFN-treated cells and blocks replication of the infecting virus soon after cell entry. It has been shown to target the viral capsids by recognizing the major capsid component, the viral ribonucleoprotein of some orthomyxo- and bunyaviruses [39–41, and Dittmann et al., unpubl. results].

MxA is easily detectable in peripheral blood lymphocytes and other cells during a viral infection [42]. Some ISGs are activated directly by viruses even in the absence of IFN signaling. In contrast, MxA gene expression depends exclusively on IFN-induced JAK-STAT signaling and cannot be stimulated directly by FLUAV infection. To formally prove this, we took advantage of human cells which were obtained from patients with inherited genetic defects in STAT1. Such cells can produce IFN but cannot

mediate the signal of the type I and type III IFN receptors [43]. As expected, no MxA expression was detectable in these STAT1-deficient cells upon infection with FLUAV or treatment with exogenous IFN [44].

A beneficial role of MxA in human populations during influenza epidemics or pandemics is difficult to assess. MxA polymorphisms have been described [45] but not investigated at a large scale, and genetic MxA defects are not known. The protective power of the human MxA GTPase is best demonstrated in MxA-transgenic mice. Human MxA was sufficient to turn susceptible Mx1-negative mice into resistant animals, even in otherwise IFN-non-responsive $IFNAR^{0/0}$ animals [46]. In addition, there is some recent circumstantial evidence that MxA might provide a barrier against trans-species transmission of avian FLUAV. We recently analyzed a range of influenza A virus strains for their sensitivities to murine Mx1 and human MxA proteins and found remarkable differences. Virus strains of avian origin were highly sensitive to Mx1, whereas strains of human origin were much more resistant. Artificial reassortments of the viral components in a minireplicon system identified the viral nucleoprotein as the main target structure of Mx proteins. Interestingly, the highly pathogenic avian H5N1 strain A/Vietnam/1203/04 was much more sensitive to the inhibitory action of the human MxA protein than the recently reconstructed 1918 H1N1 'Spanish flu' virus [47]. These findings suggest that Mx proteins provide a formidable hurdle that hinders FLUAV of avian origin to cross the species barrier to humans. The observed insensitivity of the 1918 virus-based polymerase complex towards the antiviral activity of human MxA is a hitherto unrecognized characteristic of the 'Spanish flu' virus which may have contributed to the high virulence of this unusual pandemic strain.

New Mouse Models of Innate Immunity

In conventional laboratory mice, the *Mx1* gene is defective [10]. Why intact *Mx1* genes are absent in most inbred mouse strains remains unresolved. Most likely, laboratory mice share the distal part of chromosome 16 with a common ancestor mouse due to a so-called 'founder effect' occurring during early mouse breeding attempts. Due to the *Mx1* gene defect, conventional inbred strains are not adequate experimental models to study the innate immune responses against FLUAV. We therefore introduced functional *Mx1* gene into laboratory mouse strains with various immune defects, and used these animals for infection studies.

Dramatically different outcomes were noted when BALB/c mice carrying or lacking functional *Mx1* genes were used for infection studies with highly pathogenic Asian H5N1 or the pandemic 1918 'Spanish flu' strain of influenza A virus (fig. 2). In both cases, 100- to 1,000-fold higher virus doses were required to induce lethal disease in Mx1-positive mice [48, 49]. The intrinsic resistance of Mx1-positive mice to the Asian H5N1 virus was further enhanced if the animals were treated with exogenous IFN

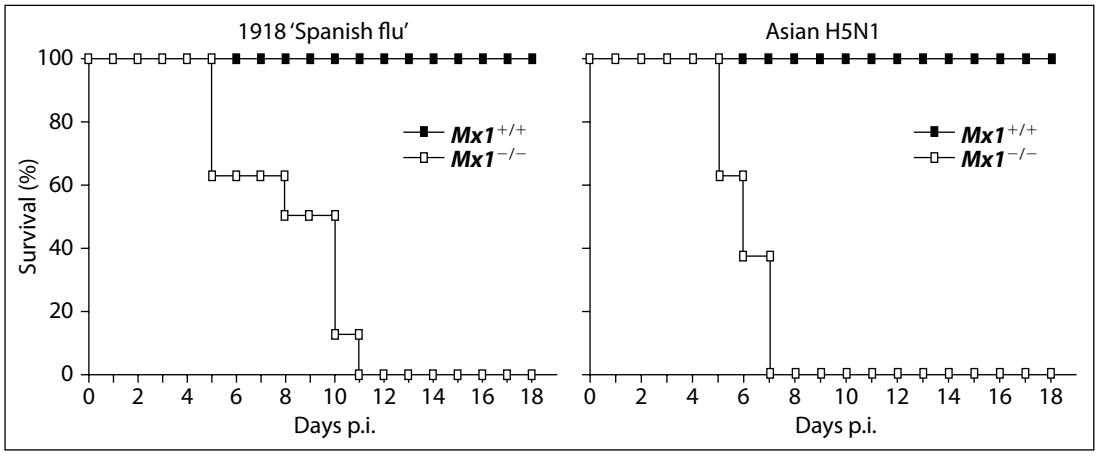


Fig. 2. Resistance of Mx1-positive mice to pandemic 1918 'Spanish flu' and highly pathogenic Asian H5N1 FLUAV. Standard BALB/c (*Mx1*$^{-/-}$) mice (open symbols) and congenic BALB.A2G-*Mx1* (*Mx1*$^{+/+}$) mice (closed symbols) were challenged by the intranasal route with 10 LD_{50} of either the pandemic 1918 strain of influenza A virus and the Asian H5N1 isolate VN1203. Survival of the animals was monitored for 18 days following infection. [Adapted from 49; copyright 2007 ASM.]

shortly before virus challenge. As expected if IFN worked by activating the Mx defense system, IFN treatment was completely ineffective in Mx1-negative mice [49].

To determine the degree of redundancy of IFN-α and IFN-β in influenza resistance, we analyzed Mx1-positive mice which either did not express functional type I IFN receptors or lacked the IFN-β gene. In the former animals, all signaling by IFN-α and IFN-β is blocked, whereas in the latter animals IFN-β is absent but IFN-α can still act. As predicted, mice lacking a functional type I IFN system *(IFNAR1*$^{0/0}$*)* were highly susceptible to challenge infections with mouse-adapted laboratory strain of H1N1 and H7N7 serotype [50]. In comparison, mice lacking only IFN-β showed a high degree of resistance which, however, was significantly less pronounced than the resistance of wild-type mice. These studies clearly showed the redundant nature of the type I IFN system and revealed that both IFN-α and IFN-β contribute to resistance against influenza virus in mice.

Until recently, the contribution of type III IFN to influenza virus resistance had not been explored. An early indication that type III IFN might play a role came from the observation that *IFNAR1*$^{0/0}$ mice lacking functional type I IFN receptors were less susceptible to certain influenza virus mutants than mice lacking the central IFN signaling molecule STAT1 [P. Staeheli, unpubl. results]. In agreement with this observation, we found that treatment of *IFNAR1*-deficient, Mx1-positive mice with recombinant IFN-λ3 resulted in robust protection against challenge with a lethal dose of wild-type influenza virus [Mordstein et al., unpubl. results]. However, Mx1-positive mice lacking

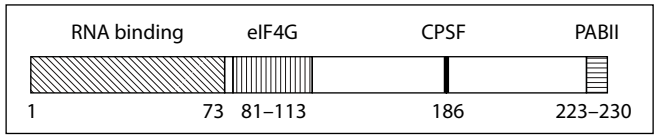


Fig. 3. NS1 is a small 26-kDa protein with distinct functional domains. NS1 features an N-terminal RNA-binding domain (positions 1–73) and a C-terminal effector domain which consists of the eIF4G-binding region (positions 81–113), the CPSF interaction site (around position 184), and the PABII-binding domain (positions 223–230).

functional type III receptors (IL-28Rα-deficient) remained fully resistant to influenza virus challenge [Mordstein et al., unpubl. results], suggesting that the protective effect of type III IFN may only become apparent when the type I IFN system has failed. In order to evaluate this concept we established Mx1-positive mice that lack both functional type I and type III receptors. We found that these animals are indeed hypersusceptible to influenza viruses. Unlike mice lacking only type I or only type III IFN receptors, the double-knockout mice died after infection with a highly attenuated influenza virus mutant that lacks the IFN-antagonistic factor NS1 [Mordstein et al., unpubl. results]. Thus, mice devoid of functional type I and type III IFN receptors seem defenseless against influenza A virus like mice lacking the central IFN signaling factor STAT1 [51].

How Does FLUAV Escape the Host Antiviral IFN Response?

Most pathogenic viruses have evolved means to escape the antiviral IFN and cytokine response of the host. Successful pathogens display numerous escape strategies allowing them to suppress IFN production, to down-modulate IFN signaling and to block the action of antiviral effector proteins [for reviews, see 52–54]. IFN antagonistic properties determine viral virulence to a great extent and may contribute to interspecies transmission of so-called emerging and re-emerging viruses, such as FLUAV.

In many instances, IFN antagonistic functions are mediated by so-called nonstructural (NS) viral proteins. These NS proteins are produced abundantly in infected cells but are not or only minimally incorporated into virus particles. Their role is confined to the cellular environment in which they exert their functions early in infection. In essence, they prepare the terrain for optimal virus growth by interacting with multiple cellular and viral factors. FLUAV has a single NS protein called NS1. It is a small protein with distinct functional domains (fig. 3) and regulates a number of critical events during influenza virus replication. NS1 directly enhances virus replication by binding and activating the cellular enzyme phosphatidylinositol 3-kinase [55, 56] and by down-regulating apoptosis [57, 58]. It also forms a trimeric complex with the eukaryotic translation initiation factor eIF4G and poly(A)-binding protein II (PABII)

to enhance initiation of viral mRNA translation [59]. It suppresses the induction of RNA interference, presumably by sequestrating small interfering RNAs [60, 61]. In addition, it has the capacity to bind dsRNA and to interact with the cleavage and polyadenylation specificity factor (CPSF) component of the cellular pre-mRNA processing machinery [62]. Given these properties, it is perhaps not too surprising that NS1 has been identified as the prototype IFN antagonist able to block IFN production and action (fig. 1) [53].

Mode of NS1 Action

It has recently become clear that NS1 exerts its anti-IFN activity in distinct ways and that the NS1 proteins of different FLUAV strains vary in their preference for one or the other pathway [63, 64]. One mechanism targets the RNA helicase RIG-I and involves the N-terminal RNA-binding domain of NS1. Binding and sequestration of dsRNA and/or interaction with RIG-I prevents downstream signaling and the activation of transcription factor IRF-3 which is crucial for IFN gene expression [65, 66] (fig. 1a). Moreover, dsRNA binding prevents activation of both the PKR and 2′-5′ OAS/RNase L enzyme systems, leading to a compromised antiviral state [67, 68]. In addition, NS1 binds directly to PKR and inhibits its activation [69]. Another mechanism of NS1 action consists in blocking posttranscriptional processing of cellular mRNAs. As a consequence, expression of cellular genes is inhibited, including those encoding IFNs and IFN-regulated genes, resulting in diminished IFN production and a reduced IFN response. This activity requires two distinct domains located in the effector region of NS1, both of which contribute to binding of NS1 to the host factor CPSF [63]. Finally, NS1 targets key components of the cellular mRNA export machinery and blocks nuclear export of host cell mRNAs, contributing to a weak IFN response [70]. Interestingly, the NS1 protein of laboratory strain A/PR/8/34 lacks a functional CPSF-binding domain [63]. Thus, unlike the NS1 proteins of the 'Spanish' influenza virus and other highly pathogenic strains which prevent the establishment of an IFN-induced antiviral state [71, 72], the NS1 protein of PR8 does not inhibit the action of IFN. Its action is rather restricted to blocking the induction of IFN in infected hosts [63].

The role of NS1 as IFN antagonist is best illustrated by mutant viruses that have a crippled NS1 gene or lack it altogether [51, 73, 74]. These NS1-deficient viruses (delNS1-FLUAV) are excellent IFN inducers and innocuous in IFN-competent hosts. They regain their pathogenic potential in IFN-non-responsive animals, indicating that their attenuation phenotype in normal hosts is linked to their inability to counteract the IFN system. For example, mice lacking the functional receptors for both type I and type III IFNs or mice with a downstream defect in STAT1 signaling are extremely susceptible and die from infection with otherwise attenuated delNS1 viruses within a few days [51, and Mordstein et al., unpubl. results].

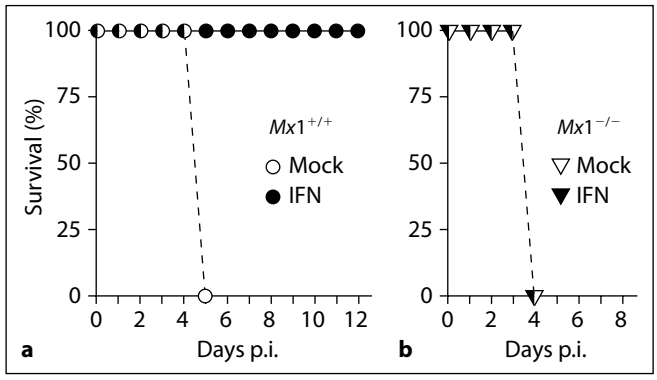


Fig. 4. Intranasal application of IFN protects *Mx1*$^{+/+}$ but not *Mx1*$^{-/-}$ mice from lethal challenge with hvPR8. (**a**) B6.A2G-*Mx1* (*Mx1*$^{+/+}$) and (**b**) C57BL/6 (*Mx1*$^{-/-}$) mice were treated with either buffer or 5×10^5 units of human IFN-αB/D (5 animals per group). Ten hours later the mice were infected with 1,000 PFU (equivalent to 100 LD_{50}) of hvPR8 and their health status was recorded for up to 14 days. [Adapted from 78; copyright 2007 National Academy of Sciences.]

Viral Replication Fitness Can Out-Compete IFN Response

All available evidence indicates that FLUAV virulence cannot be attributed to a single factor but is multifactorial. It requires an ideal 'viral gene constellation', i.e. an optimal combination of several genetic traits [75, 76]. The optimal combination is usually the product of evolutionary pressure which optimizes functional interactions between viral components and between viral and host factors. A number of virulence determinants have been identified in addition to NS1. These comprise receptor binding and cleavability of the viral hemagglutinin (HA), activity of the neuraminidase (NA), compatibility between HA and NA and the efficacy of the viral polymerase complex, among others [77].

We have recently characterized an exceptional FLUAV strain with extraordinary high virulence for IFN-competent Mx1-positive mice [78]. This highly virulent A/PR/8/34 virus (designated hvPR8) had a low LD_{50} of only 10 plaque-forming units (PFU) for both Mx1-positive and standard Mx1-negative laboratory mice. (In contrast, the LD_{50} of the standard PR8 strain is about 10^6 PFU for Mx1-positive mice and approximately 10^3 PFU for Mx1-negative mice.) Yet, hvPR8 was fully IFN-sensitive. IFN treatment protected Mx1-positive (but not Mx1-negative) mice from the lethal outcome of infection (fig. 4). Also, hvPR8 apparently did not induce less type I IFN than comparable FLUAV strains. How, then, could hvPR8 escape the IFN-induced Mx defense mechanism? A thorough analysis using recombinant parental and reassortant viruses (originating from hvPR8 and its standard counterpart) indicated that high virulence resulted from extremely fast virus multiplication in the infected lungs. Fast growth was caused by a combination of viral genes encoding the viral surface proteins HA and NA

and by the viral polymerase complex. Most likely, the virus was faster due to the combined effects of quick virus entry, high speed of intracellular replication and fast release. We therefore propose that a high virus multiplication speed may outrun a timely antiviral response of the immunocompetent host. It is conceivable that viruses in general may take advantage of the fact that the antiviral defense system is not permanently active but requires some time to get induced and established during infection.

Acknowledgement

We thank Friedemann Weber, Adolfo Garcia-Sastre and Terrence Tumpey for stimulating discussions. Own work described in this review was supported by a grant from the German Science Foundation (DFG) and by EU grant FLUINNATE 044161.

References

1 Palese P: Influenza: old and new threats. Nat Med 2004;10:S82–S87.
2 Webster RG, Peiris M, Chen H, Guan Y: H5N1 outbreaks and enzootic influenza. Emerg Infect Dis 2006;12:3–8.
3 Isaacs A, Lindenmann J: Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci 1957;147:258–267.
4 Lindenmann J: Interferon und inverse Interferenz. Z Hyg 1960;146:287–309.
5 Weber F, Kochs G, Haller O: Inverse interference: how viruses fight the interferon system. Viral Immunol 2004;17:498–515.
6 Haller O, Kochs G: Interferon-induced Mx proteins: dynamin-like GTPases with antiviral activity. Traffic 2002;3:710–717.
7 Haller O, Staeheli P, Kochs G: Interferon-induced Mx proteins in antiviral host defense. Biochimie 2007;89:812–818.
8 Haller O, Frese M, Rost D, Nuttall PA, Kochs G: Tick-borne Thogoto virus infection in mice is inhibited by the orthomyxovirus resistance gene product Mx1. J Virol 1995;69:2596–2601.
9 Staeheli P, Haller O, Boll W, Lindenmann J, Weissmann C: Mx protein: constitutive expression in 3T3 cells transformed with cloned Mx cDNA confers selective resistance to influenza virus. Cell 1986;44:147–158.
10 Staeheli P, Grob R, Meier E, Sutcliffe JG, Haller O: Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. Mol Cell Biol 1988;8:4518–4523.
11 Ank N, West H, Paludan SR: IFN-lambda: novel antiviral cytokines. J Interferon Cytokine Res 2006;26:373–379.
12 Uze G, Monneron D: IL-28 and IL-29: newcomers to the interferon family. Biochimie 2007;89:729–734.
13 Onoguchi K, Yoneyama M, Takemura A, Akira S, Taniguchi T, Namiki H, Fujita T: Viral infections activate types I and III interferon genes through a common mechanism. J Biol Chem 2007;282:7576–7581.
14 Yoneyama M, Fujita T: Function of RIG-I-like receptors in antiviral innate immunity. J Biol Chem 2007;282:15315–15318.
15 Benedict CA, Ware CF: RIGing a virus trap. Nat Med 2005;11:929–930.
16 Honda K, Taniguchi T: IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 2006;6:644–658.
17 Hiscott J, Lin R, Nakhaei P, Paz S: MasterCARD: a priceless link to innate immunity. Trends Mol Med 2006;12:53–56.
18 Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G: 5′-Triphosphate RNA is the ligand for RIG-I. Science 2006;314:994–997.
19 Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C: RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science 2006;314:997–1001.
20 Basler CF, Garcia-Sastre A: Sensing RNA virus infections. Nat Chem Biol 2007;3:20–21.
21 Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR: Double-stranded RNA is produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. J Virol 2006;80:5059–5064.

22 Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, Randall RE: The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, MDA-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci USA 2004;101: 17264–17269.
23 Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T: The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 2004;5:730–737.
24 Uematsu S, Akira S: Toll-like receptors and type I interferons. J Biol Chem 2007;282:15319–15323.
25 Kumar H, Kawai T, Kato H, Sato S, Takahashi K, Coban C, Yamamoto M, Uematsu S, Ishii KJ, Takeuchi O, Akira S: Essential role of IPS-1 in innate immune responses against RNA viruses. J Exp Med 2006;203:1795–1803.
26 Levy DE, Darnell JE Jr: Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 2002;3: 651–662.
27 Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD: How cells respond to interferons. Annu Rev Biochem 1998;67:227–264.
28 Platanias LC: Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 2005;5:375–386.
29 Van Boxel-Dezaire AH, Rani MR, Stark GR: Complex modulation of cell type-specific signaling in response to type I interferons. Immunity 2006;25:361–372.
30 Williams BR: PKR; a sentinel kinase for cellular stress. Oncogene 1999;18:6112–6120.
31 Silverman RH: A scientific journey through the 2–5A/RNase L system. Cytokine Growth Factor Rev 200718:381–388.
32 Zhou A, Paranjape JM, Der SD, Williams BR, Silverman RH: Interferon action in triply deficient mice reveals the existence of alternative antiviral pathways. Virology 1999;258:435–440.
33 Chelbi-Alix MK, Quignon F, Pelicano L, Koken MH, de The H: Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. J Virol 1998;72:1043–1051.
34 Engelhardt OG, Sirma H, Pandolfi PP, Haller O: Mx1 GTPase accumulates in distinct nuclear domains and inhibits influenza A virus in cells that lack promyelocytic leukaemia protein nuclear bodies. J Gen Virol 2004;85:2315–2326.
35 Chee AV, Lopez P, Pandolfi PP, Roizman B: Promyelocytic leukemia protein mediates interferon-based anti-herpes simplex virus 1 effects. J Virol 2003;77:7101–7105.
36 Haller O: Inborn resistance of mice to orthomyxoviruses. Curr Top Microbiol Immunol 1981;92: 25–52.
37 Staeheli P, Haller O: Interferon-induced Mx protein: a mediator of cellular resistance to influenza virus. Interferon 1987;8:1–23.
38 Arnheiter H, Frese M, Kambadur R, Meier E, Haller O: Mx transgenic mice – animal models of health. Curr Top Microbiol Immunol 1996;206:119–147.
39 Kochs G, Haller O: Interferon-induced human MxA GTPase blocks nuclear import of Thogoto virus nucleocapsids. Proc Natl Acad Sci USA 1999;96: 2082–2086.
40 Kochs G, Janzen C, Hohenberg H, Haller O: Antivirally active MxA protein sequesters La Crosse virus nucleocapsid protein into perinuclear complexes. Proc Natl Acad Sci USA 2002;99:3153–3158.
41 Reichelt M, Stertz S, Krijnse-Locker J, Haller O, Kochs G: Missorting of La Crosse virus nucleocapsid protein by the interferon-induced MxA GTPase involves smooth ER membranes. Traffic 2004;5: 772–784.
42 Roers A, Hochkeppel HK, Horisberger MA, Hovanessian A, Haller O: MxA gene expression after live virus vaccination: a sensitive marker for endogenous type I interferon. J Infect Dis 1994;169:807–813.
43 Dupuis S, Jouanguy E, Al-Hajjar S, Fieschi C, Al-Mohsen IZ, Al-Jumaah S, Yang K, Chapgier A, Eidenschenk C, Eid P, Al Ghonaium A, Tufenkeji H, Frayha H, Al-Gazlan S, Al-Rayes H, Schreiber RD, Gresser I, Casanova JL: Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. Nat Genet 2003;33:388–391.
44 Holzinger D, Jorns C, Stertz S, Boisson-Dupuis S, Thimme R, Weidmann M, Casanova JL, Haller O, Kochs G: Induction of MxA gene expression by influenza A virus requires type I or type III interferon signaling. J Virol 2007;81:7776–7785.
45 Knapp S, Yee LJ, Frodsham AJ, Hennig BJ, Hellier S, Zhang L, Wright M, Chiaramonte M, Graves M, Thomas HC, Hill AV, Thursz MR: Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR. Genes Immun 2003;4:411–419.
46 Hefti HP, Frese M, Landis H, Di Paolo C, Aguzzi A, Haller O, Pavlovic J: Human MxA protein protects mice lacking a functional alpha/beta interferon system against La Crosse virus and other lethal viral infections. J Virol 1999;73:6984–6991.
47 Dittmann J, Stertz S, Grimm D, Steel J, García-Sastre A, Haller O, Kochs G: Influenza A virus strains differ in sensitivity to the antiviral action of Mx-GTPase. J Virol 2008;82:3624–3631.
48 Salomon R, Staeheli P, Kochs G, Yen HL, Franks J, Rehg JE, Webster RG, Hoffmann E: *Mx1* gene protects mice against the highly lethal human H5N1 influenza virus. Cell Cycle 2007;6:2417–2421.

49 Tumpey TM, Szretter KJ, Van Hoeven N, Katz JM, Kochs G, Haller O, Garcia-Sastre A, Staeheli P: The *Mx1* gene protects mice against pandemic 1918 and highly lethal human H5N1 influenza viruses. J Virol 2007;10818–10821.

50 Koerner I, Kochs G, Kalinke U, Weiss S, Staeheli P: Protective role of beta interferon in host defense against influenza A virus. J Virol 2007;81:2025–2030.

51 Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T: Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 1998;252:324–330.

52 Haller O, Kochs G, Weber F: The interferon response circuit: induction and suppression by pathogenic viruses. Virology 2006;344:119–130.

53 Garcia-Sastre A, Biron CA: Type 1 interferons and the virus-host relationship: a lesson in detente. Science 2006;312:879–882.

54 Goodbourn S, Didcock L, Randall RE: Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J Gen Virol 2000;81: 2341–2364.

55 Hale BG, Jackson D, Chen YH, Lamb RA, Randall RE: Influenza A virus NS1 protein binds p85beta and activates phosphatidylinositol-3-kinase signaling. Proc Natl Acad Sci USA 2006;103:14194–14199.

56 Ehrhardt C, Wolff T, Pleschka S, Planz O, Beermann W, Bode JG, Schmolke M, Ludwig S: Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. J Virol 2007;81:3058–3067.

57 Zhirnov OP, Konakova TE, Wolff T, Klenk HD: NS1 protein of influenza A virus down-regulates apoptosis. J Virol 2002;76:1617–1625.

58 Zhirnov OP, Klenk HD: Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. Apoptosis 2007;12:1419–1432.

59 Burgui I, Aragon T, Ortin J, Nieto A: PABP1 and eIF4GI associate with influenza virus NS1 protein in viral mRNA translation initiation complexes. J Gen Virol 2003;84:3263–3274.

60 Bucher E, Hemmes H, de Haan P, Goldbach R, Prins M: The influenza A virus NS1 protein binds small interfering RNAs and suppresses RNA silencing in plants. J Gen Virol 2004;85:983–991.

61 Li WX, Li H, Lu R, Li F, Dus M, Atkinson P, Brydon EW, Johnson KL, Garcia-Sastre A, Ball LA, Palese P, Ding SW: Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proc Natl Acad Sci USA 2004;101:1350–1355.

62 Chen Z, Li Y, Krug RM: Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3′-end processing machinery. EMBO J 1999;18: 2273–2283.

63 Kochs G, Garcia-Sastre A, Martinez-Sobrido L: Multiple anti-interferon actions of the influenza A virus NS1 protein. J Virol 2007;81:7011–7021.

64 Hayman A, Comely S, Lackenby A, Hartgroves LC, Goodbourn S, McCauley JW, Barclay WS: NS1 proteins of avian influenza A viruses can act as antagonists of the human alpha/beta interferon response. J Virol 2007;81:2318–2327.

65 Guo Z, Chen LM, Zeng H, Gomez JA, Plowden J, Fujita T, Katz JM, Donis RO, Sambhara S: NS1 protein of influenza A virus inhibits the function of intracytoplasmic pathogen sensor, RIG-I. Am J Respir Cell Mol Biol 2007;36:263–269.

66 Mibayashi M, Martinez-Sobrido L, Loo YM, Cardenas WB, Gale M Jr, Garcia-Sastre A: Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J Virol 2007;81:514–524.

67 Bergmann M, Garcia-Sastre A, Carnero E, Pehamberger H, Wolff K, Palese P, Muster T: Influenza virus NS1 protein counteracts PKR-mediated inhibition of replication. J Virol 2000;74:6203–6206.

68 Min JY, Krug RM: The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2′-5′ oligo(A) synthetase/RNase L pathway. Proc Natl Acad Sci USA 2006;103:7100–7105.

69 Li S, Min JY, Krug RM, Sen GC: Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. Virology 2006;349:13–21.

70 Satterly N, Tsai PL, van Deursen J, Nussenzveig DR, Wang Y, Faria PA, Levay A, Levy DE, Fontoura BM: Influenza virus targets the mRNA export machinery and the nuclear pore complex. Proc Natl Acad Sci USA 2007;104:1853–1858.

71 Geiss GK, Salvatore M, Tumpey TM, Carter VS, Wang X, Basler CF, Taubenberger JK, Bumgarner RE, Palese P, Katze MG, Garcia-Sastre A: Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc Natl Acad Sci USA 2002;99: 10736–10741.

72 Seo SH, Hoffmann E, Webster RG: Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 2002;8:950–954.

73 Talon J, Salvatore M, O'Neill RE, Nakaya Y, Zheng H, Muster T, Garcia-Sastre A, Palese P: Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. Proc Natl Acad Sci USA 2000;97: 4309–4314.

74 Kochs G, Koerner I, Thiel L, Kothlow S, Kaspers B, Ruggli N, Summerfield A, Pavlovic J, Stech J, Staeheli P: Properties of H7N7 influenza A virus strain SC35M lacking interferon antagonist NS1 in mice and chickens. J Gen Virol 2007;88:1403–1409.
75 Rott R, Orlich M, Scholtissek C: Correlation of pathogenicity and gene constellation of influenza A viruses. III. Non-pathogenic recombinants derived from highly pathogenic parent strains. J Gen Virol 1979;44:471–477.
76 Oxford JS, McGeoch DJ, Schild GC, Beare AS: Analysis of virion RNA segments and polypeptides of influenza A virus recombinants of defined virulence. Nature 1978;273:778–779.
77 Noah DL, Krug RM: Influenza virus virulence and its molecular determinants. Adv Virus Res 2005;65:121–145.
78 Grimm D, Staeheli P, Hufbauer M, Koerner I, Martinez-Sobrido L, Solorzano A, Garcia-Sastre A, Haller O, Kochs G: Replication fitness determines high virulence of influenza A virus in mice carrying functional Mx1 resistance gene. Proc Natl Acad Sci USA 2007;104:6806–6811.

Otto Haller, MD
Abteilung Virologie, Institut für Medizinische Mikrobiologie und Hygiene
Universität Freiburg, DE–79008 Freiburg (Germany)
Tel. +49 761 203 6534, Fax +49 761 203 6626, E-Mail otto.haller@uniklinik-freiburg.de

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 210–224

Signaling to Life and Death: Influenza Viruses and Intracellular Signal Transduction Cascades

Stephan Ludwig[a] · Oliver Planz[b] · Stephan Pleschka[c] · Thorsten Wolff[d]

[a]Institute of Molecular Virology, Centre of Molecular Biology of Inflammation, Westfälische Wilhelms University, Münster; [b]Institute of Immunology, Friedrich-Loeffler-Institute, Tübingen; [c]Institute of Medical Virology, Justus Liebig University, Giessen, and [d]Robert-Koch-Institute, Berlin, Germany

Abstract

Influenza viruses successfully replicate in birds and mammals. To support their replication these pathogens extensively manipulate host-cell functions [reviewed in 1]. At the same time the infected cell activates defense mechanisms to fight the invader. These processes are mostly mediated by different intracellular signaling cascades that regulate a variety of events in the infected cell including expression of cellular antiviral genes and cytokines. The relevance of these signaling responses for the pathogenicity of avian influenza viruses was recently highlighted by the fact that infections with highly pathogenic avian H5N1 isolates result in a hyperinduction of cytokine genes ('cytokine storm') which correlates with a hyperactivation of certain signaling pathways, such as the p38 MAP kinase pathway. Thus, intracellular signaling events are at the tip of the balance between efficient virus replication and effective antiviral responses and may well determine the aggressiveness of a viral infection. Here we will review recent advances regarding signaling processes leading to the induction of the type I interferon response as well as findings towards viral exploitation of the PI3K, NF-κB and MAPK signaling pathways.

Signaling Events Executing the Type I Interferon Response

The type I interferon (IFN) system is one of the most powerful innate defenses of vertebrate cells which limits the replication and spread of viral pathogens including avian and human influenza viruses [reviewed in 2]. The IFNs are antiviral cytokines that also activate various immune cells such as dendritic cells and have therefore additional importance for the initiation of adaptive immune reactions [3]. Type I IFN comprises mainly the family members IFN-α, IFN-β and IFN-ω that were all described in mammalian species. The molecular cloning of avian type I IFN genes started not before the 1990s, although the IFN activity was discovered more than 50 years ago during analyses

of embryonated chicken eggs infected with influenza viruses [4]. Therefore, the majority of studies have focused on mammalian type I IFN. However, there is a good body of evidence showing that the avian IFN homologues share essentially the same functions in spite of some structural divergence [5, 6].

Many different cell types are capable of producing type I IFN, allowing them to respond immediately against an invading virus and to prepare neighboring cells for the imminent attack of a pathogen. Secreted IFN-α and IFN-β bind to a common IFN-α/β receptor (IFNAR) which by signaling through the JAK-STAT pathway leads to the formation of the trimeric transcription factor ISGF3 that in turn up-regulates a multitude of latent host genes. Many of those IFN-induced gene products, such as the Mx protein or p56, have strong antiviral activities by themselves [2]. Others, like the 2′-5′-oligoadenylate synthetases (OAS) and the protein kinase PKR require the additional cofactor dsRNA for activation after virus infection. Thus, IFN-treated cells establish a so-called antiviral state in which many viruses cannot efficiently replicate. The potency of the IFN-α/β system is illustrated by the high sensitivity of IFNAR$^{-/-}$ mice to viral infections [7]. However, during co-evolution with their hosts probably all natural viruses have evolved gene products that interfere with the IFN-α/β system at either the induction or effector level [8]. Thus, even viruses that are naturally strong IFN inducers are able to grow efficiently if they prevent or circumvent the antiviral state induced by IFN.

The antiviral effector activities of type I IFN described above have been characterized quite extensively [2]. However, it was not until recently that the cellular factors initiating the induction of IFNs were described. Fibroblastoid and epithelial cells respond to viral nucleic acids produced during virus infection such as dsRNA or single-stranded RNAs carrying 5′-triphosphate (fig. 1) [9–11]. These molecules are recognized by the related RNA helicases MDA5 and RIG-I, respectively, which triggers a signaling module that leads to the activation of type I IFN genes by the latent transcription factors IRF-3/-7, NF-κB and ATF-2/c-Jun (fig. 1) [12]. The RNA helicases interact via two caspase recruitment domains (CARD) with the mitochondrial IFN-β promoter stimulator 1 (IPS-1) protein [13, 14], which is thought to mediate activation of the transcription factors IRF-3 and IRF-7 through phosphorylation of the Iκ-B kinase family members TBK-1 or IKK-ε [15]. IPS-1 also activates NF-κB that participates in the induction of IFN-β and pro-inflammatory cytokine genes [16]. The intracellular detection of influenza virus may not rely on RIG-I in some myeloid cells, in which single-strand RNA viruses are detected within an endosomal compartment by toll-like receptors [13]. However, influenza viruses usually do not propagate in these cells.

Suppression of Type I IFN Induction Is a Prerequisite of Avian Influenza A Viruses Pathogenicity

Influenza virus propagation is sensitive to IFN activities and therefore these viruses not only induce type I IFN through their genomic RNAs carrying 5′-triphosphate

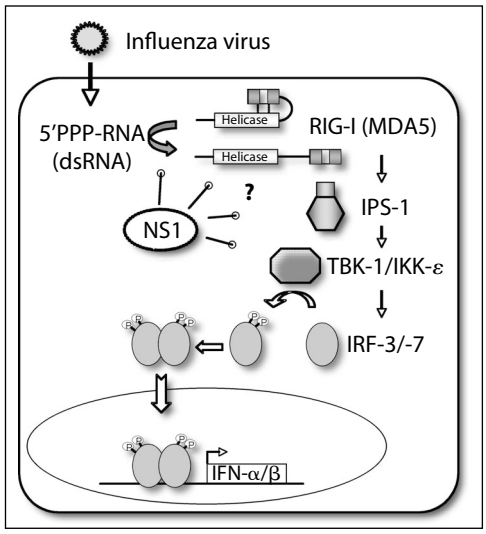


Fig. 1. The canonical pathway of IFN-α/β induction during influenza virus infection. Productive virus infection is accompanied by generation of 5′-triphosphate RNA and dsRNA that is recognized by the RNA helicases RIG-I or MDA5, respectively. This leads, via the adapter protein IPS-1, to the phosphorylation and activation of the key transcription factors IRF3 and/or IRF7 by the protein kinases TBK-1 and/or IKK-ε. Phosphorylated IRF-3/-7 dimerizes and translocates to the nucleus, where it becomes engaged in the activation of IFN-α/β genes. The influenza virus NS1 protein is known to inhibit activation of IRF-3/-7 by blocking RNA-dependent upstream signals via direct interaction with the RNA sensor RIG-I. However, NS1 interference with other levels of the activation cascade cannot be excluded.

groups, but at the same time also antagonize the production of these cytokines. This is accomplished through the viral non-structural 26-kDa NS1 protein that is abundantly expressed in infected cells (fig. 1). Thus, influenza viruses of human and avian origin with truncated or deleted NS1 genes were shown to be much stronger IFN inducers compared to the wild type and this correlated with a pronounced attenuation in animal experiments [17–19]. Two recent comparative analyses of closely related virus pairs emphasized the important role of NS1 for the virulence of low and highly pathogenic avian influenza viruses. The LPAIV strain A/turkey/Oregon/71 virus (H7N3) expressing full-length NS1 of 230 amino acids caused more severe lesions in the tissues of 4-week-old chickens compared to a virus variant encoding a truncated NS1 protein of only 124 amino acids [17]. The wild-type strain replicated to high titers in chicken kidney and lung after intravenous inoculation and was transmissible to non-infected cage mates, which did not occur with the variant virus expressing the shortened NS1 protein that also induced about 20-fold more IFN in primary chicken embryo cells (CEC). Similarly, an elegant reverse genetic study of highly pathogenic H5N1 viruses isolated from diseased domestic geese in the Guandong Province, China, in 1996, demonstrated that a single alanine to valine exchange at NS1 position 149 renders the virus apathogenic to chicken despite the

presence of a multibasic cleavage site within the hemagglutinin [20]. This was paralleled by a conversion of the low to a high IFN inducing phenotype of the virus in CEC. Interestingly, the introduction of the Ala 149→Val mutation into the NS1 gene of the A/Goose/Guangdong/1/96 virus that infects, but usually does not kill geese, also eliminated its capacity to produce morbidity in these birds. These data confirm that IFN suppression by the NS1 protein is another essential attribute of avian influenza virus virulence in addition to the insertion of multiple basic amino acids at the cleavage site of the HA.

How does the viral NS1 protein function to inhibit IFN induction? According to a previous model, the dsRNA-binding capacity of NS1 interferes with recognition of IFN-inducing viral nucleic acids by cellular sensors (such as RIG-1 or MDA5). However, this concept may have to be revised, since recombinant influenza viruses expressing dsRNA-binding defective NS1 proteins were recently shown to be capable of suppressing IFN induction, at least to some extent [21, 22]. Further studies demonstrated that the NS1 protein inhibits RIG-I-dependent IFN induction and also forms an immunoprecipitable complex with RIG-I [11, 23]. Therefore, NS1 may suppress IFN induction through a protein-protein interaction with RIG-I or another factor of this signaling module (fig. 1). The dsRNA-binding activity of NS1 may be required in the inhibition of antiviral enzymes such as PKR and 2′-5′-OAS [21, 24]. This is beneficial for the virus, because PKR activation leads to a sustained arrest in cellular translation whereas the synthesis of 2′-5′ oligo(A) chains activates the latent RNase L that degrades single-strand RNA. NS1 proteins expressed by some virus strains were also described to inhibit the maturation of cellular pre-mRNAs raising the possibility that this activity additionally reduces the production of IFN-α/β in infected cells [25].

Phosphatidylinositol-3-Kinase and Influenza Virus Infection

Phosphatidylinositol-3-kinase (PI3K) is an intracellular signaling mediator that consists of a regulatory (p85) and an enzymatic subunit (p110) and exhibits both a protein kinase and a lipid kinase activity [26]. Both subunits exist in several isoforms and the heterodimeric kinase regulates various cellular processes, such as cell metabolism, proliferation, and survival [27]. PI3K activation leads to the generation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2) in the membrane, which functions as a second messenger to recruit pleckstrin homology (PH) domain-containing proteins, such as the kinase Akt/PKB (fig. 2) and phosphoinositide-dependent kinase 1 (PDK-1). Akt/PKB is a major PI3K effector and gets further activated by phosphorylation at Thr308 and at Ser473.

The PI3K/Akt pathway kinase has mainly been discussed as a suppressor of apoptosis during viral infections [28]. However, more recent data has shed light on a novel function of PI3K in the context of RNA-induced IFN expression. It was shown that PI3K is involved in phosphorylation and activation of IRF-3, a major regulator of

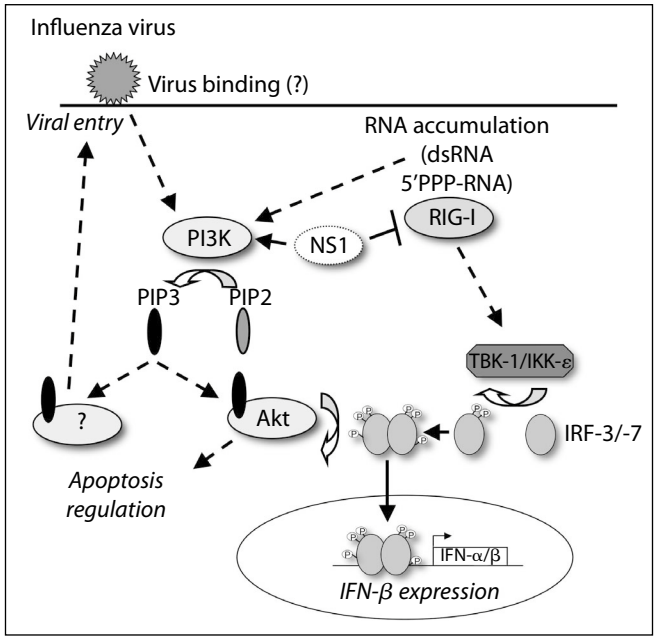


Fig. 2. Activation and function of the PI3K/Akt pathway in influenza virus-infected cells. Three mechanisms of activation of the PI3K/Akt pathway have been reported in cells productively infected with influenza A virus. In a very early phase of infection there is a transient activation that occurs most likely due to binding of virus particles to receptors at the cell surface. Later in infection the pathway can be activated by accumulation of dsRNA-like nucleic acids or by binding of the viral NS1 protein to p85, the regulatory subunit of PI3K. While it was reported that early activation of the pathway is required for virus entry, late activation appears to serve two concurrent functions. While the kinase is involved in regulation of IRF-3 activation by targeting an additional phosphorylation site on IRF-3 needed for full activation there is also a virus-supportive function by preventing premature apoptosis induction.

IFN-β transcription, in response to engagement of the dsRNA sensor TLR3 (fig. 2) [29]. Interestingly this occurred independently of TBK-1 or IKK-ε and most likely was mediated via phosphorylation of a different target phosphorylation site on IRF-3 [29]. After first indications that this pathway is also activated in influenza virus-infected cells [30] it has finally been shown that inhibition of PI3K or block of its effector PIP3 results in a misphosphorylation of IRF-3 and impaired transcriptional activation of the IFN-β promoter upon infection with avian and human influenza virus strains [31].

However, besides this antiviral activity, PI3K also exhibits virus-supportive functions, both at the early and late stages of the influenza virus infection cycle (fig. 2) [31–34]. The first evidence came from studies where progeny virus titers were determined from infected cells in which PI3K or PIP3 signaling was inhibited prior to infection. Progeny virus titers from these cells were significantly reduced compared to

wild-type cells, clearly indicating that this signaling event is beneficial for virus propagation. In further studies it was observed that entry of the virus particles was hampered in cells when PI3K and PIP3 signaling was blocked prior to infection. This has led to the intriguing conclusion that the very early entry of influenza virus particles does not occur constitutively, but is at least in part a signaling-regulated event (fig. 2) [31].

In addition to that it seems that the pathway also supports virus growth in late stages of the replication cycle since inhibition of PI3K as late as 2 h post-infection still led to reduced progeny virus titers [31]. In subsequent analysis it was shown that viral PI3K/Akt activation contributes to the inhibition of premature apoptosis in the infected cell (fig. 2) by blocking the pro-apoptotic activity of the Akt effectors caspase 9 and glycogen synthase kinase-3 [32, 34]. This might not be the only effect of PI3K since another parallel study showed that in cells treated with the PI3K inhibitor LY294002 viral RNA and protein synthesis as well as nuclear export of the RNPs was impaired [33].

Although PI3K could be activated by RNA [29] and all RNA activated cellular signaling pathways identified so far were shown to be suppressed by the influenza viral NS1 protein [for review, see 35], this was the opposite for the PI3K/Akt pathway. In cells infected with a virus mutant lacking the NS1 protein, PI3K/Akt activation was not enhanced but barely detectable [31]. This has led to the striking suggestion that NS1 itself may be an inducer of PI3K activation, a hypothesis that has been subsequently verified in four independent and parallel studies [32, 34, 36, 37]. All three sets of experiments demonstrated that NS1 expression alone is sufficient to induce PI3K/Akt activation (fig. 2). Furthermore, the viral protein was observed to interact with the regulatory subunits of PI3K, p85α and β, most likely leading to its activation. Several domains of the NS1 have been assigned to be responsible for interaction and activation of PI3K by the use of deletion or point mutants of the protein. This includes the RNA-binding domain [32], a domain between amino acids 181 and 185 in the C-terminus [32] and a conserved tyrosine residue at amino acid position 89 [36, 37]. The latter observation suggested that the phosphorylated tyrosine Y89 may represent a SH2-binding site that would bind to the respective SH2 domain of p85. This has been challenged by the observation that NS1 was not found to be tyrosine-phosphorylated, and mutation of the respective site in a recombinant virus would not directly modulate induction of apoptosis [pers. unpubl. observation, and R. Randall, pers. commun.]. This parallels the findings that a mutation of tyrosine 89 to phenylalanine in recombinant viruses did not significantly affect virus titers [our own unpublished observations]. Thus, the detailed mode of NS1 binding to p85 is still elusive and under further investigation.

Taken together, the PI3K/PIP3 module can be activated by early virus binding to the cell surface as well as late in the infection cycle via RNA accumulation and/or expression of the viral NS1 protein (fig. 2). Accordingly, PI3K appears to fulfill multiple functions early and late in the infection cycle, including IRF-3 activation, regulation of virus entry and inhibition of premature apoptosis (fig. 2).

Influenza Virus Infection and Mitogen-Activated Protein Kinase Cascades

Mitogen-activated protein kinase (MAPK) cascades are important signaling pathways that convert extra- or intracellular signals into cellular responses [reviewed in 38]. These signaling modules regulate proliferation, differentiation, cell activation and immune responses. Four different members, organized in separate cascades have been identified so far: (I) ERK (extracellular signal regulated kinase), (II) JNK (Jun-N-terminal kinase), (III) p38 and (IV) ERK5 (fig. 3). Different isoforms are known for each MAPK. All these enzymes are activated by phosphorylation via an upstream MAPK kinase (MAPKK, MEKs or MKKs) (fig. 3). The MAPKs ERK1/2 are activated by the MAPKKs MEK1/2 that are controlled by the MAPKKK Raf. Raf, MEK and ERK form the prototype module of a MAPK pathway. The MAPKs p38 and JNK in turn are activated by the MAPKKs MKK3/6 and MKK4/7, respectively, and are predominantly activated by pro-inflammatory cytokines and certain environmental stress conditions. ERK5 is activated by the MAPKK MEK5 (fig. 3). This kinase module is activated by both mitogens and certain stress inducers.

All four MAPK cascades have been shown to be activated upon infection with a variety of avian or human influenza virus strains [39–41, and V. Korte and S. Ludwig, unpubl. data]. Recent work has led to a better understanding of the importance of these signaling pathways for influenza virus replication, especially of the Raf/MEK/ERK-cascade.

Activation of p38 after influenza virus infection has been linked to expression of chemokines, such as RANTES and IL-8 that are involved in the attraction of eosinophils and neutrophils, respectively [39]. Furthermore, a role for p38 in the viral induction of cytokines, most prominently TNF, was suggested in cells infected with highly pathogenic avian influenza viruses (HPAIV) from the H5N1 subtype [42]. Here a hyperinduction of p38 by the H5N1 virus could be observed compared to cells infected by contemporary H1N1 or H3N2 strains [42] while inhibition of p38 in these infected cells resulted in a suppression of cytokine induction [42]. This has led to the assumption that hyperinduction of p38 may strongly contribute to the hypercytokinemia observed upon infection with HPAIV of the H5N1 subtype.

Interestingly, inhibition of p38 MAPK by specific inhibitors or a dominant negative mutant of the p38 activator MKK6 results in reduced virus titers indicating that p38 activity might positively contribute to the replication process [V. Korte and S. Ludwig, unpubl. data].

Induction of the MAPK JNK pathway upon influenza virus infection results in early activation of the AP-1 (activator protein 1) transcription factors. This has been observed in a variety of permissive cell lines infected with different virus strains and requires productive replication and accumulation of viral RNA. Together with NF-κB and IRF-3 the AP-1 factors are critical for regulation of the expression of IFN-β, one of the most potent antiviral cytokines (fig. 3). Accordingly, inhibition of the cascade

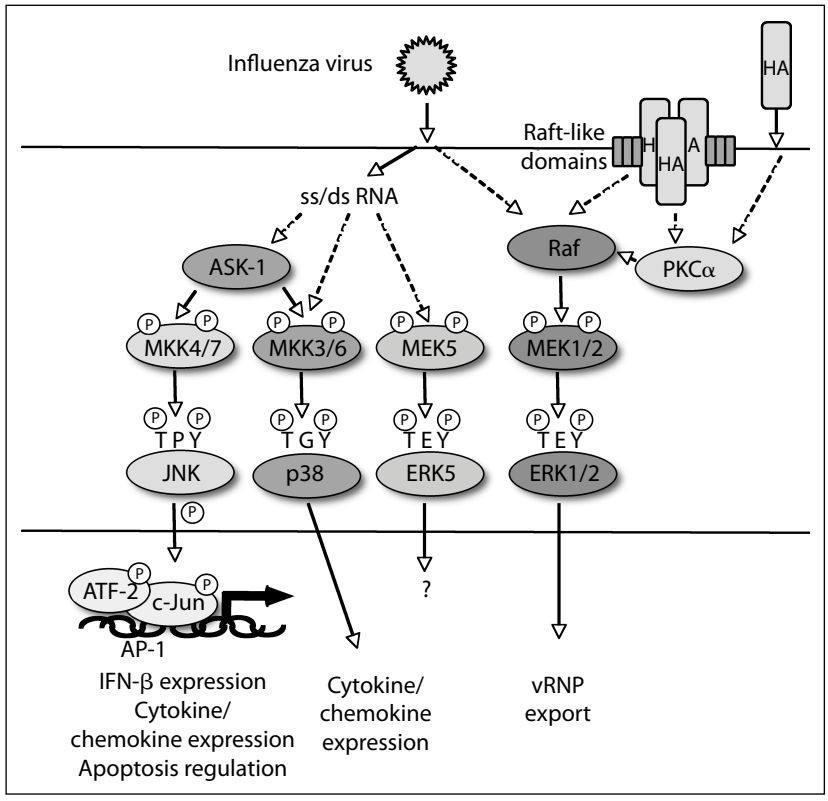


Fig. 3. Schematic representation of influenza virus-induced MAP kinase cascades and their potential function in the infected cells. MAP kinases are activated by dual phosphorylation at threonine (T) and tyrosine (Y) in a dual phosphorylation motif with the minimum sequence TXY that differs between the different family members. During influenza virus infection, p38, JNK and ERK5 MAPK cascades are primarily activated by accumulation of dsRNA-like viral nucleic acids in the infected cell. The Raf/MEK/ERK signaling cascade is activated early and late in the infection cycle. While early activation presumably is initiated by binding of the virus particle to the cell surface, late activation is mediated by incorporation of the hemagglutinin (HA) into cholesterol-rich membrane domains, also known as lipid rafts. The net impact of each of the signaling pathways on viral replication, as determined by the use of dominant-negative mutants and specific inhibitors, is also given in the figure. While the Raf/MEK/ERK cascade, the p38 pathway and different PKC isoforms exhibit virus-supportive functions, the JNK pathway acts primarily antiviral. The ERK5 pathway, although activated upon infection, has no effect on viral replication. Abbreviations: MKK = MAP kinase kinase; MEK = MAPK/ERK kinase; ERK = extracellular signal-regulated kinase; JNK = c-Jun N-terminal kinase; ASK-1 = apoptosis signal-regulated kinase-1; ATF-2 = activating transcription factor-2; PKC = protein kinase C.

resulted in impaired transcription from the IFN-β promoter and an enhanced virus production. Thus, activation of the JNK pathway appears to be part of the antiviral response to an influenza virus infection [40].

The activation of ERK upon productive influenza virus infection [39] also contributes to virus-induced cytokine production, however at the same time it appears to support viral replication [41]. Strikingly, specific blockade of the pathway strongly

impaired growth of avian and human influenza A viruses as well as human B-type viruses [41, 43]. Conversely, virus titers are enhanced in cells with an activated Raf/MEK/ERK pathway [43, 44]. This has not only been demonstrated in cell culture, but also in vivo in infected mice expressing constitutively active Raf kinase in the alveolar epithelial cells. Enhanced virus replication was observed in cells expressing the transgene [44]. Thus, activation of the Raf/MEK/ERK pathway is required for efficient virus growth. With regard to the underlying molecular mechanisms it was shown that inhibition of the cascade led to nuclear retention of the viral ribonucleoprotein (RNP) complexes in late stages of the replication cycle. This suggests that the pathway controls the active nuclear export of RNPs, most likely due to interference with the activity of the viral nuclear export protein NEP [41]. So far the detailed mechanism of how ERK regulates RNP export is unsolved. There are two likely scenarios: either it occurs directly via phosphorylation of a viral protein involved in RNP transport or by control of a cellular export factor. Although in the initial studies no alteration of the overall phosphorylation status of the NP, M and NEP proteins was observed [41], there are now first indications that certain phosphorylation sites of the NP indeed are affected by MEK inhibition [S. Pleschka, unpubl. data]. With regard to a cellular factor that might be involved there is the first evidence that the Raf/MEK/ERK cascade specifically regulates nuclear export of certain cellular RNA-protein complexes [45]. Thus, the ERK pathway may regulate phosphorylation of both the viral NP and of a yet unknown cellular factor to specifically mediate RNP export of influenza A and B viruses.

The findings led to the hypothesis that active RNP export is an induced rather than a constitutive event. While the RNPs have to reside in the nucleus for sufficient replication and transcription of the viral genome in early stages, they have to be exported from the nucleus late in the replication cycle for the incorporation into budding progeny virions at the cell membrane. This coincides well with a late activation of ERK in the viral life cycle. Thus a regulative mechanism can be predicted which raises the question how timely activation of this regulatory pathway can be achieved. A substantial advance has been made by the demonstration that membrane accumulation of the viral HA protein and its tight association with lipid-raft domains triggers protein kinase C-α (PKC-α)-dependent activation of the ERK cascade late in the infection cycle and thereby induces RNP export (fig. 3) [46]. These findings are strongly supported by results indicating that clustering of raft-associated HA in the external membrane leaflet modulates diffusion and signaling of H-Ras at the internal leaflet, which, besides PKC-α, is also a major upstream activator of Raf [47]. As HA together with NA, M2 and M1 forms electron-dense patches at the site of virus membrane budding, signaling components at the inner membrane layer could be affected leading to the activation of the Raf/MEK/ERK pathway. ERK activation by HA membrane accumulation therefore may represent an autoregulative mechanism that coordinates timing of RNP export to a point when all viral components are ready for virus budding.

It should also be mentioned here that infections with H5N1 influenza viruses have been reported to hyperinduce ERK activity [42], suggesting that the pathway may contribute to the replication capacity of these viruses.

Requirement of Raf/MEK/ERK activation for efficient influenza virus replication may suggest that this pathway can be considered a cellular target for potent antiviral approaches. A significant antiviral action against both A- and B-type viruses in cell culture [43] or infected mice [48, 49, and O. Planz, S. Pleschka and S. Ludwig, unpubl. data] could be demonstrated for commercially available MEK inhibitors. Furthermore, the compounds showed a surprisingly little toxicity, not only in cell culture [41, 43, 50] or mice [51], but also in clinical trials for the use as anticancer agent [52]. Furthermore, MEK inhibitors did not exhibit any tendency to induce generation of resistant virus variants [43].

Influenza A Virus Infection and the NF-κB Signaling Pathway

The transcription factor nuclear factor-κB (NF-κB) is a key player in the regulation of a large variety of events in the cell. The NF-κB/IκB family of transcription factors promotes the expression of well over 150 different genes, such as cytokine or chemokine genes, or genes encoding adhesion molecules or anti- and pro-apoptotic proteins [53]. Apart from its function as regulator of the expression of inflammatory cytokines, chemokines, immunoreceptors, adhesion molecules and apoptosis it is commonly activated upon virus infection [54]. NF-κB is not a single factor but represents a family of dimeric transcription factors that belong to the Rel family which include five members: NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB and c-Rel. Dimers containing RelA, RelB, or c-Rel are transcriptional activators whereas homodimers of p50 and p52, which are devoid of a transcription activation domain, function as repressors.

In non-stimulated cells, NF-κB is sequestered in an inactive form in the cytoplasm by the inhibitor of κB (IκB). The most common mode of NF-κB activation includes successive phosphorylation and degradation of IκB and translocation of the transcriptionally active factors, such as p65/p50 or p50/c-rel heterodimers, to the nucleus to exert their biological function [reviewed in reference 55]. The phosphorylation event is mediated by the IκB kinase 2 (IKK2) that forms a complex with the enzymatically active IKK1 and the scaffold protein NEMO/IKK-γ. This activation cascade is called the classical or canonical NF-κB pathway. The non-canonical or alternative pathway involves phosphorylation-dependent processing of the factor p100/NF-κB2 to p52 via activation of the NF-κB inducing kinase NIK that activates IKK1 [55]. This pathway, that results in nuclear translocation of p52/RelB heterodimers, is important for secondary lymphoid organogenesis and for the maturation of the adaptive immune response.

Influenza virus infection results in the activation of the classical NF-κB pathway [reviewed in 1, 48] although the level of activation is kept in a certain limit due to the

action of the viral NS1 protein [56]. Nevertheless, the induced activity is sufficient to control expression of a variety of genes [57, 58]. Viral induction of NF-κB follows the classical NF-κB pathway and involves activation of IKK2 [57–59]. The activation mechanism overlaps with the pathways leading to IRF-3 activation via sensors of ssRNA [60, 61] or dsRNA [62]. In addition to that, NF-κB activation can also be achieved by overexpression of the viral hemagglutinin (HA), nucleoprotein (NP) or M1 proteins [59, 63]. As gene expression of many pro-inflammatory or antiviral cytokines, such as IFN-β or TNF-α, is controlled by NF-κB [52], the concept emerged that IKK and NF-κB are essential components in the innate immune response to virus infections [62]. Accordingly, influenza virus-induced IFN-β promoter activity is strongly impaired in cells expressing transdominant negative mutants of IKK2 or IκB-α [56, 57].

Nevertheless, IKK and NF-κB also have virus-supportive functions that appear to be dominant over the antiviral activity of the factor in the context of an influenza virus infection. Two independent studies demonstrated that influenza viruses exhibit higher levels of replication in cells where NF-κB was preactivated [57, 64]. Conversely, a dramatic reduction of influenza virus titers could be observed in cells where NF-κB signaling was impaired. This is different from the situation with other RNA viruses, e.g. Borna disease virus (BDV) where constitutive activation of NF-κB clearly leads to a drop in virus titers [65]. The beneficial function on influenza virus replication was shown to be at least in part due to the NF-κB-dependent expression of pro-apoptotic factors, such as TNF-related apoptosis inducing ligand (TRAIL) or FasL [57]. Inhibition of virus-induced expression of these factors results in strongly impaired viral growth. These findings link the pro-influenza action of NF-κB to the induction of apoptosis. Accordingly, influenza virus propagation was strongly impaired in the presence of caspase inhibitors [66]. Mechanistically, the block in virus propagation appeared to be due to the retention of viral RNP complexes in the nucleus preventing proper formation of progeny virus particles [66]. However, the underlying mechanism is different from RNP retention observed upon treatment with inhibitors of the Raf/MEK/ERK cascade since it rather affects a passive export mode that appears to be caspase-dependent [65].

The strong viral need for NF-κB activity suggests that this pathway may be suitable as a target for antiviral intervention. To this end, it has been shown that pharmacological inhibitors of NF-κB impair viral replication in vivo without toxic side effects or the tendency to induce resistant virus variants [67]. One of these compounds, acetylsalicylic acid (ASA), also known as aspirin, is a widely used drug with a well-known capacity to inhibit NF-κB [68]. It was shown that ASA efficiently blocked influenza virus replication in vitro and in vivo in a mechanism that follows the previously suspected chain of events. This involves impaired expression of pro-apoptotic factors, subsequent inhibition of caspase activation as well as block of caspase-mediated nuclear export of viral RNPs [67]. Since ASA showed no toxic side effects or the tendency to induce resistant virus variants, existing salicylate-based aerosolic drugs

may be suitable as anti-influenza agents. Besides the results obtained upon blockade of the Raf/MEK/ERK cascade, this is another demonstration that specific targeting of a cellular factor is a suitable approach for anti-influenza virus intervention.

Conclusion and Outlook

The last couple of years have seen a plethora of findings that helped to unravel some of the sensing mechanisms and cellular signaling responses to influenza virus infections. However, we are still in an early phase of a rapidly evolving field. One example to underline this statement is the issue of PI3K involvement in influenza virus replication. While this signaling mediator was not on the influenza virologists map before 2006, within one year five different publications unraveled new modes of signaling activation and functions of this kinase within the infected cell. Similar rapid advances have been made in the field of structural requirements and sensors of viral RNA. With regard to avian influenza there are now first findings suggesting that the hyper-induction of cellular signaling pathways is responsible for the cytokine burst observed upon HPAIV infections. In addition, an altered signaling modulation by the NS1 proteins of HPAIV has also been reported. Thus aberrant signaling appears to strongly contribute to the pathogenicity of these viruses. The insights gained into the cellular mechanisms required for efficient viral replication of HPAIV may be very useful with respect to novel antiviral strategies in humans infected by HPAIV. Since most of the signaling pathways that have been identified to be exploited by the virus are at the same time major regulators of the cytokine response, inhibitors of these pathways may target viral replication directly as well as indirectly by interfering with the cytokine burst in HPAIV infections. Furthermore, these types of drugs would prevent the problem of viral resistance that frequently occurs upon direct attack of viral components. Thus, viral dependencies on cellular functions create opportunities to design novel antiviral strategies by targeting specific host cell functions essential for efficient virus replication. First promising candidate compounds have been proven efficient in animal models and other attempts will certainly follow.

References

1 Ludwig S, Pleschka S, Wolff T: A fatal relationship – influenza virus interactions with the host cell. Viral Immunol 1999;12:175–196.

2 Samuel CE: Antiviral actions of interferons. Clin Microbiol Rev 2001;14:778–809.

3 Garcia-Sastre A, Biron CA: Type 1 interferons and the virus-host relationship: a lesson in detente. Science 2006;312:879–882.

4 Isaacs A, Lindenmann J: Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci 1957;147:258–267.

5 Sekellick MJ, Lowenthal JW, O'Neil TE, Marcus PI: Chicken interferon types I and II enhance synergistically the antiviral state and nitric oxide secretion. J Interferon Cytokine Res 1998;18:407–414.

6 Staeheli P, Puehler F, Schneider K, Gobel TW, Kaspers B: Cytokines of birds: conserved functions – a largely different look. J Interferon Cytokine Res 2001;21:993–1010.

7 Hwang SY, Hertzog PJ, Holland KA, Sumarsono SH, Tymms MJ, Hamilton JA, Whitty G, Bertoncello I, Kola I: A null mutation in the gene encoding a type I interferon receptor component eliminates antiproliferative and antiviral responses to interferons α and β and alters macrophage responses. Proc Natl Acad Sci USA 1995;92:11284–11288.
8 Garcia-Sastre A: Identification and characterization of viral antagonists of type I interferon in negative-strand RNA viruses. Curr Top Microbiol Immunol 2004;283:249–280.
9 Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G: 5′-Triphosphate RNA is the ligand for RIG-I. Science 2006;314:994–997.
10 Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S: Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 2006;441:101–105.
11 Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C: RIG-I-mediated antiviral responses to single-stranded RNA bearing 5′-phosphates. Science 2006;314:997–1001.
12 Wathelet MG, Lin CH, Parekh BS, Ronco LV, Howley PM, Maniatis T: Virus infection induces the assembly of coordinately activated transcription factors on the IFN-β enhancer in vivo. Mol Cell 1998;1:507–518.
13 Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S: Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005;23:19–28.
14 Kumar H, Kawai T, Kato H, Sato S, Takahashi K, Coban C, Yamamoto M, Uematsu S, Ishii KJ, Takeuchi O, Akira S: Essential role of IPS-1 in innate immune responses against RNA viruses. J Exp Med 2006;203:1795–1803.
15 Hiscott J, Lin R, Nakhaei P, Paz S: MasterCARD: a priceless link to innate immunity. Trends Mol Med 2006;12:53–56.
16 Xu LG, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB: VISA is an adapter protein required for virus-triggered IFN-β signaling. Mol Cell 2005;19:727–740.
17 Cauthen AN, Swayne DE, Sekellick MJ, Marcus PI, Suarez DL: Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. J Virol 2007;81:1838–1847.
18 Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T: Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 1998;252:324–330.
19 Kochs G, Koerner I, Thiel L, Kothlow S, Kaspers B, Ruggli N, Summerfield A, Pavlovic J, Stech J, Staeheli P: Properties of H7N7 influenza A virus strain SC35M lacking interferon antagonist NS1 in mice and chickens. J Gen Virol 2007;88:1403–1409.
20 Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H: The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol 2006;80:11115–11123.
21 Dauber B, Schneider J, Wolff T: Double-stranded RNA binding of influenza B virus nonstructural NS1 protein inhibits protein kinase R but is not essential to antagonize production of α/β interferon. J Virol 2006;80:11667–11677.
22 Donelan NR, Basler CF, Garcia-Sastre A: A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of β interferon and is attenuated in mice. J Virol 2003;77:13257–13266.
23 Opitz B, Rejaibi A, Dauber B, Eckhard J, Vinzing M, Schmeck B, Hippenstiel S, Suttorp N, Wolff T: IFN-β induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. Cell Microbiol 2007;9:930–938.
24 Hatada E, Saito S, Fukuda R: Mutant influenza viruses with a defective NS1 protein cannot block the activation of PKR in infected cells. J Virol 1999;73:2425–2433.
25 Noah DL, Twu KY, Krug RM: Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3′ end processing of cellular pre-mRNAS. Virology 2003;307:386–395.
26 Dhand R, Hiles I, Panayotou G, Roche S, Fry MJ, Gout I, Totty NF, Truong O, Vicendo P, Yonezawa K, et al: PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. EMBO J 1994;13:522–533.
27 Neri LM, Borgatti P, Capitani S, Martelli AM: The nuclear phosphoinositide 3-kinase/AKT pathway: a new second messenger system. Biochim Biophys Acta 2002;1584:73–80.
28 Cooray S: The pivotal role of phosphatidylinositol 3-kinase-Akt signal transduction in virus survival. J Gen Virol 2004;85:1065–1076.
29 Sarkar SN, Peters KL, Elco CP, Sakamoto S, Pal S, Sen GC: Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded RNA signaling. Nat Struct Mol Biol 2004;11:1060–1067.
30 Guillot L, Le Goffic R, Bloch S, Escriou N, Akira S, Chignard M, Si-Tahar M: Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. J Biol Chem 2005;280:5571–5580.

31 Ehrhardt C, Marjuki H, Wolff T, Nurnberg B, Planz O, Pleschka S, Ludwig S: Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence. Cell Microbiol 2006;8:1336–1348.
32 Ehrhardt C, Wolff T, Pleschka S, Planz O, Beermann W, Bode JG, Schmolke M, Ludwig S: Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. J Virol 2007;81:3058–3067.
33 Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y: Effect of the phosphatidylinositol 3-kinase/Akt pathway on influenza A virus propagation. J Gen Virol 2007;88:942–950.
34 Zhirnov OP, Klenk HD: Control of apoptosis in influenza virus-infected cells by up-regulation of Akt and p53 signaling. Apoptosis 2007;12:1419–1432.
35 Ludwig S: Exploited defense – how influenza takes advantage of antiviral signaling responses. Future Virology 2007;2:91–100.
36 Hale BG, Jackson D, Chen YH, Lamb RA, Randall RE: Influenza A virus NS1 protein binds p85β and activates phosphatidylinositol-3-kinase signaling. Proc Natl Acad Sci USA 2006;103:14194–14199.
37 Shin YK, Liu Q, Tikoo SK, Babiuk LA, Zhou Y: Influenza A virus NS1 protein activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by direct interaction with the p85 subunit of PI3K. J Gen Virol 2007;88:13–18.
38 Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH: Mitogen-activated protein kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153–183.
39 Kujime K, Hashimoto S, Gon Y, Shimizu K, Horie T: p38 mitogen-activated protein kinase and c-jun-NH2-terminal kinase regulate RANTES production by influenza virus-infected human bronchial epithelial cells. J Immunol 2000;164:3222–3228.
40 Ludwig S, Ehrhardt C, Neumeier ER, Kracht M, Rapp UR, Pleschka S: Influenza virus-induced AP-1-dependent gene expression requires activation of the JNK signaling pathway. J Biol Chem 2001;276:10990–10998.
41 Pleschka S, Wolff T, Ehrhardt C, Hobom G, Planz O, Rapp UR, Ludwig S: Influenza virus propagation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. Nat Cell Biol 2001;3:301–305.
42 Lee DC, Cheung CY, Law AH, Mok CK, Peiris M, Lau AS: p38 mitogen-activated protein kinase-dependent hyperinduction of tumor necrosis factor α expression in response to avian influenza virus H5N1. J Virol 2005;79:10147–10154.
43 Ludwig S, Wolff T, Ehrhardt C, Wurzer WJ, Reinhardt J, Planz O, Pleschka S: MEK inhibition impairs influenza B virus propagation without emergence of resistant variants. FEBS Lett 2004;561:37–43.
44 Olschlager V, Pleschka S, Fischer T, Rziha HJ, Wurzer W, Stitz L, Rapp UR, Ludwig S, Planz O: Lung-specific expression of active Raf kinase results in increased mortality of influenza A virus-infected mice. Oncogene 2004;23:6639–6646.
45 Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN: TNF-α induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 2000;103:1071–1083.
46 Marjuki H, Alam MI, Ehrhardt C, Wagner R, Planz O, Klenk HD, Ludwig S, Pleschka S: Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase C α-mediated activation of ERK signaling. J Biol Chem 2006;281:16707–16715.
47 Eisenberg S, Shvartsman DE, Ehrlich M, Henis YI: Clustering of raft-associated proteins in the external membrane leaflet modulates internal leaflet H-ras diffusion and signaling. Mol Cell Biol 2006;26:7190–7200.
48 Ludwig S, Planz O, Pleschka S, Wolff T: Influenza-virus-induced signaling cascades: targets for antiviral therapy? Trends Mol Med 2003;9:46–52.
49 Klumpp K: Recent advances in the discovery and development of anti-influenza drugs. Expert Opin Ther Pat 2004;14:1153–1168.
50 Planz O, Pleschka S, Ludwig S: MEK-specific inhibitor U0126 blocks spread of Borna disease virus in cultured cells. J Virol 2001;75:4871–4877.
51 Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC, Tecle H, Barrett SD, Bridges A, Przybranowski S, Leopold WR, Saltiel AR: Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. Nat Med 1999;5:810–816.
52 Cohen P: Protein kinases – the major drug targets of the twenty-first century? Nat Rev Drug Discov 2002;1:309–315.
53 Pahl HL: Activators and target genes of Rel/NF-κB transcription factors. Oncogene 1999;18:6853–6866.
54 Hiscott J, Kwon H, Genin P: Hostile takeovers: viral appropriation of the NF-κB pathway. J Clin Invest 2001;107:143–151.
55 Bonizzi G, Karin M: The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol 2004;25:280–288.
56 Wang X, Li M, Zheng H, Muster T, Palese P, Beg AA, Garcia-Sastre A: Influenza A virus NS1 protein prevents activation of NF-κB and induction of α/β interferon. J Virol 2000;74:11566–11573.

57 Wurzer WJ, Ehrhardt C, Pleschka S, Berberich-Siebelt F, Wolff T, Walczak H, Planz O, Ludwig S: NF-κB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propagation. J Biol Chem 2004;279:30931–30937.
58 Bernasconi D, Amici C, La Frazia S, Ianaro A, Santoro MG: The IκB kinase is a key factor in triggering influenza A virus-induced inflammatory cytokine production in airway epithelial cells. J Biol Chem 2005;280:24127–24134.
59 Flory E, Kunz M, Scheller C, Jassoy C, Stauber R, Rapp UR, Ludwig S: Influenza virus-induced NF-κB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IκB kinase. J Biol Chem 2000;275:8307–8314.
60 Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S: Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526–1529.
61 Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, Terai K, Matsuda M, Inoue J, Uematsu S, Takeuchi O, Akira S: Interferon-α induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. Nat Immunol 2004; 5:1061–1068.
62 Chu WM, Ostertag D, Li ZW, Chang L, Chen Y, Hu Y, Williams B, Perrault J, Karin M: JNK2 and IKKβ are required for activating the innate response to viral infection. Immunity 1999;11:721–731.
63 Pahl HL, Baeuerle PA: Expression of influenza virus hemagglutinin activates transcription factor NF-κB. J Virol 1995;69:1480–1484.
64 Nimmerjahn F, Dudziak D, Dirmeier U, Hobom G, Riedel A, Schlee M, Staudt LM, Rosenwald A, Behrends U, Bornkamm GW, Mautner J: Active NF-κB signalling is a prerequisite for influenza virus infection. J Gen Virol 2004;85:2347–2356.
65 Bourteele S, Oesterle K, Pleschka S, Unterstab G, Ehrhardt C, Wolff T, Ludwig S, Planz O: Constitutive activation of the transcription factor NF-κB results in impaired borna disease virus replication. J Virol 2005;79:6043–6051.
66 Wurzer WJ, Planz O, Ehrhardt C, Giner M, Silberzahn T, Pleschka S, Ludwig S: Caspase 3 activation is essential for efficient influenza virus propagation. EMBO J 2003;22:2717–2728.
67 Mazur I, Wurzer WJ, Ehrhardt C, Pleschka S, Puthavathana P, Silberzahn T, Wolff T, Planz O, Ludwig S: Acetylsalicylic acid blocks influenza virus propagation via its NF-κB-inhibiting activity. Cell Microbiol 2007;9:1683–1694.
68 Yin MJ, Yamamoto Y, Gaynor RB: The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(κ)B kinase-β. Nature 1998;396:77–80.

Stephan Ludwig
Institute of Molecular Virology
Von-Esmarch Strasse 56, D–48149 Münster (Germany)
Tel. +49 2518 257 791, Fax +49 2518 357 793, E-Mail ludwigs@uni-muenster.de

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 225–251

Cytokines in the Pathogenesis of Highly Virulent Influenza Viruses in Humans

Kristien Van Reeth

Laboratory of Virology, Ghent University, Gent, Belgium

Abstract

Some influenza viruses are much more virulent for humans than contemporary human H1 or H3 subtype influenza viruses. Examples include the highly pathogenic H5N1 avian influenza virus and the 1918 'Spanish' influenza virus. There is now convincing evidence that H5N1, and probably also the 1918 virus, induces an overwhelming and sustained production of a series of pro-inflammatory cytokines and chemokines in the host, which frequently leads to acute respiratory distress syndrome and death. This theory is supported by investigations of human H5N1 patients and by mouse infection studies with H5N1 and the reconstructed 1918 virus. Still, we are only starting to understand the pathogenesis of H5N1 in humans, the precise roles of cytokines and the viral determinants of cytokine hyperinduction. Most important, experimental studies in this area have yielded many conflicting results, especially the studies on the role of type I IFNs. This chapter aims to review our current knowledge on the role of cytokines during human infection with H5N1 or other highly virulent influenza viruses, but it also highlights controversies and unresolved questions. I write from the perspective of a medical virologist, therefore in vivo studies that link cytokine findings with virological and clinico-pathological data get most attention.

Introduction

The outcome of an influenza infection in humans can vary substantially depending on the immune status of the host, physiologic and environmental factors and, not at least, the virus strain. Contemporary human H1 or H3 influenza viruses generally cause only transient disease or even a subclinical infection. But occasionally particularly virulent strains arise that may cause worldwide pandemics such as the 1918 'Spanish' influenza virus, or lead only to a limited number of human infections, such as the highly pathogenic avian H5N1 influenza viruses. It has been known for some time that many of the typical influenza symptoms are not caused directly by the virus,

but by the release of a series of cytokines by the host in response to the virus infection. In recent years there is growing evidence that the highly pathogenic H5N1 virus has a greater propensity to induce cytokines than the usual human influenza viruses and that a 'cytokine storm' in the lungs of H5N1 patients probably accounts for the extreme virulence of H5N1 for humans. This new insight has boosted studies on the cytokine response to H5N1 and other highly virulent influenza viruses in human patients, animal models and cell culture. This chapter aims to review these studies and their significance for a better understanding of viral pathogenesis. It is far from a complete overview of all the existing literature, and in vivo studies that link cytokine findings with virological and clinico-pathological data get most attention. I write from the perspective of a medical virologist and I try to make a sound interpretation of only a selection of studies, many of which yield seemingly conflicting results.

Some terms that are used throughout this paper are defined here to avoid confusion. An 'avian influenza (AI) virus' can be any influenza virus of avian origin, including viruses of various subtypes and of either low or high pathogenicity for chickens. It can be isolated directly from birds or after passage in humans or experimental animals. The designations 'highly pathogenic (HP)' and 'low pathogenic (LP)' refer to pathogenicity for chickens, unless otherwise mentioned. 'H5N1' is used for HP H5N1 AI viruses isolated in Southeast Asia between 1997 and now. H5N1 isolates are often referred to as 'avian isolates' (isolated directly from birds) or 'human isolates' (isolated from humans who became infected through contact with birds). 'H5' or 'H7' influenza viruses include viruses with any NA subtype, they can be either HP or LP for chickens. 'Human' or 'swine' influenza viruses are viruses of H1N1, H3N2 and H1N2 subtypes that are currently circulating in human or swine populations. The human viruses are sometimes referred to as 'contemporary', 'common', 'usual' or 'seasonal' influenza viruses. 'Recombinant' influenza viruses are obtained by reverse genetics: they are either reassortants with one or more gene segments of a highly virulent virus in a background of another influenza virus, complete reconstructed viruses like the 1918 virus, or viruses with specific desired amino acid substitutions or deletions.

Cytokines of the Innate Immune System

Cytokines – from the Greek words 'cytos' (cell) and 'kinein' (to move) – form a group of small-to-medium size (5–100 kDa) proteins or glycoproteins that act as intercellular communication signals. They are released by various cells in the body, usually in response to an activating stimulus, and induce responses through binding to specific receptors. They have critical roles in haematopoiesis, inflammation and the development and maintenance of immune responses. Over 100 cytokines have been cloned and characterized since the 1980s and their number is still growing. Anyone who wants to understand the role of cytokines in health and disease must be aware of

some typical cytokine properties: (1) Most cytokines can be induced by a variety of stimuli and viruses or viral infections are just one of these. The processes of cytokine gene transcription, translation to protein and secretion are complex, and cytokine mRNA and protein levels do not necessarily correlate. Cytokine findings are therefore highly dependent on the sensitivity and nature of the assays used. (2) Cytokines are usually secreted in minute amounts, at pg to ng per ml concentrations. They primarily act on cells in the immediate vicinity of the producer cells and cytokine secretion may be extremely transient. The detection of cytokines therefore frequently requires precise sampling and/or collection of local secretions. (3) 'Pleiotropy' and 'redundancy' are the two key words describing cytokine action. That is, most cytokines exert multiple biological activities and there are very few biological responses that are mediated by only one cytokine. Cytokines typically work in networks or cascades and a given cytokine may both stimulate or inhibit the production of several others. This also explains the multitude of additive and synergistic effects between cytokines. Not surprisingly, the production and actions of cytokines are very context-dependent and they are influenced by the type of producer and target cells, as well as by the presence of other cytokines. Consequently, in vivo cytokine studies in humans or animals are most relevant for the pathogenesis of disease and only a few selected in vitro data are discussed in this chapter.

The reader is referred to alternative texts for a more comprehensive overview on cytokines [1, 2]. Table 1 only lists some cytokines and chemokines that may be produced during the acute stage of a virus infection and a selection of their activities. All of them can be produced by cells of the innate immune system or by non-immune cells. The 'innate' cytokines are also known as 'initial', 'early' or '(pro-) inflammatory' cytokines and many of them are released within the first 24 h of infection. The chemokines have chemoattractant properties and they fall mostly into two broad groups that act on different sets of receptors and thus have different target cells. The CC chemokines have two adjacent cysteines near the amino terminus, while the CXC chemokines have another amino acid between the two equivalent cysteine residues. The actions of all these cytokines are sometimes compared to a double-edged sword. Indeed, they are indispensable for the innate immune response to virus infections and many of them also enhance adaptive immune responses. The same cytokines though are frequently responsible for a short period of disease. Neutrophil infiltration is such a typical cytokine-driven effect that contributes both to host defence and to the tissue destruction seen in local sites of infection. The production of cytokines during pulmonary virus infections like influenza is mainly restricted to the lung and diffusion of cytokines across the blood-alveolar barrier is rather limited. Still, the local production of some cytokines can also have long-distance effects like fever and other effects on the brain. In extreme cases, local cytokine production becomes uncontrolled or overwhelming and high amounts of cytokines reach the circulation, which can be life-threatening. The systemic release of TNF-α, for example, causes vasodilatation and loss of plasma volume owing to increased vascular permeability,

Table 1. Important innate cytokines and chemokines with their major effects and assigned roles in the mouse model of influenza

Cytokine			Selected activities	Proven effects in mouse model of influenza (ref)
group (class)	abbreviation	full name; alternative names		
Innate cytokines	IFN-α/β	Interferon-α/β; type 1 interferons[1]	Induces antiviral activity in a wide variety of cell types. Broad immunomodulatory effects: e.g. stimulation of growth and cytolytic function of NK cells, increased expression of major histocompatibility complex (MHC) class I molecules. Induces fever and symptoms of general malaise	15, 16, 19, 12, 8
	IFN-γ	Interferon-γ; type 2 interferon	Antiviral activity. Factor involved in macrophage activation. Inducer of class II MHC molecules. Promotes adhesion of T-helper cells to vascular endothelium	16, 5, 23, 8
	TNF-α	Tumour necrosis factor-α; cachectin	Induces adhesion molecules on vascular endothelial cells. Activates neutrophils. Induces inflammatory response and fever and initiates catabolism of muscle and fat (cachexia). Can induce several other cytokines. Potentiates lysis of some virus-infected cells	15, 3
	IL-1α and β	Interleukin-1α and β; endogenous pyrogen	Activates T cells and induces growth factors and inflammatory mediators. Induces adhesion molecules on vascular endothelial cells. Affects brain to produce fever and causes metabolic wasting (cachexia). Induces acute-phase protein synthesis in the liver	11, 12, 18
	IL-6	Interleukin-6	Stimulates B cell proliferation and antibody production. Costimulator of T cells. Induces fever, stimulates hepatocytes to synthesize acute-phase proteins	10
	IL-10	Interleukin-10	Anti-inflammatory cytokine. Inhibits synthesis of pro-inflammatory cytokines and suppresses cell-mediated immunity	

Table 1. (continued)

Cytokine			Selected activities	Proven effects in mouse model of influenza (ref)
group (class)	abbreviation	full name; alternative names		
	IL-12	Interleukin-12	Potent regulator of cell-mediated immune responses. Induces IFN-γ production by NK and T cells	14
	IL-18	Interleukin-18	Enhances IFN-γ production and NK cell cytotoxicity	7, 13, 20
CXC-type chemokines	IL-8	Interleukin-8; CXCL8	Attracts neutrophils into inflammatory sites. Activation and degranulation of neutrophils	
	IP-10	Interferon-γ-induced protein-10; CXCL10	Attracts activated T cells and monocytes	22
	MIG	Monokine-induced by interferon-γ; CXCL9	Attracts activated T cells (similar to IP-10)	
	MIP-2	Macrophage inflammatory protein-2	Chemotactic factor for neutrophils	17
CC-type chemokines	MCP-1	Monocyte chemotactic protein-1; CCL2	Chemoattracts and activates monocytes, activated T cells, basophils, NK cells and immature dendritic cells	21
	MCP-2	Monocyte chemotactic protein-2; CCL8	Chemoattractant for monocytes and activated T cells	
	RANTES	Regulated upon activation, normal T-cell expressed and secreted; CCL5	Broad specificity chemotactic factor, similar to MCP-1	22
	MIP-1α	Macrophage inflammatory protein-1α; CCL3	Chemoattractant for macrophages, T cells, NK cells, B cells	6
	MIP-1β	Macrophage inflammatory protein-1β; CCL4	Chemoattractant for macrophages, T cells, NK cells, B cells	

[1] The type 1 IFNs comprise multiple IFN-α subtypes, e.g. 14 in humans, but only a single IFN-β subtype.
CXCL = CXC chemokine ligand; CCL = CC chemokine ligand.

leading to shock. TNF-α also triggers disseminated intravascular coagulation with depletion of clotting factors and consequent bleeding. This can explain why acute respiratory distress syndrome (ARDS) may be followed by multiple organ failure and death.

The very first studies of the cytokine response to influenza virus were performed in mice after intranasal inoculation with mouse-adapted A/Puerto Rico/8/34 (H1N1) influenza virus [3, 4]. These studies are not discussed here because of space limitations. Also, the pathogenesis and clinical picture in mice differ from those in humans. Mice of most strains show a drop in body temperature instead of fever, and the infection is almost invariably lethal. Regular inbred mice carry defective genes for the IFN-induced antiviral protein Mx1, which can in part explain their susceptibility to influenza. On the other hand, the mouse model of influenza has been very useful to examine the role of individual cytokines using specific antibodies or antagonists or gene-deleted 'knock-out' mice lacking specific cytokines or their receptors. References to such studies [5–23] are also given in table 1. These studies have generally confirmed the involvement of several cytokines in both disease severity and antiviral defence, but depletion of an individual cytokine often had only marginal effects. This may be due in part to the redundancy of the biological effects of cytokines, i.e. the fact that other cytokines with overlapping biological activities can compensate for the loss of a given cytokine. Furthermore, different results may be seen in mice with identical cytokine deficiencies. In IL-18 gene knock-out mice, for example, virus clearance has been found to be delayed [7], enhanced [20] or minimally affected [13].

Cytokines as Mediators of Influenza Symptoms in Humans and in Pigs

Humans and swine are among the most important mammalian influenza virus hosts. Influenza A viruses of identical subtypes – H1N1, H3N2 and H1N2 – are currently circulating in both species, though the swine influenza viruses (SIVs) are antigenically and genetically different from their human counterparts. The pathogenesis of the infection is also very similar in humans and in pigs. In both species, the virus replicates in epithelial cells of the entire respiratory tract, notably the nasal mucosa, tonsils, trachea and lungs. Virus clearance is extremely rapid and nasal virus shedding lasts for only 5–6 days. Viraemia or virus isolation from extra-respiratory tissues is very rare.

Influenza infections in humans range in severity from asymptomatic infections to serious illness with both upper and lower respiratory tract symptoms. Typical symptoms are fever, chills, headache, sore throat, myalgias, malaise and anorexia [reviewed in 24, 25]. The infection is rarely fatal in young people, but mortality can occur in people older than 65 years and in those with underlying medical conditions. Although no direct comparative data for man are available, several findings strongly

argue for a greater susceptibility of the lower than the upper respiratory tract [reviewed in 24, 26, 27] and influenza is a significant cause of pneumonia in naive individuals, i.e. infants and young children undergoing first infection and all age groups during influenza pandemics. The predominance of mild upper respiratory and systemic signs during seasonal influenza infections in adults may be explained by partial pre-existing immunity from previous infections. Despite the systemic symptoms, most efforts to detect virus replication outside the respiratory tract were unsuccessful. Only a few rare and mainly fatal cases revealed low quantities of infectious virus in the blood, internal organs, brain and cerebrospinal fluid [reviewed in 24]. While infection of the gastrointestinal tract is a constant finding in many bird species, it has never been documented in infections with human influenza viruses.

The team of Dr Hayden at University of Virginia has undertaken experimental influenza infection studies in humans to quantitate levels of a broad range of cytokines in nasal washes and in the circulation by commercial ELISAs. Intranasal H1N1 influenza virus inoculation of such volunteers resulted in nasal virus excretion from 1 until 7 days post-inoculation, fever and signs of upper respiratory disease with a peak on day 2. Most cytokines tested for were clearly increased in nasal lavage fluids, including IFN-α, IFN-γ, TNF-α, IL-6, IL-8, IL-10, MIP-1α, MIP-1β and MCP-1 [28, 29]. In an initial kinetic study, IFN-α and IL-6 were most closely associated with both virus titres and disease. TNF-α and IL-8 peaked later and were considered less important in the initiation of the disease. Other cytokines, such as RANTES and IL-1β, showed no increase at all. The cytokine responses in plasma or serum were either much smaller than those in nasal lavage fluids, which was the case for IL-6 and TNF-α, or undetectable [29]. Prophylactic treatment with antivirals could prevent the infection, abrogate local cytokine and chemokine responses and prevent disease [28]. Increases in cytokines and chemokines have recently also been demonstrated in acute-phase plasma samples of patients who were hospitalized with severe complicated H1N1 influenza virus infection [30]. IL-6, IL-8, IP-10, MIG and MCP-1 were significantly correlated with viral RNA quantities in nasopharyngeal aspirates and IL-6 in particular was linked with prolonged hospitalization.

The pathogenesis of SIV is reviewed in Olsen et al. [31]. In the author's laboratory we inoculate caesarean-derived colostrum-deprived (CDCD) pigs intratracheally with H1N1 SIV to reproduce the pneumonia and lower respiratory symptoms that are so typical of 'swine flu' and to study the involvement of cytokines [reviewed in 32]. Unlike human volunteers and most conventional pigs, CDCD pigs have no pre-existing immunity to influenza virus and they are also negative for other swine pathogens. The pigs are euthanized sequentially during the very acute stage of the infection and bronchoalveolar lavage (BAL) fluids are collected to determine cytokine levels by bioassays or commercial swine-specific ELISAs. Within 24 h post-inoculation, we found highly significant increases in IFN-α, IFN-γ, TNF-α, IL-1, IL-6, IL-8 and IL-12. The peak levels of most cytokines were tightly associated with the peak of lung virus titres, neutrophil infiltration in the lungs and disease. By 2 and 3 days post-inoculation,

both virus and cytokine titres showed an up to 100-fold reduction and the pigs already started to recover. There were several interesting parallels with the cytokine findings in influenza-infected human volunteers. First, IFN-α and IL-6 were also most strongly correlated with virus titres and disease. Second, serum cytokine levels in the pigs were either undetectable or 100-fold lower than those in BAL fluids. Finally, the cytokine response to a SIV infection was strongly reduced or absent in experiments with pigs that had been previously vaccinated against SI, and this was associated with the reduction of virus replication and disease [33]. Other, comparative SIV infection experiments with the less invasive intranasal or aerosol inoculation routes have highlighted the importance of high viral loads in the lungs for the induction of cytokines and disease.

The same cytokines thus appear to mediate the typical influenza symptoms in humans and in pigs. One must realize though that the extremely rapid virus clearance in uncomplicated influenza virus infections is likely also due to cytokines like IFN-α and IL-6. In the author's opinion, the pig is a valuable influenza virus model to study the role of cytokines in influenza pneumonia and lower respiratory tract disease. Pigs are natural influenza virus hosts and the pathogenesis of H1 and H3 SIVs resembles that of human influenza. Due to its close physiologic and anatomic resemblance to man, the pig is an increasingly important model for biomedical research. More and more cytokine and immunological reagents for the pig are also becoming available.

Overproduction of Pro-Inflammatory Cytokines May Provide a Clue for the Unusual Severity of H5N1 Infection in Humans

The previous paragraphs describe the cytokine profile during infection of humans or pigs with common H1 or H3 influenza virus subtypes, which usually cause a mild and transient disease. This situation contrasts with the rare infections of humans with HP H5N1 AI viruses, for which the case-fatality rate is approximately 50%. The H5N1 virus has crossed the species barrier to infect humans on at least three occasions in recent years: in Hong Kong in 1997 (18 cases with 6 deaths), in Hong Kong in 2003 (2 cases with 1 death) and in the current outbreaks that began in December 2003 and were first recognized in January 2004. Since that time, the virus has become endemic in poultry in several countries in Asia. At this time of writing a total of 334 human H5N1 cases with 205 deaths have been reported to the World Health Organization [34]. Fortunately, the H5N1 virus does not easily cross from birds to infect humans and the virus still lacks the capacity to spread efficiently between humans. Most human H5N1 cases had a history of very close contact with infected poultry, usually within a week before the onset of clinical signs. Inhalation of infectious droplets and self-inoculation of the conjunctiva or upper respiratory tract mucosa are likely the most common routes of infection. The first and most prominent clinical signs are a high fever (≥38°C) and influenza-like symptoms, but diarrhoea and gastrointestinal

signs can also occur [35]. Symptoms of involvement of the lower respiratory tract – respiratory distress, difficulty in breathing and a crackling sound when inhaling – are typical. Many patients develop ARDS followed by multiple organ dysfunction and death.

The pathogenesis of H5N1 in humans is not yet fully understood. The virus clearly has a tropism for the respiratory tract and for the lungs in particular. Virus-infected cells have been demonstrated in type 2 pneumocytes in the alveoli and in epithelial cells of the trachea [36, 37]. Almost all patients develop a primary viral pneumonia with diffuse alveolar damage, interstitial pneumonia, focal haemorrhages and bronchiolitis. Initial in vitro work with respiratory tissues of humans has shown that H5N1 attaches preferably to epithelial cells in the lower respiratory tract, namely type 2 pneumocytes and epithelial cells in terminal bronchioles, whereas attachment becomes progressively more rare towards the trachea [38, 39]. This was in line with the distribution pattern of the AI virus receptor, which is rare in the nasal epithelium, trachea or bronchi of humans, but more abundant on type 2 pneumocytes [38, 40]. It was therefore believed that the H5N1 virus has difficulty to replicate in the upper region of the respiratory tract of humans, and this was seen as an explanation for the fact that H5N1 spreads inefficiently to and between humans. However, the H5N1 virus was recently shown to replicate in vitro in human nasopharyngeal tissues, despite the lack of AI virus receptors on these cells [41]. In addition, significant numbers of virus-infected tracheal epithelial cells were lately detected in 2 fatal H5N1 cases [36] and this also contrasts with the relative lack of AI virus receptors on those cells. In fact, it remains uncertain whether H5N1 has a predilection for the lower airways and whether its cell tropism differs from that of contemporary human influenza viruses. There is a strong need for comparative quantitative investigations of the replication of human and avian viruses in human respiratory tract cultures and for more virological examinations of H5N1 autopsy cases.

Another question is to what extent H5N1 can spread beyond the respiratory tract. In contrast to the disseminated infection found in birds and some other mammals, H5N1 has never been detected in the spleen, heart, liver, kidneys, pancreas, adrenal glands or bone marrow of infected humans [36, 37]. Still, infectious virus or viral RNA has been recovered from serum or plasma, cerebrospinal fluid and gastrointestinal samples of some H5N1 patients [36, 37, 42–44]. Virus positive cells have recently been demonstrated in the intestinal epithelium by in situ hybridization (ISH) and in neurons in the brain by ISH and immunohistochemistry (IHC) [36]. This finding confirms that the H5N1 virus has the potential to replicate actively in the gastrointestinal tract and central nervous system, but it remains unknown whether this commonly occurs and how the virus reaches these organs. Furthermore, several other findings point towards a greater tropism for the respiratory tract. As an example, the H5N1 virus is much more frequently detected in nasal and throat swabs than in rectal swabs or plasma, from which it is only occasionally recovered [44]. In several autopsy cases there was no documentation of virus spread beyond the respiratory

tract and the most severe lesions are seen in the lungs [45, 46]. While it remains possible that H5N1 has a broader tissue tropism in humans than the common human influenza A virus subtypes, definite proof for this is lacking. In this context, it is often overlooked that H1N1 and H3N2 influenza viruses have also been isolated from extra-respiratory sites in some fatal cases of human influenza pneumonia [reviewed in 24]. There are even some reports of gastrointestinal disorders and encephalopathy during normal seasonal influenza, especially in young children [reviewed in 47]. It has also been suggested that H5N1 virus clearance is delayed, but this as well remains to be confirmed.

The clinico-pathological features of human H5N1 infection are compatible with an overproduction of pro-inflammatory cytokines like TNF-α and this has prompted researchers at Hong Kong University to undertake in vitro cytokine induction studies in human primary monocyte-derived macrophages [48], as well as in human primary bronchial epithelial cells and alveolar type 2 pneumocytes [49]. They studied cytokine gene expression in these cells by DNA microarrays and/or quantitative RT-PCR, and the secretion of some cytokines was confirmed by ELISA. When compared to H1N1 and H3N2 human influenza viruses, H5N1 isolates from humans in 1997 or 2004 were clearly more potent inducers of a whole series of cytokines and chemokines, including IFN-β, TNF-α, IL-6, IP-10, MIP-1α, MIP-1β, MCP-1 and RANTES. IFN-β and TNF-α were most notable in alveolar macrophages. IFN-β, IP-10, IL-6 and RANTES were most remarkable in lung epithelial cells, which failed to produce TNF-α or IL-1β.

There is also evidence for an overproduction of pro-inflammatory cytokines in patients infected with H5N1. Unusually high serum ELISA concentrations of one or more cytokines including IFN-γ, TNF-α, IL-6, IP-10, MIG and soluble IL-2 receptor were found in limited investigations of H5N1 patients in Hong Kong in 1997 [46] and in 2003 [45] and in Thailand in 2004 [37]. Most of these patients were examined relatively late in the course of illness, which may account for the fact that some cytokines were undetectable. In a very interesting and larger study in Vietnam in 2004–05, as many as 16 H5N1 patients, including 8 fatal cases, were examined for cytokine protein levels in plasma using cytometric bead-array assays [44]. The study also included 8 H1 or H3 influenza patients and a number of uninfected controls. Levels of IP-10, MIG, IL-8, IL-10, IL-6 and IFN-γ were all higher in H5N1 patients than in those with seasonal influenza. The first three cytokines as well as MCP-1 were even higher in fatal than in non-fatal H5N1 cases. The authors of this study concluded that the fatal outcome of human influenza A H5N1 is associated with hypercytokinaemia, as well as with high pharyngeal viral loads. Indeed, viral RNA levels in throat swabs were also highest in the fatal H5N1 cases, followed by non-fatal cases and H1 or H3 influenza patients, and viral loads were tightly correlated with plasma cytokine and chemokine levels. Rectal swabs and serum only tested positive for viral RNA in the fatal H5N1 cases, and it was suggested that detection of virus in the circulation reflects an overall high viral burden rather than extra-respiratory virus replication.

This particularly high viral burden in humans infected with H5N1 is at least one explanation for the observed hypercytokinaemia, because cytokine induction is generally viral dose-dependent. Though lung cytokine levels in human H5N1 cases remain relatively unknown, it is accepted that the initial production of cytokines occurs in the lungs, where the virus seems to replicate to the greatest extent and where it causes the most prominent pathology. Cytokine levels in serum therefore represent possibly only a fraction of those in the lungs [37, 44].

In summary, there are still many gaps in our knowledge of the pathogenesis of H5N1 in humans and the exact differences with H1 or H3 influenza virus infections. The current view is that excessive replication of H5N1 in the human respiratory tract induces a much more intense and sustained cytokine production in the lungs. This uncontrolled or 'aberrant' cytokine response, as it is also called, is no longer beneficial to the host but causes excessive lung damage and, in many cases, ARDS and multiple organ failure without the need for extra-pulmonary viral dissemination.

Lessons from Mouse Infection Studies with Highly Pathogenic AI Viruses

The cytokine response to HP H5 as well as H7 AI viruses isolated from humans has also been examined in the mouse model. Such viruses can replicate efficiently in the lungs of mice without the prior adaptation that is usually required for human influenza viruses. After intranasal inoculation of mice, viral antigen has been visualized in the bronchial epithelium, in bronchiolar lumens in association with necrotic debris, and in the nasal epithelium [50, 51]. On the other hand, AI viruses that are HP for chickens or humans differ in their pathogenicity and replicative potential in mice [51–55]. Some isolates are LP for mice: their replication is restricted to the respiratory tract, they are non-lethal and the infection is resolved by 7–9 days post-inoculation. Other isolates, in contrast, are HP. They tend to replicate more rapidly and excessively in the lungs and cause a systemic infection with virus spread to the blood, brain and several internal organs. Death occurs 7–10 days post-inoculation with such isolates. Viral antigen has been sporadically detected in cardiac myofibers, in hepatocytes in the liver and in mononuclear cells in the spleen [53, 55, 56], but there is no massive replication and necrosis in internal organs as is the case with HPAI viruses in poultry [57]. Neurovirulence is a characteristic feature of the AI viruses that are HP for mice. Virus titres in the brain are usually higher than those in the blood and internal organs, though lower than those in lungs, and viral antigen has been demonstrated in glial cells and neurons, often in association with microscopic lesions and necrotic cells [51, 53–56, 58]. The H5N1 virus appears to spread to the brain via the neural pathway after replication in epithelial cells of the nasal mucosa and lower respiratory tract [58], though haematogenous spread to the brain cannot be completely excluded [51]. H5N1 viruses isolated from Hong Kong residents in 1997 have been used most frequently in mouse infection studies and they are designated as Hong Kong

H5N1/97 viruses. The HK/483 and HK/485 isolates were HP for mice, HK/486 was LP and HK/156 appeared to be of intermediate pathogenicity [51, 53]. In this section, the designations 'lethal' or 'virulent' refer to lethality and virulence for mice.

But why are some HPAI viruses so lethal for mice and do cytokines play a role here? To solve this question, several researchers have tested cytokine levels in serum, BAL fluids, lung or brain homogenates by commercial ELISAs, and some have compared viruses of varying pathogenicity for mice. According to some studies, AI viruses cause disease and death in mice through their dramatic effect on the lungs, and cytokine production locally in the lungs triggers a neutrophil-predominant inflammatory response and the ARDS that frequently results in multiple organ dysfunction. Xu et al. [59] successfully reproduced 'acute respiratory distress' after intranasal inoculation of mice with a chicken H5N1 virus isolated in China in 2002. The virus replicated to high virus titres in the lungs and induced severe gross and microscopic lung lesions with infiltration of alveolar walls and bronchiolar lumina with inflammatory cells, necrosis of lung epithelial cells, oedema and haemorrhages and a dramatic increase in neutrophils in BAL fluids. The liver, kidneys, heart and brain were virus-positive for shorter time periods and they showed relatively mild microscopic and no gross lesions. The mice developed prominent respiratory distress, progressive and severe hypoxaemia and most of them died by 6–8 days post-inoculation, when high levels of TNF-α and IL-6 were present in BAL fluids. Dybing et al. [50] found similar lung lesions in mice infected with Hong Kong H5N1/97 isolates from humans or chickens and the mice died without evidence for extra-respiratory virus replication or pathology. Some older, non-Hong Kong-origin H5N1 viruses that were HP for chickens generally caused milder lung lesions in mice and were non-lethal. Cytokine levels in the lungs were not examined, but serum levels of the anti-inflammatory cytokine transforming growth factor-β (TGF-β) were lower for the lethal than for the non-lethal viruses. It is thus possible that the decreased levels of TGF-β and the subsequent effect on other cytokines may enhance disease, but this is just one of many possible scenarios. Differential induction of cytokines was also observed in a study with nine H7 AI viruses of differing virulence for mice [52]. The single virus that was highly lethal for mice, an HP H7N7 isolate from a fatal human case in The Netherlands in 2003, induced higher levels of TNF-α, IFN-α and IFN-β in the lungs than all other viruses examined. In addition, the elevated levels of TNF-α and IFN-β were sustained until the death of these mice. This study also showed a correlation between H7 virus titres in the lungs and virulence for mice, but lung lesions were not studied. Another cytokine study with recombinant viruses bearing the NS gene of Hong Kong H5N1/97 viruses is discussed further in this article [60]. That study also argues for a role of high pulmonary concentrations of inflammatory cytokines, notably IL-1α, IL-1β, IL-6, IFN-γ and MIP-2, and reduced concentrations of the anti-inflammatory IL-10 in the severe disease induced by H5N1/97 infection.

According to other studies, neurovirulence is a key factor in the pathogenicity of AI viruses for mice. H7 or H5 isolates that were more pathogenic in the mouse model

appeared to spread more readily to the brain [51, 53–55] and virus titres in the brain peaked immediately before death. Non-suppurative encephalitis and neurological symptoms such as altered gait were found in some [53, 58] but not in all studies [55]. In addition, the team of Drs J. Katz and T. Tumpey has found high levels of IL-1β, TNF-α, IFN-γ and the chemokines MIP-1α and MIP-2 in brain homogenates of mice infected with the highly virulent, neurotropic HK/483 isolate [55]. They propose that these cytokines are produced locally within the brain by infiltrating inflammatory cells or resident brain cells. The cytokines peaked just before the mice died and they were undetectable in the brain of mice inoculated with non-lethal viruses. All this suggests that neurotropism and cytokines in the brain may at least contribute to the virulence of AI viruses in mice. On the other hand, cytokine production in the brain does not per se occur with any AI virus that is virulent and neurotropic for mice, as shown in the study with H7 AI viruses [52]. The source and significance of cytokines in the brain therefore need further investigation.

While they found high levels of cytokines in the brain of mice infected with HK/483, Dr Tumpey and colleagues [55] reported on diminished production of IL-1β, IFN-γ and MIP-1α in the lungs. This finding contrasts with all other studies, but another study by the same research group suggests that some nuance of this statement is in order and that cytokine titres may vary between experiments and/or between mouse strains [61]. In addition, both studies showed high lung levels of TNF-α, IL-6 and the neutrophil chemoattractant MIP-2 in HK/483-infected mice [55, 61]. Other features of highly virulent H5N1 infections in mice are a marked and sustained reduction in the number of white blood cells, primarily lymphocytes, [55, 59] and of CD4+ and CD8+ T cells in the lungs, and more apoptotic cells in the lungs and spleen [55]. This led to the postulation that the H5N1 virus has a destructive effect on lymphocytes, which leads to less recruitment and activation of virus-specific T cells and disturbed virus clearance, but this is just a hypothesis and there are no indications so far that H5N1 induces deficient antibody or T-cell responses. Apoptosis was also detected in the lungs of human H5N1 patients [62], but its significance for the pathogenesis is not clear.

By far the most clear and useful lesson from the mouse cytokine studies is that cytokine inhibition does not protect against death from H5N1 infection. Proof for this comes from two recent studies with cytokines gene (receptor) knock-out mice [61, 63]. In an infection study with the pair of Hong Kong H5N1/97 viruses with disparate pathogenicity for mice, the kinetics and extent of infection in the lungs and extra-respiratory tissues, microscopic lesions, weight loss and mortality were studied [61]. The kinetics and outcome of infection with either virus were essentially unaffected in mice deficient in IL-6 or MIP-1α. In contrast, mice deficient in the IL-1 receptor displayed delayed clearance of the less virulent HK/486 virus. Mice deficient in TNF receptor 1 or treated with specific TNF-α antibody showed a slight delay in weight loss and death after infection with the more virulent HK/483 virus, but all of them eventually died. These results suggest that TNF-α may contribute to early disease

severity, whereas IL-1 may play a role in viral clearance late in H5N1 virus infection. In the second study, weight loss and mortality after infection with the A/Vietnam/1203/04 H5N1 virus were similar in wild-type mice or mice deficient in TNF-α, one or both TNF receptors, IL-6 or the chemokine MCP-1 [63]. Treatment with glucocorticoids before and/or after H5N1 infection also did not reduce the lethality of the infection. Glucocorticoids have diverse and broad effects on the immune system including the suppression of cytokines, but the effects on individual cytokine levels were not reported in this particular study. Because of the redundancy of the cytokine network, the negative results with cytokine gene knock-out mice do not necessarily mean that cytokines are unimportant and it is possible that depletion of more than one cytokine would have more substantial effects. The authors of the second study also recognize the need to dissect the contribution of each cytokine to factors other than weight loss and mortality, the single parameters they examined. From the therapeutic viewpoint, however, therapies that target the virus rather than cytokines may be preferable.

Whether the mouse model faithfully represents human H5N1 infection is still unclear. The central nervous system and systemic involvement that are so typical of highly virulent H5N1 infection in mice may only be a rare complication in H5N1-infected humans. Furthermore, virulence and pathogenesis of H5N1 in mice frequently do not agree with that in ferrets as shown by studies with the HK/483 and HK/486 isolates in the ferret [64]. In comparative infection studies with 1997 and 2004 human H5N1 isolates, the relative virulence for humans was also slightly better reflected in the ferret than in the mouse model [51, 54]. Most H5N1 isolates of human origin are lethal for ferrets and they cause a systemic infection with spread to the brain and neurological symptoms [54, 64, 65]. Unexpectedly, virus was also isolated from the brains of ferrets inoculated with common human H3N2 influenza viruses, which do not spread beyond the respiratory tract in humans and failed to induce neurological signs in ferrets. This exemplifies the need to better study the neurotropism and neurovirulence of various influenza viruses in different hosts. One explanation for the differences in pathogenicity between the mouse and ferret models may be that 'the mouse is an inbred animal, in which case only a few molecular differences between H5N1 viruses could result in substantial phenotypic differences in this genetically homogeneous population', as stated by Dr J. Katz and colleagues [64]. According to these researchers, the inbred mouse may be a convenient model to further investigate the molecular basis of pathogenesis, while the ferret may be more useful to investigate the contribution of host factors. One shortcoming of the ferret model is the lack of commercial species-specific cytokine reagents and assays, but it should be possible to detect ferret cytokines by simple biological assays.

Alternative H5N1 infection models are non-human primates and swine. H5N1 infection studies in pigtailed macaques have been restricted to the HK/156 isolate, for which the tissue tropism and lethality varied in different mouse infection studies [51, 53]. The macaques were inoculated via combined intratracheal, oropharyngeal and

intraconjunctival routes [66, 67]. The clinical signs – fever and acute respiratory distress – of H5N1 infection in macaques strongly resembled those in humans and were more severe than those seen with a contemporary human H3N2 influenza virus. Unlike in mice, neurological symptoms did not occur in H5N1-inoculated macaques and there was no convincing evidence for virus replication outside the respiratory tract. The lungs were clearly the major site of H5N1 virus replication and multiple organ dysfunction was presumably due to diffuse alveolar damage and ARDS. As for the ferret, there are no specific cytokine reagents for monkeys, but genomic technologies have become very popular to study the host response to virus infection in this species.

Pigs are also susceptible to HPAI viruses, both in nature and experimentally [reviewed in 68], and the pig is a good model to study cytokine profiles in the lung. In the few available experimental studies in pigs, HP H5 or H7 AI viruses seemed to replicate less efficiently than the typical SIVs and they did not induce disease. However, detailed pathogenesis studies are lacking and they are urgently needed.

Altogether, it is likely that the detailed pathogenesis of H5N1 will differ in different animal species and none of these species may fully reproduce all features of H5N1 in humans. Still, these very differences offer an opportunity to gain insights in the pathogenetic features that contribute to H5N1 virus virulence. It is therefore hoped that the cytokine response to H5N1 will also be studied in non-murine models in the future.

Parallels between H5N1 and the 1918 Pandemic Influenza Virus

There appear to be interesting parallels between the pathogenesis of H5N1 in humans and that of the 1918 'Spanish' H1N1 influenza virus. The 1918 virus killed around 40 million people worldwide. Its most striking feature was the unusually high death rate among healthy adults aged 15–34 years [reviewed in 69], which has not occurred in any prior or subsequent influenza pandemic or epidemic. Modern histopathological analysis of autopsy samples from human influenza cases from 1918 revealed significant damage to the lungs with acute, focal bronchitis and alveolitis, massive pulmonary oedema and haemorrhage, and marked destruction of the respiratory epithelium. There is no real evidence for virus replication or pathology outside the respiratory tract, and death was likely due to pneumonia and respiratory failure.

The complete genomic sequence of the 1918 virus has now been deduced, but the sequence information as such could not explain the extraordinary virulence of the virus. Using reverse genetics, scientists can now make influenza viruses with any desired gene constellation starting from cloned DNA. This technique was first used to generate reassortant viruses with the HA and/or NA genes from the 1918 virus in the genetic background of contemporary human H1N1 viruses [70] and the complete 1918 virus was reconstructed in 2005 [71]. Such viruses have been tested for their

behaviour in mice [70–73] and the complete 1918 virus has also been tested in macaques [74]. In comparison with controls infected with the current human H1N1 viruses, animals infected with the 1918-like viruses generally showed higher virus titres in the lungs, more severe pulmonary lesions and higher death rates. Severe necrotizing bronchitis and alveolitis, massive recruitment of inflammatory cells and neutrophils in particular, haemorrhages and alveolar oedema – as reported for patients who succumbed to the 'Spanish' influenza – were seen in both mice and monkeys [70–73]. There was no evidence for systemic infection in mice [70, 73], while the macaques occasionally showed low virus titres in the heart or spleen but not in the colon, liver, kidneys or brain [74]. The macaques also developed 'acute respiratory distress' and only the lungs showed severe macroscopic lesions. Mortality therefore appears to result from infection and pathology in the lungs in both animal models.

Several studies link the pulmonary damage and high lethality to an enhanced ability of the 1918-like viruses to induce pro-inflammatory cytokines and chemokines in the lungs [70–72, 74]. In mice infected with reassortant human influenza viruses possessing the HA and/or NA of the 1918 virus lung ELISA levels of a whole series of cytokines and chemokines were substantially higher than in mice infected with control viruses [70, 73]. Many of these cytokines have also been found in the circulation of H5N1 patients or in H5N1-infected primary human cells, as there are TNF-α, IL-1β, IL-6, IL-12, IL-18, IFN-γ, granulocyte colony-stimulating factor and a list of chemokines. Neutrophils are the predominant inflammatory cells in the lungs of mice infected with 1918-like viruses and they are thought to make important contributions to the severe pulmonary damage and lethality. As could be expected, however, the neutrophils are also essential to control virus replication and spread and this was clearly shown by depletion methods [71].

Importantly, the complete reconstructed 1918 virus caused more rapid and severe disease in mice than viruses bearing only one or few genes from the 1918 virus [72]. The host response to such viruses has been examined in mice [72] and macaques [74] and these studies are considered most relevant for the pathogenesis of the 1918 virus in humans. Both studies largely relied on DNA microarrays to analyse gene expression changes in the lungs after inoculation with the 1918 virus or a contemporary human influenza virus. Microarray gene expression analysis is a powerful new technique that allows monitoring expression levels of thousands of genes simultaneously. The data are analysed through advanced computer programmes, and the genes are usually grouped based on their potential functions, e.g. IFN-related genes, cell death genes, T-helper-1 or -2 genes etc. One difficulty is the often poor to moderate correlation between the relative expression abundances of a gene and its biologically active protein product. For many genes the biological functions are even completely unknown. This makes the interpretation of gene expression analysis particularly complex and the reader is referred to other texts for a detailed description of the findings with the reconstructed 1918 virus [75, see also Palese et al., this volume]. In both

studies the 1918 virus induced a much more substantial increase in the expression of genes involved in inflammation, oxidative stress, and necrosis than the common H1N1 virus, and this correlated with more severe disease and lung pathology. Limited cytokine ELISA investigations of 1918-infected animals also showed increases in IP-10, TNF-α, MIP-1α and MIP-2 in the lungs of mice and in IL-6 in the sera of macaques. More interesting still was the difference in the kinetics of gene expression in the lungs of macaques infected with the contemporary H1N1 versus the 1918 virus. The latter virus triggered a much more sustained expression of genes and a prolonged virus replication. This supports the notion that persistent elevation of inflammatory-response genes could account for the massive inflammation in the respiratory tract of animals infected with the 1918 virus.

Mechanisms of Cytokine Hyperinduction by H5N1 and Other Highly Virulent Influenza Viruses

One of the ultimate goals of many influenza researchers is to identify the viral genes responsible for cytokine hyperinduction by highly virulent influenza viruses. But for the time being, these viral determinants remain an enigma and we are just starting to understand how viruses in general induce the production of innate cytokines in cells of the host [reviewed in 1]. It is now known that viral 'pathogen-associated molecular patterns' (PAMPs) are recognized through a network of cellular proteins, including particular toll-like receptors (TLRs) or other cytoplasmic proteins. This results in the activation of specific transcriptional signalling pathways that converge on the promoters of the cytokine genes. Among such viral PAMPs are dsRNA, which is often produced in infected cells during viral gene expression, viral ssRNA, viral glycoproteins and viral ribonucleoproteins. The processes of viral receptor binding and cellular entry can also trigger cytokine induction pathways. It seems only logical therefore that a given virus may use more than one PAMP and hence activate many signalling pathways. Which of the receptors is essential for the cellular response depends on both the virus and the type of host cell. Though sometimes produced in clusters, different cytokines are likely induced via at least partly different pathways. In addition, some cytokines are induced indirectly as a result of the production of other cytokines. All this means that it is an oversimplification to think of cytokine hyperinduction as the effect of a single viral gene or gene sequence. Therefore only a few preliminary findings will be summarized here. Any findings relating to type I IFN induction are covered in the next section.

Based on studies with H5N1 viruses in macrophages and lung epithelial cells of human origin, it was concluded that cytokine induction was dependent on viral replication since UV inactivated virus had no effect [48, 49]. The higher cytokine levels induced by the Hong Kong H5N1/97 viruses compared to human H1 or H3 influenza viruses could not be explained by a better growth of H5N1 in these sys-

tems, as all viruses showed a similar infectivity and replication kinetics. The H5N1/97 virus was later shown to be a stronger inducer of the p38 mitogen-activated protein kinase (MAPK) pathway, which is involved in expression of several inflammatory cytokines and chemokines, and this was associated with TNF-α hyperinduction [76]. Initial experiments with H5N1 NS gene reassortants in macrophages pointed to the NS1 gene as crucial to the high TNF-α-inducing phenotype [48], but further investigations and more recent studies with reassortants that had specific combinations of H5N1 genes also suggest a role of other viral genes [M. Peiris, pers. commun.].

Reverse genetics has also been used to examine the contribution of specific viral gene segments to cytokine induction and pathogenicity of highly virulent influenza viruses in animal models. A mouse infection study, which is discussed in detail in the next section, partly supports a role for the H5N1 NS gene as a cytokine inducer [60]. Pulmonary concentrations of several pro-inflammatory cytokines were clearly higher in mice infected with a reassortant containing the H5N1/97 NS gene in a background of A/Puerto Rico/8/34 than in control PR/8-infected mice. The concentrations of TNF-α, however, were too low to allow conclusions about the effect on TNF-α production. A second reassortant with the NS gene of a 2001 avian virus that is closely related to human H5N1 isolates from 2003 induced negligible levels of most cytokines when compared to PR/8, and an explanation for this is still lacking. In comparable studies with reassortant viruses bearing 1918 influenza virus genes, the HA and/or NA have been associated with high induction of cytokines and chemokines [70, 71] but TNF-α was hyperinduced in only one of both studies. The PB1-F2 protein, the eleventh and most recently discovered influenza viral protein, has also been associated with increased cytokine induction and virulence of both Hong Kong/97 H5N1 and 1918 influenza viruses in the mouse model [77]. One caveat here is that observations of cytokine hyperinduction in vivo may simply reflect more extensive replication of the respective viruses, and this seems to apply to all studies mentioned.

Altogether, the mechanisms of cytokine hyperinduction by H5N1 and other influenza viruses are still a black box. Research on this issue is confounded by several factors. It is likely, for example, that cytokine induction by influenza is multigenic and that different genes are involved in different influenza viruses. The high cytokine levels in H5N1 patients and experimental animals are probably also due to the high replication efficiency of the virus, next to an intrinsic greater cytokine-inducing capacity.

The Ambiguous Role of Interferons in the Pathogenicity of AI Viruses

There are at least two reasons why type I IFNs deserve a separate discussion here. First, the IFN response to influenza viruses has been better studied than the production of

other cytokines. Second, many research findings are contradictory or confusing and their true relevance for the pathogenesis of influenza is puzzling. During the last few years we have also gained many new insights in the various pathways viruses can use to induce type I IFNs [reviewed in 78]. Some of these pathways involve double-stranded RNA (dsRNA), a by-product of viral replication for both RNA and DNA viruses. dsRNA is now known to bind to 'sensors' in the cell, namely Toll-like receptor 3 and RNA-binding helicases. This interaction will set in motion the activation of a series of transcription factors that regulate transcription of IFN genes. dsRNA is also essential for the activation of at least two of the IFN-induced antiviral proteins – protein kinase R (the R stands for 'activated by dsRNA') and the 2′-5′-oligoadenylate synthetases. The single true non-structural protein of the influenza A virus, NS1, was one of the first and best studied examples of a viral IFN antagonist. The NS1 protein appears to counteract the host's IFN response in various ways. Several of these mechanisms have something to do with the capacity of NS1 to bind to and sequester dsRNA so that it is no longer available for recognition by the cellular sensors, thereby blocking the induction of IFN, or for stimulation of antiviral proteins [reviewed in 79]. Consequently, H1N1 influenza viruses from which the NS1 gene is (partially) deleted replicate poorly in IFN-competent cell cultures or embryonated eggs. Such viruses also replicate less efficiently in mice [80, 81] and swine [82] and they were less pathogenic in both species.

These findings have recently been extended to AI viruses of H5 and H7 subtypes. Three independent research groups have used various approaches to make deletions or alterations in the NS1 genes of AI viruses [83–85] and this resulted in viruses with an enhanced IFN-inducing capacity in cell culture compared to the original parental viruses. NS1 also appeared to be involved in the replication efficiency and/or virulence of the AI viruses in chickens. As an example, an H7N7 recombinant with a complete NS1 deletion could not be recovered from pharyngeal swabs of intratracheally inoculated chickens, in contrast to the wild-type virus [84]. A mutant with a 10-nucleotide deletion in the NS1, as well as a few amino acid differences in three other genes, was obtained by serial egg passage of a turkey H7N3 isolate [83]. Unlike the parental virus, the mutant could barely be detected in tracheal or cloacal swabs or in upper respiratory tract tissues of experimentally infected chickens and it induced less severe necrosis of the respiratory epithelium. Still, the parental viruses used in this and in the previous study were also LP for chickens. Chinese researchers have worked with a pair of H5N1 viruses isolated from geese in Guangdong in 1996, which differed in sequence by only five amino acids mapping to the PA, NP, M1 and NS1 genes [85]. One virus caused a systemic infection after intranasal inoculation of chickens and was HP, the other virus was unable to replicate in chickens. They created (four) single-gene recombinants with one of the sequence-differing gene(s) from the non-pathogenic virus and the remaining seven gene segments from the HP virus. This way they could prove that the NS1 gene of the non-pathogenic virus inhibited the replication of the HP virus in chickens, while the substitution of the PA, NP or M

gene had no effect. The other way round, only the recombinant containing the NS1 gene of the HP virus in a background of the non-pathogenic virus could replicate in chickens. It must be said though that virus titres were much lower than for the complete HP virus and disease and death were rare. The latter finding is in keeping with the view that virulence of influenza viruses is multigenic. Based on their findings in cell cultures the authors of all three studies propose that the NS1 deletion mutants or the reassortant with NS1 from the LP virus are attenuated in chickens because they enhance the synthesis of IFN in the infected lungs. They did not, however, analyse the production of IFN or other cytokines during the time course of the infection and this is a major flaw in most animal studies with NS1 mutants. It will thus take further study to determine the exact mechanisms of the diminished replication of NS1 mutants, and several factors other than the IFN response may account for this. It is of interest in this regard that some NS1 mutant viruses grew about 100 times less well than the parental virus in Vero cells that are incapable of IFN production [84, 86]. This in itself indicates that NS1 has additional effects on the host cell that are not related to the production or action of IFN.

But do NS1 and/or a suppressed IFN response contribute to the unusual virulence of H5N1 in humans or other mammals? Two in vitro studies in human lung cell lines partly support this theory. Hayman et al. [87] used an indirect approach and they transfected A549 cells, a human alveolar basal epithelial cell line, with a reporter gene under the control of the IFN-β promoter and with the NS1 genes of various HP and LP AI virus subtypes. Upon a subsequent infection with Sendai virus, all NS1 proteins blocked the activation of the IFN-β promoter to the same extent and with equal efficiency as the human Victoria/75 (H3N2) virus. In spite of this, infection of A549 cells with the AI viruses induced variable, and in some cases rather high, amounts of secreted IFN-β. This implies that other determinants than the NS1 will also contribute to the variation in IFN induction. In another study in polarized human bronchial epithelial (Calu-3) cells, two H5N1 isolates from humans in Thailand in 2004 elicited lower amounts of secreted IFN-β than the human Panama/99 (H3N2) virus, but the reduction was only transient for one of both H5N1 isolates [88]. Both studies somehow contrast with the previously mentioned studies in primary human macrophages and alveolar and bronchiolar epithelial cells [48, 49], in which all human H5N1 isolates tested were much stronger inducers of IFN-β than human H1N1 or H3N2 viruses. Similarly, H7 AI viruses of high pathogenicity for mice induced high ELISA levels of both IFN-α and IFN-β in the mouse lung [52].

The possible effects of the AI virus NS1 on the IFN-mediated antiviral effects have also been studied, and the results of these studies are equally confusing. In initial experiments by Dr Webster's team, human Hong Kong H5N1/97 viruses and contemporary avian H5N1 viruses were found to be insensitive to the antiviral effects of IFN-α, IFN-γ or TNF-α in a continuous porcine lung cell line, in contrast to control human, swine and non-H5 AI viruses [89, 90]. The investigators then used reverse

genetics to create two reassortant viruses with the NS gene of either a human 1997 or an avian 2001 H5N1 isolate in a background of the A/Puerto Rico/8/34 (H1N1) laboratory strain. Cell culture experiments with these reassortants confirmed the role of the H5N1 NS gene in the observed antiviral resistance. These findings led to the exciting hypothesis that the NS gene of the H5N1 virus allows the virus to escape from the antiviral effects of cytokines and that this property accounts for its extraordinary virulence. It may be noted in passing that no such antiviral resistance was observed when human Calu-3 cells were pretreated with IFN-β and then challenged with H5N1 isolates from Thai patients in 2004 [88]. The two H5N1 NS gene recombinants have also been tested for their pathogenicity in mice and in miniature pigs [60, 89, 90]. The H5N1/97 NS gene reassortant was HP in these in vivo experiments and this was associated with the presence of Glu at position 92 of the NS1 protein, which was also required for antiviral resistance in vitro. Pigs inoculated with this virus shed virus in nasal secretions for a longer period than those inoculated with the parental PR/8 virus, and their body temperature and weight losses were higher [89]. In the mouse model, both the PR/8 virus and the reassortant replicated to high titres in the lungs and were HP for mice, but the reassortant virus required 2 more days to be cleared from the lungs [60]. Contrary to expectations, the H5N1/01 NS gene reassortant replicated to lower virus titres in the respiratory tract of both species and it did not induce disease, though it has a similar NS gene as a human 2003 lethal isolate. Thus, H5N1 NS gene reassortants that behaved similar in cell culture experiments exerted opposite pathological effects in vivo in both mouse and pig models. All this probably means that escape from the antiviral effects of cytokines is not the main mechanism of the high virulence associated with the NS gene of H5N1/97 origin, if it occurs at all in vivo. As in many other animal studies, the IFN response and its correlation with virus titres have not been examined in pig or mouse experiments with the H5N1 NS gene reassortants. Lung levels of five other pro-inflammatory cytokines though were significantly higher in mice infected with the more virulent H5N1/97 NS reassortant than in those infected with the attenuated H5N1/01 NS reassortant [60]. These data document that differences in the NS gene can affect the induction of a whole series of cytokines other than IFN.

All in all, it is quite clear that NS1 is an important influenza virus virulence factor, but it is premature to conclude that virulence is due to the suppression of the IFN response by NS1. As already mentioned, many researchers have even found an excessive production of type I IFNs in in vitro or in vivo studies with H5N1 [48, 49] or other influenza viruses [reviewed in 91, see also 29, 32, 52]. Moreover, excessive IFN levels often correlate with disease severity and this raises the question as to whether the antiviral or harmful effects of IFN will prevail in the in vivo situation. The experimental studies on the interaction between NS1 and type I IFNs make use of cell types that may not correspond to the IFN-producing cells in the host, they focus on a single IFN induction and signalling pathway and usually also on a single type I IFN subtype, i.e. IFN-β. The major IFN-producing cells and induction pathways in the

Table 2. Some outstanding questions on the pathogenesis of H5N1 in mammals and the role of cytokines herein

Research topic	Specific questions
Organ and cell tropism of H5N1 in humans	– Does the virus have a greater tropism for the lower than for the upper respiratory tract? – How frequent is extra-respiratory spread and pathology, spread to central nervous system and encephalopathy in particular? – Is virus replication prolonged? – Precise differences with the pathogenesis of contemporary H1 and H3 influenza viruses in naive individuals
Pathogenesis of H5N1 in various outbred animal models (e.g. ferrets, macaques, pigs)	– Further studies on organ and cell tropism, kinetics of replication, pathology and clinical signs – Primary sites of virus replication – Modes of virus dissemination within the animal – Differences and similarities with pathogenesis of H5N1 in humans
Cytokine response to H5N1 (animal models)	– Cytokine profile in the lungs; cytokines that are secreted first or secondary to other cytokines – Cell types responsible for the production of some major cytokines like TNF-α, IL-6, chemokines – Cytokine production at sites other than the lungs – Relationship between virus replication, levels of individual cytokines, pathology and disease
Role of IFNs in the pathogenesis of H5N1 (animal models)	– Kinetics of type I IFN secretion in the lungs; proportion of different type I IFN subtypes, i.e. IFN-α versus IFN-β – Nature of cells producing IFN-α and β; major viral determinants and pathways of IFN induction – Pathogenesis of viruses with differences or deletions in NS1: effects on production of type I IFNs, other cytokines, virus replication etc. – Differences with IFN response during H1N1 or H3N2 infections

influenza-infected host remain to be discovered and this would be of great help for the design and interpretation of future in vitro studies. Finally, it is noteworthy that viral proteins that counteract the IFN response have been identified in many if not most viruses [78, 79]. The SARS coronavirus is another example of a highly virulent virus that inhibits the production and signalling of type I IFNs in vitro. Surprisingly, however, high levels of type I IFNs were induced in the lungs of SARS coronavirus infected macaques [92].

Outlook and Conclusions

Writing a review on the role of cytokines in the pathogenesis of highly virulent influenza viruses is a daunting task. It is clear by now that an overproduction of innate cytokines and chemokines in human H5N1 patients is crucial to the development of ARDS and the high fatality. But yet there are many confusing and conflicting research data when it comes to the production and importance of individual cytokines and the mechanisms of cytokine induction by influenza viruses. Perhaps the best example is the data on the type I IFN response and the role of the viral NS1 protein herein. Some researchers found that NS1 counteracts IFN production and they believe that the shortage of this important antiviral cytokine is responsible for the excessive replication and extraordinary virulence of some influenza viruses. Other researchers, in contrast, found very high amounts of IFN in influenza-infected subjects. In their opinion, exactly these high IFN levels are responsible for the prominent fever and systemic signs. Part of the confusion is related to some inherent properties of cytokines: their redundant and pleiotropic actions, the importance of the local cellular and cytokine milieu for their production and actions, etc. Consequently, no two experimental systems will yield the same results and many issues are difficult to resolve.

Other questions though may find an answer in the future through more intensive research and just a few examples are shown in table 2. These include basic questions on the cell and tissue tropism of H5N1 in humans and different outbred animal species, which will also help to understand the values and limits of different animal models. Another important issue is the nature of the cytokine-producing cells in the H5N1-infected host, which can be determined by IHC or ISH. Such information is highly useful for the design of future in vitro studies, which are now performed in cell types that may be irrelevant to pathogenesis. There is also a need to study the cytokine profile to H5N1, and the link with virus replication, pathology and disease in outbred animals. Modern techniques like DNA microarrays make it possible to study expression levels of hundreds of cytokine and host genes at once, but nevertheless data interpretation has never been more complicated. Genomic analyses are therefore best combined with other simple, methods for the detection of cytokines and inflammatory markers that are more biologically relevant.

References

1 Biron CA, Sen GC: Innate responses to viral infections; in Knipe SM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Strauss SE (eds): Field's Virology. Philadelphia, Lippincott Williams & Williams, 2007, pp 249–278.

2 Meager T: The Molecular Biology of Cytokines. Chichester, Wiley, 1998.

3 Hennet T, Ziltener HJ, Frei K, Peterhans E: A kinetic study of immune mediators in the lungs of mice infected with influenza A virus. J Immunol 1992;149: 932–939.

4 Vacheron F, Rudent A, Perin S, Labarre C, Quero AM, Guenounou M: Production of interleukin-1 and tumour necrosis factor activities in bronchoalveolar washings following infection of mice by influenza virus. J Gen Virol 1990;71:477–479.
5 Baumgarth N, Kelso A: In vivo blockade of γ-interferon affects the influenza virus-induced humoral and the local cellular immune response in lung tissue. J Virol 1996;70:4411–4418.
6 Cook DN, Beck MA, Coffman TM, Kirby SL, Sheridan JF, Pragnell IB, Smithies O: Requirement of MIP-1α for an inflammatory response to viral infection. Science 1995;269:1583–1585.
7 Denton AE, Doherty PC, Turner SJ, La Gruta NL: IL-18, but not IL-12, is required for optimal cytokine production by influenza virus-specific CD8+ T cells. Eur J Immunol 2007;37:368–375.
8 Durbin JE, Fernandez-Sesma A, Lee CK, Rao TD, Frey AB, Moran TM, Vukmanovic S, Garcia-Sastre A, Levy DE: Type I IFN modulates innate and specific antiviral immunity. J Immunol 2000;164:4220–4228.
9 Hussell T, Pennycook A, Openshaw PJ: Inhibition of tumor necrosis factor reduces the severity of virus-specific lung immunopathology. Eur J Immunol 2001;31:2566–2573.
10 Kozak W, Poli V, Soszynski D, Conn CA, Leon LR, Kluger MJ: Sickness behavior in mice deficient in interleukin-6 during turpentine abscess and influenza pneumonitis. Am J Physiol 1997;272:R621–R630.
11 Kozak W, Zheng H, Conn CA, Soszynski D, van der Ploeg LH, Kluger MJ: Thermal and behavioral effects of lipopolysaccharide and influenza in interleukin-1β-deficient mice. Am J Physiol 1995;269: R969–R977.
12 Kurokawa M, Imakita M, Kumeda CA, Shiraki K: Cascade of fever production in mice infected with influenza virus. J Med Virol 1996;50:152–158.
13 Liu B, Mori I, Hossain MJ, Dong L, Takeda K, Kimura Y: Interleukin-18 improves the early defence system against influenza virus infection by augmenting natural killer cell-mediated cytotoxicity. J Gen Virol 2004;85:423–428.
14 Monteiro JM, Harvey C, Trinchieri G: Role of interleukin-12 in primary influenza virus infection. J Virol 1998;72:4825–4831.
15 Peper RL, Van Campen H: Tumor necrosis factor as a mediator of inflammation in influenza A viral pneumonia. Microb Pathog 1995;19:175–183.
16 Price GE, Gaszewska-Mastarlarz A, Moskophidis D: The role of α/β and γ interferons in development of immunity to influenza A virus in mice. J Virol 2000; 74:3996–4003.
17 Sakai S, Kawamata H, Mantani N, Kogure T, Shimada Y, Terasawa K, Sakai T, Imanishi N, Ochiai H: Therapeutic effect of anti-macrophage inflammatory protein 2 antibody on influenza virus-induced pneumonia in mice. J Virol 2000;74:2472–2476.
18 Schmitz N, Kurrer M, Bachmann MF, Kopf M: Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. J Virol 2005;79:6441–6448.
19 Traynor TR, Majde JA, Bohnet SG, Krueger JM: Interferon type I receptor-deficient mice have altered disease symptoms in response to influenza virus. Brain Behav Immun 2007;21:311–322.
20 Van der Sluijs KF, Van Elden LJR, Arens R, Schuurman R, Florquin S, Kwakkel J, Akira S, Jansen HM, Lutter R, Van Der Polls T: Enhanced viral clearance in interleukin-18 gene-deficient mice after pulmonary infection with influenza A virus. Immunology 2005;114:112–120.
21 Wareing MD, Lyon A, Inglis C, Giannoni F, Charo I, Sarawar SR: Chemokine regulation of the inflammatory response to a low-dose influenza infection in CCR2$^{-/-}$ mice. J Leukoc Biol 2007;81:793–801.
22 Wareing MD, Lyon AB, Lu B, Gerard C, Sarawar SR: Chemokine expression during the development and resolution of a pulmonary leukocyte response to influenza A virus infection in mice. J Leukoc Biol 2004;76:886–895.
23 Wohlleben G, Erb KJ: The absence of IFN-γ leads to increased Th2 responses after influenza A virus infection characterized by an increase in CD4+ but not CD8+ T cells producing IL-4 or IL-5 in the lung. Immunol Lett 2004;95:161–166.
24 Wright PF, Webster RG: Orthomyxoviruses; in Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE (eds): Field's Virology. Philadelphia, Lippincott-Raven, 2001, pp 1533–1579.
25 Zambon M: The pathogenesis of influenza in humans. Rev Med Virol 2005;11:227–241.
26 Betts RF, Douglas RG: Influenza virus; in Mandell GL, Douglas RG, Benett JE (eds): Principles and Practice of Infectious Diseases. New York, Churchill-Livingstone, 1990, pp 1306–1325.
27 Glezen WP, Couch RB: Influenza viruses; in Fraser RG, Paré JA (eds): Diagnosis of Diseases of the Chest. Philadelphia, Saunders, 1990, pp 419–449.
28 Fritz RS, Hayden FG, Calfee DP, Cass LM, Peng AW, Alvord WG, Strober W, Straus SE: Nasal cytokine and chemokine responses in experimental influenza A virus infection: results of a placebo-controlled trial of intravenous zanamivir treatment. J Infect Dis 1999;180:586–593.
29 Hayden FG, Fritz R, Lobo MC, Alvord W, Strober W, Straus SE: Local and systemic cytokine responses during experimental human influenza A virus infection. Relation to symptom formation and host defense. J Clin Invest 1998;101:643–649.

30 Lee N, Wong CK, Chan PK, Lun SW, Lui G, Wong B, Hui DS, Lam CW, Cockram CS, Choi KW, Yeung AC, Tang JW, Sung JJ: Hypercytokinemia and hyperactivation of phospho-p38 mitogen-activated protein kinase in severe human influenza A virus infection. Clin Infect Dis 2007;45:723–731.
31 Olsen CW, Brown IH, Easterday BC, Van Reeth K: Swine influenza; in Straw BE, Zimmerman JJ, d'Allaire S, Taylor D J (eds): Disease of Swine. Oxford, Blackwell, 2006, pp 469–482.
32 Van Reeth K, Van Gucht S, Pensaert M: In vivo studies on cytokine involvement during acute viral respiratory disease of swine: troublesome but rewarding. Vet Immunol Immunopathol 2002;87:161–168.
33 Van Reeth K, Van Gucht S, Pensaert M: Correlations between lung proinflammatory cytokine levels, virus replication, and disease after swine influenza virus challenge of vaccination-immune pigs. Viral Immunol 2002;15:583–594.
34 http://www.who.int/csr/disease/avian_inflenza/en/
35 Beigel JH, Farrar J, Han AM, Hayden FG, Hyer R, de Jong MD, Lochindarat S, Nguyen TK, Nguyen TH, Tran TH, Nicoll A, Touch S, Yuen KY: Writing Committee of the World Health Organization (WHO) Consultation on Human Influenza A/H5. N Engl J Med 2005;353:1374–1385.
36 Gu J, Xie Z, Gao Z, Liu J, Korteweg C, Ye J, Lau LT, Lu J, Gao Z, Zhang B, McNutt MA, Lu M, Anderson VM, Gong E, Yu AC, Lipkin WI: H5N1 infection of the respiratory tract and beyond: a molecular pathology study. Lancet 2007;370:1137–1145.
37 Uiprasertkul M, Puthavathana P, Sangsiriwut K, Pooruk P, Srisook K, Peiris M, Nicholls JM, Chokephaibulkit K, Vanprapar N, Auewarakul P: Influenza A H5N1 replication sites in humans. Emerg Infect Dis 2005;11:1036–1041.
38 Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y: Avian flu: influenza virus receptors in the human airway. Nature 2006;440:435–436.
39 Van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RAM, Osterhaus AD, Kuiken T: H5N1 virus attachment to lower respiratory tract. Science 2006;312:399.
40 Yao L, Korteweg C, Hsueh W, Gu J: Avian influenza receptor expression in H5N1-infected and noninfected human tissues. FASEB J 2008;22:733–740.
41 Nicholls JM, Chan MC, Chan WY, Wong HK, Cheung CY, Kwong DL, Wong MP, Chui WH, Poon LL, Tsao SW, Guan Y, Peiris JS: Tropism of avian influenza A (H5N1) in the upper and lower respiratory tract. Nat Med 2007;13:147–149.
42 Chutinimitkul S, Bhattarakosol P, Srisuratanon S, Eiamudomkan A, Kongsomboon K, Damrongwatanapokin S, Chaisingh A, Suwannakarn K, Chieochansin T, Theamboonlers A, Poovorawan Y: H5N1 influenza A virus and infected human plasma. Emerg Infect Dis 2006;12:1041–1043.
43 De Jong MD, Bach VC, Phan TQ, Vo MH, Tran TT, Nguyen BH, Beld M, Le TP, Truong HK, Nguyen VV, Tran TH, Do QH, Farrar J: Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. N Engl J Med 2005;352:686–691.
44 De Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha do Q, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J: Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 2006;12:1203–1207.
45 Peiris JS, Yu WC, Leung CW, Cheung CY, Ng WF, Nicholls JM, Ng TK, Chan KH, Lai ST, Lim WL, Yuen KY, Guan Y: Re-emergence of fatal human influenza A subtype H5N1 disease. Lancet 2004;363:617–619.
46 To KF, Chan PK, Chan KF, Lee WK, Lam WY, Wong KF, Tang NL, Tsang DN, Sung RY, Buckley TA, Tam JS, Cheng AF: Pathology of fatal human infection associated with avian influenza A H5N1 virus. J Med Virol 2001;63:242–246.
47 Kristensson K: Avian influenza and the brain – comments on the occasion of resurrection of the Spanish flu virus. Brain Res Bull 2006;68:406–413.
48 Cheung CY, Poon LL, Lau AS, Luk W, Lau YL, Shortridge KF, Gordon S, Guan Y, Peiris JS: Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet 2002;360:1831–1837.
49 Chan MC, Cheung CY, Chui WH, Tsao SW, Nicholls JM, Chan YO, Chan RW, Long HT, Poon LL, Guan Y, Peiris JS: Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir Res 2005;6:135.
50 Dybing JK, Schultz-Cherry S, Swayne DE, Suarez DL, Perdue ML: Distinct pathogenesis of Hong Kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. J Virol 2000;74:1443–1450.
51 Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM: A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol 1999;73:5903–5911.

52 Belser JA, Lu X, Maines TR, Smith C, Li Y, Donis RO, Katz JM, Tumpey TM: Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. J Virol 2007;81:11139–11147.
53 Gao P, Watanabe S, Ito T, Goto H, Wells K, McGregor M, Cooley AJ, Kawaoka Y: Biological heterogeneity, including systemic replication in mice, of H5N1 influenza A virus isolates from humans in Hong Kong. J Virol 1999;73:3184–3189.
54 Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, Nguyen DC, Szretter KJ, Chen LM, Thawatsupha P, Chittaganpitch M, Waicharoen S, Nguyen DT, Nguyen T, Nguyen HH, Kim JH, Hoang LT, Kang C, Phuong LS, Lim W, Zaki S, Donis RO, Cox NJ, Katz JM, Tumpey TM: Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J Virol 2005;79:11788–11800.
55 Tumpey TM, Lu X, Morken T, Zaki SR, Katz JM: Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. J Virol 2000;74:6105–6116.
56 Nishimura H, Itamura S, Iwasaki T, Kurata T, Tashiro M: Characterization of human influenza A (H5N1) virus infection in mice: neuro-, pneumo- and adipotropic infection. J Gen Virol 2000;81:2503–2510.
57 Swayne DE, Halvorson DA: Influenza; in Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougald LR, Swayne DE (eds): Diseases of Poultry. Ames, Iowa State Press, 2003, pp 135–160.
58 Tanaka H, Park CH, Ninomiya A, Ozaki H, Takada A, Umemura T, Kida H: Neurotropism of the 1997 Hong Kong H5N1 influenza virus in mice. Vet Microbiol 2003;95:1–13.
59 Xu T, Qiao J, Zhao L, Wang G, He G, Li K, Tian Y, Gao M, Wang J, Wang H, Dong C: Acute respiratory distress syndrome induced by avian influenza A (H5N1) virus in mice. Am J Respir Crit Care Med 2006;174:1011–1017.
60 Lipatov AS, Andreansky S, Webby RJ, Hulse DJ, Rehg JE, Krauss S, Perez DR, Doherty PC, Webster RG, Sangster MY: Pathogenesis of Hong Kong H5N1 influenza virus NS gene reassortants in mice: the role of cytokines and B- and T-cell responses. J Gen Virol 2005;86:1121–1130.
61 Szretter KJ, Gangappa S, Lu X, Smith C, Shieh WJ, Zaki SR, Sambhara S, Tumpey TM, Katz JM: Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. J Virol 2007;81: 2736–2744.l
62 Uiprasertkul M, Kitphati R, Puthavathana P, Kriwong R, Kongchanagul A, Ungchusak K, Angkasekwinai S, Chokephaibulkit K, Srisook K, Vanprapar N, Auewarakul P: Apoptosis and pathogenesis of avian influenza A (H5N1) virus in humans. Emerg Infect Dis 2007;13:708–712.
63 Salomon R, Hoffmann E, Webster RG: Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. Proc Natl Acad Sci USA 2007;104:12479–12481.
64 Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM: Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J Virol 2002;76:4420–4429.
65 Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen TD, Hanh TH, Puthavathana P, Long HT, Buranathai C, Lim W, Webster RG, Hoffmann E: Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol 2005;79:2191–2198.
66 Kuiken T, Rimmelzwaan GF, Van Amerongen G, Osterhaus AD: Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques *(Macaca fascicularis)*. Vet Pathol 2003;40:304–310.
67 Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD: Pathogenesis of influenza A (H5N1) virus infection in a primate model. J Virol 2001;75:6687–6691.
68 Van Reeth K: Avian and swine influenza viruses: our current understanding of the zoonotic risk. Vet Res 2007;38:243–260.
69 Taubenberger JK, Palese P: The origin and virulence of the 1918 'Spanish' influenza virus; in Kawaoka Y (ed): Influenza Virology: Current Topics. Norfolk, Caister Academic Press, 2006, chapt 211, pp 299–312.
70 Kobasa D, Takada A, Shinya K, Hatta M, Halfmann P, Theriault S, Suzuki H, Nishimura H, Mitamura K, Sugaya N, Usui T, Murata T, Maeda Y, Watanabe S, Suresh M, Suzuki T, Suzuki Y, Feldmann H, Kawaoka Y: Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature 2004;431:703–707.
71 Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Pantin-Jackwood MJ, Schultz-Cherry S, Solorzano A, Van Rooijen N, Katz JM, Basler CF: Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol 2005;79:14933–14944.
72 Kash JC, Tumpey TM, Proll SC, Carter V, Perwitasari O, Thomas MJ, Basler CF, Palese P, Taubenberger JK, Garcia-Sastre A, Swayne DE, Katze MG: Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. Nature 2006;443:578–581.

73 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A: Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 2005;310:77–80.
74 Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M, Feldmann F, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H, Kawaoka Y: Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 2007;445: 319–323.
75 Korth MJ, Kash JC, Baskin CR, Katze MG: Insights into influenza virus-host interactions through global gene expression profiling: cell culture systems to animal models; in Kawaoka Y (eds): Influenza Virology: Current Topics. Norfolk, Caister Academic Press, 2006, pp 341–357.
76 Lee DC, Cheung CY, Law AH, Mok CK, Peiris M, Lau AS: p38 mitogen-activated protein kinase-dependent hyperinduction of tumor necrosis factor-α expression in response to avian influenza virus H5N1. J Virol 2005;79:10147–10154.
77 Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P: A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog 2007;3: 1414–1421.
78 Garcia-Sastre A, Biron CA: Type 1 interferons and the virus-host relationship: a lesson in detente. Science 2006;312:879–882.
79 Garcia-Sastre A: Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. Virology 2001;279: 375–384.
80 Donelan NR, Basler CF, Garcia-Sastre A: A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of β-interferon and is attenuated in mice. J Virol 2003;77:13257–13266.
81 Ferko B, Stasakova J, Romanova J, Kittel C, Sereinig S, Katinger H, Egorov A: Immunogenicity and protection efficacy of replication-deficient influenza A viruses with altered NS1 genes. J Virol 2004;78: 13037–13045.
82 Solorzano A, Webby RJ, Lager KM, Janke BH, Garcia-Sastre A, Richt JA: Mutations in the NS1 protein of swine influenza virus impair anti-interferon activity and confer attenuation in pigs. J Virol 2005;79:7535–7543.
83 Cauthen AN, Swayne DE, Sekellick MJ, Marcus PI, Suarez DL: Amelioration of influenza virus pathogenesis in chickens attributed to the enhanced interferon-inducing capacity of a virus with a truncated NS1 gene. J Virol 2007;81:1838–1847.
84 Kochs G, Koerner I, Thiel L, Kothlow S, Kaspers B, Ruggli N, Summerfield A, Pavlovic J, Stech J, Staeheli P: Properties of H7N7 influenza A virus strain SC35M lacking interferon antagonist NS1 in mice and chickens. J Gen Virol 2007;88:1403–1409.
85 Li Z, Jiang Y, Jiao P, Wang A, Zhao F, Tian G, Wang X, Yu K, Bu Z, Chen H: The NS1 gene contributes to the virulence of H5N1 avian influenza viruses. J Virol 2006;80:11115–11123.
86 Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T: Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 1998;252:324–330.
87 Hayman A, Comely S, Lackenby A, Hartgroves LC, Goodbourn S, McCauley JW, Barclay WS: NS1 proteins of avian influenza A viruses can act as antagonists of the human alpha/beta interferon response. J Virol 2006;81:2318–2327.
88 Zeng H, Goldsmith C, Thawatsupha P, Chittaganpitch M, Waicharoen S, Zaki S, Tumpey TM, Katz JM: Highly pathogenic avian influenza H5N1 viruses elicit an attenuated type i interferon response in polarized human bronchial epithelial cells. J Virol 2007;81:12439–12449.
89 Seo SH, Hoffmann E, Webster RG: Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med 2002;8:950–954.
90 Seo SH, Hoffmann E, Webster RG: The NS1 gene of H5N1 influenza viruses circumvents the host anti-viral cytokine responses. Virus Res 2004;103:107–113.
91 Krug RM, Yuan WM, Noah DL, Latham AG: Intracellular warfare between human influenza viruses and human cells: the roles of the viral NS1 protein. Virology 2003;309:181–189.
92 De Lang A, Baas T, Teal T, Leijten LM, Rain B, Osterhaus AD, Haagmans BL, Katze MG: Functional genomics highlights differential induction of antiviral pathways in the lungs of SARS-CoV-infected macaques. PLoS Pathog 2007;3:1129–1141.

Kristien Van Reeth
Laboratory of Virology, Ghent University
Salisburylaan 133, BE–9820 Merelbeke (Belgium)
Tel. +32 9 264 7369, Fax +32 9 264 7495, E-Mail kristien.vanreeth@ugent.be

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 252–271

Antivirals and Resistance

Alan J. Hay[a] · Patrick J. Collins[a] · Rupert J. Russell[b]

[a]Virology Division, National Institute for Medical Research, London, and [b]Interdisciplinary Centre for Human and Avian Influenza Research, University of St Andrews, St Andrews, Fife, UK

Abstract

There are two classes of anti-influenza drugs – one class targets the M2 proton channel whereas the other class targets the surface glycoprotein neuraminidase. This review discusses the mechanism of action of both classes and details the problem of the emergence of drug resistance mutations from an evolutionary and structural perspective.

Introduction

Two classes of specific anti-influenza drugs have been developed to date: the amino-adamantanes, amantadine and rimantadine, developed in the early 1960s [1] and targeted against the M2 proton channel of influenza A viruses [2], and the more recently developed inhibitors of the viral neuraminidase, zanamivir and oseltamivir [3, 4] (fig. 1). Prior to the late 1990s, application of the former class of drugs was limited, with the possible exception of the Soviet Union, where rimantadine was reported to be widely used. There were several contributory factors, including restriction for treatment of influenza A, since the drugs are not effective against influenza B, and concerns about effectiveness and central nervous system complications, particularly associated with amantadine, which was also used in the treatment of parkinsonism. The propensity for resistance emergence also reduced enthusiasm for widespread use in a seasonal context, and the recent prevalence of amantadine resistance among AH3N2 and AH1N1 viruses circulating worldwide [5], as well as among AH5N1 viruses [6], which may be associated with more extensive application of the drug in China and other countries, has led to specific recommendations against the use of this class of drug against seasonal influenza. In contrast, resistance to the neuraminidase inhibitors (NAIs) emerges less readily, especially since some of the more frequent mutations substantially compromise virus infectivity and transmission.

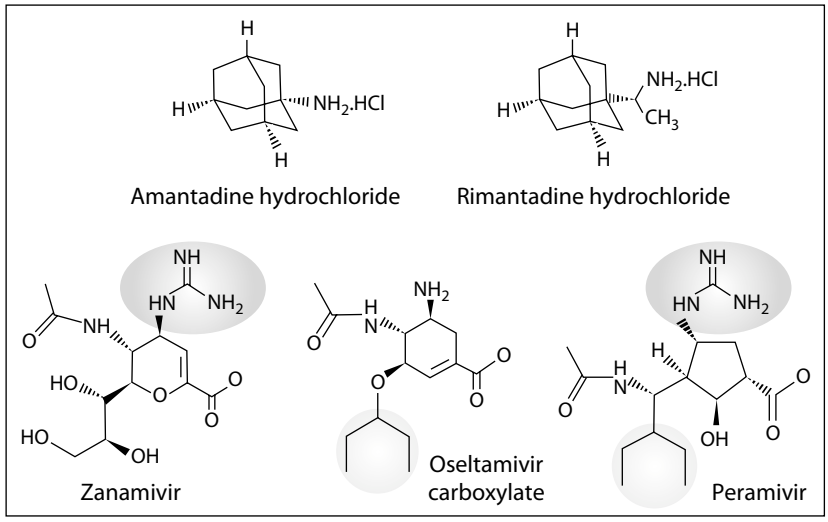


Fig. 1. Structures of inhibitors of the M2 proton channel of influenza A and the neuraminidases of influenza A and B viruses. The guanidino and ethylpropoxy substituents of zanamivir and oseltamivir, respectively, both present in peramivir, are highlighted.

However, with the spectre of the next pandemic looming and recent examples of the more frequent emergence of oseltamivir resistance associated with treatment of children in Japan [7] and H5N1-infected patients [8], the impact on the effectiveness of drug treatment of mutations which reduce drug susceptibility as well as those which cause high resistance is of major concern, especially in relation to the drugs stockpiled as the first line of defence to counter a pandemic.

M2 Channel Inhibitors: Amantadine and Rimantadine

The aminoadamantanes amantadine (Symmetrel) and its derivative rimantadine (Flumadine) represent the first class of antivirals clinically approved for treatment of influenza A infection. Clinical studies showed that the efficacy of amantadine and rimantadine when used prophylactically was 70–90% [9]. Although also effective therapeutically [10], drug-resistant mutants emerge frequently in both human [11, 12] and avian [13] hosts.

Mechanism of Drug Action and Molecular Basis of Resistance

Influenza A and B viruses each contain a small integral membrane protein, M2 and BM2, respectively, which are minor components of the virus membrane, and form homotetrameric pH-activated proton-selective channels [14, 15]. M2 plays two roles in influenza replication [2]. The M2 channel mediates an influx of protons into the

Table 1. Amino acid substitutions in the M2 proteins of natural isolates of amantadine- and rimantadine-resistant influenza A viruses

Virus subtype	Amino acid residue			
	26	27	30	31
Human				
H1N1	Leu>Phe	Val>Ala		Ser>Asn
H3N2	Leu>Phe	Val>Ala	Ala>Val/Thr	Ser>Asn
Avian				
H5N1		Val>Ala/Gly	Ala>Ser	Ser>Asn
H5N2	Leu>Phe	Ile>Ser/Thr	Ala>Ser/Thr	Ser>Asn
H7N2		Val>Ala	Ala>Ser	Ser>Asn
H9N2		Val>Ala	Ala>Thr	Ser>Asn
Swine				
H1N1	Leu>Phe	Val>Ala	Ala>Ser	Ser>Asn
H1N2	Leu>Phe		Ala>Ser	Ser>Asn
H3N2	Leu>Phe			Ser>Asn

infecting virion during virus entry, which facilitates the low pH dissociation of the viral ribonucleoprotein (vRNP) from the matrix protein and its release into the cytoplasm for transport into the cell nucleus to initiate replication [16]. Also, in highly pathogenic avian influenza viruses (H5 and H7), the M2 proton channel acts at a later stage in infection by reducing the pH of the *trans* Golgi network [17]. This is necessary to prevent exposure of HA1/HA2 of these pathogenic avian viruses, cleaved by furin-like proteases within the *trans* Golgi, to a pH that could trigger prematurely the low pH conformational change in haemagglutinin (HA) [18].

Location of amantadine resistance mutations in the transmembrane (TM) domain of the M2 protein identified both the target and mechanism of action of the drugs [19]. Single amino acid substitutions at five residues, 26, 27, 30, 31 or 34 which line the pore of the channel, have been shown to confer resistance to amantadine and rimantadine in vitro and/or in vivo, depending on the virus (table 1; fig. 2). These mutations in general cause cross-resistance between amantadine, rimantadine and analogous inhibitors, and attempts to develop alternative, complementary inhibitors of M2, effective against these resistant mutants, have been unsuccessful. The consistent correlation between the presence of these mutations and high drug resistance provides the basis for the simple, routine screening of resistance to amantadine and rimantadine by sequence analysis of the M gene encoding the short stretch of M2 sequence, particularly suited to pyrosequencing [5].

Structural studies of the M2 channel have indicated that the residues altered in drug-resistant viruses line the channel, and available data, including NMR studies of

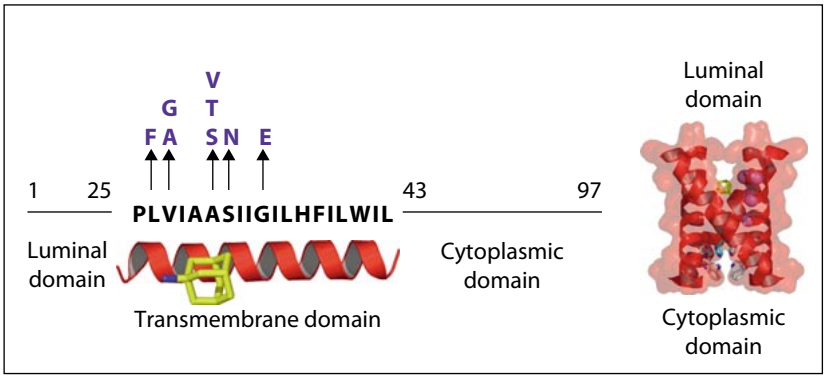


Fig. 2. Schematic diagram of the transmembrane domain of the influenza A M2 channel, showing the locations of single amino acid substitutions (LHS) which have been principally responsible for conferring resistance to amantadine, their positions (purple spheres) relative to the His37/Trp41 motif in a schematic model of the tetrameric channel (RHS) and the inferred location of amantadine (yellow) binding.

interaction of amantadine with a TM peptide, are consistent with drug binding within the channel in the vicinity of residues 27–34 [20, 21]. Furthermore, the drug binds to the channel with a stoichiometry of one molecule per tetrameric channel, but does not act as a simple non-competitive blocker; rather it acts allosterically, possibly inducing structural alterations in the channel which abrogate channel activation [22, 23]. Whereas most resistance mutations inhibit amantadine binding, some, especially those affecting residue 27, appear to permit drug binding but prevent inhibition of channel activity [24].

Although some resistance mutations selected in vitro in certain viruses have been shown to affect the channel activity of M2 [25], most of the more common mutations emerging naturally, e.g. V27A and S31N, in human H3N2 and H1N1 viruses and in avian viruses have been shown to have little effect on the infectivity, virulence or transmissibility of the viruses in ferrets [26] or birds [13], respectively. The lack of adverse consequences of these mutations is amply demonstrated by the recent prevalence of these mutations in human [5], swine [27] and certain avian viruses including H5N1 [6, 28] (table 1).

Emergence of Drug Resistance

Early observations indicated a low incidence (<1%) of primary resistance to amantadine among human influenza A viruses circulating before 1995 [29]. Furthermore, there were few reports of amantadine resistance among human or animal viruses prior to their emergence in European swine viruses in the mid-1980s [27], and there was no indication that the reported extensive use of rimantadine in Russia over more than two decades was associated with spread of resistant viruses.

Clinical studies of the effectiveness of rimantadine revealed, however, that rimantadine-resistant viruses were frequently shed by children treated with drug [11], and were frequently recovered from index patients and transmitted to family members in households treated with rimantadine [12]. As a consequence, post-exposure prophylaxis of families is only recommended in the absence of treatment of index cases. Use of the drugs to control influenza among nursing home residents has also shown a high frequency of isolation of resistant viruses, of greater than 30% and as high as 80%, resulting in treatment failure [30]. Although in such instances prior to 2000 there was little evidence of spread of the resistant viruses to the wider community, the recent emergence of amantadine-resistant H3N2 viruses in China and Hong Kong in 2002 [31] and their spread worldwide indicates that resistance is retained in the absence of selective drug pressure and is not detrimental to epidemiological potential. In addition, there is evidence from amantadine resistance of a few viruses isolated between 1933 and 1937 that the H1N1 viruses circulating in the human population at that time, prior to the advent of the drugs, may have possessed significant amantadine resistance.

Phylogenetic analyses of recent AH3N2 viruses showed that amantadine resistance was principally associated with an AH3N2 variant, represented by A/Wisconsin/67/2005, which predominated during 2005–2006, such that in some countries resistance approached 100%. The reduction in the proportion of resistant viruses during 2006–2007 was associated with the emergence of drug-sensitive variants, which appear to have acquired, by genetic reassortment, 'sensitive' M genes close to those of amantadine-sensitive variants previously prominent during 2005–2006, rather than by reversion of the resistance mutation (fig. 3B). The emergence of amantadine resistance among a recently emerging variant of H1N1 viruses (fig. 3A) occurred independently and was not due to reassortment between H1N1 and H3N2 viruses. These independent events suggest a recent increase in selective pressure, likely due to the more widespread use of amantadine in China and other countries, and emphasize the tolerability of the resistance mutations in both subtypes of human viruses. The S31N mutation has been the most prominent determinant of resistance, also among swine and avian viruses.

Coincidentally, some of the AH5N1 viruses which re-emerged in poultry in Asia in 2003, including those which caused two human infections in Hong Kong, possessed amantadine resistance mutations in M2. In particular, most of the clade 1 H5N1 viruses, which were prevalent in Vietnam, Thailand and Cambodia during 2004–2005, causing numerous human infections, were resistant to amantadine (fig. 3C) [6]. On the other hand, H5N1 viruses isolated from poultry outbreaks in Japan and Korea in late 2003 did not possess resistance mutations, nor did the 'Qinghai Lake' viruses which spread to the Middle East, Europe and Africa from mid-2005 onwards. An intermediate frequency of resistance was reported among H5N1 viruses isolated in China and Hong Kong since 2002 [6]. Although H5N1 viruses isolated in Indonesia during 2004–2005 were mainly sensitive to amantadine, those isolated from both

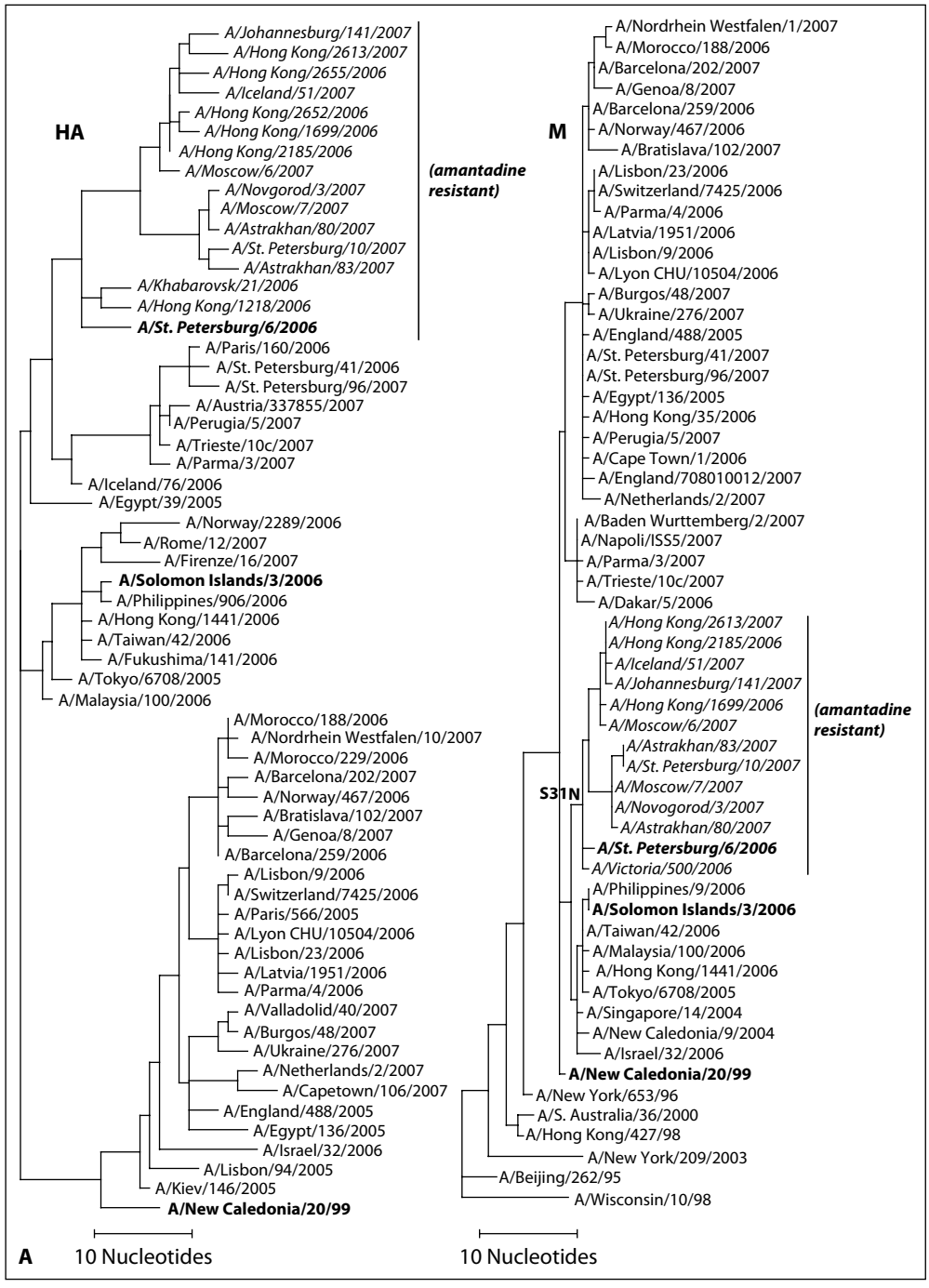


Fig. 3. **A** and **B**, phylogenetic relationships between the HA and M genes of amantadine-sensitive and resistant influenza A viruses circulating during 2005–2007. **A.** Emergence of a subgroup of amantadine-resistant H1N1 viruses (in italics) represented by ***A/St. Petersburg/6/2006***, within the **A/Solomon Islands/3/2006** clade. **B.** Evolutionary relationships associated with changes in amantadine sensitivity of H3N2 viruses. Viruses circulating during 2004 to 2006 with sequences close to those of

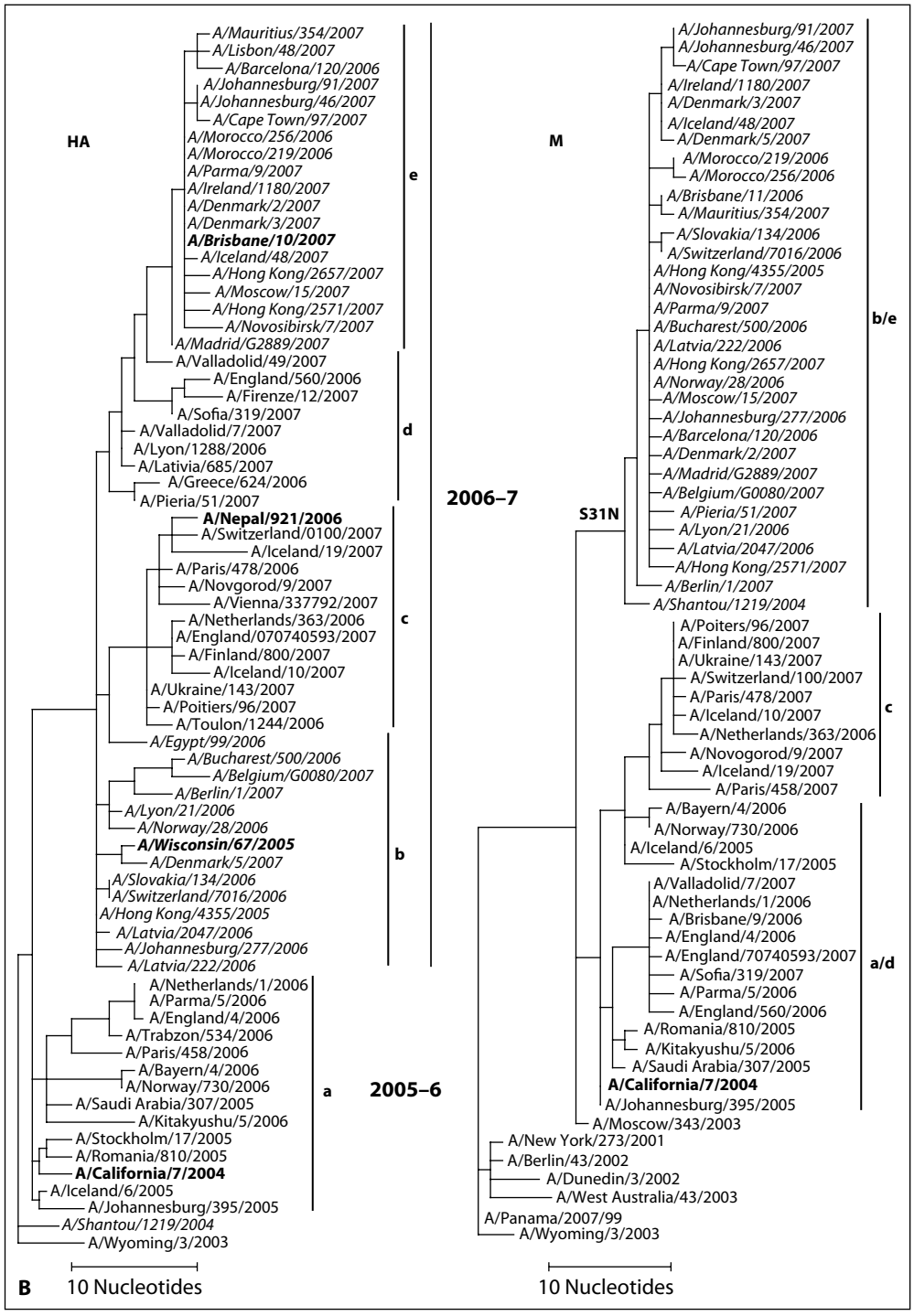
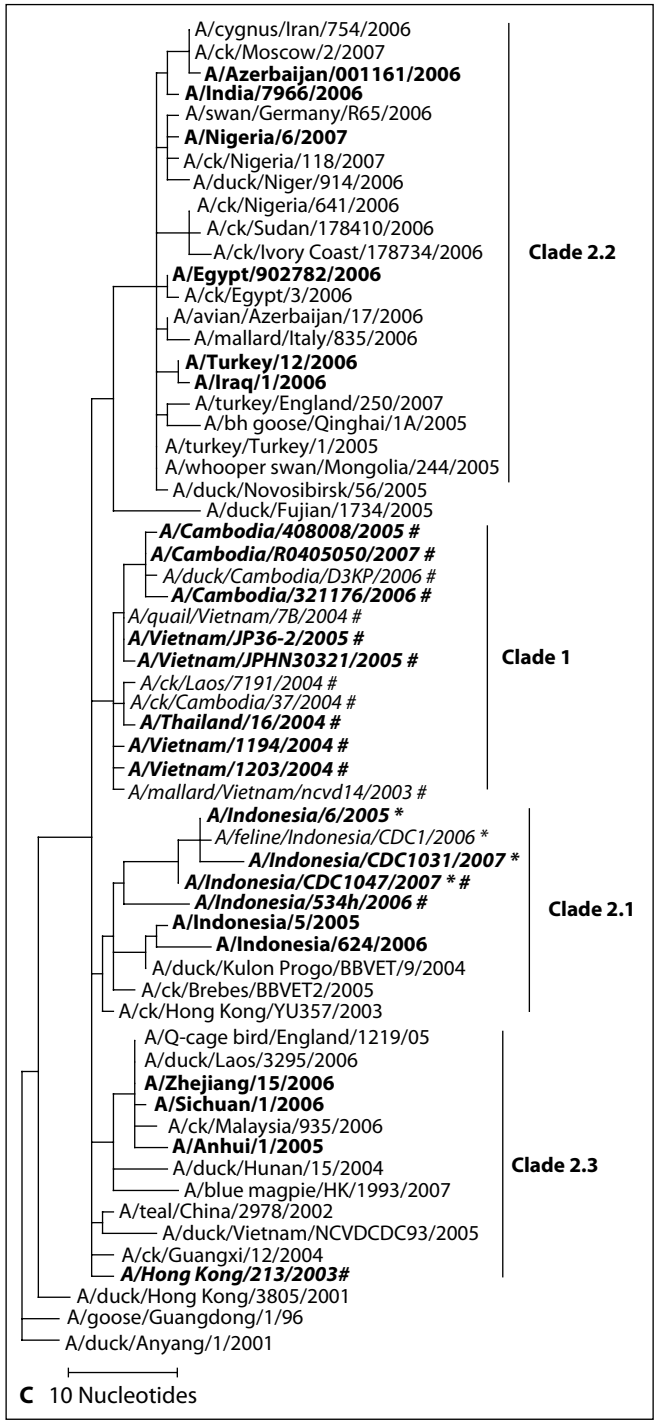


Fig. 3. (continued)



A/California/7/2004 (a) were mainly sensitive, whereas those with sequences close to ***A/Wisconsin/67/2005*** (b) were mainly resistant to amantadine (italics) and possessed asparagine 31 in the M2 protein. A number of variants of the latter viruses (c-e) emerged during 2006–2007; whereas those represented by ***A/Brisbane/10/2007*** (e) possessed M genes similar to the earlier '***A/Wisconsin/67/2005***' viruses and retained the amantadine resistance marker Asn 31, other variants, such as those represented by **A/Nepal/921/2006** (c) possessed 'sensitive' M genes (lacking resistance mutations in M2) closely related to those of the earlier **'A/ California/7/2004'** viruses, apparently acquired by reassortment. Phylogenetic subgroups are labelled a-e to indicate correspondence of HA and M genes. Representative viruses are shown in bold typeface. **C.** Phylogenetic relationships among the M genes of clade 1 and 2 H5N1 viruses, which have been associated with human infection. Sequences of human isolates are shown in bold typeface. Amantadine resistant M genes are in italics; # and * indicate the presence of Asn31 and Ala27 resistance markers, respectively, in the M2 protein. Sequences were determined by the authors, obtained from the database or provided by others, as indicated in Acknowledgements.

poultry and human cases of H5N1 during 2006–2007 have shown a high level (>80%) of resistance [32].

In the context of pandemic viruses and the possibility that they might emerge by genetic reassortment in an intermediate host, such as the pig, the amantadine resistance of swine viruses is of particular significance. Amantadine resistance was initially detected among European swine H1N1 and H3N2 viruses circulating in the mid-1980s, and this phenotype has been retained in more recently isolated H1N1, H1N2 and H3N2 viruses [27]. Amantadine resistance has also been observed among H3N2 viruses isolated in Hong Kong between 1999 and 2002, H1N1 and H9N2 viruses isolated in Korea in 2004, and among H1N1, H1N2 and H3N2 swine viruses circulating in North America (table 1). These latter viruses are of particular interest since many were the result of genetic reassortment between swine, avian and human viruses, possessing the H3 and N2 of recent human viruses [33], and demonstrate the potential for generating novel drug-resistant subtypes with pandemic potential.

Neuraminidase Inhibitors: Zanamivir and Oseltamivir

The virus neuraminidase (NA) performs a complementary role to that of the HA in virus replication. It removes the terminal sialic acid present on cellular receptors to which the HA binds and on the virus glycoproteins to facilitate release and dispersal of progeny virus particles. In addition, cleavage of sialic acid from mucins in the respiratory tract removes these potential non-specific inhibitors of virus infection.

Two drugs, zanamavir and oseltamivir, have been developed against the virus NA, based on the X-ray crystal structure of the enzyme [3, 4]. They act specifically against all subtypes of influenza A NAs and influenza B NA [34] and have been licensed for therapeutic and prophylactic use [10, 35]. They differ in the ring structure and the active substituents (fig. 1), which target different parts of the enzyme and provide the basis for complementary resistance profiles. The guanidino group of zanamivir interacts with a pocket within the catalytic site formed by the acidic residues glutamic acid 119, aspartic acid 151 and glutamic acid 227, with an affinity of binding some 1,000-fold greater than that of the substrate [3]. The hydrophobic ethylpropoxy moiety of oseltamivir increases affinity by a similar amount by binding to a hydrophobic pocket in the active site exposed following reorientation of glutamic acid 276 [4]. A third compound, peramivir, which is still under clinical development, possesses both active substituents [36]. All three compounds have K_i values within the nanomolar range. The IC_{50}s reported for inhibition of different A subtype NAs varied within the ranges of 2–30 nM for zanamivir, 2–69 nM for oseltamivir and 1–4 nM for peramivir [34]. The IC_{50} for oseltamivir inhibition of influenza B NA is somewhat higher than those for the NAs of human H1N1 and H3N2 viruses, possibly contributing to reduced effectiveness of this drug against influenza B [37]. Although all three drugs have been shown to protect mice against lethal infection by highly pathogenic H5N1 viruses,

zanamivir failed to protect chickens against virulent infections by some other avian viruses possessing different NA subtypes, suggesting that locally acting drug is less effective in treating disseminated infection [38]. In this respect, higher doses of oseltamivir were shown to be required for effective treatment of more virulent H5N1 infections of mice and ferrets [39].

Structure of Influenza Neuraminidases

Influenza NA is a homotetrameric molecule with 4-fold symmetry, with each monomer consisting of six topologically identical four-stranded antiparallel β-sheets that are themselves arranged like the blades of a propeller [40]. Sialic acid binds in a deep pocket on the surface of the molecule roughly in the middle of each monomer, and the amino acids in this pocket, which form the active site, including Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371 and Tyr406 (numbering according to N2 sequence is used throughout), are highly conserved across all NA subtypes [41]. The three arginine residues, Arg118, Arg292 and Arg371, bind the carboxylate of the substrate sialic acid, Arg152 interacts with the acetamido substituent of the substrate, and Glu276 forms hydrogen bonds with the 8- and 9-hydroxyl groups of the substrate. Other conserved residues that provide a framework to support the structure of the catalytic site include Glu119, Arg156, Trp178, Ser179, Asp/Asn198, Ileu222, Glu227, His274, Glu277, Asn294 and Glu425.

Influenza A NA sequences, however, fall into two distinct phylogenetic groups and, although crystal structures of N1, N4 and N8 of group 1 and N2 and N9 of group 2 all have the same homotetrameric conformation, they possess group-specific differences in the active site [42]. The main conformational differences between the two groups are centred on the 150-loop (residues 147–152) and the 150 cavity, adjacent to the active site, of group 1 NAs (fig. 4). The conformation of the 150-loop is such that the α carbon of valine 149 in group 1 is about 7 Å distant from that of the equivalent isoleucine in group 2, and the side chain points away rather than towards the active site. In addition, there is a difference of 1.5 Å in the positions of the conserved aspartic acid 151 side chains, and the carboxylate of the nearby conserved glutamic acid 119 points in approximately the opposite direction to that in group 2, such as to increase the width at that point of the active site cavity of group 1 NAs by about 5 Å. These features, together with the location of glutamine 136 3.5 Å lower at the base of the cavity, produce the 10 × 5 × 5 Å 150-cavity adjacent to the active site, in the 'open form' of group 1 NAs, but not in the 'closed form' of group 2 NAs.

Inhibitor Binding

Crystal structures of zanamivir, oseltamivir and peramivir in complex with N2 or N9 showed that only minor conformational changes occur in the active site of group 2 NAs upon inhibitor binding [43]. For example, in unliganded N9 the carboxylate of Glu276 faces into the active site cavity, but upon oseltamivir binding Glu276 adopts a

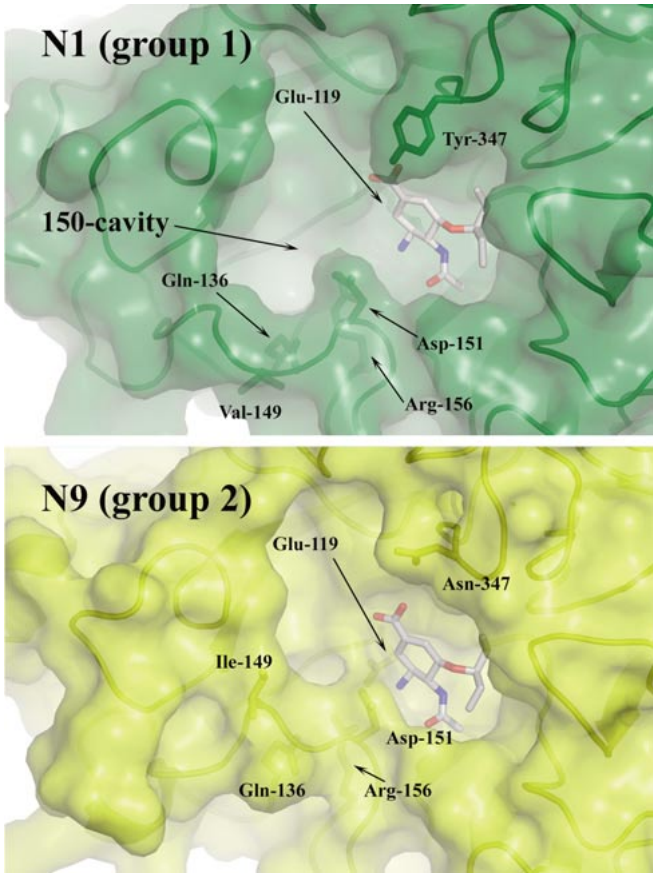


Fig. 4. Oseltamivir bound to the open conformation of N1 (group 1) (green) and to the closed conformation of N9 (group 2) (yellow) NAs.

conformation that points the carboxylate away from the active site so that it now makes a bidentate interaction with the guanidinium group of Arg224. In so doing the hydrophobic CB and CG of Glu276 move towards the C6-linked hydrophobic moiety of oseltamivir.

When group 1 NAs are incubated in zanamivir, oseltamivir or peramivir, however, the 150-loop changes its conformation so that it closely resembles the 'closed' conformation of group 2 NAs, both in the presence and absence of inhibitor [42]. The presence of the 150-cavity, therefore, does not significantly influence binding of these drugs to the two groups of NAs, but does provide the basis for development of alternative inhibitors with constituents which target the 150 cavity, since it has been shown under certain conditions oseltamivir can bind to group 1 NAs without inducing the conformational change of the 150-loop (fig. 4).

Drug Resistance

Emergence of resistance to NA inhibitors, either in vitro or in vivo in animal experiments or treated human patients have occurred far less readily than to the M2 inhibitors [44]. Initial in vitro studies showed that in many instances mutations were initially selected in the HA. These, in general, reduced the receptor binding affinity of the HA and as a consequence reduced the requirement for an active NA and conferred cross-resistance to the other NA inhibitors. Mutations in NA tended to be selected after further cell culture passage in the presence of drug, directly reducing inhibition of the enzyme. These mutations in NA appeared to be of more significance than those in HA in reducing drug susceptibility in animal models [45, 46]. Furthermore, mutations in NA have been observed to be principally responsible for emergence of drug resistance in patients treated with oseltamivir [35]. There are fewer examples of resistance associated with zanamivir treatment; however, this may reflect to some extent lower drug use and fewer clinical studies. The principal amino acid substitutions associated with resistance in drug-treated patients are listed in table 2. With the exception of the R152K mutation, selected from a zanamivir-treated case of influenza B, which conferred cross-resistance to all three drugs, the other mutations were selected in response to oseltamivir treatment and, in general, caused much less reduction in susceptibility to zanamivir. Their influence on susceptibility to peramivir was more varied. The complementarity of oseltamivir and zanamivir in this respect, as a means of reducing or overcoming resistance emergence, is particularly pertinent to the recent widespread stockpiling of drugs for pandemic use.

Type and Subtype-Specific Differences in Resistance Mutations in NA

In addition to type-specific differences, resistance mutations, particularly to oseltamivir, show NA group (and subtype) specificity in their resistance profiles, in that different mutations are principally responsible for resistance of influenza A N1 and N2 subtypes (table 2). Structural analyses of the sites of mutation have revealed both a basis for resistance and the observed group specificity.

The mutation R292K causes high resistance of group 2 NAs to oseltamivir, but has little effect on group 1 NAs. The effect of this mutation on N2 has been the subject of a detailed crystallographic analysis which revealed that the resistance results from the loss of a hydrogen bond to the carboxylate group of oseltamivir [43]. The structure of group 1 NAs complexed with oseltamivir revealed the likely reason why this mutation does not affecting binding to N1 NAs (fig. 5A). A conserved tyrosine residue at position 347 in group 1 NAs makes an additional hydrogen bond to the carboxylate group of the inhibitor that cannot be made by the equivalent residues in group 2 NAs. This additional hydrogen bond interaction, possible only in group 1 NAs, compensates for the loss of the interaction associated with the R292K mutation.

Glutamic acid 119 is strictly conserved in all NAs and forms a hydrogen bond with the hydroxyl group of sialic acid, the amino group of oseltamivir and the guanidino group of zanamivir. This residue undergoes a conformational change upon inhibitor

Table 2. Drug susceptibility of influenza A and B viruses recovered from oseltamivir- or zanamivir-treated patients

Virus type/ subtype	Drug treatment	NA[1] substitution	Drug susceptibility[2]		
			oseltamivir	zanamivir	peramivir
AH3N2	Oseltamivir	R292K	R	'R'	R
		E119V	R	S	S
		E119V+I222V	R	S	'R'
		N294S	'R'	S	S
		Δ244-247	R	S	
AH1N1	Oseltamivir	H274Y	R	S	R
AH5N1	Oseltamivir	H274Y	R	S	
		N294S	'R'	S	
B	Oseltamivir	D198N	'R'	'R'	S
	Zanamivir	R152K	R	R	R

[1]Amino acids numbered according to N2 sequence.
[2]Determined by NA assay. R = High level resistance; 'R' = intermediate reduction in drug susceptibility; S = relatively little change in drug susceptibility.

binding to group 1 NAs but does not in group 2 NAs. Several mutations in N2 NAs (Gly, Ala or Asp), as well as in N9 and B NAs, have been selected by passage in cell culture in the presence of zanamivir, resulting in resistance to zanamivir, presumably due to elimination of the hydrogen bond, but retaining sensitivity to oseltamivir [47]. In contrast, the E119V mutation conferring resistance to oseltamivir retains sensitivity to zanamivir. Mutations at this position have not yet arisen in the group 1 NAs, however the mutation I117V which has been associated with reduced sensitivity to oseltamivir in N1 NA [32] is located adjacent to Glu119. The isoleucine side chain points away from the active site and packs against a number of other hydrophobic residues; mutation to a valine could disrupt this packing and lead to a change in the conformation of the loop in which Glu119 sits.

The mutation H274Y leads to high resistance of N1 NAs against oseltamivir but has little effect on N2 NAs [48]. Inspection of the structures of the group 1 NAs in complex with oseltamivir, and comparison with equivalent group 2 NA complexes, suggests that this clade-dependent behaviour is mediated by the differential effect of this mutation on the conformation of Glu276. There appear to be at least two contributory factors as to why this mutation can be accommodated in N2 but not in N1 NAs [42]. Firstly, the 270-loop (approaching residue 273) in N1 makes a tighter turn than the equivalent loop in N9. Secondly, in N1, but not in N9, there is a conserved tyrosine residue at position 252

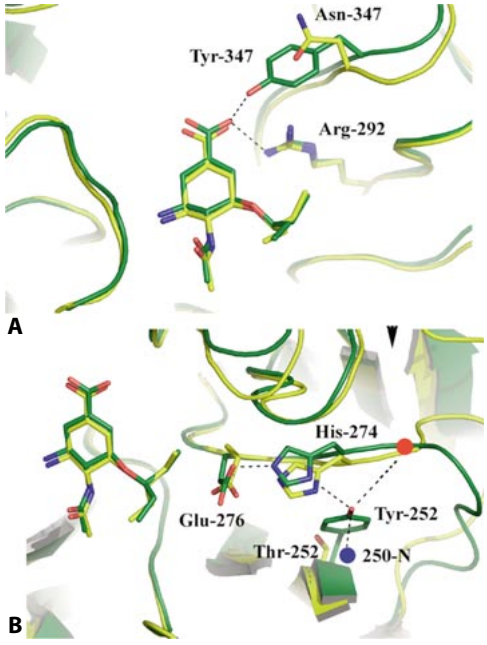


Fig. 5. Differences in the structures of group 1 (green) and group 2 (yellow) NAs provide explanations for subtype-specific differences in oseltamivir resistance mutations of N1 and N2 NAs. **A** A H-bond between the carboxylate group of oseltamivir and the conserved Tyr 347 of N1 complements the loss of a H-bond with Arg292 on substitution by lysine, to abrogate reduced binding of the drug by the Arg292 Lys mutant of N2. **B** In N1, the introduction of the bulkier Tyr residue at 274 may cause displacement of Glu276, interfering with binding of the ethylpropoxy substituent of oseltamivir, whereas the different conformation of the 270-loop together with the smaller Thr252 residue in group 2 NAs provides more space to accommodate Tyr 274 without perturbing Glu276. Structures are of the NAs of A/Vietnam/1203/2004 (H5N1; in green) and of NWS/G70C (H1N9; in yellow). Residues are numbered according to the N2 sequence.

that makes hydrogen bonds to main chain groups at position 273 (CO) and 250 (NH) and to the histidine side chain at 274, while this histidine also hydrogen bonds through its other side chain nitrogen with Glu276 (fig. 5B). The substitution of the bulkier tyrosine residue at position 274 in N1 can only be accommodated by the new side chain moving towards, and partially displacing, Glu276. In contrast, in group 2 NAs, the much smaller threonine residue at position 252 leaves space for the introduced tyrosine to occupy without perturbing Glu276. Furthermore, replacement of Tyr252 by histidine in the NAs of clade 1 H5N1 viruses appears to account for their increased sensitivity to oseltamivir, relative to NAs of clade 2 H5N1 viruses which possess Tyr252 [49].

Disruption of the Hydrophobic Pocket

The hydrophobic ethylpropoxy substituent at the C6 position of the cyclohexene core of oseltamivir packs into a hydrophobic pocket in the NA active site, providing

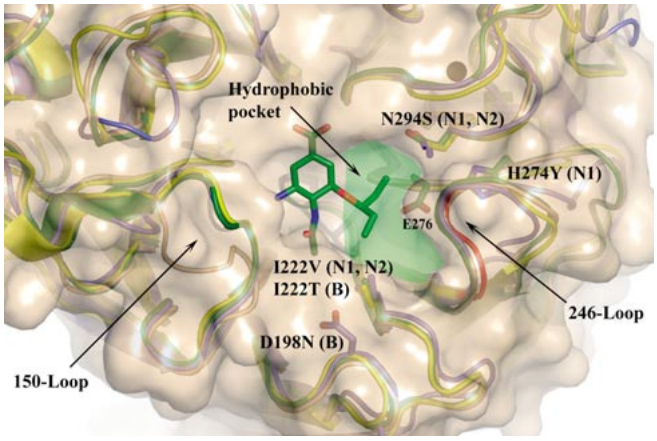


Fig. 6. Locations of mutations which reduce oseltamivir susceptibility by affecting interaction of the drug with the hydrophobic pocket of the active site of NA. The structures of N1 open (gold) and closed (green) conformations, N2 (yellow) and B (blue) NAs are superimposed. The deletion of residues 244–247 of N2 is shown in red. Residues are numbered according to N2 sequences.

favourable van der Waals contacts. A number of mutations that give rise to reduced susceptibility to oseltamivir are located in this hydrophobic pocket (fig. 6). An isoleucine at position 222 is strictly conserved in all NAs and packs against the hydrophobic ethylpropoxy substituent of oseltamivir. Mutation to valine in N1 and N2 or threonine in influenza B NA reduces the interactions between NA and oseltamivir and consequently the binding of oseltamivir may be compromised [50].

A more dramatic alteration of the hydrophobic pocket occurs upon the deletion of residues 244–247 in an N2 NA with reduced susceptibility to oseltamivir [51]. These residues form one edge of the active site, and a highly conserved alanine at 246 sits in the hydrophobic pocket. Their deletion would firstly remove some stabilising hydrophobic interactions and also result in the hydrophobic ethylpropoxy substituent becoming more exposed to solvent.

The mutation N294S confers reduced susceptibility of both N1 and N2 to oseltamivir [7, 52]. Asn294 forms a hydrogen bond to the main chain carbonyl of Ala246 that would lead to stabilisation of the 246-loop that contains Ala246. A serine at position 294 would not be able to form this hydrogen bond and therefore could lead to disruption of the 246-loop. As noted above, deletion of this loop does indeed result in reduced susceptibility to oseltamivir. Asn294 also forms a hydrogen bond to the strictly conserved Arg292 which interacts with the carboxylate group of oseltamivir. Once again, a serine at position 294 would eliminate this hydrogen bond. It is unlikely though that this loss results in the reduced susceptibility to oseltamivir of the N294S mutant NA, as it would also be expected that would lead to reduced

susceptibility to zanamivir. Zanamivir, however, has a hydrophilic glycerol substituent rather the hydrophobic ethylpropoxy of oseltamivir; thus disruption of the 246-loop would have less impact on its binding.

Two NA mutations that have arisen in influenza B patients which reduce susceptibility to both oseltamivir and zanamivir are R152K and D198N, the latter also in conjunction with I222T [50, 53, 54]. Arg152 forms an electrostatic interaction with Glu119 and thus stabilises the position of this key residue, and also is within hydrogen bonding distance to the guanidino group of zanamivir. Mutation of this residue to a lysine would remove the potential to form both of these hydrogen bonds. The aliphatic side chain of Asp198 packs against Ileu222 and stabilises the conformation of the hydrophobic pocket. The terminal acidic group of Asp198 forms an ion-pair to Arg149 which in turn forms a hydrogen bond to the oxygen in the acetamide substituent of sialic acid, oseltamivir and zanamivir. A D198N mutation, although isosteric, would eliminate the electrostatic interaction and may alter the conformation of the conserved Arg149. Additionally, the I222T mutation would now allow a hydrogen bond to be formed between Asn198 and Thr222 which could further alter the conformation of Arg149.

Consequences of Resistance Mutations in NA

In contrast to the tolerability of amantadine resistance mutations and their epidemiological significance, a number of the common NAI resistance mutations in NA have been shown to adversely affect the properties of the enzyme and the infectivity, virulence and/or transmissibility of the mutant virus, such that early considerations concluded that resistance to NAIs, particularly zanamivir, was unlikely to be of significant clinical consequence. For example, a number of studies showed that mutations in NA of Glu119 to Gly, Ala or Asp, Arg292 to Lys or Arg152 to Lys in flu B compromised enzyme activity and virus replication [46]. Furthermore, studies of infection in mice or ferrets have shown that the most common mutation conferring resistance to oseltamivir in H3N2 viruses, R292K, severely impairs their infectivity, virulence and transmissibility [55]. On the other hand, the oseltamivir-resistant mutation E119V was shown be stable and to have little effect on the growth or transmissibility of the mutant H3N2 viruses [56]. The common mutation in N1-containing viruses, H274Y, was observed to be intermediate and variable in its effects on different viruses [57]. Thus, whereas this mutation was observed to reduce the infectivity of some H1N1 viruses, recent studies showed that H5N1 viruses possessing either the H274Y or N294S mutation retained the high pathogenicity of the wild-type virus in mice [58]. The recent emergence of oseltamivir resistance among H1N1 viruses circulating in different parts of the world during late 2007-early 2008, the proportion of which was as high as 70% among Norwegian isolates, demonstrates that viruses carrying the H274Y mutation can readily transmit between individuals, be maintained in the absence of drug pressure, and become epidemiologically important [63].

Drug Combinations and Other Developments

Recent concern about the impact of drug resistance has stimulated renewed interest in the effectiveness of combinations of the different types of anti-influenza drugs and their ability to reduce the emergence of resistance. Earlier studies of amantadine or rimantadine in combination with the broad-spectrum antiviral ribavirin exhibited enhanced effectiveness in vitro and in vivo in a mouse model, compared with the individual drugs. More recently, for example, combinations of the NA inhibitors, zanamivir, oseltamivir or peramivir, with rimantadine were shown to interact both additively and synergistically in inhibiting the yield of H1N1 and H3N2 viruses in cell culture [59]. Furthermore, combinations of amantadine and oseltamivir were shown to reduce the emergence of drug resistance of H1N1, H3N2 and H5N1 viruses passaged in cell culture compared to either drug used individually. Such combinations were also shown to be more effective than monotherapy in protecting mice against lethal infection by drug-sensitive H5N1 virus and did not result in emergence of resistant viruses [60]. These studies highlight the potential of combination therapy to enhance therapeutic efficacy and circumvent resistance emergence and the need to determine optimum regimens for complementary use of the different anti-influenza drugs.

The small armoury of anti-influenza drugs and the limitations posed by acquisition of drug resistance emphasize the need for additional effective drugs. Arbidol, a drug shown to affect the fusion activity of the HA of some viruses, is widely used against influenza in Russia, although the basis of its clinical efficacy is not clear [61]. Clinical development of other anti-NA drugs, including peramivir and 'long-lasting' zanamivir dimers [62], is being pursued. Although inhibitors of other targets, in particular HA and polymerase, e.g. T-705, have been identified, few are being progressed clinically.

Acknowledgements

We acknowledge the contribution of the members of the WHO Global Influenza Surveillance Network and particularly those who provided unpublished HA and M gene sequence data, some of which is included in figure 3, related to the amantadine susceptibility of recent human influenza H1N1 and H3N2 variants: T. Popow-Kraupp, Vienna, Austria; L. Nielsen, Copenhagen, Denmark; B. Schweiger, Berlin, Germany; F. Ansaldi and P. Crovari, Genoa, Italy; S. Puzelli and I. Donatelli, Rome, Italy; P. D'Agaro and C. Campello, Trieste, Italy; O. Hungnes, Oslo, Norway; H. Rebelo de Andrade, Lisbon, Portugal; T. Besselaar, Johannesburg, South Africa; M. Stivers and M.N. Brytting, Stockholm, Sweden; A. Lackenby, J. Ellis and M. Zambon, London, UK, and M. Bennett and V. Gregory, NIMR.

References

1 Davies WL, Grunert RR, Haff RF, McGahen JW, Neumayer EM, Paulshock M, Watts JC, Wood TR, Hermann EC, Hoffmann CE: Antiviral activity of 1-adamantanamine (amantadine). Science 1964;144: 862–863.

2 Hay AJ: The action of adamantanamines against influenza A viruses: inhibition of the M2 ion channel protein. Semin Virol 1992;3:21–30.

3 Von Itzstein M, Wu WY, Kok GB, Pegg MS, Dyason JC, Jin B, Van Phan T, Smythe ML, White HF, Oliver SW: Rational design of potent sialidase-based inhibitors of influenza virus replication. Nature 1993; 363:418–423.

4 Kim CU, Lew W, William MA, Liu H, Zhang I, Swaminathan S, Bischofberger N, Chen MS, Mendel DB, Tai CY, Laver G, Stevens RC: Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. J Am Chem Soc 1997;119:681–690.

5 Deyde VM, Xu X, Bright RA, Shaw M, Smith CB, Zhang Y, Shu Y, Gubareva LV, Cox NJ, Klimov AI: Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. J Infect Dis 2007;196:249–257.

6 Cheung C-L, Rayner JM, Smith GJD, Wang P, Naipospos TSP, Zhang J, Yuen K-Y, Webster RG, Peiris JSM, Guan Y, Chen H: Distribution of amantadine-resistant H5N1 avian influenza variants in Asia. J Infect Dis 2006;193:1626–1629.

7 Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, Hayden FG, Sugaya N, Kawaoka Y: Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet 2004;364:759–765.

8 De Jong MD, Tran TT, Truong HK, Vo MH, Smith GJD, Nguyen VC, Bach VC, Phan TQ, Do QH, Guan Y, Peiris JSM, Tran TH, Farrar J: Oseltamivir resistance during treatment of influenza A (H5N1) infection. N Engl J Med 2005;353:2667–2672.

9 Dolin R, Riechman RC, Madore HP, Maynard R, Linton PN, Webber-Jones J: A controlled trial of amantadine and rimantadine in the prophylaxis of influenza A infection. N Engl J Med 1982;307: 580–584.

10 Hayden FG, Pavia AT: Antiviral management of seasonal and pandemic influenza. J Infect Dis 2006; 194:119–126.

11 Belshe RB, Smith MH, Hall CB, Betts R, Hay AJ: Genetic basis of resistance to rimantadine emerging during treatment of Influenza virus infection. J Virol 1988;62:1508–1512.

12 Hayden FG, Belshe RB, Clover RD, Hay AJ, Oakes MG, Soo W: Emergence and apparent transmission of rimantadine-resistant influenza A virus in families. N Engl J Med 1989;321:1696–1702.

13 Bean WJ, Threlkeld SC, Webster RG: Biologic potential of amantadine-resistant influenza A virus in an avian model. J Infect Dis 1989;159:1050–1056.

14 Chizhmakov IV, Geraghty FM, Ogden DC, Hayhurst A, Antoniou M, Hay AJ: Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukemia cells. J Physiol 1996;494:329–336.

15 Mould JA, Paterson RG, Takeda M, Ohigashi Y, Venkatarman P, Lamb RA, Pinto LH: Influenza B virus BM2 protein has an ion channel activity that conducts protons across membranes. Dev Cell 2003; 5:175–184.

16 Martin K, Helenius A: Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell 1991;67:117–130.

17 Sugrue RJ, Bahadur G, Zambon MC, Hall-Smith M, Douglas AR, Hay AJ: Specific structural alteration of the influenza haemagglutinin by amantadine. EMBO J 1990;9:3469–3476.

18 Steinhauer DA, Wharton SA, Skehel JJ, Wiley DC, Hay AJ: Amantadine selection of a mutant influenza virus containing an acid-stable hemagglutinin glycoprotein: evidence for virus-specific regulation of the pH of glycoprotein transport vesicles. Proc Natl Acad Sci USA 1991;72:191–194.

19 Hay AJ, Wolstenholme AJ, Skehel JJ, Smith MH: The molecular basis of the specific anti-influenza action of amantadine. EMBO J 1985;4:3021–3024.

20 Duff KC, Gilchrist PJ, Saxena AM, Bradshaw JP: Neutron diffraction reveals the site of amantadine blockade in the influenza A M2 ion channel. Virology 1994;202:287–293.

21 Hu J, Asbury T, Achuthan S, Li C, Bertram R, Quine JR, Fu R, Cross TA: Backbone structure of the amantadine-blocked trans-membrane domain M2 proton channel from Influenza A virus. Biophys J 2007;92:4335–4343.

22 Czabotar PE, Martin SR, Hay AJ: Studies of structural changes in the M2 proton channel of influenza A virus by tryptophan fluorescence. Virus Res 2004; 99:57–61.

23 Okada A, Miura T, Takeuchi H: Protonation of histidine and histidine-tryptophan interaction in the activation of the M2 ion channel from influenza A virus. Biochemistry 2001;40:6053–6060.

24 Astrahan P, Kass I, Cooper MA, Arkin IT: A novel method of resistance for influenza against a channel-blocking antiviral drug. Proteins 2004;55:251–257.
25 Grambas S, Bennett MS, Hay AJ: Influence of amantadine resistance mutations on the pH regulating function of influenza A viruses. Virology 1992;191: 541–549.
26 Sweet C, Hayden FG, Jakeman KJ, Grambas S, Hay AJ: Virulence of rimantadine-resistant human influenza A (H3N2) viruses in ferrets. J Infect Dis 1991;164:969–972.
27 Marozin S, Gregory V, Cameron K, Bennett M, Valette M, Aymard M, Foni E, Barigazzi G, Lin Y, Hay AJ: Antigenic and genetic diversity among swine influenza A H1N1 and H1N2 viruses in Europe. J Gen Virol 2002;83:735–745.
28 Ilyushina NA, Govorkova EA, Webster RG: Detection of amantadine-resistant variants among avian influenza viruses isolated in North America and Asia. Virology 2005;341:102–106.
29 Ziegler T, Hemphill ML, Ziegler ML, Perez-Oronoz G, Klimov AI, Hampson AW, Regnery HL, Cox NJ: Low incidence of rimantadine resistance in field isolates of influenza A viruses. J Infect Dis 1999;180:935–939.
30 Degelau J, Somani SK, Cooper SL, Guay DR, Crossly KB: Amantadine-resistant influenza A in a nursing facility. Arch Intern Med 1992;152:390–392.
31 Bright RA, Medina M-J, Xu X, Perez-Oronoz G, Wallis TR, Davis XM, Povinelli L, Cox NJ, Klimov AI: Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005:a cause for concern. Lancet 2005; 366:1175–1181.
32 Hurt AC, Selleck P, Komadina N, Shaw R, Brown L, Barr IG: Susceptibility of highly pathogenic A (H5N1) avian influenza viruses to the neuraminidase inhibitors and adamantanes. Antiviral Res 2007;73:228–231.
33 Olsen CW: The emergence of novel swine influenza viruses in North America. Virus Res 2002;85:199–210.
34 Govorkova EA, Leneva IA, Goloubeva OG, Bush K, Webster RG: Comparison of the efficacies of RWJ-270201, zanamivir and oseltamivir against H5N1, H9N2 and other avian influenza viruses. Antimicrob Agents Chemother 2001;45:2723–2732.
35 Ward P, Small I, Smith J, Suter P, Dutkowski R: Oseltamivir (Tamiflu) and its potential for use in the event of an influenza pandemic. J Antimicrob Chemother 2005;55(suppl 1):5–21.
36 Babu YS, Chand P, Bantia S, Kotian P, Dehghani A, El-Kattan Y, Lin TH, Hutchison TL, Elliott AJ, Parker CD, Ananth SL, Horn LL, Laver GW, Montgomery JA: BCX-1812 (RWJ-270201): Discovery of a novel, highly potent, orally active, and selective influenza neuraminidase inhibitor through structure-based drug design. J Med Chem 2000;43:3482–3486.
37 Kawai N, Ikematsu H, Iwaki N, Maeda T, Satoh I, Hirotsu N, Kashiwagi S: A comparison of the effectiveness of oseltamivir for the treatment of influenza A and influenza B: a Japanese multicenter study of the 2003–2004 and 2004–2005 influenza seasons. Clin Infect Dis 2006;43:439–444.
38 Gubareva LV, Penn CR, Webster RG: Inhibition of the replication of avian influenza viruses by the neuraminidase inhibitor 4-guanidino-2,4-dideoxy-2,3-dehydro-N-acetylneuraminic acid. Virology 1995; 212:323–330.
39 Govorkova EA, Ilyushina NA, Bolz DA, Douglas A, Yilmaz N, Webster RG: Efficacy of oseltamivir therapy in ferrets inoculated with different clades of H5N1 influenza viruses. Antimicrob Agents Chemother 2007;51:1414–1424.
40 Varghese JN, Laver WG, Colman PM: Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9 Å resolution. Nature 1983;303: 35–40.
41 Colman PM, Varghese JN, Laver WG: Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature 1983;303:41–44.
42 Russell RJ, Haire LF, Stevens DJ, Collins PJ, Lin YP, Blackburn GM, Hay AJ, Gamblin SJ, Skehel JJ: The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. Nature 2006;443:45–49.
43 Varghese JN, Smith PW, Sollis SL, Blick TJ, Sahasrabudhe A, McKimm-Breschkin JL, Colman PM: Drug design against a shifting target: A structural basis for resistance to inhibitors in a variant of influenza virus neuraminidase. Structure 1998;6: 735–746.
44 Hayden FG: Antiviral resistance in influenza viruses-implications for management and pandemic response. N Engl J Med 2006;354:785–788.
45 Blick TJ, Sahasrubudhe A, McDonald M, Owens IJ, Morley PJ, Fenton RJ, McKimm-Breschkin JL: The interaction of neuraminidase and hemagglutinin mutations in influenza virus in resistance to guanidino-Neu5Ac2en. Virology 1998;246:95–103.
46 McKimm-Breschkin JL: Resistance of influenza viruses to neuraminidase inhibitors – a review. Antiviral Res 2000;47:1–17.
47 Blick TJ, Tiong T, Sahasrabudhe A, Varghese JN, Colman PM, Hart GJ, Bethell RC, McKimm-Breschkin JL: Generation and characterization of an influenza virus neuraminidase variant with decreased sensitivity to the neuraminidase-specific inhibitor 4-guanidino-Neu5Ac2en. Virology 1995;214: 475–484.
48 Wang MZ, Tai CY, Mendel DB: Mechanism by which mutations at His274 alter sensitivity of influenza A virus N1 neuraminidase to oseltamivir carboxylate and zanamivir. Antimicrob Agents Chemother 2002;46:3809–3816.

49 Rameix-Welti MA, Agou F, Buchy P, Mardy S, Aubin JT, Veron M, van der Werf S, Naffakh N: Natural variation can significantly alter the sensitivity of influenza A (H5N1) viruses to oseltamivir. Antimicrob Agents Chemother 2006;50:3809–3815.
50 Hatakeyama S, Sugaya N, Ito M, Yamazaki M, Ichikawa M, Kimura K, Kiso M, Shimizu H, Kawakami C, Koike K, Mitamura K, Kawaoka Y: Emergence of influenza B viruses with reduced sensitivity to neuraminidase inhibitors. JAMA 2007;297: 1435–1442.
51 Dr. Maria Zambon, Centre for Infection, HPA, Colindale, London, UK. Personal Communication.
52 Normile D, Enserink M: Avian influenza. With change in the seasons, bird flu returns. Science 2007; 315:448.
53 Gubareva LV, Matrosovich MN, Brenner MK, Bethell RC, Webster RG: Evidence for zanamivir resistance in an immunocompromised child infected with influenza B virus. J Infect Dis 1998;178:1257–1262.
54 Gubareva LV: Molecular mechanisms of influenza virus resistance to neuraminidase inhibitors. Virus Res 2004;103:199–203.
55 Herlocher ML, Carr J, Ives J, Elias S, Truscon R, Roberts N, Monto AS: Influenza virus carrying an R292K mutation in the neuraminidase gene is not transmitted in ferrets. Antiviral Res 2002;54:99–111.
56 Yen HL, Herlocher LM, Hoffmann E, Matrosovich MN, Monto AS, Webster RG, Govorkova EA: Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. Antimicrob Agents Chemother 2005;49:4075–4084.
57 Herlocher ML, Truscon R, Elias S, Yen H-L, Roberts NA, Ohmit SE, Monto AS: Influenza viruses resistant to the antiviral drug oseltamivir: transmission studies in ferrets. J Infect Dis 2004;190:1627–1630.
58 Yen H-L, Ilyushina NA, Solomon R, Hoffman E, Webser RG, Govorkova EA: Neuramindase inhibitor-resistant recombinant A/Vietnam/1203/04 (H5N1) influenza viruses retain their replication efficiency and pathogenicity in vitro and in vivo. J Virol 2007; 81:12418–12426.
59 Govorkova EA, Fang H-B, Tan M, Webster RG: Neuraminidase inhibitor-rimantadine combinations exert additive and synergistic anti-influenza virus effects in MDCK cells. Antimicrob Agents Chemother 2004;48:4855–4863.
60 Ilyushina NA, Hoffman E, Salomon R, Webster RG, Govorkva EA: Amantadine-oseltamivir combination therapy for H5N1 influenza virus infection in mice. Antiviral Ther 2007;12:363–370.
61 Uchaikin V, Kharlamova S, Cheshik S: Arbidol and amixin application as an etiotropic influenza and common cold therapy in children. Pediatrics 2004;5: 73–77.
62 Macdonald SJF, Watson KG, Cameron R, Chalmers DK, Demaine DA, Fenton RJ, Gower D, Hamblin JN, Hamilton S, Hart GJ, Inglis GGA, Jin B, Jones HT, McConnell DB, Mason AM, Nguyen V, Owens IJ, Parry N, Reece PA, Shanahan SE, Smith D, Wu W-Y, Tucker SP: Potent and long-acting dimeric inhibitors of influenza virus neuraminidase are effective at a once a week dosing regimen. Antimicrob Agents Chemother 2004;48:4542–4549.
63 Lackenby A, Hungnes O, Dudman SG, Meijer A, Paget WJ, Hay AJ, Zambon MC: Emergence of resistance to oseltamivir among influenza A(H1N1) viruses in Europe. Euro Surveill 2008;13:pii–8026.

Alan J. Hay
Virology Division, National Institute for Medical Research
The Ridgeway, Mill Hill, London NW7 1AA (UK)
Tel. +44 20 8816 2141, Fax +44 20 8906 4477, E-Mail ahay@nimr.mrc.ac.uk

Klenk H-D, Matrosovich MN, Stech J (eds): Avian Influenza.
Monogr Virol. Basel, Karger, 2008, vol 27, pp 272–286

The 1918 Influenza Pandemic: Lessons from the Past Raise Questions for the Future

John Steel · Peter Palese

Department of Microbiology, Mount Sinai School of Medicine, New York, N.Y., USA

Abstract

The 'Spanish' influenza pandemic of 1918–1919 was the most severe in recorded history, affecting approximately 25% of the world's population and killing in the order of 50 million people. Subsequent influenza pandemics of the 20th century have been less severe. An understanding of the mechanisms underlying the severity of the 1918 pandemic could potentially help to reduce the extent of future pandemics. To this end, the entire 1918 virus and viruses bearing combinations of 1918 genes have been reconstructed through reverse genetics techniques. The availability of a viable 1918 strain has enabled researchers to investigate the viral and host factors underlying the extreme pathogenicity of the 1918 virus. These studies and others have revealed many features regarding the unusual epidemiology, pathogenicity, replication and transmission of the 1918 virus, and aid us in predicting the severity of future pandemic outbreaks.

Introduction

During interpandemic periods, seasonal peaks of morbidity and mortality in humans are attributed to annual outbreaks of influenza A virus. Pandemics occur much less frequently and are associated with the introduction of an antigenically novel subtype of influenza virus into the human population. In 1918–19, the most deadly influenza pandemic in recorded history spread globally, killing an estimated 50 million people (2.5% mortality), a significant proportion of which were otherwise healthy young adults [1–4]. Novel influenza A subtypes were responsible for two subsequent pandemics in 1957 and 1968 causing an estimated 1 million (overall mortality 0.02%) and 0.5 million deaths (overall mortality 0.01%), respectively [5].

Insight into the viruses responsible for these pandemic events would allow us to anticipate the emergence of pandemic strains and limit the extent of future pandemics or abrogate them altogether. Until recently, examination of the virus which caused the

1918 pandemic was hampered by the absence of an isolate from that period. In addition, due to genetic drift, descendants of the 1918 virus do not possess the extreme virulence exhibited by the original strain, limiting their value for research into the pathogenesis of this virus. These limitations were overcome when an isolate derived from the pandemic of 1918 was recreated using reverse genetics techniques [6]. The viral gene sequences were elucidated from fragments of RNA found in preserved lung tissue which belonged to victims of the 1918 influenza [7]. Comparison of the 1918 influenza A virus with other pandemic viruses can shed light on the virological determinants of pandemic potential. Conversely, study of the differences between the 1918 and other pandemic viruses may explain the extreme severity of the 1918 virus and possibly enables us to predict the magnitude of future pandemics.

Origins of Pandemic Viruses

Reassortment

The main reservoir of influenza A virus subtypes is wild aquatic avian species [8]. Typically, avian viruses do not efficiently infect human hosts, and are obligated to adapt in order to spread within the human population.

Genetic reassortment is one mechanism by which antigenically novel influenza viruses acquire the ability to circulate within the human population. The influenza strains responsible for the 1957 and 1968 pandemics arose by reassortment [9, 10], whereby previously circulating human viruses acquired novel gene segments encoding surface (antigenic) and internal protein products from an avian virus. Antigenic novelty permitted the resulting viruses to evade immune recognition in human hosts. In combination with further adaptations which allowed efficient spread in humans, the lack of immune recognition of these pandemic viruses is believed to have contributed significantly to their increased virulence [11]. Human influenza viruses are known to undergo selection pressure from immune surveillance and they are therefore required to evolve in order to persist in the human host [12, 13].

Phylogenetic analysis of the 1918 virus has been interpreted as showing that most or all of the gene segments are of avian origin [7, 14]. However, the phylogenetic data currently available appears not sufficiently robust to determine precisely how the 1918 virus arose [15, 16]; a definitive answer to the question of whether the virus was derived directly from a wholly avian source, or was the product of a reassortment event, requires sequencing of human influenza viruses predating 1918.

The Intermediate Mixing Vessel

In order for a reassortment event to occur, viruses of avian and human origin must co-infect a permissive host. Such reassortment has been observed in pigs in a laboratory

setting, and it has therefore been proposed that these animals act as intermediate hosts for the adaptation of viruses of avian origin to humans [17, 18, also see chapter by Scholtissek]. It should be noted that, while there are numerous incidences of human H1 and H3 influenza viruses adapting to swine [10], only sporadic examples of swine influenza virus isolation from humans exist [19, 20]. In this light, the likelihood that swine act as intermediate hosts may be called into question. An alternative hypothesis argues that an unknown avian intermediate host exists, permitting reassortment and subsequent adaptation of avian viruses to the human host. In support of this hypothesis, it has been demonstrated that quail can sustain the growth of avian H9N2 viruses with α-2,6 receptor-binding specificity (human receptor specificity) and furthermore, that the gastrointestinal tract of this species contains receptors which allow the binding of both avian and human-adapted viruses [21, 22]. Alternatively, there may be no intermediate host, but rather that reassortment or adaptation occurs directly within humans.

Defining the Minimal Adaptive Changes

Defining the minimal adaptive changes necessary for viral adaptation to the human host is a key component in understanding how pandemic strains emerge. In addition to the ability to evade neutralizing antibody-mediated immunity through the acquisition of a novel subtype of HA, it is almost certain that novel viruses require further changes in order to burgeon into pandemic strains. These changes are most likely polygenic in nature [11] and include adaptations to the host which improve transmissibility, increase replicative efficiency and optimize tissue tropism.

A critical step in host adaptation is alteration of the receptor-binding specificity from that of avian viruses to that of human viruses [see chapter by Matrosovich et al.]. The binding of influenza virus to the sialic acid receptors on host cells is mediated directly by a subset of amino acid residues present in the HA protein. The identities of these amino acids are conserved among avian isolates, and correlate with the binding of avian viruses to α-2,3-linked sialic acid [23, 24]. In mammalian viruses, these amino acids differ, conferring affinity for α-2,6-linked sialic acid receptors.

The adaptations that allowed the 1918 virus to flourish have not been fully characterized to date [6], but the recognition of human-type receptors by the HA has been shown to be important [25, 26]. Elucidation of the crystal structure of the 1918 HA [27, 28] has allowed a comparison with the structures of avian H5 HA and human H3 HA proteins. In terms of the overall structure of the receptor-binding pocket, the 1918 HA appears similar to the avian HA. However, comparison of the coding sequences of the HA gene from five independent strains of 1918 virus has demonstrated that, while they share greater than 99% sequence identity, they differ at residue 225 [29]. Three strains share a Gly to Asp change at this position, which

Table 1. Critical amino acids for the receptor-binding specificity of the influenza HA[1]

Viral HA	Amino acid position (H3 numbering)						α-2,3 binding	α-2,6 binding
	77	138	86	190	194	225		
A/South Carolina/1/18	D	A	P	D	L	D	–	+++
A/New York/1/18	D	A	P	D	L	**G**	++	++
Avian H1 HA consensus	D	A	P	**E**	L	**G**	+++	–

[1]The avian consensus sequence of the H1 HA was determined by comparing human and avian HA sequences [24, 25]. The above amino acids are conserved in most avian H1 HA sequences. Boldface type indicates a change from the A/South Carolina/1/18 HA sequence.

diverges from the avian consensus. Furthermore, the five sequences demonstrate a consistent divergence from the avian consensus at amino acid 190, adopting the Glu to Asp change which is observed in H1 subtype viruses that have adapted to swine (table 1).

The contribution of the variant amino acids at positions 190 and 225 to human type receptor-binding specificity has been investigated [25, 26]. It was demonstrated that the A/South Carolina/1/18 strain, which encodes Asp190 and Asp225, possesses preferential α-2,6-linked receptor specificity. The A/New York/1/18 strain, which encodes Asp190 and Gly225, binds to both α-2,3- and α-2,6-linked sialic acids. Thus, two virulent strains of virus were circulating in 1918 which differed in their receptor-binding specificity. Interestingly, reversion of Asp190 to the avian consensus, Glu190, is sufficient to convert the mixed binding specificity of the A/New York/1/18 strain hemagglutinin to exclusive α-2,3-linked binding (table 1) [25]. Therefore, the minimal change required to alter receptor specificity of the 1918 virus from human- to avian-type comprises one amino acid (Asp190 to Glu190) [25, 26].

Relevance of Receptor-Binding Preference to Transmission

The transmissibility of recombinant 1918 viruses encoding the variant HA proteins was subsequently tested in a ferret model [30]. Introduction of the two avian consensus amino acids which abolish α-2,6 binding in the A/South Carolina/1/18 strain abrogated transmission in ferrets. Notably, this virus retained virulence and high replication rates in the upper respiratory tract of these animals. Similarly, relatively low transmission efficiency of the virus which binds both α-2,3- and α-2,6-linked sialic acid suggests that a predominant α-2,6-binding configuration is required for optimal transmission of the 1918 virus [30].

Unusual Aspects of the 1918 Virus: Epidemiology and Pathogenesis

The 1918 pandemic was exceptional in many aspects, including the rate of spread, compression of three waves of disease in a short span of time, distribution of disease burden, and pathological features of infection. The causative strain arose between March and April of 1918 and proceeded to spread with chilling efficiency across highly populated regions of the USA, Europe, and Asia [31] and sparsely populated areas such as Alaska and the Pacific Islands. Overall, up to 500 million people across the globe (25% of the world population) are thought to have been clinically infected with influenza virus [2, 3].

The Three Waves of the Pandemic

The rapid progression of the 1918 pandemic is one of its alarming features [32]. Following an initial wave in the spring of 1918, during which the virus exhibited suboptimal virulence, the main wave of the pandemic struck in the fall of 1918, wherein the virus demonstrated its full capacity for virulence. A final wave of influenza with variable severity was experienced in many places in early 1919 [32]. Despite analysis of the complete genome sequence of the 1918 virus, no obvious features explain the unusual epidemiology. However, as each of the elucidated sequences is derived from viruses which circulated during the second wave of the pandemic, a comparison with sequences from the less virulent first wave may shed more light on the molecular determinants of pathogenicity. One possible explanation for the three closely spaced waves is that the surface proteins of the virus drifted more rapidly than is normally seen with other strains of epidemic and pandemic influenza viruses. Alternatively, the pattern of infection may be attributable to undetermined features of the virus which rendered it unusually effective at escaping the host immune response.

Burden of Disease

The 1918 virus was associated with high mortality rates in the very young ($<$1 year of age), and notably in healthy young adults (ages 15–35) who accounted for approximately half of all deaths, and more than 99% of the excess deaths [33, 34]. This high death rate among adults is one of the pandemic's most conspicuous features. Mortality rates were lower among people aged 35–65 years, but higher again in people $>$65 years. The atypical, W-shaped, mortality curve is unique to the 1918 virus, and contrasts with an illustrative U-shaped curve for mortality observed in 1915 and other years (fig. 1). In this more traditional curve, there are few fatalities

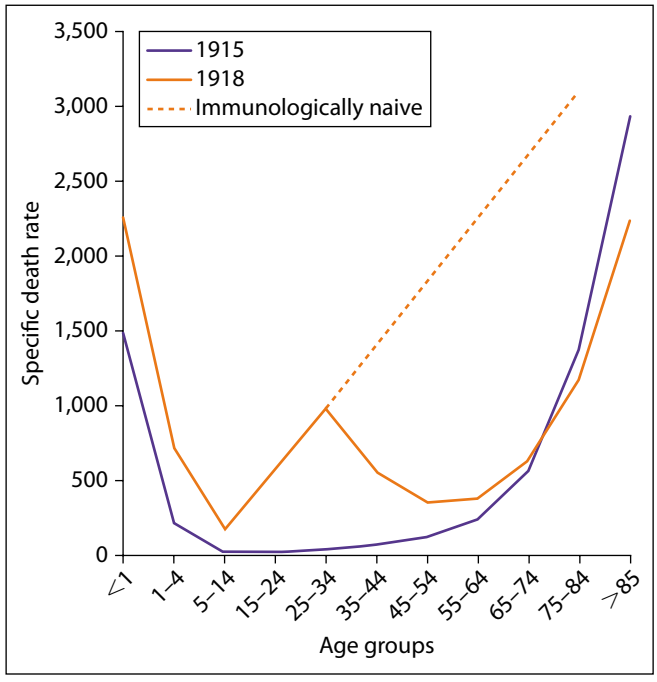


Fig. 1. Reported influenza/pneumonia deaths from 1915 and 1918, expressed as a function of age. A typical U-shaped curve is observed for 1915, whereas the striking increase in death rates among persons aged 15–44 results in a W-shaped curve for 1918. It is hypothesized that a V-shaped curve would have resulted had the population in 1918 been immunologically naive (dotted line). Specific death rate is per 100,000 persons in each indicated age group [5, 35, 51].

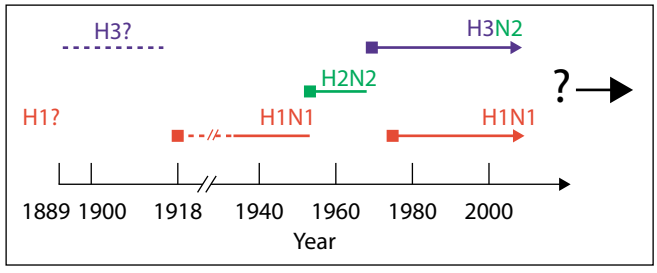


Fig. 2. History of influenza A virus subtypes known to have circulated in the human population. Currently H1N1 (hemagglutinin, subtype 1; neuraminidase, subtype 1) and H3N2 subtypes co-circulate, whereas from 1968 to 1977 only the latter were in circulation. From 1957 to 1968 the only circulating subtype was H2N2. Between 1918 and 1957, H1N1 strains were observed. The broken line indicates that no isolates are available. H1 subtype strains are postulated to have circulated until 1889 [35] and indirect evidence [5, 52] suggests an introduction of H3 strains thereafter.

in the age group 5–50, consistent with a population with previous antigenic exposure. The W-shape mortality curve of the 1918 pandemic may be attributed to the virulence of the virus combined with the immunologically naive state of the population [35]. This hypothesis suggests that there was an H1-like virus in circulation before 1889 (fig. 2), and that partial cross protection in the age group born before 1889 actually lowered the case fatality rate, preventing the 1918 pandemic from being even more severe. Such a V-shaped mortality curve (fig. 1) is in fact common to most lytic viral infections in immunologically naive humans when plotted against age groups [5]. For example, the case fatality rate of epidemic measles on the Faroe Islands in 1846 follows a V-like shape with a trough in the 10–19 age group [36].

One piece of circumstantial evidence gives weight to the hypothesis that H1 viruses circulated before 1889, providing partial protection to the older population in 1918. People younger than 25 years of age were most affected by the outbreak of an H1N1 influenza virus in 1977 which was genetically almost identical to strains circulating in 1950 [37]. The circulating H1N1 viruses were replaced in 1957 by an H2N2 virus subtype. The protection of older members of the population in 1977 suggests that there is long-lasting subtype specific immunity in individuals who have been infected by a virus many years earlier.

Severity of Illness

The 1918 pandemic also surpassed subsequent pandemics of the 20th century in terms of severity of disease, with a mortality rate of approximately 2%, in contrast to mortality rates of less than 0.1% during the 1957 and 1968 pandemics. During interpandemic periods, individuals at the extremes of age or with underlying chronic disease typically suffer from complications of influenza virus infection. These include lower respiratory tract viral infection, secondary bacterial infection, central nervous system complications, and in the case of H5N1 infection, multiorgan failure. Mortality is generally higher in such cases. In contrast, otherwise healthy individuals experience a self-limiting febrile illness [38].

Once again, the 1918 influenza was exceptional. Although many victims of the 1918 influenza died of pneumonia associated with secondary bacterial infections [39], a significant proportion of deaths resulted from acute pulmonary edema or massive pulmonary hemorrhage, which progressed rapidly after the onset of symptoms [40]. Histopathology of lung tissue derived from individuals who succumbed to the 1918 virus demonstrated acute bronchiolitis, alveolitis and bronchopneumonia [34]. Autopsy series performed in 1918 repeatedly revealed pathology confined to the respiratory tract, with death due to respiratory failure [40], consistent with infection of the lung by a well-adapted, highly replicating influenza virus.

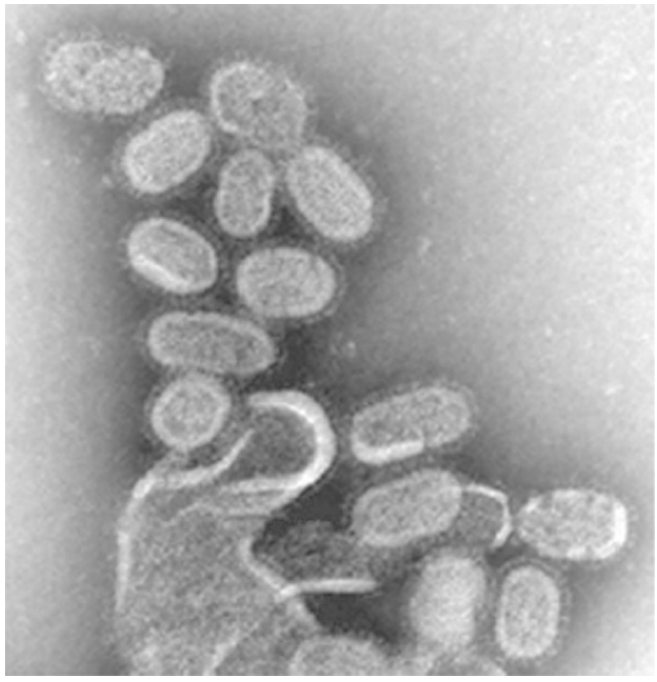


Fig. 3. Transmission electron micrograph of the reconstructed influenza A/South Carolina/1/18 virus. Visualization of the ultrastructural features of the 1918 influenza virion indicates that roughly spherical, rather than filamentous, particles predominate. Surface projections visible on the virions are composed of the hemagglutinin and neuraminidase glycoproteins. A dense envelope is prominent, composed of a cell derived lipid membrane surrounding a layer of virus encoded matrix protein. Within this envelope lies the encapsidated RNA genome of each virion (image provided courtesy of T. Tumpey and C. Goldsmith, CDC, Atlanta, Ga.).

Virulence of the 1918 Influenza Virus in Model Systems

In order to study the properties associated with the 1918 virus, reverse genetics has been employed to generate a virus bearing all eight segments of the A/South Carolina/1/18 strain of 1918 virus (fig. 3), as well as recombinant viruses bearing selected 1918 genes in a background of a contemporary H1 influenza virus (A/Texas/36/91) [6]. Using several animal model and cell culture systems, it has been possible to demonstrate the exceptional virulence and growth of the A/South Carolina/1/18 virus (table 2).

In human bronchial epithelial cells, the virus grew to high titers, even in the absence of exogenous trypsin. This phenomenon has been associated with increased tissue tropism of other H1 viruses [41], and may be related to the high pathogenicity of the 1918 virus. In contrast to contemporary mammalian H1 viruses, the 1918 virus is able to kill embryonated chicken eggs. Lethality in eggs is typically a specific attribute of avian H1 viruses, however the 1918 virus was lethal at a low infectious

Table 2. Virulence characteristics of the 1918 virus in various animal and cell culture systems

	Virus			
	1918	1918 5: Tx 3	1918 2: Tx 6	A/Texas/36/91
				
Percent weight loss of mice[1,2]	22	14.5	15.9	0.7
Lung titers in mice[2,3]	8.0	6.0	ND	3.0
Egg LD50[4], $\log_{10}$ PFU/ml	1.5	>7	>7	>7
Mouse LD50[5], $\log_{10}$ PFU	3.3	5.5	4.75	>6
Titer in Calu-3 cells[6], $\log_{10}$ EID/ml	8.7	6.5	ND	6.8

ND = Not determined.

[1]Percent weight loss of Balb/c mice on day 4 post-infection (n = 13) [6].

[2]Mice were intranasally infected with 10^6 PFU of the appropriate virus [6].

[3]Viral titers in lung homogenates of Balb/c mice on day 4 post-infection (n = 4) [6].

[4]Embryo viability was determined by daily candling [6]. ELD_{50} titer was calculated by the method of Reed and Muench [53].

[5]Mice were inoculated with 10-fold serial dilutions of virus. Mice were monitored daily for weight loss. Animals losing more than 25% of their body weight were euthanized. MLD_{50} titer was calculated by the method of Reed and Muench [53].

[6]Titer of virus released from apically infected human bronchial epithelial cells 24 h after infection with an MOI of 0.01 [6].

dose, illustrating the exceptional virulence of the 1918 virus. Recombinant viruses composed of selected 1918 gene segments in an A/Texas/36/91 background demonstrated that the 1918 polymerase complex and HA genes were associated with the virulence observed in chicken eggs [6].

The virus also proved extremely pathogenic in mice, killing animals in as little as 3 days, and generating up to 13% loss of body weight in only 2 days. Substitution of the HA gene of the 1918 virus for that of A/Texas/36/91 resulted in a virus which was no longer lethal in mice, and a reduction in lung virus titer of around 100-fold was observed, although this virus still replicated to significantly higher levels than the A/Texas/36/91 control virus. Similarly, an approximately 100-fold level of attenuation in growth and reduction of lethality was observed with a recombinant virus which substituted the A/Texas/36/91 polymerase complex for that of the 1918 virus [6]. Thus, in mice, as in embryonated chicken eggs, the HA gene and polymerase complex function as determinants of pathogenicity.

The high virulence of the HA gene segment from 1918 has been further confirmed by studies in which infection of mice with viruses containing the HA and NA genes of 1918 virus in a background of A/WSN/33 genes [42] or the 1918 HA gene alone in a background of contemporary human viruses (A/Kawasaki/173/01, A/Memphis/8/88) [43] have been uniformly fatal, while substitution of the HA and NA genes with those of contemporary viruses in the same backgrounds demonstrated no pathogenicity in mice.

Histological examination of mouse lungs infected with the 1918 influenza virus revealed necrotizing bronchitis and bronchiolitis, and moderate to severe alveolitis with accompanying peribronchial and alveolar edema. This is similar to what was reported in human patients during the 1918 pandemic. Interestingly, as is presumed to be the case with human victims of the 1918 virus, there was no evidence of viral replication outside of the lungs of mice. The molecular basis of this restricted tissue tropism remains unresolved. There was a predominant infiltration of neutrophils and macrophages into the lungs of mice, and alveolitis was associated with neutrophils. These findings and an increase in expression of cytokines and chemokines was observed in mice infected with viruses containing the 1918 HA and NA genes [44]. Depletion of either neutrophils or especially macrophages, resulted in a reduced increase in cytokine and chemokine production. However, it was also shown that depletion of these cells lead to uncontrolled growth and virulence of the pathogenic virus bearing the 1918 HA and NA. Thus, although the immune cells were involved in producing a pathological immune response, they were also necessary for clearance of the virus.

Experimental inoculation of cynomolgus macaques with the 1918 virus has also recently been carried out [45]. As with previous animal models, the virus demonstrated exceptional virulence in macaques, with animals developing symptoms 24 h post-infection, and all individuals requiring euthanasia by day 8 due to severity of disease. Symptoms included depression, anorexia, and respiratory dysfunction which progressed to acute respiratory distress syndrome. Infection was characterized by the presence of high titers of virus in both upper and lower respiratory tissues, which did not clear during the course of infection, in contrast to animals infected with a contemporary H1 influenza virus (A/Kawasaki/173/01). Thus, with respect to gross pathology, the infection of macaques represents a faithful model of the infection observed in humans in 1918. Interestingly, although the lung was the only organ to exhibit gross pathologic lesions, virus was isolated from the heart and spleen of 1918 virus-infected animals.

Contribution of NS1 to Pathogenicity

The pathogenesis of 1918 influenza virus observed in macaques was accompanied by upregulation of host proinflammatory cytokines and chemokines, notably IL-6 and CCL-11 [45]. Similarly to what was seen in the mouse model [44], the chemokines CXCL6 and CXCL-1, which are important in neutrophil activation and recruitment,

were also upregulated. In contrast, there was a marked downregulation of IFN-α genes and interferon-stimulated genes, suggesting the induction of an altered antiviral response in the lungs of macaques. This downregulation of the antiviral response is similar to that seen during infection of human epithelial cells with virus which contains the 1918 NS1 [46]. There was a notable absence in the induction of RIG-I and MDA-5 proteins during infection with 1918 virus, which was not seen during A/Kawasaki/173/01 virus infection, suggesting a pivotal role for the NS1 protein in the immunomodulatory activity of the 1918 virus. NS1 is a known immunomodulator, and RIG-I is a target of its activity [47].

Experiments with influenza viruses lacking the NS1 gene have shown that the NS1 protein has interferon antagonist activity [48]. Assessment of the contribution of the NS1 protein to the fitness of the 1918 virus suggests that NS1 operates in a species specific manner. In recombinant viruses where the 1918 NS1 replaced the mouse-adapted A/WSN/33 NS1, the resultant virus was attenuated in mice [49]. However, this same virus demonstrated the ability to block the interferon response in infected human epithelial lung cells [46]. These data are consistent with the hypothesis that the actions of the NS1 protein are host-dependent. It is possible that the NS1 proteins of different strains determine differential virulence in different species.

In summary, the above studies have suggested roles for several genes in the pathogenesis of the 1918 virus. The coordinated expression of the polymerase genes, which appear to confer a high replicative capacity on the virus in many host systems, the HA gene, which is associated with an uncontrolled and extreme inflammatory response, and the NS1 gene, which demonstrates the ability to markedly downregulate the interferon response in both human and simian model systems, may contribute significantly to the pathogenesis of the virus. In combination with the ability of the virus to spread efficiently between human hosts, presumably due at least in part to the receptor-binding specificity of the virus, these pathogenicity determinants have contributed to produce the most virulent human influenza virus in recorded history.

Prophylaxis and Treatment of 1918 Infection

It is possible that a 1918-like pandemic virus may re-emerge, either naturally or through ill intent, thus it is prudent to consider antiviral therapies which could combat the outbreak, particularly during the period prior to vaccine availability for mass distribution. In this light, it has been shown that replication of a recombinant virus containing the 1918 M segment was blocked in cell culture by the currently available M2 ion channel inhibitors, amantadine and rimantadine, similarly, replication of the virus was shown to be inhibited in mice by rimantadine [42]. In addition, the growth of recombinant viruses containing the 1918 NA or both the 1918 HA and NA segments was effectively inhibited in cell culture by the neuraminidase inhibitors oseltamivir and zanamivir, and in mice by oseltamivir [42] (fig. 4).

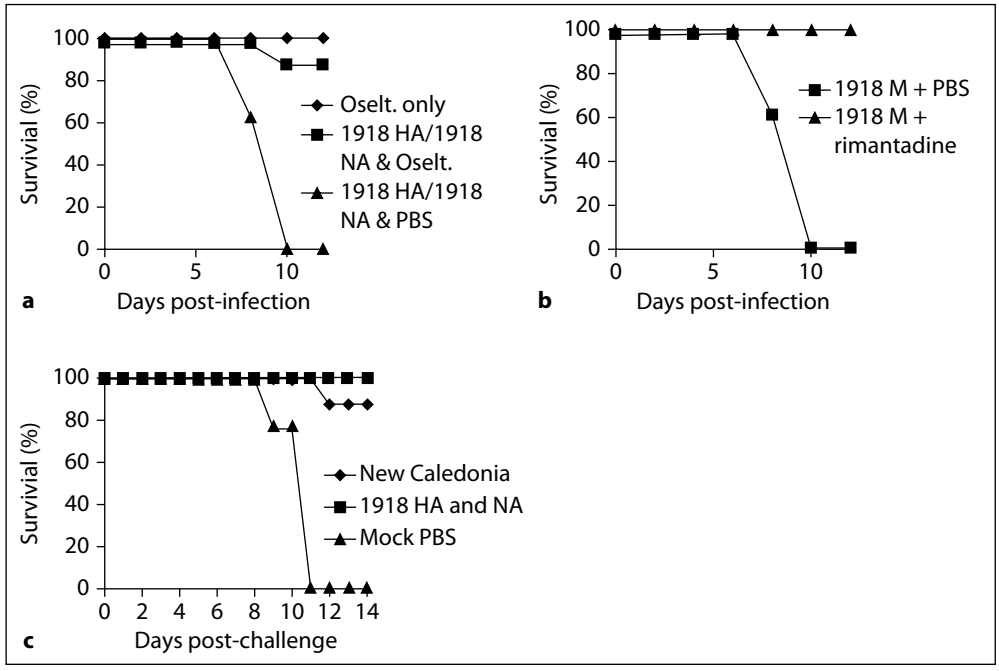


Fig. 4. Effectiveness of currently approved antiviral drugs against viruses carrying 1918 genes. **a** Oral administration of oseltamivir protects mice from death due to infection with influenza virus containing the 1918 HA and NA genes. The percentage of mice surviving intranasal infection is shown as a function of time post-infection. **b** Rimantadine protects mice from infection with a lethal dose of an influenza virus containing the 1918 virus M segment. Survival of mice infected with the WSN: 1918 M virus is compared for mock treatment with PBS or treatment with rimantadine [42]. **c** The protective efficacy of H1N1-inactivated virus vaccine in mice against lethal challenge with an 1918 HA/NA:WSN recombinant influenza virus. Groups of Balb/C mice received a single i.m. injection of H1N1 vaccine or PBS mock vaccine. 22 days post-vaccination, mice were challenged intranasally with 100 LD_{50} of the 1918 HA/NA:WSN recombinant virus. Mice were monitored daily for survival [50].

In order to control an outbreak of 1918-like influenza in the longer term, a prophylactic vaccine would be desired. Immunization of mice with an inactivated vaccine containing a contemporary H1N1 HA led to partial protection from death upon challenge with a virus containing the 1918 HA and NA genes (fig. 4) [50]. This indicates that, in mice, partial immune protection can be achieved by vaccination with homologous subtype virus. It was also observed that full protection of mice could be attained by immunization with an inactivated vaccine containing the 1918 HA and NA proteins. Thus, antivirals, currently available inactivated vaccines, and reverse genetics-based inactivated vaccines appear to be effective against the 1918 strain. Together, these data predict that an effective antiviral strategy could rapidly be developed to control a re-emergent 1918 virus in the human population.

Conclusion

Until recently, the extreme virulence of the 1918 pandemic influenza virus was a matter of historical debate. Through the successful reconstruction of the 1918 virus, an understanding of the virulence of the virus is being gained. It has been demonstrated that the coordinated expression of the 1918 gene constellation results in uniquely high virulence of this virus in several animal models. The characterization of the 1918 virus may aid in predicting the timing and scale of future pandemics. Namely, information obtained through the study of the 1918 virus will allow estimation of the threat posed by new influenza strains, assessment of the prophylactic and therapeutic measures necessary to control an anticipated outbreak, and quantification of the magnitude of public health needs. Further questions remain to be answered, such as from where did the virus originate? It may be necessary to sequence further strains of influenza originating before 1918, should they be available, in order to obtain a definitive answer. We are also left to consider what caused the virus to produce three such rapid waves of disease and what was the basis of the exceptional transmissibility of the virus? Continuing research efforts into this most deadly of influenza viruses are required to address these issues.

Acknowledgements

We thank Samira Mubareka for critical reading of the manuscript. Work done in the laboratory of the author (P.P.) was partially supported by NIH grant PO1AI058113, the Northeast Biodefense Center (U54 AI057158), the Centre for Investigating Viral Immunity and Antagonism (CIVIA) (U19 AI62623), and grants R01AI8998 and P01AI52106. P.P. is a senior scholar of the Ellison Medical Foundation.

References

1 Palese P, Shaw ML: Orthomyxoviridae: the viruses and their replication; in Knipe DM, Howley PM (eds): Fields Virology. Philadelphia, Lippincott Williams & Wilkins, 2006, pp 1648–1689.
2 Frost W: Statistics of influenza morbidity. Public Health Rep 1920;35:584–597.
3 Burnet C, Clark E: Influenza: A Survey of the Last 50 Years in the Light of Modern Work on the Virus of Epidemic Influenza. Melbourne, MacMillan, 1942.
4 Johnson NP, Mueller J: Updating the accounts: global mortality of the 1918–1920 'Spanish' influenza pandemic. Bull Hist Med 2002;76:105–115.
5 Palese P, Tumpey TM, Garcia-Sastre A: What can we learn from reconstructing the extinct 1918 pandemic influenza virus? Immunity 2006;24:121–124.
6 Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solorzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, Garcia-Sastre A: Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 2005;310:77–80.
7 Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG: Characterization of the 1918 influenza virus polymerase genes. Nature 2005;437:889–893.
8 Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y: Evolution and ecology of influenza A viruses. Microbiol Rev 1992;56:152–179.
9 Kawaoka Y, Krauss S, Webster RG: Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol 1989;63:4603–4608.

10 Wright PF, Neumann G, Kawaoka Y: Orthomyxoviruses; in Knipe DM, Howley PM (eds): Fields Virology. Philadelphia, Lippincott Williams & Wilkins, 2006, pp 1692–1739.
11 Kilbourne ED: Influenza pandemics in perspective. JAMA 1977;237:1225–1228.
12 Buonagurio DA, Nakada S, Parvin JD, Krystal M, Palese P, Fitch WM: Evolution of human influenza A viruses over 50 years: rapid, uniform rate of change in NS gene. Science 1986;232:980–982.
13 Fitch WM, Leiter JM, Li XQ, Palese P: Positive darwinian evolution in human influenza A viruses. Proc Natl Acad Sci USA 1991;88:4270–4274.
14 Fanning TG, Slemons RD, Reid AH, Janczewski TA, Dean J, Taubenberger JK: 1917 avian influenza virus sequences suggest that the 1918 pandemic virus did not acquire its hemagglutinin directly from birds. J Virol 2002;76:7860–7862.
15 Gibbs MJ, Gibbs AJ: Molecular virology: was the 1918 pandemic caused by a bird flu? Nature 2006;440: E8–E10.
16 Antonovics J, Hood ME, Baker CH: Molecular virology: was the 1918 flu avian in origin? Nature 2006; 440:E9–E10.
17 Scholtissek C, Hinshaw VS, Olsen CW: Influenza in pigs and their role as the intermediate host; in Nicholson KG, Webster RG, Hay AJ (eds): Textbook of Influenza. Oxford, Blackwell Sciences, 1998, pp 137–145.
18 Scholtissek C: Source for influenza pandemics. Eur J Epidemiol 1994;10:455–458.
19 Dacso CC, Couch RB, Six HR, Young JF, Quarles JM, Kasel JA: Sporadic occurrence of zoonotic swine influenza virus infections. J Clin Microbiol 1984;20:833–835.
20 Rota PA, Rocha EP, Harmon MW, Hinshaw VS, Sheerar MG, Kawaoka Y, Cox NJ, Smith TF: Laboratory characterization of a swine influenza virus isolated from a fatal case of human influenza. J Clin Microbiol 1989;27:1413–1416.
21 Wan H, Perez DR: Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology 2006;346:278–286.
22 Wan H, Perez DR: Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. J Virol 2007;81:5181–5191.
23 Gambaryan AS, Tuzikov AB, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Bovin NV, Matrosovich MN: Specification of receptor-binding phenotypes of influenza virus isolates from different hosts using synthetic sialylglycopolymers: non-egg-adapted human H1 and H3 influenza A and influenza B viruses share a common high binding affinity for 6′-sialyl(*N*-acetyllactosamine). Virology 1997;232:345–350.
24 Matrosovich MN, Gambaryan AS, Teneberg S, Piskarev VE, Yamnikova SS, Lvov DK, Robertson JS, Karlsson KA: Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. Virology 1997;233:224–234.
25 Glaser L, Stevens J, Zamarin D, Wilson IA, Garcia-Sastre A, Tumpey TM, Basler CF, Taubenberger JK, Palese P: A single amino acid substitution in 1918 influenza virus hemagglutinin changes receptor binding specificity. J Virol 2005;79:11533–11536.
26 Stevens J, Blixt O, Glaser L, Taubenberger JK, Palese P, Paulson JC, Wilson IA: Glycan microarray analysis of the hemagglutinins from modern and pandemic influenza viruses reveals different receptor specificities. J Mol Biol 2006;355:1143–1155.
27 Stevens J, Corper AL, Basler CF, Taubenberger JK, Palese P, Wilson IA: Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. Science 2004;303:1866–1870.
28 Gamblin SJ, Haire LF, Russell RJ, Stevens DJ, Xiao B, Ha Y, Vasisht N, Steinhauer DA, Daniels RS, Elliot A, Wiley DC, Skehel JJ: The structure and receptor binding properties of the 1918 influenza hemagglutinin. Science 2004;303:1838–1842.
29 Reid AH, Janczewski TA, Lourens RM, Elliot AJ, Daniels RS, Berry CL, Oxford JS, Taubenberger JK: 1918 influenza pandemic caused by highly conserved viruses with two receptor-binding variants. Emerg Infect Dis 2003;9:1249–1253.
30 Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, Garcia-Sastre A: A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science 2007;315:655–659.
31 Jordan EO: Epidemic Influenza: A Survey. Chicago, American Medical Association, 1927.
32 Barry JM: The Great Influenza: The Epic Story of the Deadliest Plague in History. New York, Viking Press, 2004.
33 Simonsen L, Clarke MJ, Schonberger LB, Arden NH, Cox NJ, Fukuda K: Pandemic versus epidemic influenza mortality: a pattern of changing age distribution. J Infect Dis 1998;178:53–60.
34 Morens DM, Fauci AS: The 1918 influenza pandemic: insights for the 21st century. J Infect Dis 2007; 195:1018–1028.
35 Palese P: Influenza: old and new threats. Nat Med 2004;10(suppl):S82–S87.
36 Panum PL: Observations made during the epidemic of measles on the Faroe Islands in the year 1846. Originally published in the Bibliothek for Læger Copenhagen, 1847;3R:270–344 (www.deltaomega.org/Panum FaroeIslands.pdf).

37 Nakajima K, Desselberger U, Palese P: Recent human influenza A (H1N1) viruses are closely related genetically to strains isolated in 1950. Nature 1978;274:334–339.
38 Glezen WP, Cherry JD: Textbook of Paediatric Infectious Diseases, ed 3. Philadelphia, Saunders, 1992, vol 2, pp 1688–1704.
39 Taubenberger JK, Morens DM: 1918 Influenza: the mother of all pandemics. Emerg Infect Dis 2006;12: 15–22.
40 Winternitz MC, Wason IM, McNamara FP: The Pathology of Influenza. New Haven, Yale University Press, 1920.
41 Li S, Schulman J, Itamura S, Palese P: Glycosylation of neuraminidase determines the neurovirulence of influenza A/WSN/33 virus. J Virol 1993;67: 6667–6673.
42 Tumpey TM, Garcia-Sastre A, Mikulasova A, Taubenberger JK, Swayne DE, Palese P, Basler CF: Existing antivirals are effective against influenza viruses with genes from the 1918 pandemic virus. Proc Natl Acad Sci USA 2002;99:13849–13854.
43 Kobasa D, Takada A, Shinya K, Hatta M, Halfmann P, Theriault S, Suzuki H, Nishimura H, Mitamura K, Sugaya N, Usui T, Murata T, Maeda Y, Watanabe S, Suresh M, Suzuki T, Suzuki Y, Feldmann H, Kawaoka Y: Enhanced virulence of influenza A viruses with the haemagglutinin of the 1918 pandemic virus. Nature 2004;431:703–707.
44 Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Pantin-Jackwood MJ, Schultz-Cherry S, Solorzano A, Van Rooijen N, Katz JM, Basler CF: Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol 2005;79:14933–14944.
45 Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M, Feldmann H, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H, Kawaoka Y: Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 2007;445:319–323.
46 Geiss GK, Salvatore M, Tumpey TM, Carter VS, Wang X, Basler CF, Taubenberger JK, Bumgarner RE, Palese P, Katze MG, Garcia-Sastre A: Cellular transcriptional profiling in influenza A virus-infected lung epithelial cells: the role of the nonstructural NS1 protein in the evasion of the host innate defense and its potential contribution to pandemic influenza. Proc Natl Acad Sci USA 2002;99:10736–10741.
47 Mibayashi M, Martinez-Sobrido L, Loo YM, Cardenas WB, Gale M Jr, Garcia-Sastre A: Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J Virol 2007;81:514–524.
48 Garcia-Sastre A, Egorov A, Matassov D, Brandt S, Levy DE, Durbin JE, Palese P, Muster T: Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology 1998;252:324–330.
49 Basler CF, Reid AH, Dybing JK, Janczewski TA, Fanning TG, Zheng H, Salvatore M, Perdue ML, Swayne DE, Garcia-Sastre A, Palese P, Taubenberger JK: Sequence of the 1918 pandemic influenza virus nonstructural gene (NS) segment and characterization of recombinant viruses bearing the 1918 NS genes. Proc Natl Acad Sci USA 2001;98:2746–2751.
50 Tumpey TM, Garcia-Sastre A, Taubenberger JK, Palese P, Swayne DE, Basler CF: Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. Proc Natl Acad Sci USA 2004;101:3166–3171.
51 Linder FE, Grove RD: Vital statistics rates in the United States 1900–1940. US Government Printing Office, 1947, p 254 www.cdc.gov/nchc/data/usus/usrates1900_40.pdf.
52 Dowdle WR: Influenza A virus recycling revisited. Bull World Health Organ 1999;77:820–828.
53 Reed LJ, Muench H: A simple method for estimating fifty percent end points. Am J Hyg 1938;27: 493–497.

Dr. Peter Palese
Department of Microbiology, Mount Sinai School of Medicine
1 Gustave L. Levy Pl., New York, NY 10029-6574 (USA)
Tel. +1 212 241 7318, Fax +1 212 722 3634, E-Mail peter.palese@mssm.edu

Author Index

Subject Index