Resurrected Pandemic Influenza Viruses*

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Key Words

pathogenesis, transmission, adaptation, virus genes

Abstract

Influenza viruses continue to pose a major global public health problem. There is a need to better understand the pathogenicity and transmission of pandemic influenza viruses so that we may develop improved methods for their prevention and control. Reconstruction of the 1918 virus and studies elucidating the exceptional virulence and transmissibility of the virus are providing exciting new insights into this devastating pandemic strain. The primary approach has been to reconstruct and analyze recombinant viruses, in which genes of the 1918 virus are replaced with genes of contemporary influenza viruses of lesser virulence. This review highlights the current status of the field and discusses the molecular determinants of the 1918 pandemic virus that may have contributed to its virulence and spread. Identifying the exact genes responsible for the high virulence of the 1918 virus will be an important step toward understanding virulent influenza strains and will allow the world to better prepare for and respond to future influenza pandemics.

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INTRODUCTION

In humans, influenza is a highly contagious respiratory disease caused by an RNA virus of the *Orthomyxoviridae* family. Influenza A viruses are classified into subtypes on the basis of the antigenicity of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). 16 HA and 9 NA subtypes are known to exist (22), and among them H1, H2, and H3 subtypes have caused pandemics. Influenza A viruses of the H1N1 subtype, descendants of the 1918 pandemic strain, circulated in humans from 1918 to 1956. After their disappearance in 1957, H1N1 viruses reappeared in the human population in 1977 and continue to cocirculate along with H3N2 subtype viruses (descendants of the 1968 pandemic strain) and influenza B viruses.

HA: hemagglutinin

NA: neuraminidase

Genetic reassortment: exchange of viral gene segments when at least two different viral genomes coinfect the same host cell

Influenza virus infection begins in the nasal and tracheal airways and can spread throughout the upper and lower respiratory tract. Clinical symptoms of an acute human influenza virus infection range from mild to severe and typically include fever, cough, headache, and malaise. For most people, the course of influenza infection is self-limiting, without requiring any medical treatment. However, human influenza viruses continue to cause substantial morbidity and mortality worldwide on an annual or near-annual basis and are responsible for approximately 250,000 to 500,000 deaths each year. Each year in the United States alone, on average, 5–20% of the population is infected, causing 200,000 hospitalizations and approximately 36,000 deaths from complications of influenza virus infection. The majority of these deaths occur in the elderly population aged >65 years (100, 101). During pandemic years, novel influenza strains have the capacity to cause severe disease and death on a global scale. It is estimated that the Spanish influenza pandemic of 1918 was responsible for approximately 500,000 deaths in the United States alone (20–50 million deaths worldwide)—a number vastly higher than the approximate combined total of 100,000 for the Asian influenza pandemic of 1957 and the Hong Kong influenza pandemic of 1968 (40). The influenza pandemic in 1918 was so exceptional in its lethality that, as a result, the average life expectancy in the United States was lowered by more than 10 years (30). For a host of reasons it is difficult to precisely determine the exact number of deaths caused by the pandemic. First and foremost, influenza was not a reportable disease in 1918–1919; the first human influenza virus strain would not be isolated for another 12 years. A confounding factor was that the etiologic agent was widely held at the time to be a bacterium called Pfeiffer's bacillus (a gram-negative bacterium now recognized as *Haemophilus influenzae*). It was not until the 1930s when retrospective serological investigations were performed among people living in those periods that the 1918 agent was linked to an influenza H1 subtype virus (6, 85). Many questions about the origin of the 1918 virus, its unusual epidemiologic features, and the basis of its virulence remain unanswered. Although genetic reassortment events led to new viruses that caused the two subsequent influenza

pandemics of 1957 and 1968 (116), it remains uncertain whether the 1918 virus was the result of adaptation of an avian virus to humans or whether it was a product of reassortment (28, 99). Identification of viral sequence data of pre-1918 human influenza samples is needed to better understand the origin of this pandemic virus.

Influenza A viruses also have a major impact on the health of domestic poultry, in which some highly pathogenic avian influenza (HPAI) strains are responsible for systemic disease with high mortality rates (see sidebar, What Makes a Virus HPAI?) (1, 95). The HPAI viruses are restricted to subtypes H5 and H7, although not all H5 and H7 viruses are highly pathogenic (96). Prior to 1997, transmission of HPAI viruses to humans was not considered a major health risk. However, during that year, HPAI viruses of the H5N1 subtype spread in poultry flocks in Hong Kong and jumped the species barrier to severely infect humans, killing 6 of the 18 documented cases (14, 18, 94). This was the first documented influenza outbreak caused by a wholly avian virus directly transmitting to humans from infected poultry and causing death. Since 2003, influenza A viruses of the H5N1 subtype have caused devastating outbreaks in poultry in Asia, Africa, and Europe, resulting in over 400 laboratory-confirmed human infections with an overall case fatality rate of approximately 60% (3). Although H5N1 viruses continue to evolve and diversify (2), they have yet to acquire the ability to transmit efficiently among humans. Although it has been over 40 years since the last influenza pandemic, there is an ever-present threat that a pandemic will result from the emergence of a new influenza strain to which humans have little immunity. The factors allowing an influenza virus to acquire pandemic capability are poorly understood, and the approach of trying to determine these factors by studying contemporary avian influenza (H5 or H7) subtype strains is a daunting task. Conversely, identifying the role of individual viral gene products and mapping the molecular determinants that influence virulence and transmission of a previous pandemic (1918) strain are feasible approaches toward understanding pandemic traits.

WHAT MAKES A VIRUS HPAI?

The intravenous pathogenicity index (IVPI) is a method described by the World Organization for Animal Health (OIE) to determine the classification of avian influenza viruses as being of high or low pathogenicity in domestic poultry (70). Chickens (4 to 8 weeks of age) are experimentally inoculated with live virus and observed daily for clinical signs of illness. Viruses are highly pathogenic (HPAI) if they cause lethality in six to eight of eight infected chickens within 10 days postinfection. Viruses that can replicate in cell culture in the absence of trypsin, or that contain multiple basic amino acids at the HA cleavage site, are also considered highly pathogenic. This classification of HPAI is independent of pathogenicity observed in mammalian models (such as mice or ferrets). Thus, mammalian models must be utilized to elucidate the pathogenicity and transmissibility of these viruses. To date, only selected viruses within the H5 and H7 subtypes have been classified as HPAI.

HPAI: highly pathogenic avian influenza

Archaevirology: the systematic study of past viruses by the recovery and examination of remaining material evidence

RECONSTRUCTION OF THE 1918 PANDEMIC INFLUENZA VIRUS

Although scientists have been keen to unlock the mysteries of the virus responsible for the 1918 pandemic, the virulent H1N1 strain could not be studied until recently, as the virus was not isolated at the time of the pandemic. Research interest in the virulence of the 1918 virus has been further prompted by the emergence of a novel, potentially pandemic strain containing the HA derived from a HPAI virus. The main questions are why was the 1918 virus so virulent, and can the molecular secrets associated with the high virulence and transmission of this H1N1 subtype virus help guide our response to future influenza pandemics?

The reconstruction of the 1918 virus first required sequence analysis of the viral genome using archaevirology. Sequencing of the eight viral gene segments was laborious, taking nine years to complete (7, 76–79, 98, 99). Scientists collected viral cDNA fragments of the 1918

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RT-PCR: reverse transcription polymerase chain reaction

Reverse genetics: a system to generate infectious virus entirely from cloned cDNAs

Plasmid: circular, double-stranded unit of DNA expressing influenza genes used to transfect 293T cells

NS: nonstructural

virus by reverse transcription polymerase chain reaction (RT-PCR) from formalin-fixed, paraffin-embedded autopsy tissues taken from victims at the time or, in one case, isolated from a frozen lung sample of a 1918 victim from Brevig Mission, Alaska (76). The coding sequences of the 1918 viral RNA segments did not reveal obvious genetic features that had previously been associated with virulence (98, 99). Therefore, it was crucial to study intact replicating virus in suitable animal models to better understand the genetic determinants responsible for 1918 virus pathogenicity and transmissibility. With the advent of reverse genetics technology for influenza viruses in 1999, infectious virus could be rescued entirely from plasmid-cloned influenza gene segments without helper virus (21). This technology made it possible to produce influenza viruses with specific sequences. With the 1918 virus gene sequences completed, cDNAs were constructed by PCR using commercially synthesized overlapping deoxyoligonucleotides corresponding to the published sequence of the 1918 influenza virus and subcloned into plasmids for virus rescue (7). In 2005, following completion of the 1918 virus coding region sequence, a reconstructed influenza virus containing all eight gene segments from the H1N1 pandemic virus was generated for the first time at the Centers for Disease Control and Prevention (103). A negative-stained transmission electron micrograph of the recreated 1918 influenza virus showed virions with typical influenza virus morphology (**Figure 1**).

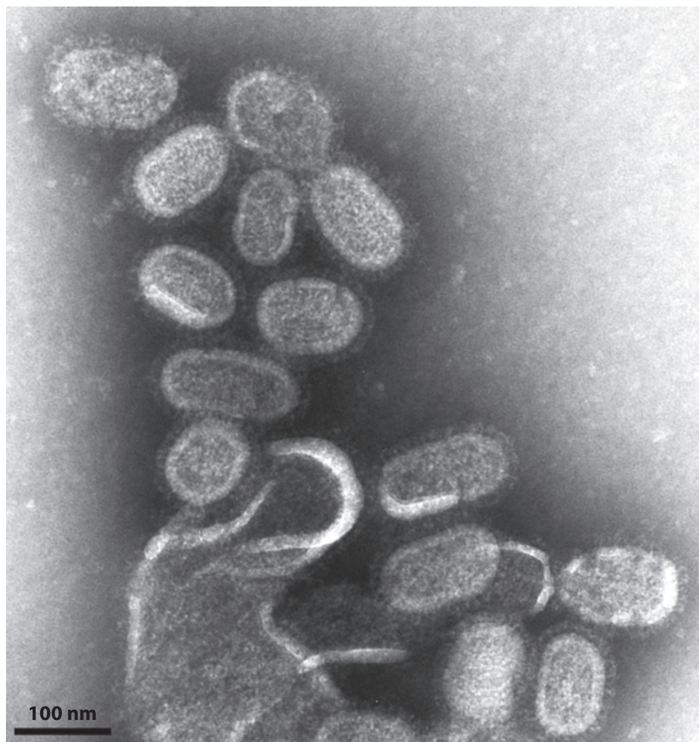

Figure 1

Negative-stained transmission electron micrograph of reconstructed 1918 virions. The prominent surface projections on the virions are composed of either HA or NA glycoproteins. Virus samples were collected from supernatants of 1918-infected Madin-Darby canine kidney (MDCK) cell cultures 18 h after infection. The solid mass in lower center contains MDCK cell debris that did not spin down (centrifugation) during the procedure. Prepared by T. Tumpey and C. Goldsmith, CDC.

NS1 PROTEIN IS NOT A CRUCIAL VIRULENCE FACTOR OF THE 1918 VIRUS

The initial sequencing of the nonstructural (NS) gene of the 1918 influenza virus in 2000 made it possible to study the role of this individual 1918 virus protein encoded in the genetic background of a commonly used influenza virus (7). NS1 protein, the product of a spliced mRNA from the influenza NS gene, functions as an antagonist to block type 1 interferon (IFN)-mediated host antiviral response following infection (25, 115). Viral infection is generally associated with double-stranded RNA (dsRNA) production. By virtue of its dsRNA binding properties, NS1 protein has been proposed to be an important determinant of influenza virus virulence because of its ability to sequester dsRNA generated during virus replication (24, 57, 97, 114). This in turn blocks the activation of 2′-5′ oligo (A) synthetase (64), which is believed to be an activator of RNaseL, an endonuclease that degrades cellular and viral RNA (20, 66, 125). NS1 has evolved additional ways to evade the antiviral effects on the host cell, including blocking activation of IFN-inducible protein kinase R (PKR) activity

during virus infection (9, 35). In the absence of NS1, PKR (a serine-threonine protein kinase) inhibits eukaryotic translation initiation factor 2 (eIF-2α) (56), thereby inhibiting viral and cellular protein synthesis and virus replication. The retinoic acid-inducible gene I (RIG-I) is required for the induction of interferon-beta (IFNβ) in response to influenza virus infection, and NS1 suppresses production of IFN by targeting and inactivating the RIG-I-dependent signaling pathway (120). Deletion of NS1 results in heightened expression of cellular genes, including RIG-I (71), involved in the antiviral response.

The concept that a strong IFN-antagonist 1918 NS1 protein may have contributed to the exceptional virulence of the pandemic virus prompted initial studies on the function of this gene (7, 26). The approach to studying the contribution of a strain-specific NS gene to virus pathogenesis has been to generate single-gene reassortant viruses through reverse genetics. On the basis of reported nucleotide sequences, a virus was reconstructed containing the NS gene of the 1918 virus in the genetic background of a common laboratory strain, A/WSN/33 (WSN;H1N1) virus. However, the introduction of the 1918 NS1 gene or the entire 1918 NS segment into the WSN virus background (1918 NS:WSN) resulted in a virus that was attenuated in mice (7). In more recent studies the genetic background influenza gene set from WSN virus was replaced with the genetic background of a nonlethal seasonal influenza H1N1 virus. This required generating a new set of rescue plasmids, in this case from A/Texas/36/91 (Tx/91:H1N1) virus, which has been characterized in humans under experimental settings (37) and is nonpathogenic in mice (106). Overall, the 1918 virus virulence observed in mice correlated with the ability of 1918 recombinant viruses to replicate efficiently in mouse lungs and human airway cells (**Table 1**). Studies with 7:1 recombinant viruses, in which the NS gene of the 1918 virus was replaced with the NS genes from Tx/91, revealed that the lethal outcome in mice did not differ significantly from mice inoculated with the parental 1918 virus. Moreover, in the reciprocal experimental approach, a 1918 1:7 recombinant virus was generated in which the Tx/91 NS virus gene was individually replaced by the 1918 NS virus gene. The 1918 NS1:Tx/91 virus did not confer a more virulent virus in mice or increase the replication efficiency of the parental Tx/91 virus in human airway cells (74). These data further suggested that the NS1 protein is not a crucial virulence factor of the 1918 virus or, alternatively, that the mouse is not an ideal model to study human NS1 virulence.

While the 1918 human NS1 may be incapable of inhibiting the murine IFN in mouse

Reassortant virus: virus that possesses gene segments from multiple strains, derived by classical reassortment or through reverse genetics

Table 1 Role of individual virus genes in the high pathogenicity phenotype of 1918 virus in mice

	1918 (7:1) Recombinant viruses[a]		1918 (1:7) Recombinant viruses	
Gene segment	Replication[b]	Virulence	Replication	Virulence
PB2	Same as 1918	High	Same as Tx/91	Low
PB1	Significantly reduced	Intermediate	Moderately elevated	Low
PA	Same as 1918	High	Same as Tx/91	Low
HA	Significantly reduced	Low	Significantly elevated	High
NA	Significantly reduced	Intermediate	Moderately elevated	Low
NP	Same as 1918	High	Same as Tx/91	Low
M	Same as 1918	High	Same as Tx/91	Low
NS	Same as 1918	High	Same as Tx/91	Low

[a]Summarized from Reference 74.

[b]Virus replication in mouse lungs.

Abbreviations: HA, hemagglutinin; M, matrix; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic protein; PB1/2, polymerase basic protein 1/2.

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cells, it may be a virulence factor in alternative models. Evidence to support this comes from microarray analysis on human lung epithelial cells infected with 1918 NS1:WSN virus (26). This virus was more effective at blocking the expression of IFN-regulated genes than the parental WSN virus was. Moreover, in a 2006 study by Salomon et al. (81) addressing the NS gene of H5N1 virus, a single-gene reassortant virus that combined the NS gene of the nonlethal A/chicken/Vietnam/C58/04 virus with the remaining seven genes from the highly virulent A/Vietnam/1203/04 virus (isolated from a fatal case) attenuated the virulence observed with the parental VN/1203 virus in ferrets, but not in mice. For the H7 subtype virus, Munster and colleagues (67) generated a reassortant virus that possessed the NS gene from the highly virulent H7N7 virus A/Netherlands/219/03, with remaining genes derived from the low virulent A/Netherlands/33/03 virus, and found that it did not result in increased pathogenicity in mice. The data suggest that the mouse model might not be suitable for experiments of this kind and that other species should be considered for studying the influenza virus NS gene. Moreover, standard inbred mouse strains lack a functional IFN-regulated *Mx1* gene, and therefore their susceptibility to influenza virus infections may not be typical of mammals.

HEMAGGLUTININ AND NEURAMINIDASE ARE REQUIRED FOR OPTIMAL VIRUS REPLICATION AND VIRULENCE OF THE 1918 PANDEMIC STRAIN

Most of the initial research emphasis on the 1918 virus was placed on the HA and NA glycoproteins because they are the major viral surface antigens of influenza A viruses and in general are important virulence factors in mammals and poultry. Early studies employing the mouse-adapted WSN influenza virus as a background virus showed that the HA and NA genes of the 1918 virus maintained the virulence of the parental WSN virus (104, 105). In contrast, the control virus possessing the HA and NA genes from a seasonal H1N1 (New Caledonia HA/NA:WSN) virus on the same WSN genetic background was not lethal to mice at any virus dose tested. These results were striking because the 1918 HA and 1918 NA genes were derived directly from a human virus without prior mouse adaptation. Typically, strains of influenza A viruses become lethal in mice only after they are adapted to growth in these animals. Subsequent studies using the genetic background of Tx/91 virus possessing the 1918 HA and NA genes (1918 HA/NA:Tx/91) or 1918 HA only (1918 HA:Tx/91) confirmed the initial results by demonstrating that these recombinant viruses replicated efficiently in the mouse lung and were lethal for this species (106). Similarly Kobasa and colleagues (48) demonstrated that the HA gene of 1918 virus enhanced the virulence of both H1N1 and H3N2 subtype viruses in mice.

The 1918 HA gene is also essential for maximum virus replication in human airway cells and for eliciting a heightened host inflammatory response (74, 106). The 1918 HA recombinant viruses induced a heavy inflammatory infiltrate into the lung marked by a predominance of neutrophils and an increase of alveolar macrophages and cytokines shortly before the death of these mice. A variety of inflammatory mediators, including complement factors (39) and chemokines (16, 86), can cause neutrophil migration. A greater expression of chemokines that activate and exert chemotactic effects on neutrophils, observed in 1918 HA/NA:Tx/91–infected mouse lungs, correlated well with the increased neutrophil influx into the lung (106). The notion that neutrophils drive the increased lung pathology and contribute to mortality following 1918 HA/NA:Tx/91 virus infection was addressed in neutrophil depletion studies. However, depletion of neutrophils after the initiation of lung inflammation had no effect on the overall disease outcome (106). On the contrary, neutrophil-depleted mice had significant weight loss, increased virus replication, and increased mortality following lethal challenge with the 1918 HA/NA recombinant virus.

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These data suggest that while neutrophils may be contributing to the overall pathogenesis, they still play a crucial role in controlling virus growth and promoting clearance of this highly virulent virus.

Among all eight gene segments tested individually, the HA was the only single 1918 virus gene able to confer a virulent phenotype when rescued on the genetic background of Tx/91 H1N1 virus (74). It is interesting to speculate on the role of the 1918 HA glycoprotein and its ability to enhance virus replication and disease of a seasonal influenza virus. The HA molecule of influenza A viruses is an integral membrane glycoprotein with multiple functions. The posttranslational cleavage at a conserved arginine residue of HA0 to generate the subunits HA1 and HA2 is necessary for virus infectivity as it activates the membrane fusion potential of the HA (88). The HA homotrimer is responsible for viral binding to sialic acid (SA)-containing receptors on host cells and mediating the subsequent fusion of virus and host membranes (via HA2 fusion) in the endosome after the virus has been taken up by endocytosis. Thus, the HA protein mediates fusion and uncoating because of low-pH-induced conformational changes; acidification converts the HA into a fusogenic conformation, thereby exposing the hydrophobic N terminus of the HA2 subunit (73). Genetic analysis of mouse-adapted variants showed a number of HA mutations, including loss of a glycosylation site and substitution in HA2, which increases the pH of fusion (34, 89). If the 1918 virus possesses an HA with an altered (elevated) pH of fusion, this could increase the rate of uncoating and thus more rapidly establish infections, resulting in higher yields of virus (46). Future studies are needed to clarify the fusion activity of the 1918 HA glycoprotein and determine if its high virulence is associated with its ability to fuse membranes at higher pH, as demonstrated for some influenza strains with increased virulence. Such studies may reveal whether the fusion activity of HA can be modulated by other viral genes and whether the pH threshold for membrane fusion is linked to receptor specificity.

Receptor specificity for the influenza virus is controlled by the glycoprotein HA and is important for host restriction of human and avian viruses. In general, avian influenza virus HAs preferentially recognize α2,3-linked SA receptors (92), which are abundant in the gastrointestinal tract of poultry and wild birds (61, 62, 111). Conversely, human influenza viruses preferentially recognize α2,6-linked SA receptors, which predominate in the upper respiratory tract of humans and some mammals, such as ferrets (17, 23, 29, 80, 118). It appears that the 1918 influenza pandemic was caused by viruses with two receptor binding variants: A/South Carolina/1/1918 (SC18) and A/Brevig Mission/1/1918, like A/London/1/1918, possess the human α2,6 SA receptor preference, whereas natural variants A/New York/1/1918 and A/London/1/1919 possess a mixed α2,6/α2,3 SA specificity (29). The 1918 reassortant viruses described above all possessed the SC18 HA. A confounding feature of the SC18 HA is its ability to attach and replicate efficiently in the murine respiratory tract (103), which contains a paucity of α2,6-linked SA receptors (36, 38). As alluded to above, human influenza viruses with α2,6 SA receptor preference generally require adaptation by sequential passage in the mouse respiratory tract before viruses can replicate efficiently and induce disease in mice. Finding the answer to how a non-mouse-adapted SC18 virus replicates and kills mice may shed light on the role of the 1918 HA in virulence.

The paradigm of avian viruses binding α2,3 linkages, and human-adapted viruses binding cells bearing glycans with α2,6 linkages, may be an oversimplification. Chandrasekaran et al. (11) showed that the presence of α2,3 or α2,6 linkage alone is not sufficient, but that the critical considerations are glycan topology in addition to glycan composition. Thus, the recognition of a specific structural topology or some unidentified receptor linkage, and not the SA linkage itself, may allow a virus possessing the SC18 HA to bind glycans in the respiratory tract of mice and to replicate efficiently (44). The binding of the HA protein may be a

Sialic acid (SA): the essential terminal sugar on the receptor for influenza type A viruses

SC18: A/South Carolina/1/1918

Glycan topology: characteristic structural features of glycans that affect receptor binding to ligands

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PB1: polymerase basic protein 1

determinant for virulence by targeting specific host cells. The SC18 HA binds specifically to goblet cells in ferret as well as human respiratory tissue(s) (90). Enhanced cell tropism, provided by the HA, could allow viruses to bind a subset of cells in the upper respiratory tract that are critical for pathogenesis.

The NA protein of influenza A virus functions as a sialidase and cleaves SA from host cells, thus promoting viral spread (72). Influenza viruses possessing low NA enzymatic activity or blocking the receptor-destroying activity of the NA result in viral aggregation at the cell surface (72, 73). A functional cooperative must exist between HA and NA surface proteins for optimal receptor binding and virus release (65). Thus, an evolutionary balance between the HA and NA results in a cooperative between the avidity of HA and the strength of NA (112, 113). A pandemic virus possessing an efficient NA could promote enhanced replication and contribute to virulence. Therefore, single gene reassortants were generated and a critical role for the NA in the high virulence of the 1918 pandemic influenza virus was identified (74). In contrast to the lethal outcome in mice infected with the eight-gene 1918 virus, mice infected with the 7:1 recombinant virus containing the Tx/91 NA were attenuated. Moreover, the reduced disease and weight loss of the Tx/91 NA:1918–infected mice correlated with the lower levels of virus replication in mouse lungs and human airway epithelial cells. Further proof that the NA gene contributes to the high virulence of the 1918 pandemic virus was provided by generating reciprocal recombinant (1:7) viruses. A Tx/91 recombinant virus expressing the 1918 NA increased the replication efficiency of the parental Tx/91 virus in primary normal human bronchial epithelial (NHBE) cells and mouse lungs (74).

A unique phenotype identified for the 1918 virus is its ability to form visible plaques on Madin-Darby canine kidney (MDCK) cells in the absence of the protease trypsin (103). The proteolytic cleavage of the HA molecule is a prerequisite for multicycle replication, and the ability of an influenza virus to replicate in the absence of trypsin has been thought to be an important determinant of influenza virus pathogenicity in mammals (32, 33). In contrast to the contemporary human Tx/91 and N.Cal/99 H1N1 viruses, which require an exogenous protease source for their multicycle replication and plaque formation, the 1918 virus and a recombinant influenza virus bearing only the 1918 NA segments (1918 NA:Tx/91) formed visible plaques without the addition of trypsin. This finding suggested that the 1918 NA activity facilitates HA cleavage. The 1918 HA and NA gene sequences do not point to any obvious genetic features that have previously been associated with the ability to replicate in the absence of trypsin; i.e., the 1918 virus possesses neither a series of basic amino acids at the HA cleavage site (as seen in highly pathogenic avian H5 or H7 influenza viruses) nor mutations (N146R or N146Y) in the NA that lead to the loss of a glycosylation site at position 130 like those that allow the WSN virus NA to sequester plasminogen (51, 98). Moreover, the systemic infection in WSN-infected mice, attributed to structural components of the NA protein (33), has not been observed with the 1918 virus in mice (103, 108). Scientists are currently considering other mechanisms of NA-mediated HA cleavability that may be relevant to the replication and virulence of the 1918 virus.

THE VIRAL PB1 GENE CONTRIBUTES TO OPTIMAL VIRULENCE OF THE 1918 PANDEMIC STRAIN

The influenza RNA polymerase complex is a heterotrimer consisting of polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) (72). The PB1 subunit is a key component of the viral RNA polymerase complex and catalyzes the sequential addition of nucleotides during RNA chain elongation. PB1 contains multiple active sites critical for the polymerization of RNA chains and also for association with PA and PB2 to form the polymerase heterotrimer (10, 31, 75). Both the 1957 and 1968 pandemic

strains are thought to have originated as reassortants, in which a novel avian HA replaced the prevailing human-adapted HA surface protein (116). Both pandemic viruses also acquired a novel polymerase subunit PB1 gene of wild waterfowl origin (45), suggesting that the acquisition of an avian PB1 gene may be a critical step in generating a pandemic virus through reassortment. The selective advantage for an influenza virus possessing an avian PB1 gene over a human PB1 gene is not entirely clear; however, the acquisition of an avian influenza PB1 gene by reassortment might provide increased transcriptional activity of the RNA-dependent RNA polymerase and result in increased virus replication efficiency of a new pandemic strain (69). As described below, the PB1 virus gene of the 1918 virus was essential for maximal replication and virulence of the pandemic virus.

In October 2005, the sequencing of the 1918 polymerase virus genes (PB1, PB2, and PA) completed the entire genetic sequence of the pandemic virus. Analysis of the 1918 polymerase protein sequences revealed striking similarities to the polymerase protein sequences found in the avian influenza consensus sequences. The 1918 PB1 protein differs from the conserved avian influenza consensus sequence by only seven amino acid residues (99). Following completion of the virus coding region sequence, an influenza virus containing all eight gene segments from the 1918 pandemic virus was rescued in 2005 (103). In vitro, the reconstructed 1918 virus replicated with extraordinary efficiency compared with contemporary seasonal influenza strains or recombinant viruses possessing seasonal Tx/91 virus genes. This difference was demonstrated by studying the apical virus release in a number of relevant cell lines including primary bronchial epithelial cells and Detroit 562 cells, derived from a human pharyngeal epithelium. In vivo, the reconstructed 1918 virus was lethal at high doses in ferrets, mice, and macaques (42, 47, 103, 108). At the high virus inoculum of 10^6 PFU, mice displayed a sudden onset of severe illness and succumbed to infection as early as 3 days following an intranasal inoculation. This was consistent with the rapid course of disease in some of its human victims who died in 1918 as a result of an overwhelming viral pneumonia (50). Virus titers in lungs of 1918 virus–inoculated mice were nearly 40,000-fold higher than those of mice infected with the contemporary Tx/91 virus (103). These studies also revealed that the virulence of the 1918 virus was largely determined by the HA and, to a lesser extent, by the polymerase gene complex.

NP: nucleoprotein

Until recently, it was not known which of the three polymerase genes contributed to the exceptional virulence of the 1918 virus or whether other virus genes also contributed to its virulence. In 2007, the 1918 1:7 recombinant viruses were generated in which the polymerase genes from Tx/91 were individually replaced by the 1918 polymerase genes. The increased replication efficiency of the 1918 PB1:Tx/91 (1:7) virus could be visually observed as a distinctly larger plaque size phenotype compared with the small plaque phenotype of the 1918 PA:Tx/91 and 1918 PB2:Tx/91 (1:7) reassortants. Moreover, the 1918 PB1:Tx/91 virus produced eightfold-greater virus release in apical supernatants as early as 12 h after infection, compared with virus production by the other polymerase reassortants and the wild-type Tx/91 virus. In contrast, infection with 1:7 reassortants, in which the 1918 nucleoprotein (NP), matrix (M), NS, or polymerase subunits PB2 and PA were individually substituted into the background of the Tx/91 virus, did not result in increased virus replication compared with the parental Tx/91 virus (**Table 1**). In the reciprocal experiments, mice infected with the 7:1 reassortant virus containing the Tx/91 PB1 gene had higher survival rates and lower lung titers than mice infected with the eight-gene 1918 virus (74) (**Table 1**). These data further illustrated the importance of the 1918 PB1 genes for optimal virus replication of this pandemic strain. Antiviral therapies directed at inhibiting the polymerase complex formation [such as recently developed competitive inhibitors of the PB1 protein (27)] should be further developed because new and better antiviral therapies are

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Cytokine storm: hypercytokinemia, or the excessive release of proinflammatory cytokines which has been proposed to be detrimental to the host

needed to deal with future pandemic and epidemic strains.

The increased virulence associated with the 1918 PB1 might be due to the PB1-F2 protein generated by an alternative reading frame. Recently, a novel protein encoded by an alternative reading frame in the PB1 gene segment has been serendipitously identified following a broad search for antigenic influenza A viral peptides encoded by alternative reading frames (13). The PB1-F2 protein has intrigued scientists ever since its initial discovery by Chen and colleagues in 2001. It can be found in various lengths in all known influenza A subtypes but is generally a 87-amino-acid peptide generated from a +1 reading frame by virtue of ribosomal scanning (124). Of note, all the contemporary H1N1 viruses have truncated PB1-F2 proteins consisting of 57 amino acids. This is relevant with respect to the 1918 PB1 (7:1 and 1:7) recombinant virus results described above. The PB1-F2 protein is truncated in Tx/91 virus and all contemporary human H1N1 viruses but is full length in the 1918 virus, and therefore the replacement of the 1918 PB1 gene may be conferring the increased virulence associated with this virus gene. Although the PB1-F2 protein is not critical for viral replication in vitro (13), it induces apoptosis by localizing to the inner and outer membranes of mitochondria. There, the PB1-F2 protein can permeabilize planar lipid membranes by oligomerizing and punching holes into the mitochondria (12). The PB1-F2 protein specifically targets and destroys alveolar macrophages, inducing apoptosis in this cell type and to a lesser extent in epithelial cells that support virus replication (12, 49). Theoretically, PB1-F2-mediated killing of professional antigen-presenting cells could impede antigen presentation to the adaptive T cell response, thus allowing for the increased pathogenicity of the virus.

Work by Zamarin and colleagues (122, 123) demonstrates the ability of PB1-F2 to affect the outcome of influenza A virus infection in mice. Knocking out the PB1-F2 protein had no effect on viral replication in tissue culture but diminished virus pathogenicity and mortality in mice. Subsequent studies by Conenello and colleagues (15) revealed the importance of amino acid position 66 in PB1-F2 in the virulence of a H5N1 virus and the 1918 pandemic virus. A recombinant H5N1 virus possessing an asparagine-to-serine change at amino acid position 66 (N66S) in the PB1 gene of the H5N1 virus A/Hong Kong/156/97, with all other genes derived from WSN virus, was significantly more virulent compared with a virus without this amino acid change. This amino acid change, N66S, was also found in the PB1-F2 segment of the 1918 virus. In the reverse experiment, mutation of S66N into the reconstructed 1918 virus resulted in reduced pathogenicity and decreased mortality and morbidity compared with the parental 1918 virus. For both the H5N1 and the 1918 PB1-F2 mutations studied, mice infected with the viruses containing a serine at position 66 had higher lung virus titers and cytokines compared with mice infected with their respective S66N mutants (15), further suggesting the importance of this amino acid in the pathogenesis of some influenza strains.

PB1-F2 has been hypothesized to cause apoptosis of immune cells, which results in a decrease in the adaptive immune response. A remarkable and underappreciated feature of lethal H5N1 virus infection has been the destructive effects on the mammalian immune system, which may be a factor contributing to the overall pathogenesis (107). This is counterintuitive and contrary to the proposed mechanism that an exaggerated immune response (cytokine storm) significantly contributes to the pathogenesis of lethal influenza virus infections. The high rate of mortality among young, healthy individuals between 15 and 34 years of age in 1918 (30, 87), a notable divergence from the burden of excess mortality among elderly adults, has raised the question of whether this was the result of a too vigorous (or overexuberant) immune response among this age group. However, the hypothesis that severe lung disease is based on induction of a cytokine storm has been difficult to prove and model in vivo, and it is clear that highly virulent H5N1 viruses

cause a decrease in peripheral blood and tissue lymphocytes.

In mice, H5N1 viruses typically fall into two main groups: a highly pathogenic phenotype with systemic replication and death (A/Hong Kong/483/97-like), and a low pathogenic phenotype with efficient respiratory viral replication without systemic spread or lethal infection (A/Hong Kong/486/97-like) (43, 107). Intranasal infection of mice with HK/483 virus resulted in a significant decrease in the total number of circulating leukocytes evident as early as day 2 postinfection. Differential blood counts demonstrated an up to 80% drop in lymphocytes by day 4 postinfection. In contrast, nonlethal HK/486-infected mice displayed only a transient drop of lymphocytes during the infectious period. There was evidence of apoptosis in the primary and secondary lymphoid organs of HK/483-infected mice, suggesting a mechanism for lymphocyte destruction (107). Thus, the lethal H5N1 viruses appear to possess the capacity to limit the induction of immune responses by targeting lymphocytes and destroying these cells. A common feature among the H5N1-infected patients with a severe or fatal outcome was a low peripheral leukocyte count, or leukopenia (19, 102, 121). In contrast, patients who did not display leukopenia upon hospital admission were more likely to recover.

Additional 1918 PB1-F2 protein studies by McAuley and colleagues (63) incorporated secondary bacterial infections into the mouse model. The premise of this work is that targeted elimination of alveolar macrophages by PB1-F2 protein may facilitate an opportunistic bacterial infection. The results show that inserting the 1918 PB1-F2 gene into the genetic background of the A/PR/8/34 virus created a recombinant virus that was more deadly than the parental PR/8 strain, with secondary bacterial infection, suggesting that the 1918 PB1-F2 gene exacerbated any resulting secondary bacterial pneumonia. The PB1-F2 protein could be looked at as a marker for virulence and incorporated as part of our general surveillance and testing for potentially pandemic viruses, as is currently done with PB2 position 627 (83, 84). A better understanding of the contribution of polymerase proteins in virulence will aid in designing drugs that target the key intersubunit binding sites of the polymerase complex and that diminish the high replication efficiency of pandemic virus strains. In addition, the design of small-molecule drugs that target PB1-F2 and prevent the detrimental effects of this protein should be considered.

1918 VIRUS TRANSMISSION

In humans, influenza viruses are expelled in respiratory secretions when an individual coughs or sneezes. Individuals become infected either through direct inhalation of large or small droplets containing viruses or by indirect contact with fomites on contaminated surfaces (4, 8, 52). A key property of seasonal and pandemic influenza virus strains is their ability to spread with high efficiency through low-titer aerosol transmission. Avian influenza viruses, which spread primarily by fecal-oral transmission among birds, lack the ability for sustained transmission in humans (117). For contemporary H5N1 viruses, other than the occasional family clusters that have provided evidence for limited person-to-person transmission (41), the majority of human infections have been the result of exposure to H5N1 virus–infected poultry. Increasing persistence and genetic diversity of H5N1 viruses in poultry (2) with concomitant human infection since 2003 have been driving the central question: What are the genetic changes necessary for an avian influenza virus to adapt to humans and acquire efficient and sustained transmission? Even with sequencing data of countless influenza virus genomes, no clear molecular signatures tell us how influenza viruses spread efficiently in the human population.

Despite classical experimentation by Andrewes and Glover as far back as 1941 (5) which determined that human influenza virus may transmit from infected ferret to uninfected ferret, the molecular basis of influenza virus transmission is not well understood. The molecular basis can be broken down into two fields of study: the complex virus-host

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interactions and viral factors that influence virus transmission. The latter factors have been the subject of recent transmission studies using a combination of reverse genetics and animal models. Initial transmission studies with the reconstructed 1918 virus in mice suggested that this species is a poor model to mimic the transmission pattern of influenza viruses among humans (54). However, both ferrets and guinea pigs are naturally susceptible to infection with influenza A viruses, and both animals have shown value in modeling influenza virus transmission (53–55, 58, 119). Moreover, the disease in ferrets resembles that seen in humans infected with influenza A viruses, and their upper respiratory tract epithelia possess a distribution of influenza virus receptors (predominant α2,6-linked SA receptors) that corresponds to what has been observed in human airway cells (111, 118).

It has been postulated that the lack of sustained human-to-human transmission of avian influenza H5N1 viruses is due to their α2,3 SA receptor binding preference (60, 82, 110). In fact, the three influenza pandemic viruses of the last century, occurring in 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2), each possessed an HA with a human α2,6 SA binding preference and is thought to have originated from an avian virus possessing an α2,3 SA binding preference (17, 92). In general, the introduction of only two amino acid mutations into the HA of the three human HA subtypes causes a switch from the avian α2,3 SA to the human α2,6 SA receptor binding preference. H2 and H3 subtypes make the switch between an α2,3 and α2,6 SA binding preference with two changes at amino acid positions 226 and 228 (Q226L and G228S) of the HA (17, 68), whereas two different mutations (E190D, G225D) in the H1 HA of the 1918 pandemic virus results in a receptor switch (29). When the known HA mutations were inserted into contemporary H5N1 viruses, it was found that mutations that caused a shift from the avian-type to human-type receptor binding specificity for the H1/H3 subtype do not cause an equivalent shift in specificity for the H5 subtype (92). These results indicate that a different combination of mutations in HA is required for the current H5N1 virus to completely shift from avian-like to human-like receptor binding preference.

It is currently unknown which additional mutations in the H5 HA would cause a shift to the human-type specificity; it becomes an overwhelming task to identify random mutations that may be required for this avian subtype virus to transmit efficiently among humans. However, the alternative approach of making mutations in the 1918 virus and carrying out transmission studies in ferrets has proved successful in identifying viral factors that influence virus transmission (**Table 2**). The ability of the 1918 pandemic virus to transmit through the

Table 2 Summary of the roles of individual virus genes in the transmission of 1918 virus in ferrets

Gene segments from 1918 virus[a,b]	Sneezing observed	Transmission[c]
All	Yes	Yes
None: rescued avian A/Duck/New York/15024/96	No	No
All: except two amino acid changes (D190E/D225G) in HA (AV18)	No	No
HA, NA	No	No
HA, NA, PB1	Occasional	No
HA, NA, PA	No	No
HA, NA, PB2	Yes	Yes

[a]Remaining gene segments derived from avian A/Duck/New York/15024/96 H1N1 virus.
[b]Summarized from References 108 and 109.
[c]Transmission through the air via respiratory droplets of A/Duck/New York/15024/96 H1N1–1918 recombinant viruses. Three ferrets were inoculated with 10^6 PFU of the indicated virus and placed in separate cages. Naïve ferrets were placed in cages adjoined to those of the inoculated ferrets, and viral shedding in the upper respiratory tract was assessed on alternating days for inoculated and naive ferrets.
Abbreviations: HA, hemagglutinin; NA, neuraminidase; PA, polymerase acidic protein; PB1/2, polymerase basic protein 1/2.

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air via respiratory droplets (108) is of particular interest, as efficient transmission via this route is a critical property of pandemic influenza strains. In 2007, a key finding showed that the binding preference for α2,6-linked SA receptors was an essential property of the 1918 pandemic strain, as generation of a mutant 1918 virus with an α2,3 binding HA (AV18) resulted in loss of transmissibility in the ferret model (108). No sneezing was noted among the AV18-inoculated ferrets through the course of the experiment, a finding consistent with the lack of significant sneezing among other viruses that do not spread to naïve ferrets (**Table 2**). However, using a series of human 1918–avian H1N1 influenza reassortant viruses, recent studies in the field demonstrated that the 1918 HA gene was necessary but not sufficient to allow transmission between ferrets if the 1918 recombinant virus possessed an avian polymerase subunit PB2. Strikingly, the 1918 PB2 protein was both necessary and sufficient for airborne transmission of a virus expressing the 1918 HA (109) (**Table 2**).

Alignment of the avian virus and 1918 virus PB2 proteins shows a number of amino acid differences that include amino acid position 627. The residue 627 contributes to host range and to the temperature sensitivity of avian viral replication in mammalian cells (59, 93). All three twentieth-century influenza pandemics were caused by viruses containing human-adapted PB2 genes, and in general lysine is present at position 627 among the human influenza viruses that do transmit efficiently, whereas a glutamic acid is found in the position among the avian influenza isolates. Transmission of the 1918 virus was abolished when a glutamic acid, the avian consensus residue, was introduced at position 627 (109). Moreover, using the guinea pig to model human influenza transmission, Steel et al. (91) identified not only the 627 residue in the PB2, but also the 701 residue as important for efficient transmission. These findings demonstrate that the adaptation of the PB2 protein is critical for the development of pandemic influenza strains from avian influenza viruses.

Identification of the PB2 protein as a critical determinant of respiratory droplet transmission in the ferret suggests a number of interesting hypotheses concerning the mechanism. Studies have shown that the H5N1 PB2 protein contributes to increased replication at lower temperatures encountered in the nasal passages of mice (36). In the ferret transmission studies, influenza viruses that transmitted efficiently were able to replicate efficiently at the lower temperature (33°C) found in the environment of the mammalian airway cells. The K627 to E mutation resulted in a substantial impairment in plaque formation at the lower temperature. Thus, it is reasonable to speculate that the 627E mutant virus is not replicating efficiently in the airway cells putatively involved in shedding the virus into the air. In addition, the 1918 HA protein, which is a determinant for direct contact transmission, binds specifically to goblet cells in ferret as well as human respiratory tissue(s) (90). Enhanced cell tropism, provided by the HA, could allow viruses to bind a subset of cells in the upper respiratory tract that are critical for transmission. An adapted PB2 may then be further required to optimize replication (at the lower temperature) following entry into these cells, thus promoting transmission. Further examination of the tissue distribution of the 1918 reassortants, as well as virus-host interactions, is needed to fully understand the mechanisms of enhanced transmission.

Respiratory droplets: droplets or droplet nuclei of indeterminate particle size expelled during coughing or sneezing

CONCLUSION

The recent events in Asia, Africa, and Europe have led to intensive planning and preparation for a potential influenza pandemic. Identification of the molecular secrets associated with the high virulence and transmissibility of the 1918 pandemic virus will offer many further avenues of investigation important for our preparedness for the next pandemic. The research detailed within this review demonstrates the great strides that have been made toward understanding the exceptional virulence of this pandemic virus as it relates to the phenotype of contemporary avian influenza strains that possess

pandemic potential. In the few short years since the reconstruction of the 1918 pandemic virus, a number of biological properties associated with this unusually virulent influenza virus have been found. The high replication efficiency of the reconstructed 1918 virus observed in mouse and human airway cells appears to be the result of molecular determinants in the HA, NA, and PB1 virus genes. The role of PB1 may reflect the polymerase activity of the PB1 protein itself or the proapoptotic viral protein, PB1-F2, generated by an alternative reading frame in the PB1 gene segment. While the role of the PB1 gene is likely to be a crucial component of 1918 virus virulence, PB2 appears to contribute to the transmissibility of this virus by allowing increased replication at lower temperatures in the airways of mammals. Although a number of selected 1918 virus genes were critical, it is most certainly the coordinated expression of all 1918 virus genes that confers the unique highly virulent, transmissible phenotype observed with this pandemic virus. Overall, such studies should provide further insight and a basis for the rational design of intervention strategies that target specific virus proteins.

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SUMMARY POINTS

1. Sequence analysis of the 1918 H1N1 viral genome and the plasmid-based reverse genetics system has allowed researchers an unprecedented opportunity to study the composition of the virus responsible for the influenza pandemic of 1918.
2. Among the eight 1918 gene segments studied, the HA, NA, and PB1 genes contributed significantly to the efficient replication and enhanced virulence of the pandemic strain.
3. The surface glycoproteins and PB2 segments of the 1918 virus are sufficient to confer virus transmissibility of an avian H1N1 virus.

FUTURE ISSUES

1. The contribution of the 1918 polymerase subunit PB1 gene to virulence is particularly significant in the context of the 1957 and 1968 pandemic viruses, each of which acquired a novel PB1 gene from the avian influenza gene pool. How does the PB1 gene provide for enhanced virus replication and virulence of the 1918 pandemic strain? A better knowledge of the structure-function relationships of PB1 and its interactions with host components such as host transcription machinery is needed for a better understanding of the overall mechanism(s).
2. A better knowledge of the mechanisms of influenza virus transmission from the standpoint of the host will help researchers to better understand the virus-host interactions and specific airway cells conferring transmission.
3. Further characterization of the avian polymerase subunit PB2 in efficient transmission of more relevant avian influenza strains such as H5 and H7 subtype viruses is needed. Multiple subtypes of H5 and H7 avian influenza viruses circulating in domestic poultry have infected more than 500 individuals in the past decade and currently represent the greatest threat to public health.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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